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Haemophilus influenzae Protocols

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The Pathogenesis of Disease Due to Nontypeable *Haemophilus influenzae*

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1. Introduction

Isolates of *Haemophilus influenzae* can be separated into encapsulated and nonencapsulated forms. Encapsulated strains express one of six structurally and antigenically distinct capsular polysaccharides, designated serotypes a–f ***(1)***. In contrast, nonencapsulated strains are defined by their inability to react with antisera against the known *H. influenzae* polysaccharide capsules and are referred to as nontypeable.

2. Epidemiology

Nontypeable *H. influenzae* is a common commensal organism in the human nasopharynx and occupies this niche as its natural habitat ***(2,3)***. The rate of nasopharyngeal colonization increases from approx 20% during the first year of life to over 50% by the age of 5–6 yrs, then remains high through adulthood ***(2)***. Children often harbor multiple strains simultaneously, whereas adults typically carry only one ***(3)***. Based on longitudinal studies, in most cases an individual carries a given strain for several weeks to mo, then loses the strain, and then acquires a new strain ***(4–6)***. Transmission generally occurs by airborne droplets or by direct contact with respiratory secretions.

Isolates of nontypeable *H. influenzae* account for 20–30% of all episodes of acute otitis media and possibly a higher percentage of recurrent episodes ***(7)***. In addition, recent studies indicate that this organism is responsible for over 40% of cases of otitis media with effusion (chronic otitis media) ***(8)***. Among patients with acute or chronic sinusitis, nontypeable *H. influenzae* is the causative agent in approx one-third ***(9,10)***. Similarly, in patients with chronic bron-

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chitis and pulmonary exacerbations, several lines of evidence implicate nontypeable *H. influenzae* as a common precipitant ***(11)***. Nontypeable *H. influenzae* is also an important cause of community-acquired pneumonia, especially in children in developing countries, in patients with underlying chronic lung disease, and among the elderly ***(12–14)***. On occasion, nontypeable *H. influenzae* causes systemic disease, with examples including meningitis, septicemia, and septic arthritis ***(15)***.

The pathogenesis of respiratory tract disease due to nontypeable *H. influenzae* involves colonization of the nasopharynx followed by contiguous spread to adjacent sites. In most cases, contiguous spread occurs in the setting of abnormalities in nonspecific host defenses. For example, viral upper respiratory infection disrupts mucociliary activity, mucosal integrity, and neutrophil function and predisposes to nontypeable *H. influenzae* otitis media, sinusitis, and pneumonia. Similarly, underlying lung diseases such as chronic bronchitis, bronchiectasis, and cystic fibrosis are associated with abnormalities in mucociliary clearance, thus predisposing to nontypeable *H. influenzae* bronchitis and pneumonia ***(16,17)***. Exposure to cigarette smoke results in goblet cell hyperplasia, mucus hypersecretion, and decreased respiratory epithelial cell ciliary function, changes that increase the likelihood of localized respiratory tract disease due to nontypeable *H. influenzae* ***(18)***.

Most cases of systemic disease due to nontypeable *H. influenzae* occur in patients with anatomic abnormalities or compromised immunity ***(15)***. For example, in patients with nontypeable *H. influenzae* meningitis, typically a communication exists between the upper respiratory tract and the central nervous system. In individuals with nontypeable *H. influenzae* bacteremic disease, a deficiency in humoral immunity is almost always present, allowing for bacterial invasion of the bloodstream, then intravascular survival, and then seeding of distant sites ***(19,20)***.

Although many cases of nontypeable *H. influenzae* disease can be treated successfully with a β-lactam antibiotic such as ampicillin or amoxicillin, resistance is becoming increasingly common, usually reflecting production of a β-lactamase ***(21,22)***. In occasional isolates, resistance results from an altered penicillin binding protein with diminished affinity for β-lactams. Resistance to other antibiotics, including trimethoprim-sulfamethoxazole, clarithromycin, and azithromycin, has also been reported ***(21)***.

Even with successful therapy, infection due to nontypeable *H. influenzae* can produce long-term complications. As an example, middle ear effusion that develops during acute otitis media can persist for several weeks or months and cause deficits in language acquisition, speech development, and cognitive function ***(23)***. The economic impact of nontypeable *H. influenzae* disease is also significant, with the cost of physician visits and prescription drugs approaching $1 billion per year in the United States alone ***(24)***. *H. influenzae* otitis

media, sinusitis, pneumonia, and bronchitis also incur societal costs related to time lost from school and work. Beyond morbidity and economic costs, nontypeable *H. influenzae* is an important cause of mortality among children in the developing world who develop pneumonia ***(25)***.

3. Bacterial Structure

Nontypeable *H. influenzae* is typical of Gram-negative bacteria and has a cell wall that consists of an inner (cytoplasmic) membrane, a periplasm, and an outer membrane. Analysis of the genome of *H. influenzae* strain Rd reveals a system of proteins that comprise the Sec machinery and presumably facilitate protein export from the cytoplasm ***(26)***. The periplasm contains several layers of peptidoglycan and a protease with homology to the *Eschericia coli* DegP (HtrA) protein ***(27)***. The outer membrane represents the interface between the organism and the human host and contains integral proteins, surface-associated proteins, and lipooligosaccharide.

The major outer membrane proteins include P1, P2, P4, P5, and P6. P1 (also called protein a) is heat-modifiable and has a molecular mass of 35 kDa at room temperature and 46–50 kDa after boiling ***(28–30)***. P2 (also called protein b/c) is the most abundant protein in the outer membrane and has a molecular mass that ranges between 36 and 42 kDa ***(30)***. Structural studies indicate that P2 forms a trimer and has porin activity, allowing molecules up to 1400 Da to pass through the membrane ***(31)***. P4 (or protein e) is a 28–30 kDa lipoprotein that has been implicated in heme uptake ***(32,33)***. P5 (or protein d) is a heat modifiable protein with homology to *E. coli* OmpA and has a molecular mass of approx 27 kDa at room temperature and 35 kDa after boiling ***(34,35)***. P6 (also called protein g or PAL) is another outer membrane lipoprotein and has a molecular mass of 16 kDa ***(28–30)***. Comparison of predicted amino acid sequences from multiple strains reveals that P1, P2, and P5 vary considerably from one strain to another ***(36–39)***. In contrast, P6 is highly conserved, with little variation in amino acid sequence from strain to strain ***(40)***. P4 contains at least one epitope that is highly conserved among strains ***(33)***.

A number of minor outer-membrane proteins exist, including PCP, D15, OMP 26, protein D, the transferrin-binding proteins (Tbp1 and Tbp2), and several heme-binding proteins. PCP is a 16-kDa protein that co-migrates with P6 and makes up approx 0.5% of the outer membrane ***(41)***. D15 is a high-mol wt (103-kDa) protein that is highly conserved among strains and is immunogenic ***(27,42)***. OMP 26 is a 26-kDa protein that elicits clearance of both homologous and heterologous strains after mucosal immunization in a rat pulmonary challenge model ***(43,44)***. Protein D is a highly conserved, surface-exposed, 42-kDa lipoprotein that was first identified based on its ability to bind IgD ***(45)***. More recent evidence indicated that protein D has glycerophosphodiester phosphodi-

esterase activity and facilitates utilization of glycerophosphorylcholine as a source of choline ***(45a)***. The transferrin-binding proteins serve as surface receptors for human transferrin and facilitate iron acquisition ***(46)***.

Lipooligosaccharide is a major component of the *H. influenzae* cell wall, accounting for roughly 4% of the dry weight of the organism ***(47)***. Similar to other nonenteric Gram-negative bacteria, *H. influenzae* lipooligosaccharide contains a lipid A moiety and an oligosaccharide core but lacks O side chains, giving rise to the term lipooligosaccharide (LOS). A given cell expresses a variety of LOS structures, with considerable variation in carbohydrate content ***(48)***.

4. Population Structure

Over the years, a number of methods have been employed to examine the genetic and evolutionary relationships between strains of nontypeable *H. influenzae*. Examples include biotyping, outer membrane protein typing, lipooligosaccharide typing, and ribotyping, among others. Perhaps most powerful is multilocus enzyme electrophoresis, a method that measures electrophoretic mobility of metabolic enzymes. In particular, chromosomally encoded enzymes are separated on a starch gel and then stained histochemically with specific substrates. Enzyme variants are identified based on differences in mobility (revealing electromorphs), which reflect changes in net electrostatic charge as a result of one or more amino acid substitutions ***(49)***. Amino acid substitutions in turn reflect changes in nucleotide sequence. With a given strain, the electrophoretic type is defined by the combination of electromorphs for the enzymes tested. Examination of a relatively large number of enzymes within a group of strains allows accurate estimates of the level of genetic relatedness between strains.

Analysis by multilocus enzyme electrophoresis indicates that encapsulated *H. influenzae* strains are clonal and can be segregated into genetically related clusters, which are grouped into two major phylogenetic divisions ***(50,51)***. Division I contains clusters of types a, b, c, d, and e strains, and division II contains clusters of types a, b, and f strains. The population structure of nontypeable *H. influenzae* is less well defined. Musser et al. examined 65 epidemiologically unrelated isolates by multilocus enzyme electrophoresis and found considerable heterogeneity, with each isolate corresponding to a unique electrophoretic type ***(52)***. Furthermore, comparison with 177 type b isolates revealed no sharing of electrophoretic types ***(52)***. These observations indicate greater genetic diversity among nontypeable *H. influenzae* than among *H. influenzae* type b and suggest that the nontypeable *H. influenzae* population structure lacks clonality. In addition, they suggest that nontypeable strains generally are not phenotypic variants of type b strains.

More recently, we studied a series of 123 pharyngeal isolates of nontypeable *H. influenzae* collected from healthy 3-yr old Finnish children ***(53)***. Among

these isolates, one was a capsule-deficient type b strain, based on Southern analysis demonstrating loss of the one functional copy of the nearly duplicated *cap*b locus. Of the remaining 122 isolates, 31% hybridized with a probe containing the *cap*b locus, suggesting that a subgroup of nontypeable strains might be more closely related to an encapsulated ancestor. To extend these observations, we examined the collection of nontypeable strains previously characterized in terms of genetic relatedness by Musser et al. ***(54)***. Roughly 15% of these strains hybridized with a probe for the *cap*b locus, in all cases due to the presence of IS*1016*, an insertion element associated with *cap* genes in division I, encapsulated strains of *H. influenzae*. Although the strains harboring an IS*1016* element do not define a distinct genetic division, they form small clusters or lineages within the larger population structure. Interestingly, these strains uniformly lack the family of adhesins most common in nontypeable strains (*see* **Subheading 5.2.2.**). Instead, almost all express a homolog of the major non-pilus adhesin in encapsulated strains of *H. influenzae* (*see* **Subheading 5.2.3.**), again supporting the conclusion that selected nontypeable strains are more closely related to encapsulated lineages.

Considered together, this information suggests a model in which the primordial *H. influenzae* strain was nonencapsulated and spawned two separate but overlapping lineages. The larger lineage acquired one set of genes involved in interactions with the host and remained nonencapsulated. The other lineage acquired a separate set of colonization factors as well as the *cap* genes and became encapsulated; subsequent mutations within the *cap* locus resulted in evolution of a relatively restricted set of nonencapsulated strains, which represent a minority of all nontypeable isolates.

5. Establishment on the Mucosal Surface

5.1. Binding to Mucus and Ciliotoxicity

Upon entering the respiratory tract, bacteria interact initially with mucus and are probably largely eliminated by the mucociliary escalator, which consists of ciliated respiratory epithelial cells and the associated mucus layer. However, in at least some circumstances, a small number of organisms may persist. Based on experiments examining interactions between nonencapsulated *H. influenzae* and nasal turbinate tissue in organ culture, residual organisms appear to form microcolonies within the mucus layer, ultimately elaborating soluble factors that cause ciliostasis, loss of cilia, and sloughing of ciliated cells ***(55)***.

Studies by Kubiet and Ramphal demonstrated that nontypeable *H. influenzae* is capable of binding to mucin, a major component of mucus ***(56)***. The mechanism of this binding remains poorly understood but probably reflects both specific and nonspecific interactions. Using a gel overlay assay, Reddy et al. found that the P2 and P5 outer membrane proteins influence binding to nasopharyn-

geal mucin ***(57)***. Additional experiments using the chinchilla model revealed that mutants lacking P5 display reduced adherence to Eustacian tube mucus ***(58)***. In the same model, purified P5 is capable of direct binding to mucus and of blocking adherence by whole organisms ***(58)***.

The nontypeable *H. influenzae* effector responsible for ciliotoxicity was initially shown to be released from the surface of the organism and to be nondialyzable, heat stable, and trypsin resistant ***(59)***. Subsequent studies by Johnson and Inzana established that LOS is the relevant substance, as purified nontypeable *H. influenzae* LOS reproduces the ciliotoxic effects of infection with whole organisms ***(60)***. In additional work, these investigators demonstrated that the lipid-free oligosaccharide fraction of LOS has no effect on ciliary activity, thus pointing to lipid A as the biologically active moiety ***(60)***. During infection due to nontypeable *H. influenzae*, LOS may remain associated with the bacterial surface or may be released ***(61)***. These two forms differ in their biological activities. Secreted LOS is 10-fold more potent in stimulating the release of monocyte-derived inflammatory mediators such as tumor necrosis factor alpha (TNF-α), interleukin 1α (IL-1α), and interleukin 6 (IL-6) ***(61)***. In addition, this form is more active in the limulus assay and is more toxic to mice ***(61)***. It remains unknown whether cell-associated LOS and secreted LOS have differential effects on ciliated respiratory epithelial cells.

Protein D is a second factor that has been implicated in ciliotoxicity ***(62)***. This protein was first identified by Raun et al. and is capable of binding certain human IgD myeloma proteins ***(45)***. Analysis of the predicted amino acid sequence revealed 67% identity with the *E. coli* periplasmic glycero-phosphodiesterase encoded by *glpQ*, and Munson and Sasaki demonstrated that protein D contains glycerophosphodiesterase phosphodiesterase activity ***(63)***. Recently, Janson and co-workers compared isogenic protein D-expressing and protein D-deficient strains in assays with human adenoidal tissue in culture and found that protein D was essential for maximal impairment of ciliary activity and damage to ciliated cells ***(62)***. Of note, protein D did not affect the number of bacteria associated with the mucosal surface. Consistent with these results, in a rat otitis media model, elimination of expression of protein D resulted in a 100-fold decrease in virulence ***(64)***. At this point, the mechanism by which protein D influences ciliated epithelium and the relevance of IgD binding and glycerophosphodiesterase phosphodiesterase activity remain unclear. However, it is intriguing to consider that the role of protein D in facilitating acquisition of choline may be important.

5.2. Adherence to Respiratory Epithelium

A fundamental step in the process of colonization involves adherence to the epithelial surface. Based on experiments with adenoidal tissue and nasal turbinates in organ culture, nontypeable *H. influenzae* adheres preferentially to nonciliated

cells and to areas of damaged epithelium. In vitro studies using isolated epithelial cells indicate that a variety of bacterial factors influence adherence.

5.2.1. Pili

Selected isolates of nontypeable *H. influenzae* express adhesive pili, which are hairlike surface appendages that extend up to 450 nm in length and are displayed circumferentially ***(65)***. As viewed by transmission electron microscopy using the quick-freeze, deep-etch technique, *H. influenzae* pili are composite structures consisting of a relatively rigid helical rod joined to a very thin tip ***(66)***. The helical rod is approx 6–7 nm in diameter and appears to be two-stranded, similar to filamentous actin and different from other well-characterized pili ***(66)***.

The *H. influenzae* gene cluster contains a total of five genes, designated *hifA–E* ***(67–71)***. *hifA* encodes the HifA major structural subunit and is transcribed divergently from the rest of the gene cluster ***(67)***. *hifB* encodes the HifB chaperone, which resides in the periplasm and serves to stabilize subunits against premature degradation and to target subunits to the outer membrane ***(66)***. *hifC* encodes the HifC protein, which shares homology with outer-membrane ushers and presumably sits in the outer membrane and facilitates the translocation of subunits across the outer membrane, allowing their incorporation into growing pili ***(69)***. *hifD* encodes the HifD minor subunit, and hifE encodes the HifE minor subunit ***(67,68)***. Based on localization by immunoelectron microscopy, both HifD and HifE appear to make up the tip fibrillum ***(66,72)***. An in-frame deletion in *hifD* results in a decreased number of pili, suggesting that HifD serves to nucleate pilus assembly ***(68)***. Antiserum against HifE eliminates pilus-mediated adherence, arguing that this protein represents the pilus adhesin ***(72)***. Consistent with this possibility, HifE is larger than most structural subunits and shares significant homology with other pilus adhesins.

The adhesive activity of *H. influenzae* pili was first appreciated in the early 1980s, when two groups independently noted a correlation between piliation and agglutination of human erythrocytes and adherence to human oropharyngeal epithelial cells ***(73,74)***. Subsequently, van Alphen demonstrated that pilus-mediated hemagglutination correlates with expression of the AnWj antigen (formerly called the Anton antigen) and is inhibited by anti-AnWj antiserum ***(75)***. Interestingly, human oropharyngeal epithelial cells lack this antigen, and pilus-dependent attachment to these cells is unaffected by anti-AnWj antiserum ***(75)***. Nevertheless, the same anti-pilus monoclonal antibody blocks agglutination of erythrocytes and attachment to oropharyngeal epithelial cells ***(76)***, suggesting that pili may recognize the same structure on both erythrocytes and epithelial cells, apparently in the context of either the AnWj antigen or some other cell membrane receptor. Consistent with this consideration, compounds containing sialyllactosylceramide, including the gangliosides GM1,

GM2, GM3, and GD1a, are able to inhibit hemagglutination as well as adherence to epithelial cells ***(77)***.

5.2.2. HMW1 and HMW2

Following the identification of *H. influenzae* pili, a series of observations suggested that nontypeable strains express nonpilus adhesins as well. In particular, several studies demonstrated that nonpiliated strains are capable of efficient adherence to cultured human epithelial cells ***(78)***. Additional experiments revealed that nonpiliated strains are capable of appreciable association with the mucosal surface of nasal turbinates in culture ***(55)***. A major clue regarding the nature of these adhesins came from work by Barenkamp and co-workers, who identified a family of surface-exposed high-molecular-weight proteins that are major targets of the serum antibody response to infection ***(79,80)***. The prototype members of this family are proteins called HMW1 and HMW2, which are related proteins expressed by the same strain ***(79)***. Overall, HMW1 and HMW2 are 80% similar and 71% identical. Interestingly, they share significant sequence similarity with the filamentous hemagglutinin agglutination (FHA) protein, an adhesin and colonization factor expressed by *Bordetella pertussis* ***(81,82)***.

The HMW1 and HMW2 adhesins are encoded by separate gene clusters, each containing three genes called *hmwA, hmwB,* and *hmwC*. The *hmw1A* and *hmw2A* genes encode the adhesins (HMW1 and HMW2), the *hmw1B* and *hmw2B* genes encode 60-kDa outer membrane proteins (HMW1B and HMW2B) that translocate the adhesins to the cell surface, and the *hmw1C* and *hmw2C* genes encode 73-kDa cytoplasmic proteins (HMW1C and HMW2C) that appear to stabilize the adhesins prior to export from the cytoplasm ***(83,84)***. Of note, the predicted amino acid sequences of HMW1B and HMW2B are 99% identical, and the predicted sequences of HMW1C and HMW2C are 97% identical. Consistent with this homology, in assays examining secretion of HMW1 and HMW2, HMW1B/HMW1C are able to substitute for HMW2B/HMW2C and vice versa ***(84)***.

The HMW1 and HMW2 adhesins are synthesized as 160-kDa and 155-kDa preproteins, respectively. In each case, a 68-amino acid N-terminal fragment directs export from the cytoplasm via the Sec machinery ***(85)***. Following cleavage between amino acids 68 and 69 by leader peptidase, the proteins are released into the periplasm ***(85)***. With both proteins, the segment between amino acids 69 and 441 serves as an intramolecular chaperone and mediates interaction with the HMW1B or HMW2B outer-membrane translocator ***(85)***. While associated with the periplasmic face of the outer membrane, HMW1 and HMW2 are cleaved between amino acids 441 and 442, resulting in mature 125-kDa and 120-kDa proteins, respectively ***(79,85)***. Subsequently, they are transported to the surface of the organism via the HMWB proteins ***(84)***.

In studies using diverse cultured human epithelial cell lines, HMW1 and HMW2 exhibit differing cellular binding specificities ***(86)***, suggesting that they

recognize distinct host cell receptor structures. More detailed experiments with Chang conjunctival cells have demonstrated that HMW1 interacts with a glycoprotein that contains N-linked oligosaccharide chains with sialic acid in an α-2,3 configuration ***(87)***. At this point, the nature of the HMW2 receptor remains unknown.

5.2.3. Hia

Approximately 25% of nontypeable strains lack proteins that belong to the HMW1/HMW2 family of proteins. Nevertheless, nearly all of these strains remain capable of efficient adherence to cultured human epithelial cells ***(54)***. Furthermore, they express high-molecular-weight proteins that are major targets of the antibody response to infection ***(80)***. With this information in mind, recent work has demonstrated that these strains express an adhesin called Hia ***(88)***. The prototype Hia protein is 115 kDa in size and is encoded by a 3.3-kb gene ***(88)***. Interestingly, Hia shares significant homology with the major nonpilus adhesin present in *H. influenzae* type b, a protein called Hsf ***(89)***. Overall, Hia and Hsf share 72% identity and 80% similarity. They are most similar at their N-terminal and C-terminal ends and also contain a conserved internal domain that is repeated three times in Hsf, thus accounting for the larger size of Hsf (approx 240 kDa).

Adherence assays with *E. coli* transformants harboring the *hia* gene demonstrate that Hia is capable of reaching the bacterial surface and mediating in vitro adherence independent of other *H. influenzae* gene products, with the probable exception of the Sec proteins, which are present in both *H. influenzae* and *E. coli* ***(88)***. Recent evidence indicates that Hia belongs to the growing family of autotransporters, IgA1 protease-like proteins present in Gram-negative bacteria ***(89a)***. However, in contrast to other well-characterized autotransporter proteins, Hia undergoes no processing event on the bacterial surface and remains cell-associated in the full-length form.

5.2.4. Hap

Mutants deficient in expression of HMW1 and HMW2 or Hia remain capable of low-level adherence to cultured human epithelial cells, suggesting that an additional adhesin exists. To address this possibility, we prepared a genomic library from a virulent clinical isolate and identified a gene called *hap*, which confers a capacity for intimate interaction with cultured epithelial cells ***(90)***. Analysis of the predicted amino acid sequence of the Hap protein reveals significant sequence homology with the *H. influenzae* and *Neisseria* IgA proteases. This homology includes the region identified as the serine protease catalytic site of the IgA1 proteases. Overall, there is 30–35% identity and 51–55% similarity between Hap and the *H. influenzae* and *N. gonorrhoeae* IgA1 proteases.

The striking homology between Hap and the IgA1 proteases reflects a similar mechanism of processing and secretion ***(91)***. In particular, Hap is synthesized as a 155-kDa precursor protein with three domains, including a typical prokaryotic signal sequence, an internal 110-kDa serine protease domain (designated Hap_s), and a 45-kDa C-terminal outer-membrane protein (designated Hap_β. The Hap signal sequence is presumed to direct export out of the cytoplasm. Subsequently, the C-terminal Hap_β domain inserts into the outer membrane and facilitates translocation of Hap_s. Once the protein is on the surface of the organism, the Hap active site serine (S243) gains catalytic activity, promoting autoproteolysis between the leucine residue at position 1036 and the asparagine at position 1037. This cleavage event releases Hap_s from Hap_β and from the bacterial surface, leaving Hap_β embedded in the outer membrane.

The Hap_s domain has adhesive activity, and in the context of full-length Hap, Hap_s is responsible for mediating interaction with host cells ***(92)***. With this information in mind, it is noteworthy that secretory leukocyte protease inhibitor (SLPI) blocks Hap autoproteolysis, resulting in increased amounts of full-length Hap and increased Hap-mediated adherence ***(92)***. SLPI is a natural component of respiratory secretions and is especially abundant in the human upper respiratory tract; it was first discovered based on its ability to block the activity of neutrophil elastase and appears to be upregulated in the setting of inflammation ***(93)***.

5.2.5. Other Adhesins

Based on bacterial overlay assays, three additional adhesive activities have been identified in nontypeable *H. influenzae*. Using thin layer chromatography, Busse et al. observed that nontypeable strains are capable of binding to phosphotidylethanolamine (PE), gangliotriosylceramide (Gg3), gangliotetraosylceramide (Gg4), sulfatoxygalactosylceramide, and, to a lesser extent, sulfatoxygalactosylglycerol ***(94)***. In additional experiments, these investigators prepared a PE affinity matrix and purified a 46-kDa protein that inhibits binding of whole bacteria to immobilized PE and Gg3 ***(94)***. More recently, Hartmann and Lingwood found that heat shock treatment resulted in a marked increase in nontypeable *H. influenzae* binding to sulfatoxygalactosylceramide and sulfatoxygalactosylglycerol ***(95)***. Additional analysis suggested that this binding is due to two Hsp 70-related heat-shock proteins ***(95)***. In work by Fakih, studies using thin-layer chromatography revealed that at least one nontypeable strain is able to bind to two minor ganglioside doublets that migrate near GM1 ***(96)***. This binding is independent of piliation ***(96)***.

Studies by Prasadarao and co-workers indicate that OapA is another protein that influences adherence to cultured human epithelial cells ***(97)***. OapA is a surface-associated protein responsible for the transparent-colony phenotype

and is required for efficient colonization in the infant rat nasopharyngeal colonization model ***(98)***. In studies with Chang cells, elimination of expression of OapA in strains Rd and H233 resulted in a three- to nine-fold decrease in attachment compared with the isogenic parental strains ***(97)***. In addition, expression of OapA by *E. coli* DH5α was associated with a three-fold increase in adherence ***(97)***.

6. Persistence on the Mucosal Surface

Following establishment on the mucosal surface, bacteria face the challenge of persisting. Persistence requires that the organism evade host immune mechanisms and acquire essential nutrients, including iron and heme.

6.1. Evasion of Host Immune Mechanisms

6.1.1. IgA1 Protease

The predominant immunoglobulin present on mucosal surfaces is IgA, a molecule that participates in host defense by agglutinating bacteria and inhibiting adherence, binding and inactivating bacterial toxins, and augmenting phagocytosis ***(99)***. Of note, IgA is unable to fix complement. Within the respiratory tract, IgA1 accounts for over 90% of all IgA present ***(99)***. Along with several other mucosal pathogens, *H. influenzae* elaborates an extracellular endopeptidase called IgA1 protease, which cleaves the hinge region of the serum and secretory forms of IgA1 and releases the antigen-binding Fab domains from the Fc portion of the molecule, thus eliminating agglutination activity ***(100)***.

Two different types of IgA1 protease have been identified in isolates of *H. influenzae*, and almost all strains express one or the other. Type 1 IgA1 protease cleaves at the peptide bond between the proline at position 231 and the serine at position 232, while type 2 IgA1 protease cleaves at the peptide bond between the proline at residue 235 and the threonine at residue 236. The specificity for a type 1 or a type 2 pattern of cleavage maps to a stretch of 125 amino acids at the N terminus of the protease. The cleavage site in IgA1 consists of an eight amino acid sequence repeated in tandem at the hinge region of the IgA1 heavy chain. IgA2 lacks this repeat, thus explaining resistance to proteolysis.

6.1.2. Invasion

Several studies suggest that nontypeable strains of *H. influenzae* are able to pass between cells and invade the subepithelial space. In addition, nontypeable organisms are capable of entering and surviving within respiratory epithelial and nonepithelial cells. In early work, Hers and Mulder examined tissue sections from patients with acute and chronic bronchitis associated with nontypeable *H. influenzae* infection and observed bacteria between epithelial

cells of the bronchi and bronchioles ***(101)***. In some cases, bacteria were found extending down to and beyond the basement membrane. Farley and co-workers reported similar results in experiments with adenoidal tissue in organ culture ***(102)***. Using scanning and transmission electron microscopy, these investigators observed disruption of tight junctions between cells within the epithelium and found clusters of bacteria between adjacent cells. More recently, van Schilfgaarde et al. examined interactions between nontypeable *H. influenzae* and polarized NCI-H292 cells and observed that bacteria were able to migrate between cells to the basolateral surface of the monolayer, a process they called paracytosis ***(103)***. Forsgren et al. examined adenoids removed from children with adenoidal hypertrophy or chronic secretory otitis media and discovered nontypeable *H. influenzae* in the reticular crypt epithelium and in macrophage-like cells in the subepithelial space ***(104)***. These organisms survived treatment with gentamicin and sometimes appeared to be dividing, suggesting that they were viable and raising the possibility that nontypeable *H. influenzae* may target macrophages to establish an intracellular niche. Experiments using in vitro models have demonstrated that nontypeable strains are also capable of entering transformed and primary respiratory epithelial cells ***(78,90,105)***. Studies with transformed cells led to the identification of the Hap adhesin, which promotes cellular invasion when expressed in a noninvasive laboratory strain of *H. influenzae* ***(90)***. However, the level of invasion due to Hap by itself is low, suggesting that other factors might also be involved. Recent evidence implicates phosphorylcholine decorating Los ***(105a)***. One possibility is that *H. influenzae* invasion into and between cells allows the organism to establish a protected niche and evade the immune system.

6.1.3. Phase Variation

Phase variation involves the reversible loss or gain of a defined surface structure and represents a common strategy employed by pathogens to persist under diverse conditions, including in the setting of the host immune response ***(106)***. A variety of nontypeable *H. influenzae* surface structures are subject to phase variation, including LOS, pili, the HMW adhesins, and heme receptors. Of note, all of these structures are immunogenic and stimulate a significant antibody response.

H. influenzae LOS is a highly variable structure, with as many as 25 different forms associated with nontypeable strain 9274 ***(48)***. LOS biosynthesis involves multiple enzymatic steps and a number of genes. Among these genes, *lic1A, lic2A, lic3A, lex-2*, and *lgtC* contain long stretches of tandem 4-bp repeats within their 5' coding region. In studies of the *lic* loci, Weiser and co-workers observed that the number of repeats varies spontaneously, generating translational frame shifts with different ATG codons falling in or out of frame ***(107)***. Such frame

shifts result in synthesis of a protein with a different N terminus or eliminate protein production altogether (when no in-frame start codon exists). By analogy to other systems, the mechanism of variation in repeat number is presumed to be slipped-strand mispairing. Changes in *lic2A* and *lic3A* influence glycotransferase activity and alter reactivity with monoclonal antibodies directed against specific LOS oligosaccharide epitopes ***(108)***. *lic2A* results in the addition of a Galα1-4Galα moiety which resembles human glycolipids and protects *H. influenzae* from antibody-mediated killing, possibly by molecular mimicry ***(109)***. *lgtC* may be involved in formation of a Galα1-4Glu moiety ***(110)***. Variation in the *lic1A* gene affects production of a choline kinase responsible for addition of phosporylcholine (ChoP) to the LOS molecule, a physical change that enhances binding of C-reactive protein and results in susceptibility to serum bactericidal activity ***(111–113)***.

H. influenzae pili promote adherence to epithelial cells and facilitate nasopharyngeal colonization. At the same time, piliation results in an augmented respiratory burst by neutrophils, and antibody against pili is associated with enhanced complement-mediated clearance ***(114)***. Accordingly, phase variation of pili provides the organism with a significant survival advantage. Insight into the mechanism underlying the shift between piliated and nonpiliated organisms came from experiments by van Ham et al., who noted that nonpiliated bacteria no longer express transcripts from either the *hifA* or the *hifB* gene ***(115)***. Additional studies demonstrated that *hifA* and *hifB* have overlapping promoters and share a common RNA polymerase binding site ***(115)***. The area of overlap contains a variable number of tandem TA dinucleotide repeats, with the number of repeats influencing spacing between the –10 and –35 regions. In *H. influenzae* strains AM20 and AM30, nonpiliated variants contain 9 TA repeats and fail to express either *hifA* or *hifB*, whereas fully piliated variants contain 10 repeats and express both *hifA* and *hifB*. Variants with an intermediate level of piliation contain 11 repeats and again express both *hifA* and *hifB*.

Levels of expression of the HMW1 and HMW2 adhesins were first noted to vary in animal immunization studies performed by Barenkamp ***(116)***. In particular, among chinchillas immunized with HMW1 and then challenged with the wild-type *H. influenzae* strain, isolates from the middle ear uniformly expressed relatively decreased amounts of HMW1 ***(116)***. Similarly, isolates from animals immunized with HMW2 uniformly expressed minimal HMW2 ***(116)***. Of note, the nucleotide sequences upstream of *hmw1A* and *hmw2A* are absolutely identical up to 311 base pairs from the start codon and include a series of 7-bp direct repeats arranged in a tandem array. Experiments by Dawid et al. demonstrated that the number of repeats undergoes spontaneous variation in a Rec-independent manner, presumably by slipped-strand mispairing ***(117)***. Increases in repeat number result in a stepwise decrease in protein expression,

and decreases in repeat number are associated with a graded increase in protein expression ***(117)***. These changes in protein expression reflect changes in levels of specific mRNA ***(117)***, suggesting that variation in repeat number influences either transcriptional activity or transcript stability.

Under aerobic growth conditions, *H. influenzae* has an absolute requirement for heme. Accordingly, the organism has developed complex systems for acquisition of heme, including heme in the form of hemoglobin:haptoglobin and heme:hemopexin. The combined work of a number of investigators suggests that strains of *H. influenzae* possess multiple heme-binding proteins. Interestingly, these proteins are commonly encoded by genes that contain 4-bp repeats within their 5' coding region, analogous to the situation with *H. influenzae* LOS. In the case of *hgpA* (also called *hhuA*), CCAA repeats vary in number and affect translational reading frame, resulting in phase-variable expression ***(118)***.

6.1.4. Antigenic Variation

Selected *H. influenzae* surface structures undergo antigenic drift, an irreversible process that involves substitution, deletion, or addition of amino acids in immunodominant regions of the relevant protein. In some cases, antigenic variation appears to be the consequence of horizontal gene transfer from another strain, with some or the entire original gene being replaced by donor DNA. These changes in amino acid sequence result in subtle structural changes, which in turn prevent recognition by previously generated host antibodies. Perhaps the clearest examples of antigenic variation in *H. influenzae* include the P2 and P5 outer-membrane proteins.

P2 is an outer-membrane porin and is strongly immunogenic during natural infection ***(119)***. Characterization of P2 from a number of nontypeable isolates indicates that this protein is highly variable in both size and amino acid sequence from one strain to another ***(37,38,120)***. In addition, variation in P2 sequence occurs within a clonal population during the course of persistent infection ***(121)***. Both in individual children with chronic otitis media and adults with chronic bronchitis due to nontypeable *H. influenzae*, specific regions of P2 vary at a relatively high frequency ***(121,122)***. These changes correspond to surface-exposed loops called loop 5 and loop 6, which are the immunodominant epitopes in the protein ***(121)***. In studies using the rabbit subcutaneous cage model, organisms are able to persist despite the development of bactericidal antibodies against P2 ***(123)***. Survival is the result of frequent nucleotide changes within the region encoding loop 6, resulting in amino acid changes that render the original antibodies no longer bactericidal ***(123)***. Work by Smith-Vaughn et al. suggests that antigenic variation in P2 also occurs by horizontal transfer of *ompP2* from other strains ***(122)***.

The *H. influenzae* P5 protein shares significant homology (approx 50%) with the *E. coli* OmpA protein, which has weak pore-forming activity and is proposed to form a β-barrel ***(35)***. Of note, immunization with P5 stimulates antibodies that are bactericidal and associated with protection against the homologous strain ***(124)***. In studies of patients with chronic bronchitis and persistent infection with a single *H. influenzae* strain, Groeneveld et al. observed that P5 undergoes variation in size over time ***(125)***, suggesting that P5 is an important target of the immune response. More recently, Duim et al. found that persistent infection is associated with acquisition of point mutations and codon deletions scattered throughout four well-defined regions of the P5 gene ***(126)***. Similar to the situation with P2, these regions encode putative surface-exposed loops ***(39,126)***.

A number of other surface proteins show significant strain-to-strain variation in sequence, suggesting spontaneous mutation perhaps in combination with horizontal exchange of large segments of DNA over time. Examples include P1, IgA1 protease, HxuA, the HMW1 and HMW2 adhesins, the Hia adhesin, and the HifA, HifD, and HifE pilus subunits.

6.2. Iron and Heme Acquisition

In the human host, iron-binding proteins maintain free iron at levels well below those required for bacterial survival and growth ***(127)***. Accordingly, in order to survive, bacteria have developed mechanisms for acquiring iron ***(128–131)***. Siderophore-mediated iron uptake involves the production and secretion of a high-affinity, low-mol-wt iron chelator, called a siderophore. Siderophores remove iron from transferrin or lactoferrin and deliver it to a bacterial iron transport. With nonsiderophore iron uptake, bacteria produce cell surface receptors that bind host iron transport molecules directly. Subsequently, the iron is removed and translocated into the bacterial cell ***(127)***.

H. influenzae lacks siderophore iron uptake systems and instead uses nonsiderophore mechanisms. In particular, *H. influenzae* can bind transferrin ***(132)*** and a variety of other iron-containing sources such as heme, ***(133)*** hemoglobin ***(134,135)***, hemoglogin:haptoglobin complex ***(136)***, and heme:hemopexin complex ***(137)***. Transferrin binding involves two outer membrane proteins, called Tbp1 and Tbp2 ***(138)***. Expression of these proteins is iron-repressible and under the control of the ferric uptake repressor protein (Fur) ***(46,139)***. Tbp1 is a 95-kDa integral outer membrane protein, while Tbp2 is a 68- to 85-kDa protein and appears to be anchored in the outer membrane via a lipid moiety ***(46)***. Based on a proposed model, Tbp2 and Tbp1 interact together with transferrin, allowing removal of iron. Subsequently, Tbp1 releases the iron into the periplasm by a TonB-dependent transport mechanism ***(46,140)***.

The next step involves the *hit* (*Haemophilus* iron transport) locus, which encodes a set of proteins that work together to transport iron across the cyto-

plasmic membrane and into the cytoplasm. The *hit* locus encodes three proteins, HitA (also called FbpA) ***(141)***, HitB, and HitC, which share similarity with the *S. marcescens* Sfu proteins involved in periplasmic iron transport ***(142)***. HitA is a periplasmic iron-binding protein and belongs to the transferrin family. HitB is a cytoplasmic permease and transports iron from the periplasm to the cytoplasm. HitC is a nucleotide-binding protein and functions in energy transduction, facilitating active transport of iron through the HitB permease and into the cytoplasm ***(143)***.

H. influenzae lacks the ability to synthesize protoporphyrin IX, which is a precursor for the biosynthesis of heme. As a result, the organism has an absolute requirement for heme or heme derivatives for growth under aerobic conditions. Within the host, free heme is sequestered by hemopexin, and hemoglobin is sequestered by haptoglobin ***(127)***. Accordingly, *H. influenzae* has developed efficient mechanisms for extracting heme and heme-containing compounds from these storage proteins.

As with iron, the system for acquisition of heme is complex and involves several components, including those that bind heme sources, such as heme:hemopexin and hemoglobin:haptoglobin, and those that mediate transfer of heme to the cytoplasm. One heme- and heme:hemopexin-binding complex consists of proteins called HxuA, HxuB, and HxuC, which are encoded by the *hxu* operon. HxuA is a 100-kDa protein that is released into the culture medium by growing cells and binds heme:hemopexin ***(137)***. HxuB is a 60-kDa outer membrane protein that shares homology with outer membrane translocators and is believed to facilitate secretion of HxuA ***(144)***. The 78-kDa HxuC has characteristics of a TonB-dependent outer-membrane protein and may be involved in the transport of heme within the cell ***(144)***. The importance of TonB in heme acquisition has been demonstrated by the inability of *tonB* mutants to utilize heme or heme:hemopexin ***(145)***. Sequence analysis of the *hxu* operon indicates that *hxuA* and *hxuB* are transcribed as a unit and independently of *hxuC*. Both *hxuC* and *hxuB* have putative *fur* boxes upstream, indicating they are regulated by iron ***(144)***.

H. influenzae is also capable of binding hemoglobin as the first step in the utilization of hemoglobin-associated heme. This binding activity has been attributed to a series of proteins, including HgpA, HgpB, and HgpC. HgpA is a 120-kDa heme-regulated outer-membrane protein that mediates binding of both hemoglobin:haptoglobin complexes and free hemoglobin ***(135,146)***. HgpB is a 115-kDa TonB-dependent outer-membrane protein involved in hemoglobin binding ***(147)***. HgpC is another TonB-dependent,120-kDa outer membrane protein and is involved in hemoglobin:haptoglobin binding ***(148)***. A fourth

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Fig. 1. *(opposite page)* Model for the pathogenesis of disease due to nontypeable *Haemophilus influenzae*. Upon entry into the nasopharynx, bacteria encounter the

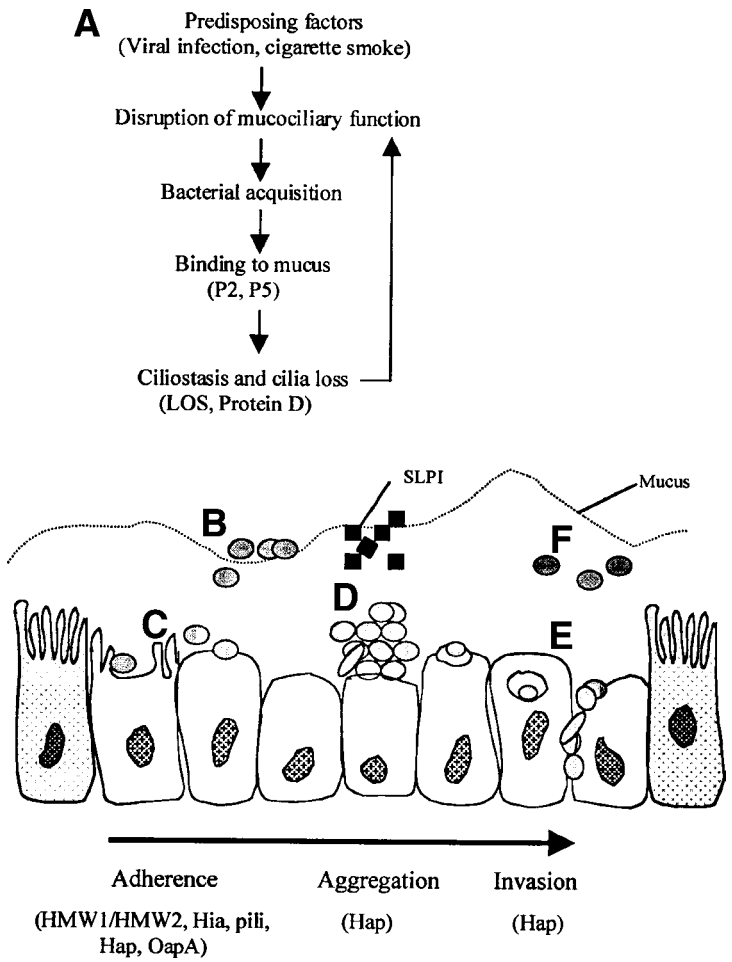

Fig. 1. *(continued)* mucociliary escalator. Viral infection, exposure to cigarette smoke, and allergic disease disrupt mucociliary function and increase the likelihood of colonization. **(A,B)** Bacteria that are not cleared bind to mucin and elaborate lipooligosaccharide (LOS), protein D, and possibly other factors, which lead to paralysis and loss of cilia, further impairing the mucociliary escalator. **(C)** Subsequently, bacteria adhere to respiratory epithelium using a number of adhesive factors, including pili, HMW1/HMW2, Hia, Hap, and OapA. **(D)** In the presence of secretory leukocyte protease inhibitor (SLPI), the Hap adhesin promotes augmented adherence and also mediates interbacterial interactions, leading to bacterial aggregation. **(E)** Hap and possibly other bacterial factors are able to promote entry into epithelial cells, providing organisms with a protected niche. In addition, occasional organisms are able to penetrate between cells via a process referred to as paracytosis. **(F)** When the concentration of SLPI is relatively diminished, Hap undergoes autoproteolysis, potentially contributing to dispersal of organisms and spread within the respiratory tract. IgA1 protease, phase variation, and antigenic drift facilitate evasion of the immune system, and acquisition of heme and iron are critical for persistence on the mucosal surface.

protein, called HhuA, also binds hemoglobin:haptoglobin complexes ***(136)***. Given that HgpA and HhuA share 90% similarity, it is possible that these two proteins represent homologs expressed in different strains ***(146)***.

7. Summary

To summarize, the pathogenesis of disease due to nontypeable *H. influenzae* involves multiple steps and the interplay of a number of bacterial and host factors, as shown in **Fig. 1.** Following entry into the upper respiratory tract, bacteria encounter the mucociliary escalator. The P2 and P5 outer-membrane proteins and probably other factors promote bacterial binding to mucus, and elaboration of LOS causes damage to ciliated cells and impairs mucociliary function. Subsequently, several adhesins, including HMW1 and HMW2, pili, Hia, Hap, and others, mediate direct adherence to nonciliated epithelial cells. Cleavage of IgA1, invasion into cells and the subepithelial space, and phase and antigenic variation facilitate evasion of local immune mechanisms. Binding and uptake of iron and heme allow organisms to persist on the respiratory mucosa despite the relative scarcity of these nutrients. In the setting of a viral infection, allergic disease, or exposure to cigarette smoke, bacteria spread from the nasopharynx to other sites within the respiratory tract and produce symptomatic disease.

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References

1. Pittman, M. (1931) Variation and type specificity in the bacterial species *Haemophilus influenzae. J. Exp. Med.* **53,** 471–493.
2. Howard, A. J., Dunkin, K. T., and Millar, G. W. (1988) Nasopharyngeal carriage and antibiotic resistance of *Haemophilus influenzae* in healthy children. *Epidemiol. Infect.* **100,** 193–203.
3. Kuklinska, D. and Kilian, M. (1984) Relative proportions of *Haemophilus* species in the throat of healthy children and adults. *Eur. J. Clin. Microbiol.* **3,** 249–252.
4. Faden, H., Duffy, L., William, A., Tonawanda/Williamsville Pediatrics, Krystofik, D.A., and Wolf, J. (1995) Epidemiology of nasopharyngeal colonization with nontypeable *Haemophilus influenzae* in the first 2 yr of life. *J. Infect. Dis.* **172,** 132–135.
5. Spinola, S. M., Peacock, J., Denny, F. W., Smith, D. L., and Cannon, J. G. (1986) Epidemiology of colonization by nontypeable *Haemophilus influenzae* in children: a longitudinal study. *J. Infect. Dis.* **154,** 100–109.
6. Trottier, S., Stenberg, K., and Svanborg-Eden, C. (1989) Turnover of nontypeable *Haemophilus influenzae* in the nasopharynges of healthy children. *J. Clin. Microbiol.* **27,** 2175–2179.

7. Giebink, G. S. (1989) The microbiology of otitis media. *Pediatr. Infect. Dis. J.* **8,** S18–S20.
8. Rayner, M. G., Zhang, Y., Ph.D., Gorry, M. C., Chen, Y., Post, J. C., and Ehrlich, G. D. (1998) Evidence of bacterial metabolic activity in culture-negative otitis medic with effusion. *JAMA* **279,** 296–299.
9. Evans, F. O., Jr., Sydnor, J. B., Moore, W. E. C., et al. (1975) Sinusitis of the maxillary antrum. *N. Engl. J. Med.* **293,** 735–739.
10. Wald, E. R., Milmoe, G. J., Bowen, A. D., Ledesma-Medina, J., Salamon, N., and Bluestone, C. D. (1981) Acute maxillary sinusitis in children. *N. Eng. J. Med.* **304,** 749–754.
11. Murphy, T. F. and Sethi, S. (1992) Bacterial infection in chronic obstructive pulmonary disease. *Am. Rev. Respir. Dis.* **146,** 1067–1083.
12. Musher, D. M., Kubitschek, K. R., Crennan, J., and Baughn, R. E. (1983) Pneumonia and acute febrile tracheobronchitis due to *Haemophilus influenzae*. *Ann. Intern. Med.* **99,** 444–450.
13. Weinberg, G. A., Ghafoor, A., Ishaq, Z., et al. (1989) Clonal analysis of *Haemophilus influenzae* isolated from children from Pakistan with lower respiratory tract infections. *J. Infect. Dis.* **160,** 634–643.
14. Shann, F. (1986) Etiology of severe pneumonia in children in developing countries. *Pediatr. Infect. Dis. J.* **5,** 247–252.
15. Krasan, G. P., Cutter, D., Block, S. L., and St. Geme, J. W., III. (1999) Adhesin expression in the matched nasopharyngeal and middle ear isolates of nontypeable *Haemophilus influenzae* from children with acute otitis media. *Infect. Immun.* **67,** 449–454.
16. Gilligan, P. H. (1991) Microbiology of airway disease in patients with cystic fibrosis. *Clin. Microbiol. Rev.* **4,** 35–51.
17. Kilbourn, J. P., Haas, H., Morris, J. F., and Samson, S. (1983) *Haemophilus influenzae* biotypes and chronic bronchitis. *Am. Rev. Respir. Dis.* **128,**1093–1094.
18. St. Geme, J. W., III (1993) Nontypeable *Haemophilus influenzae* disease: epidemiology, pathogenesis, and prospects for prevention. *Infect. Agents Dis.* **2,** 1–16.
19. Farley, M. M., Stephens, D. S., Brachman, P. S., Jr., Harvey, R. C., Smith, J. D., and Wenger, J. D. (1992) Invasive *Haemophilus influenzae* disease in adults. A prospective population-based surveillance. *Ann. Intern. Med.* **116,** 806–812.
20. Musher, D. M., Kubitschek, K. R., Crennan, J., and Baughn, R. E. (1983) *Haemophilus influenzae* in patients with dysgammaglobulinemia. *J. Clin. Microbiol.* **17,** 143–145.
21. Pichichero, M. E. and Pichichero, C. L. (1995) Persistent acute otitis media I. Causative pathogens. *Pediatr. Infect. Dis. J.* **14,**178–183.
22. Barry, A. L., Phaller, M. A., Fuchs, P. C., and Packer, R. R. (1994) In vitro activities of 12 orally administered agents against four species of bacterial respiratory pathogens from US medical centers in 1992 and 1993. *Antimicrob. Agents Chemother.* **38,** 2419–2425.
23. Teele, D. W., Klein, J. O., Chase, C., Menyuk, P., and Rosner, B. (1990) Otitis media in infancy and intellectual ability, school achievement, speech, and language at age 7 years. *J. Infect. Dis.* **162,** 685–694.

24. Stool, S. E. and Field, M. J. (1989) The impact of otitis meida. *Pediatr. Infect. Dis. J.* **8,** S11–S14.
25. Monto, A. S. (1989) Acute respiratory infection in children of developing countries: challenge of the 1990's. *Rev. Infect. Dis.* **11,** 498–505.
26. Fleischman, R. D., Adams, M. D., White, O., et al. (1995) Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. *Science* **269,** 496–512.
27. Loosmore, S. M., Yang, Y.-P., Oomen, R., Shortreed, J. M., Coleman, D. C., and Klein, M. H. (1998) The *Haemophilus inlfuenzae* HtrA protein is a protective antigen. *Infect. Immun.* **66,** 899–906.
28. Barenkamp, S. J., Munson, R. S., Jr., and Granoff, D. M. (1981) Subtyping isolates of *Haemophilus influenzae* type b by outer-membrane protein profiles. *J. Infect. Dis.* **143,** 668–676.
29. Loeb, M. R. and Smith, D. H. (1980) Outer membrane protein composition in disease isolates of *Haemophilus influenzae*: pathogenic and epidemiological implications. *Infect. Immun.* **30,** 709–717.
30. Murphy, T. F., Dudas, K. C., Mylotte, J. M., and Apicella, M. A. (1983) A subtyping system for nontypeable *Haemophilus influenzae* based on outer-membrane proteins. *J. Infect. Dis.* **147,** 838–846.
31. Coulton, J. W., Chin, A. C., and Vachon, V. (1992) Recombinant porin of *Haemophilus influenzae* type b. *J. Infect. Dis.* **165,**S188–S191.
32. Reidl, J. and Mekalanos, J. J. (1996) Lipoprotein *e*(P4) essential for hemin uptake by *Haemophilus influenzae*. *J. Exp. Med.* **183,** 621–629.
33. Green, B. A., Farley, J. E., Quinn-Dey, T., Deich, R. A., and Zlotnick, G. W. (1991) The *e* (P4) outer membrane protein of *Haemophilus influenzae*: biologic activity of anti-e serum and cloning and sequencing of the structural gene. *Infect. Immun.* **59,** 3191–3198.
34. Munson, R. S., Jr. and Granoff, D. M. (1985) Purification and partial characterization of outer membrane proteins P5 and P6 from *Haemophilus influenzae* type b. *Infect. Immun.* **49,** 544–5490.
35. Munson, R. S., Jr., Grass, S., and West, R. (1993) Molecular cloning and sequence of the gene for outer membrane protein P5 of *Haemophilus influenzae*. *Infect. Immun.* **61,** 4017–4020.
36. Munson, R. S., Jr., Grass, S., Einhorn, E., Bailey, C., and Newell, C. (1989) Comparative analysis of the structure of the outer membrane protein P1 genes from major clones of *Haemophilus influenzae* type b. *Infect. Immun*. **57,** 3300–3305.
37. Bell, J., Grass, S., Jeanteur, D., and Munson, R. S., Jr. (1994) Diversity of the P2 protein among nontypeable *Haemophilus influenzae* isolates. *Infect. Immun.* **62,** 2639–2643.
38. Sikkema, D. J. and Murphy, T. F. (1992) Molecular analysis of the P2 porin protein of nontypeable *Haemophilus influenzae. Infect. Immun.* **60,**5204–5211.
39. Webb, D.C. and Cripps, A.W. (1998) Secondary structure and molecular analysis of interstrain variability in the P5 outer-membrane protein of non-typeable *Haemophilus influenzae* isolated from diverse anatomical sites. *J. Med. Microbiol.* **47,** 1059–1067.

40. Nelson, M. B., Munson, R. S., Jr., Apicella, M. A., Sikkema, D. J., Molleston, J. P., and Murphy, T.F. (1991) Molecular conservation of the P6 outer membrane protein amoung strains of *Haemophilus influenzae*: analysis of antigenic determinants, gene sequences, and restriction fragment length polymorphisms. *Infect. Immun.* **59,** 2658–2663.
41. Deich, R. A., Anilionis, A., Fulginiti, J., et al. (1990) Antigenic conservation of the 15,000-dalton outer membrane lipoprotein PCP of *Haemophilus influenzae* and biologic activitiy of anti-PCP antisera. *Infect. Immun.* **58,** 3388–3393.
42. Thomas, W. R., Callow, M. G., Dilworth, R. J., and Audesho, A .A. (1990) Expression in *Escherichia coli* of a high-molecular-weight protective surface antigen found in non-typeable and type b *Haemophilus influenzae. Infect. Immun.* **58,** 1909–1913.
43. Kyd, J. M. and Cripps, A. W. (1998) Potential of a novel protein, OMP26, from nontypeable *Haemophilus influenzae* to enhance pulmonary clearance in a rat model. *Infect. Immun.* **66,** 2272–2278.
44. El Adhami, W., Kyd, J. M., Bastin, D. A., and Cripps, A. W. (1999) Characterization of the gene for OMP26 from nontypeable *Haemophilus influenzae* and immune repsonses to the recombinant protein. *Infect. Immun.* **67,** 1935–1942.
45a. Fan, X., Goldfine, H., Lysenko, E., and Weiser, J. N. (2001) The transfer of choline from the host to the bacterial cell surface requires *glpQ* in *Haemophilus influenzae*. *Mol. Microbiol.* **41,** 1029–1036.
45. Raun, M., Akkoyunlu, M., Grubb, A., and Forsgren, A. (1990) A novel bacterial surface protein with affinity for human IgD. *J. Immunol.* **145,** 3379–3384.
46. Gray-Owen, S. D., Loonsmore, S., and Schryvers, A. B. (1995) Identification and characterization of genes encoding the human transferrin-binding proteins from *Haemophilus influenzae. Infect. Immun.* **63,** 1201–1210.
47. Flesher, A. R. and Insel, R. A. (1978) Characterization of lipopolysaccharide of *Haemophilus influenzae. J. Inect. Dis.* **138,** 719–730.
48. Rahman, M. M., Gu, X.-X., Tsai, C.-M., Kolli, V. S. K., and Carlson, R. W. (1999) The structural heterogeneity of the lipooligosaccharide (LOS) expressed by pathogenic nontypeable *Haemophilus influenzae* strain NTHi 9274. *Glycobiol.* **9,** 1371–1380.
49. Porras, O., Caugant, D. A., Lagergard, T., and Svanborg-Eden, C. (1986) Application of multilocus enzyme electrophoresis to *Haemophilus influenzae. Infect. Immun.* **53,** 71–78.
50. Musser, J. M., Kroll, J. S., Granoff, D. M., et al. (1990) Global genetic structure and molecular epidemiology of encapsulated *Haemophilus influenzae. Rev. Infect. Dis.* **12,** 75–111.
51. Musser, J. M., Kroll, J. S., Moxon, E. R., and Selander, R. K. (1988) Clonal population structure of encapsulated *Haemophilus influenzae. Infect. Immun.* **56,** 1835–1845.
52. Musser, J. M., Barenkamp, S. J., Granoff, D. M., and Selander, R. K. (1986) Genetic relationships of serologically nontypeable and serotype b strains of *Haemophilus influenzae. Infect. Immun.* **52,** 183–191.
53. St. Geme, J. W., III, Takala, A., Esko, E., and Falkow, S. (1994) Evidence for capsule gene sequences among pharyngeal isolates of nontypeable *Haemophilus influenzae. J. Infect. Dis.* **169,** 337–342.

54. St. Geme, J. W., III, Kumar, V. V., Cutter, D., and Barenkamp, S. J. (1998) Prevalence and distribution of the *hmw* and *hia* genes and the HMW and Hia adhesins among genetically diverse strains of nontypeable *Haemophilus influenzae*. *Infect. Immun.* **66,** 364–368.
55. Read, R., Wilson, R., Rutman, A., Lund, V., Todd, H., Brian, A., Jeffery, P., and Cole, P. (1991) Interaction of nontypeable *Haemophilus influenzae* with human respiratory mucosa in vitro. *J. Infect. Dis.* **163,** 549–558.
56. Kubiet, M. and Ramphal, R. (1995) Adhesion of nontypeable *Haemophilus influenzae* from blood and sputum to human tracheobronchial mucins and lactoferrin. *Infect. Immun.* **63,** 899–902.
57. Reddy, M. S., Bernstein, J. M., Murphy, T. F., Faden, H. (1996) Binding between outer membrane proteins of nontypeable *Haemophilus influenzae* and human nasopharyngeal mucin. *Infect. Immun.* **64.** 1447–1449.
58. Miyamoto, N. and Bakaletz, L. O. (1996) Selective adherence of non-typeable *Haemophilus influenzae* (NTHi) to mucus or epithelial cells in the chinchilla Eustachian tube and middle ear. *Microb Pathog.* **21,** 343–356.
59. Denny, F. W. (1974) Effect of a toxin produced by *Haemophilus influenzae* on ciliated respiratory epithelium. *J. Infect. Dis.* **129,** 93–100.
60. Johnson, A. P. and Inzana, T. J. (1986) Loss of ciliary activity in organ cultures of rat trachea treated with lipo-oligosaccharide from *Haemophilus influenzae*. *J. Med. Microbiol.* **22,** 265–268.
61. Gu, X.-X., Tsai, C.-M., Apicella, M. A., and Lim, D. (1995) Quantitation and biological properties of released and cell-bound forms of lipooligosaccharides from nontypeable *Haemophilus influenzae*. *Infect. Immun.* **63,** 4115–4120.
62. Janson, H., Carlen, B., Cervin, A., et al. (1999) Effects on the ciliated epithelium of protein D-producing and -nonproducing nontypeable *Haemophilus influenzae* in nasopharyngeal tissue cultures. *J. Infect. Dis.* **180,** 737–746.
63. Munson, R. S., Jr., and Sasaki, K. (1993) Protein D, a putative immunoglobulin D-binding protein produced by *Haemophilus influenzae*, is a glycerophosphodiester phosphodiesterase. *J. Bacteriol.* **175,** 4569–4571.
64. Janson, H., Melhus, A., Hermansson, A., and Forsgren, A. (1994) Protein D, the glycerophosphophodiesterase from *Haemophilus influenzae* with affinity for human immunoglobulin D, influences virulence in a rat otitis model. *Infect. Immun.* **62,** 4848–4854.
65. Stull, T. L., Mendelman, P. M., Haas, J. E., Schoenborn, M. A., Mack, K. D., and Smith, A. L. (1984) Characterization of *Haemophilus influenzae* type b pili. *Infect. Immun.* **46,** 787–796.
66. St. Geme, J. W., III, Pinker, J. S., Krasan, G. P., et al. (1996) *Haemophilus influenzae* pili are composite structures assembled via the HifB chaperone. *Proc. Natl. Acad. Sci. USA* **93,** 11913–11918.
67. van Ham, S. M., van Alphen, L., Mooi, F. R., and van Putten, J. P. (1994) The fimbrial gene cluster of *Haemophilus influenzae* type b. *Mol. Microbiol.* **13,** 673–684.
68. McCrea, K. W., Watson, W. J., Gilsdorf, J. R., and Marrs, C. F. (1994) Idenfication of *hifD* and *hifE* in the pilus gene cluster of *Haemophilus influenzae* type b strain Eagan. *Infect. Immun.* **62,** 4922–4928.

69. Watson, W. J., Gilsdorf, J. R., Tucci, M. A., McCrea, K. W., Forney, L. J., and Marrs, C. F. (1994) Identification of a gene essential for piliation in *Haemophilus influenzae* type b with homology to the pilus assembly platform genes of Gram-negative bacteria. *Infect. Immun.* **62,** 468–475.
70. Geluk, F., Eijk, P. P., van Ham, S. M., Jansen, H. M., and van Alphen, J. (1998) The fimbria gene cluster of nontypeable *Haemophilus influenzae*. *Infect. Immun.* **66,** 406–417.
71. Mhlanga-Mutangadura, T., Morlin, G., Smith, A. L., Eisenstark, A., and Golomb, M. (1998) Evolution of the major pilus gene cluster of *Haemophilus influenzae*. *J. Bacteriol.* **180,** 4693–4703.
72. McCrea, K. W., Watson, W. J., Gilsdorf, J. R., and Marrs, C. F. (1997) Identification of two minor subunits in the pilus of *Haemophilus influenzae*. *J. Bacteriol.* **179,** 4227–4231.
73. Pichichero, M. E., Anderson, P., Loeb, M., and Smith, D. H. (1982) Do pili play a role in pathogenicity of *Haemophilus influenzae* type b? *Lancet.* **ii,** 960–962.
74. Guerina, N. G., Langermann, S., Clegg, H. W., Kessler, T. W., Goldmann, D. A., and Gilsdorf, J. R. (1982) Adherence of piliated *Haemophilus influenzae* type b to human oropharyngeal cells. *J. Infect. Dis.* **146,** 564.
75. van Alphen, L., Poole, J., and Overbeeks, M. (1986) The Anton blood group antigen is the erythrocyte receptor for *Haemophilus influenzae. FEMS Microbiol. Lett.* **37,** 69–71.
76. van Alphen, L., van den Berghe, N., Geelen-van den Broek, L. (1988) Interaction between *Haemophilus influenzae* human erythrocytes and oropharyngeal epithelial cells is mediated by a common fimbrial epitope. *Infect. Immun.* **56,** 1800–1856.
77. van Alphen, L., Geelen-van den Broek, L., Blaas, L., van Ham, M., and Dankert, J. (1991) Blocking of fimbria-mediated adherence of *Haemophilus influenzae* by sialyl gangliosides. *Infect. Immun.* **59,** 4473–4477.
78. St. Geme, J. W., III and Falkow, S. (1990) *Haemophilus influenzae* adheres to and enters cultured human epithelial cells. *Infect. Immun.* **58,** 4036–4044.
79. Barenkamp, S. J. and Leininger, E. (1992) Cloning, expression, and DNA sequence analysis of genes encoding nontypeable *Haemophilus influenzae* high-molecular-weight proteins related to filamentous hemagglutinin of *Bordatella pertussis. Infect. Immun.* **60,** 1302–1313.
80. Barenkamp, S. J. and Bodor, F. F. (1990) Development of serum bactericidal activity following nontypeable *Haemophilus influenzae* acute otitis media. Pediatr. *Infect. Dis. J.* **9,** 333–339.
81. Relman, D. A., Domenighini, M., Tuomanen, E., Rappuoli, R., and Falkow, S. (1989) Filamentous hemagglutinin of *Bordetella pertussis*: nucleotide sequence and crucial role in adherence. *Proc. Natl. Acad. Sci., USA* **86,** 2637–2641.
82. Kimura, A., Mountzouros, K. T., Relman, D. A., Falkow, S., and Cowell, J. L. (1990) *Bordetella pertussis* filamentous hemagglutinin: evaluation as a protective antigen and colonization factor in a mouse respiratory infection model. *Infect. Immun.* **58,** 7–16.

83. Barenkamp, S. J. and St. Geme, J. W., III. (1994) Genes encoding high-molecular-weight adhesion proteins of nontypeable *Haemophilus influenzae* are part of gene clusters. *Infect. Immun.* **62,** 3320–3328.
84. St. Geme, J.W., III and Grass, S. (1998) Secretion of the *Haemophilus influenzae* HMW1 and HMW2 adhesins involves a periplasmic intermediate and requires the HMWB and HMWC proteins. *Mol. Microbiol.* **27,** 617–630.
85. Grass, S. and St. Geme, J.W., III (2000) Maturation and secretion of the nontypeable *Haemophilus influenzae* HMW1 adhesin: roles of the N-terminal and C-terminal domains. *Mol. Microbiol.* **36,** 55–67.
86. Hultgren, S. J., Abraham, S., Caparon, M., Falk, P., St. Geme, J.W., III, and Normark, S. (1993) Pilus and nonpilus bacterial adhesins: assembly and function in cell recognition. *Cell* **73,** 887–901.
87. St. Geme, J. W., III (1996) The HMW1 adhesin of nontypeable *Haemophilus influenzae* recognizes sialylated glycoprotein receptors on cultured human epithelial cells. *Infect. Immun.* **62,** 3881–3889.
88. Barenkamp, S. J. and St. Geme, J.W., III. (1996) Identification of a second family of high-molecular-weight adhesion proteins expressed by non-typeable *Haemophilus influenzae. Mol. Microbiol.* **19,** 1215–1223.
89. St. Geme, J. W., III, Cutter, D., and Barenkamp, S. J. (1996) Characterization of the genetic locus encoding *Haemophilus influenzae* type b surface fibrils. *J. Bacteriol.* **178,** 6281–6287.
89a. St. Geme, J. W., III and Cutter, D. (2000) The *Haemophilus influenzae* Hia adhesin is an autotransporter protein that remains uncleaved at the C terminus and fully cell associated. *J. Bacteriol.* **182,** 6005–6013.
90. St. Geme, J. W., III, de la Morena, M .L., and Falkow, S. (1994) *Haemophilus influenzae* IgA protease-like protein promotes intimate interaction with human epithelial cells. *Mol. Microbiol.* **14,** 217–233.
91. Hendrixson, D. R., de la Morena, M. L., Stathopoulos, C., and St. Geme, J.W., III (1997) Structural determinates of processing and secretion of the *Haemophilus influenzae* Hap protein. *Mol. Microbiol.* **26,** 505–518.
92. Hendrixson, D. R. and St. Geme, J.W., III (1998) The *Haemophilus influenzae* Hap serine protease promotes adherence and aggregation, potentiated by a soluble host protein. *Mol. Cell.* **2,** 741–750.
93. Seemuller, U., Arnold, M., Fritz, H., et al. (1986) The acid stable proteinase inhibitor of human mucous secretions (HUSI-I, anitleukoprotease). *FEBS Lett.* **199,** 43–48.
94. Busse, J., E. Hartmann, E., and Lingwood, C. A. (1997) Receptor affinity purification of a lipid-binding adhesin from *Haemophilus influenzae*. *J. Infect. Dis.***175,** 77–83.
95. Hartmann, E. and Lingwood, C. (1997) Brief heat shock treatment induces a long-lasting alteration in the glycolipid receptor binding specificity and growth rate of *Haemophilus influenzae. Infect. Immun.* **65,** 1729–1733.

96. Fakih, M. G., Murphy, T. F., Pattoli, M. A., Berenson, C. S. (1997) Specific binding of *Haemophilus influenzae* to minor gangliosides of human respiratory epithelial cells. *Infect. Immun.* **65,** 1695–1700.
97. Prasadarao, N. V., Lysenko, E., Wass, C. A., Kim, K. S., and Weiser, J. N. (1999) Opacity-associated protein A contributes to the binding of *Haemophilus influenzae* to Chang epithelial cells. *Infect. Immun.* **67,** 4153–4160.
98. Weiser, J. N., Chong, S. T. H., Greenberg, D., and Fong, W. (1995) Identification and characterization of a cell envelope protein of *Haemophilus influenzae* contributing to phase variation in colony opacity and nasopharyngeal colonization. *Mol. Microbiol.* **17,** 555–564.
99. Brandtzaeg, P. (1992) Humoral immune response patterns of human mucosae: Induction and relation to bacterial tract infections. *J. Infect. Dis.* **165** S167–S176.
100. Mulks, M. H., Kornfeld, S. J., and Plaut, A. G. (1980) Specific proteolysis of human Iga by *Streptococcus pneumoniae* and *Haemophilus influenzae. J. Infect. Dis.* **141,** 450–456.
101. Hers, J. F. P. and Mulder, J. (1953) The mucosal epithelium of the respiratory tract in muco-purulent bronchitis caused by *Haemophilus influenzae. J. Pathol. Bacteriol.* **66,** 103–108.
102. Farley, M. M., Stephens, D. S., Mulks, M. H., et al. (1986) Pathogenesis of IgA1 protease-producing and -nonproducing *Haemophilus influenzae* in human naspharyngeal organ cultures. *J. Infect. Dis.* **154,** 752–759.
103. van Schilfgaarde, M., van Alphen, L., Eijk, P., Everts, V., and Dankert, J. (1995) Paracytosis of *Haemophilus influenzae* through cell layers of NCI-H292 lung epithelial cells. *Infect. Immun.* **63,** 4729–4737.
104. Forsgren, J., Samuelson, A. S., Ahlin, A., Jonasson, J., Rynnel-Dagöö, B., and Lindberg, A. (1994) *Haemophilus influenzae* resides and multiplies intracellularly in human adenoid tissue as demonstrated by in situ hybridization and bacterial viability assay. *Infect. Immun.* **62,** 673–679.
105. Ketterer, M R., Shao, J. Q., Hornick, D. B., Buscher, B., Bandi, V. K., and Apicella, M. A. (1999) Infection of primary human bronchial epithelial cells by *Haemophilus influenzae*: macropinocytosis as a mechanism of airway epithelial cell entry. *Infect. Immun.* **67,** 4161–4170.
105a. Swords, W. E., Buscher, B. A., Ver Steeg, K., et al. (2000) Non-typeable *Haemophilus influenzae* adhere to and invade humanbronchial epithial cells via an interaction of lipooligosaccharide with the PAF receptor. *Mol. Microbiol.* **37,** 13–27.
106. Henderson, I. R., Owen, P., and Nataro, J. P. (1999) Molecular switches-the ON and OFF of bacterial phase variation. *Mol. Microbiol.* **33,** 919–932.
107. Weiser, J. N., Maskell, D. J., Butler, P. D., Lindgerg, A. A., and Moxon, E.R. (1990) Characterization of repetitive sequences controlling phase variation of *Haemophilus influenzae* lipopolysaccharide. *J. Bacteriol* **172,** 3304–3309.
108. Gulig, P. A., Patrick, C. C., Hermanstofer, L., McCracken, G. H., and Hansen, E. J. (1987) Conservation of epitopes in the oligosaccharide portion of the

lipooligosac-charide of *Haemophilus influenzae* type b. *Infect. Immun.* **55,** 513–520.
109. Weiser, J. N. and Pan, N. (1998) Adaptation of *Haemophilus influenzae* to acquired and innate humoral immunity based on phase variation of lipopolysaccharide. *Mol. Microbiol.* **30,** 767–775.
110. Hood, D. W., Deadman, M. E., Allen, T., et al. (1996) Use of the complete genome sequence information of *Haemophilus influenzae* strain Rd to investigate lipopolysaccharide biosynthesis. *Mol. Microbiol.* **22,** 951–965.
111. Weiser, J. N., Shchepetov, M., and Chong, S. T. H. (1997) Decoration of lipopolysaccharide with phosphorylcholine: A phase-variable characteristic of *Haemophilus influenzae*. *Infect. Immun.* **65,** 943–950.
112. Weiser, J. N., Pan, N., McGowan, K. L., Musher, D., Martin, A., and Richards, J. (1998) Phosphorylcholine on the lipopolysaccharide of *Haemophilus influenzae* contributes to persistence in the respiratory tract and sensitivity to serum killing mediated by C-reactive protein. *J. Exp. Med.* **187,** 631–640.
113. Lysenko, E., Richards, J. C., Cox, A. D., et al. (2000) The position of phosphorylcholine on the lipopolysaccharide of *Haemophilus influenzae* affects binding and sensitivity to C-reactive protein-mediated killing. *Mol. Microbiol.* **35,** 234–245.
114. Tosi, M. F., Anderson, D. C., Barrish, J., Mason, E. O., Jr., and Kaplan, S. L. (1985) Effect of piliation on interactions of *Haemophilus influenzae* type b with human polymorphonuclear leukocytes. *Infect. Immun.* **47,** 780–785.
115. van Ham, S. M., van Alphen, L., Mooi, F. R., and van Putten, J. P. M. (1993) Phase variation of *H. influenzae* fimbriae: Transcriptional control of two divergent genes through a variable combined promoter region. *Cell.* **73,** 1187–1196.
116. Barenkamp, S. J. (1996) Immunization with high-molecular-weight adhesion proteins of nontypeable *Haemophilus influenzae* modifies experimental otitis media in chinchillas. *Infect. Immun.* **64,** 1246–1251.
117. Dawid, S., Barenkamp, S. J., and St. Geme, J.W., III (1999) Variation in expression of the *Haemophilus influenzae* HMW adhesins: A prokaryotic system reminiscent of eukaryotes. *Proc. Natl. Acad. Sci. USA* **96,** 1077–1082.
118. Ren, Z., Jin, H., Whitby, P. W., Morton, D. J., and Stull, T. L. (1999) Role of CCAA nucleotide repeats in regulation of hemoglogin and hemoglobin-haptoglobin binding protein genes of *Haemophilus influenzae*. *J. Bacteriol.* **181,** 5865–5870.
119. Murphy, T. F. and Bartos, L. C. (1988) Human bactericidal antibody response to outer membrane protein P2 of nontypeable *Haemophilus influenzae*. *Infect. Immun.* **56,** 2673–2679.
120. Forbes, K. J., Bruce, K. D., Ball, A., and Pennington, T. H. (1992) Variation in length and sequence of porin (*ompP2*) alleles of non-encapsulated *Haemophilus influenzae*. *Mol. Microbiol.* **6,** 2107–2112.
121. Duim, B., van Alphen, L., Eijk, P., Jansen, H.M., and Dankert, J. (1994) Antigenic drift of non-encapsulated *Haemophilus influenzae* major outer membrane protein P2 in patients with chronic bronchitis is caused by point mutations. *Mol. Microbiol.* **11,** 1181–1189.
122. Smith-Vaughan, H. C., Sriprakash, K. S., Mathews, J. D., and Kemp, D. J. (1997) Nonencapsulated *Haemophilus influenzae* in Aboriginal infants with otits media:

Prolonged carriage of P2 porin variants and evidence for horizontal P2 gene transfer. *Infect. Immun.* **65,** 1468–1474.
123. Duim, B., Vogel, L., Puijk, W., et al. (1996) Fine mapping of outer membrane protein P2 antigenic sites which vary during persistent infection by *Haemophilus influenzae*. *Infect. Immun.* **64,** 4673–4679.
124. Quigley-Reape, G., Delmastro, M. J., and Zlotnick, G. W. (1995) Biological properties of antisera of purified P5 of nontypeable *Haemophilus influenzae*. in *Abstracts 95th General Meeting of the American Society of Microbiology,* Washington, D.C. p. 239.
125. Groeneveld, K., van Alphen, L., Eijk, P. E., Jansen, H. M., and Zanen, H. C. (1988) Changes in outer membrane proteins of nontypeable *Haemophilus influenzae* in patients with chronic obstructive pulmonary disease. *J. Infect. Dis.* **158,** 360–365.
126. Duim, B., Bowler, L. D., Eijk, P. P., Jansen, H. M., Dankert, J., and van Alphen, L. (1997) Molecular variation in the major outer membrane protein P5 gene of nonencapsulated *Haemophilus influenzae* during chronic infections. *Infect. Immun.* **65,** 1351–1356.
127. Otto, B. R., Verweij-van, A. M. J. J., and MacLaren, D. M. (1992) Transferrins and heme-compounds as iron sources for pathogenic bacteria. *Crit. Rev. Microbiol.* **18,** 217–233.
128. Cross, J. H. (1984) The relationship of plasmid-mediated iron transport and bacterial virulence. *Annu. Rev. Microbiol.* **38,** 69–89.
129. Weinberg, E. D. (1978) Iron and infection. *Microbiol. Rev.* **42,** 45–66.
130. Schryvers, A. B. and Gray-Owen, S. (1992) Iron acquisition in *Haemophilus influenzae*: receptors for human transferrin. *J. Infect. Dis.* **165,** S103–S104.
131. Holbein, B. E. (1981) Enhancement of Neisseria meningitidis infection in mice by addition of iron bound to transferrin. *Infect. Immun.* **34,** 120–126.
132. Herrington, D. A. and Sparling, P. F. (1985) *Haemophilus influenzae* can use human transferrin as a sole source for required iron. *Infect. Immun.* **48,** 248-251.
133. Hanson, M.S., Slaughter, C., and Hansen, E.J. (1992) The *hbpA* gene of *Haemophilus influenzae* type b encodes a heme-binding lipoprotein conserved among heme-dependent Haemophilus species. *Infect. Immun.* **60,** 2257–2266.
134. Frangipane, M. E., Morton, D. J., Wooten, J. A., Pozsgay, J. M., and Stull, T. L. (1994) Binding of human hemoglogin by *Haemophilus influenzae*. *FEMS Microbiol. Lett.* **118,** 243–248.
135. Jin, H., Ren, Z., Pozsgay, J. M., Elkins, C., Whitby, P. W., Morton, D. J., Stull, and T. L. (1996) Cloning of a DNA fragment encoding a heme-repressible hemoglogin-binding outer membrane protein from *Haemophilus influenzae. Infect. Immun.* **64,** 3134–3141.
136. Maciver, I., Latimer, J. L., Liem, H. H., Müller-Eberhard, U., Hrkal, Z., and Hansen, E. J. (1996) Identification of an outer membrane protein involved in utilization of hemoglogin-haptoglobin complexes by nontypeable *Haemophilus influenzae*. *Infect. Immun.* **64,** 3703–3712.
137. Cope, L. D., Thomas, S. E., Latimer, J. L., Slaughter, C. A., Müller-Eberhard, U., and Hansen, E. J. (1994) The 100 kDa haem:haemopexin-binding protein of *Haemophilus influenzae*: Structure and localization. *Mol. Microbiol.* **13,** 863–873.

138. Schryvers, A. B. (1989) Identification of the transferrin- and lactoferrin-binding proteins in *Haemophilus influenzae*. *J. Med. Microbiol.* **29,** 121–130.
139. Hasan, A. A., Holland, J., Smith, A., and Williams, P. (1997) Elemental iron does repress transferrin, haemopexin and haemoglobin receptor expression in *Haemophilus influenzae*. *FEMS Microbiol. Lett.* **150,** 19–26.
140. Jarosik, G. P., Maciver, I., and Hansen, E. J. (1995) Utilization of transferrin-bound iron by *Haemophilus influenzae* requires an intact tonB gene. *Infect. Immun.* **63,** 710–713.
141. Kirby, S. D., Gray-Owen, S. D., and Schryvers, A. B. (1997) Characterization of a ferric-finding protein mutant in *Haemophilus influenzae*. *Mol. Microbiol.* **25,** 979–987.
142. Angerer, A., Gaisser, S., and Braun, V. (1990) Nucleotide sequences of the *sfuA, sfuB,* and *sfuC* genes of *Serratia marcescens* suggest a periplasmic-binding-protein-dependent iron transport mechanism. *J. Bacteriol.* **172,** 572–578.
143. Sanders, J. D., Cope, L. D., and Hansen, E. J. (1994) Identification of a locus involved in the utilization of iron by *Haemophilus influenzae*. *Infect. Immun.* **62,** 4515–4525.
144. Cope, L. D., Yogev, R., Müller-Eberhard, U., and Hansen, E. J. (1995) A gene cluster involved in the utilization of both free heme and heme:hemopexin by *Haemophilus influenzae* type b. *J. Bacteriol.* **177,** 2644–2653.
145. Jarosik, G. P., Sanders, J. D., Cope, L. D., Müller-Eberhard, U., and Hansen, E. J. (1994) A functional *tonB* gene is required for both utilization of heme and virulence expression by *Haemophilus influenzae* type b. *Infect. Immun.* **62,** 2470–2477.
146. Jin, H., Ren, Z., Whitby, P. W., Morton, D. J., and Stull, T. L. (1999) Characterization of *hgpA*, a gene encoding a haemoglobin/haemoglogin-haptoglobin-binding protein of *Haemophilus influenzae*. *Microbiol.* **145,** 905–914.
147. Ren, Z., Jin, H., Morton, D. J., and Stull, T. L. (1998) *hgpB*, a gene encoding a second *Haemophilus influenzae* hemoglogin- and hemoglobin-haptoglobin-bindign protein. *Infect. Immun.* **66,** 4733–4741.
148. Morton, D. J., Whitby, P. W., Jin, H., Ren, Z., and Stull, T. L. (1999) Effect of multiple mutations in the hemoglobin- and hemoglobin-haptoglobin-binding proteins, HgpA, HgpB, and HgpC, of *Haemophilus influenzae* type b. *Infect. Immun.* **67,** 2729–2739.

2

The Pathogenesis of Disease Due to Type b *Haemophilus influenzae*

Ruth Aubrey and Christoph Tang

1. Introduction

1.1. Haemophilus influenzae

Haemophilus influenzae is a Gram-negative bacterium that was first described by Pfeiffer in 1892 ***(1)***. This ubiquitous, human-specific organism was originally thought to be the etiologic agent of "influenza." However, *H. influenzae* was not consistently isolated from autopsied lungs of individuals who had died during the influenza pandemic in 1918. The confusion that existed about the relationship between the prevalence of *H. influenzae* and human disease was relieved when Pittman discovered that strains of this bacterium could be divided into two groups, encapsulated (typeable) and nonencapsulated (nontypeable) strains ***(2)***. Pittman further distinguished six encapsulated types of *H. influenzae*, designated a–f, by the serological specificities of their capsular polysaccharide ***(2)***.

Mainly type b (Hib) strains cause serious invasive diseases including meningitis and septicemia, as well as epiglottitis, cellulitis, septic arthritis, pneumonia and empyema ***(3,4)***. Nonencapsulated strains cause otitis media, sinusitis, conjunctivitis, and acute lower respiratory tract infections, the latter causing many millions of deaths mainly in developing countries. Infants between the ages of 12 and 36 mo are most at risk of disease caused by *H. influenzae*. During this time, infants are no longer protected by transplacentally acquired maternal antibodies and have not developed their own serum antibodies ***(5)***.

Interest in the pathogenesis of *H. influenzae* infection waned with the advent of antimicrobial chemotherapy. Whereas the mortality of meningitis caused by *H. influenzae* in North America was almost 100% in 1930 ***(6)***, with the advent of antibiotics the mortality fell to 5–10% by the mid-1960s ***(4,7)***. However, at

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least 30% of survivors were left with serious defects such as deafness, blindness, seizures, and mental retardation ***(8–11)***.

During the 1970s, antibiotic resistant strains of Hib arose and were propagated ***(12)***. By 1991 it was estimated that 11% of isolates of *H. influenzae* type b had acquired resistance to ampicillin, the most common antibiotic used at the time to treat infection by this organism ***(13)***. Jenner and colleagues reported the emergence of chloramphenicol-resistant strains of *H. influenzae* in 1990 ***(14)***.

H. influenzae has gained prominence not only as an important pathogen but also as the subject of fundamental genetic and molecular research. In the early 1950s, *H. influenzae* was found to be the second example (after the pneumococcus) of a naturally transformable organism. Then, in the late 1960s, Hamilton Smith's research on homologous recombination in *H. influenzae* led directly to the discovery of type II restriction endonucleases, which underlie much of recombinant DNA technology. Molecular and immunological research focusing on the polysaccharide capsule led to the development and commercial release of the first polysaccharide-conjugate vaccine in 1989. And finally, in 1995, *H. influenzae* became the first free-living bacterium to have its genome completely sequenced ***(15)***. This remarkable feat marked the beginning of widespread efforts to determine the whole genome sequences of many other bacteria, and initiated the application of genomics and bioinformatics in microbiological research.

1.2. Microbiology

Haemophilus belongs to the family Pasteurellaceae, which contains two other genera, *Actinobacillus* and *Pasteurella*. Bacteria belonging to this family are small (1×0.3 μm), non-spore-forming coccobacilli that have fastidious growth requirements, often needing supplemented media for isolation. The name of the genus, *Haemophilus* (meaning blood-loving) refers to the specific dependence of this organism on heme-related molecules for growth under aerobic conditions. The morphology of *H. influenzae* in clinical specimens is variable, ranging from coccobacilli to long filaments ***(16)***.

1.3. Classification of Typeable H. influenzae Strains

To understand the epidemiology of disease caused by typeable *H. influenzae*, investigators searched for marker systems to discriminate between strains. In the early 1980s, clinical Hib isolates were classified on the basis of variation in the electrophoretic mobility patterns of the major outer-membrane proteins (OMPs) ***(17–19)*** and lipopolysaccharide (LPS) ***(20,21)***. Hib strains were later assigned to one of four groups on the basis their reactivity with LPS-specific monoclonal antibodies (mAbs).

Musser and colleagues endeavored to measure genetic diversity and the evolutionary relationship between *H. influenzae* strains ***(22,23)***. Isolates were classified by multilocus enzyme electrophoresis (MLEE), in which the polymorphisms in essential metabolic enzymes were used to estimate genetic divergence from a presumed common ancestor. Over time, genes encoding essential metabolic enzymes accumulate neutral mutations that are not subject to selection. One hundred and seventy-seven type b strains, mostly isolated from the blood or cerebrospinal fluid of North American children with invasive disease, were grouped into several genetically distinct clusters or electrophoretic types (ETs), each of which showed strong nonrandom association of alleles ***(22)***. Strains having identical or very similar ETs were isolated from geographically distinct regions over a period of up to 40 yr. It was concluded that the population of type b strains was relatively clonal and that those causing most of the invasive disease episodes were a restricted subset of the genotypes of the species as a whole. Indeed, most of the North American isolates belonged to two closely related ETs and were distinct from a collection of isolates obtained from several European countries ***(24)***. These findings suggested an epidemiological pattern in which 'successful' type b clones swept through a host population and became hyperendemic over a period of yr, analogous to changes in *Neisseria meningitidis* populations ***(25,26)***.

More recently, the employment of techniques such as multilocus sequence typing (MLST) (Fiel and Spratt, personal communication), ribotyping, and RNA sequencing (Goldstein, personal comunication) has revealed substantial diversity within typeable and nontypeable strains of *H. influenzae*, which has raised doubts regarding the clonality of the species as a whole.

2. Colonization and Invasion

The pathogenesis of *H. influenzae* has been investigated through case studies and by using animal and in vitro models of infection. The investigation of *H. influenzae* has been a paradigm for understanding the pathogenesis of bacterial meningitis in general. Several distinct stages have been recognized during *Haemophilus* invasion.

2.1. Colonization of the Upper Respiratory Tract

H. influenzae typically colonizes human respiratory mucosal surfaces and occasionally the female genital tract ***(27)***. Children are more likely to be colonized than adults ***(28)***. Approximately 5% of healthy individuals are colonized with strains of serotypes a–f ***(28)***. From the nasopharynx, organisms are transmitted from one individual to another by airborne droplets or by direct contact with secretions ***(29)***. The primary interaction between *Haemophilus* and humans was shown in human organ cultures to be mediated by the binding of the bacte-

rium to mucin ***(30,31)***. In in vitro culture systems such as oropharyngeal epithelial cells, pili were shown to mediate adherence and were therefore proposed to be involved in colonization ***(32,33)***. It was later reported that nonpiliated *H. influenzae* also demonstrated significant levels of attachment to mammalian cells in vitro mediated by outer-membrane proteins (OMPs) ***(34,35)***. It has been shown by transmission electron microscopy (TEM) that, in the human nasopharyngeal organ culture system, both piliated and nonpiliated Hib attach themselves selectively to nonciliated epithelial cells ***(34)***. It was recently reported that specific gangliosides (sialylated glycosphingolipids) of human respiratory epithelial cells and of human macrophages serve as host receptors for *H. influenzae* ***(36)***.

It is speculated that during long-term carriage of *H. influenzae* the organism resides intracellularly. First, it is known that colonization is difficult to eradicate with antimicrobials ***(37)*** unless agents are used that are active within human cells, such as rifampicin and quinolones. Also, in vitro studies have shown that *H. influenzae* can be taken up by, and survive within, human epithelial cells ***(35)*** and macrophages ***(38,39)***.

2.2. Invasion of the Epithelium

In in vitro culture systems, strains of *H. influenzae* have been shown to exhibit a strong tropism for mucus, cause the breakdown of tight junctions of epithelial cells, and cause the sloughing off of ciliated cells and ciliostasis ***(29,40–42)***. Furthermore, this damage to epithelial cells led to exposure of the nonluminal cell surfaces underlying basal cells and the basement membrane ***(29)***. The strains demonstrated a much greater association with these surfaces than with intact epithelia ***(29)***. Despite the preferred intercellular route of entry into and through the mucosa, intracellular invasion has been observed ***(35,43)***.

2.3. Invasion of the Blood Stream

During the mid to late 1970s it was not known whether the transmission of *H. influenzae* to the central nervous system (CNS) occurred by spread from the nasopharynx along the olfactory nerve fibers or by the hematogenous route. To investigate this, isogenic strains of *H. influenzae* differing in antibiotic resistance were intranasally inoculated into rats. One strain was identified in the blood and cerebral spinal fluid (CSF) ***(44)*** demonstrating that the hematogenous route was taken by this organism. Furthermore, the occurrence of meningitis after an intranasal inoculation of *H. influenzae* in rats was shown to be directly related to the intensity of bacteremia ***(45)***.

Detailed investigations of the direct interactions between *H. influenzae* and endothelial cells were undertaken using the human umbilical vein endothelial cells (HUVECs) model ***(46)***. Using TEM, phagocytic ingestion of the organism by these cells could be visualized. It was shown that, upon internalization, the

bacteria remained viable within endothelial cell vacuoles and were then translocated within the vacuoles across the cell, emerging at the opposite surface.

As originally reported by Rubin and Moxon ***(47)***, it is now accepted that the passage of *H. influenzae* from the subepithelial tissue to the bloodstream occurs through direct invasion of capillaries supplying the epithelial tissue.

2.4. Survival in the Bloodstream

Innate and adaptive humoral immune responses elicited by the host renders the bloodstream a hostile environment in which *H. influenzae* must survive and replicate to cause prolonged infection. It has been shown that more than 90% of a population of *H. influenzae* cells inoculated intravascularly (iv) into the adult rat are cleared within a few minutes ***(48)***. Interestingly, it was shown that the remaining subpopulation that evaded clearance replicated sufficiently to invade the meninges of the rat and cause meningitis ***(48)***. Factors that aid the survival of subpopulations or variants of *H. influenzae* in the bloodstream are discussed later in this chapter. The clearance of encapsulated *H. influenzae* from blood involves deposition of C3 on the bacterium, and is independent of the later complement components, C5–C9. Bacteria are then removed from the circulation following phagocytosis by tissue macrophages. The type b capsule inhibits the initial binding of C3, thereby reducing uptake by phagocytic cells ***(49)***. The type b capsule appears to be more efficient than other capsular types at preventing bacterial clearance. This may largely account for the preponderance of type b strains among invasive disease isolates.

2.5. Invasion of the CNS

Specific interactions are believed to occur between *H. influenzae* and the blood-brain barrier (BBB). The BBB is a single layer of unique endothelial cells, which is largely responsible for the maintenance of biochemical homeostasis within the CNS ***(50)***. These endothelial cells exhibit continuous tight junctions and a marked paucity of pinocytosis ***(51)***. Interactions between *H. influenzae* and the BBB have been investigated in in vivo models such as the infant rat model of meningitis ***(52)***, and in in vitro BBB models such as the bovine microvascular endothelial cell model ***(51)***. The models show that *H. influenzae* cells adhere to the BBB and then translocate across or between the cellular tight junctions, to enter the CSF. In the rat, live or heat-killed Hib increase pinocytosis and disrupt interendothelial tight junctions ***(52,53)***. Damage to the BBB enhances *H. influenzae* entry into the CSF. Once in the CSF, the population of *H. influenzae* may continue to expand and infect the meninges of the brain, causing meningitis.

3. Virulence Determinants of *H. influenzae*

Each step in the pathogenesis of *H. influenzae* infection appears to depend on the expression of a combination of several specific virulence determinants.

These determinants include outer-membrane proteins (OMPs), pili, IgAl proteases, lipopolysaccharide (LPS), and capsule. It has been shown that many of these virulence determinants elicit a protective immune response to *H. influenzae* in rats and humans ***(17,54,55)*** and are relatively conserved between strains of this organism ***(56–58)***. They have therefore been investigated as vaccine candidates against diseases caused by *H. influenzae* ***(54,57)***.

3.1. OMPs

Strains of *H. influenzae* express between 10 and 20 OMPs ***(59)*** ranging from 16- to 98-kDa in size. The combination of expressed proteins varies between strains ***(60)***. The most abundant OMP of Hib is the porin protein, P2 ***(60,61)***. Cope and colleagues reported that P2 contributes to virulence of Hib as an isogenic mutant of a virulent Hib strain, incapable of synthesizing P2, was avirulent in the infant rat ***(62)***. This protein interacts with LPS ***(63)***. The P5 protein is thought to be involved in the invasion of the mucosal epithelium as inactivation of the P5 gene results in a decrease in bacteremia following intranasal inoculation of infant rats ***(64)***. The P6 and 98K OMPs have also been shown to be immunogenic in humans ***(54,65)*** and protective, as anti-P6 and anti-98K antibodies protect infant rats from *H. influenzae* disease ***(54,55)***.

3.2. Pili

H. influenzae pili are 4.7–18.0 nm in diameter, between 209 and 453 nm in length, and possess a hollow core ***(66,67)***. They appear as relatively thick flexible rods with a short, thinner fibrillum at the top, similar to *Eschericia coli* Pap pili. The pilus rods are composed of polymerized pilin proteins, which show slight interstrain variability in migration by polyacrylamide gel electrophoresis (PAGE) ***(67)***. At least 16 pili are expressed per bacterial cell ***(66)*** and are distributed in a peritrichous manner ***(68)***.

Pili appear to mediate bacterial adherence to mucosal surfaces and hence facilitate respiratory tract colonization. Anderson and co-workers observed that a piliated *H. influenzae* strain showed stronger adherence to buccal epithelial cells and was more effective in colonizing rats following intranasal inoculation than its nonpiliated variant ***(69)***. It was later shown that infant rats inoculated by the ip or iv route with piliated *H. influenzae* type b had decreased levels of bacteremia compared to rats inoculated with nonpiliated variants ***(70)***. Furthermore, piliated *H. influenzae* were shown to stimulate enhanced opsonization-dependent phagocytosis by neutrophils ***(71)***. It appears, then, that the expression of pili is important during the colonization stage of pathogenesis but detrimental at systemic stages.

The expression of pili in *H. influenzae*, like that in other organisms, is phase-variable ***(72)***. A single copy of the pilin locus, comprising *hifA* to *hifE* is present

in most *Haemophilus* strains studied, with the notable exception of Rd, the strain whose entire genome was sequenced and which is 1.8 Mb in size ***(15)***, 0.3 Mb smaller than the prototypic pathogenic strain, Eagan. The promoter regions of the two divergently transcribed pilus genes, *hifA* (encoding the major pilus subunit) and *hifB* (encoding the pilus chaperone) overlap. Tandem repeats of the dinucleotide 5'-TA-3' are located between the −10 and −35 regions of both promoters ***(73)***. The spacing between the –10 and –35 sequences is altered by changes in the number of repeats within this promoter region. This is thought to alter the efficiency of RNA polymerase binding and hence gene expression ***(74)***. A subset of piliated colonizing *H. influenzae* switch to the nonpiliated form by an alteration in TA repeat number between generations, and nonpiliated variants are presumed to have selective advantage for invasive disease ***(34)***.

3.3. Immunoglobulin Al Proteases

Immunoglobulin A1 (IgAl) protease is constitutively secreted by a number of mucosal pathogens, including *Neisseria meningitidis, N. gonorrhoeae*, and *Streptococcus pneumoniae* as well as *H. influenzae* ***(75,76)***. The *Haemophilus* IgAl proteases are serine type enzymes that are synthesized as 169-kDa proteins ***(75,77)***. The activity of IgAl proteases in cleaving and inactivating human IgAl, the predominant secretory antibody in the upper respiratory tract ***(78)*** is believed to facilitate colonization ***(79)***. IgAl proteases specifically cleave one out of four peptide bonds located within a limited amino acid sequence of the hinge region of the α chain of human IgAl, including the secretory form (S-IgAl). Thereby the antibody molecules are left as intact Fab (monomeric) fragments devoid of the Fc portion, which is particularly responsible for the protective properties of this immune factor ***(80)***. Upon cleavage, the 50-kDa C-terminal domain of the IgAl protease remains in the bacterial outer membrane, while the proteolytically active N terminus is secreted. For *H. influenzae*, at least two classes of IgAl proteases have been described based on cleavage at either a prolyl-seryl (designated type 1) or four amino acids away at a prolyl-threoryl bond (type 2) ***(81–83)***. In addition to differences in cleavage specificity, these proteins display considerable polymorphism and antigenic variation, so that more than 30 types have been described based on serological responses in humans ***(84,85)***.

3.4. Lipopolysaccharide

Lipopolysaccharide (LPS) is the major component of the outer membrane of Gram-negative bacteria. A hydrophobic lipid moiety, lipid A, constitutes about 60% (w/w) of the LPS of *H. influenzae* ,while the remainder of this molecule consists of hydrophilic polysaccharide ***(86)***. Lipid A is embedded in the outer membrane, while the polysaccharide portion extends outward from the

bacterial surface. As well as being an extremely important surface-exposed immunogen, LPS is essential for the integrity and functioning of the cell membrane. Unlike that of enteric bacteria, the LPS of *H. influenzae* lacks an O-antigen and thus consists of a simple set of monosaccharides ***(87)***.

3.4.1. Structure of LPS of H. influenzae

The lipid A of *H. influenzae* consists of a β-(1,6)-1inked D-glucosamine disaccharide substituted by phosphate groups at C-1 of the reducing, and C-4 of the non-reducing D-glucosamine. The diglucosamine is further substituted by four molecules of (R)-3-hydroxytetradecanoic acid linked by amide linkages at the 2 and 2' positions and ester linkages at the 3 and 3' positions. The two 3-hydroxytetradecanoic acid molecules on the nonreducing glucosamine carry a further two tetradecanoic acid molecules on their hydroxy groups, so that there are six fatty acid molecules in all ***(88)***. This pattern is very similar to that of *Escherichia coli*, which differs only in that the 2'-amide linked 3-hydroxytetradecanoic acid is substituted by dodecanoic acid ***(89,90)***. It appears that the structure of lipid A in different strains of *H. influenzae* is relatively conserved. Up to 50% of a large collection of Hib strains were shown to share at least one epitope in the lipid A region as determined by reactivity with a Hib lipid A-directed Mab ***(91)***. Mass spectrometric (MS) and nuclear magnetic resonance (NMR) analyses of the LPS of several strains of *H. influenzae* reveal that lipid A is linked via a single 2-keto-3-deoxyoctulosonic acid (KDO) to a conserved triheptose backbone ***(92–96)***. From this backbone there is interstrain variation in the presence, number, arrangement, and linkages of hexose sugars, phosphate groups, and sialic acid. The structure of LPS of two Hib strains, RM7004 (Richards, personal communication) and RM 153 ***(95)*** is shown in **Fig. 1.**

3.4.2. Involvement of LPS in Pathogenesis

Despite absence of the O-antigen, the LPS of *H. influenzae* plays an important role in pathogenesis. Isogenic strains with mutations in single LPS genes and therefore differing in LPS structure were constructed, and survival was compared in the infant rat ***(97–99)***. Likewise, natural LPS variants, isolated by LPS-specific Mabs ***(100,101)***, as well as chemically ***(62)*** or genetically mutagenized strains ***(102)*** with altered LPS, were compared with parental strains for survival. The findings confirmed that LPS indeed contributes to the virulence of *H. influenzae*.

The role of LPS in causing the symptoms of meningitis has also been shown by inoculating both rabbits and rats with Hib LPS alone rather than the whole organism ***(53,103)***. Following intracisternal inoculation of LPS, a dose- and time-dependent increase in BBB permeability was observed and a correlation established between CSF pleocytosis and BBB permeability. There was a close

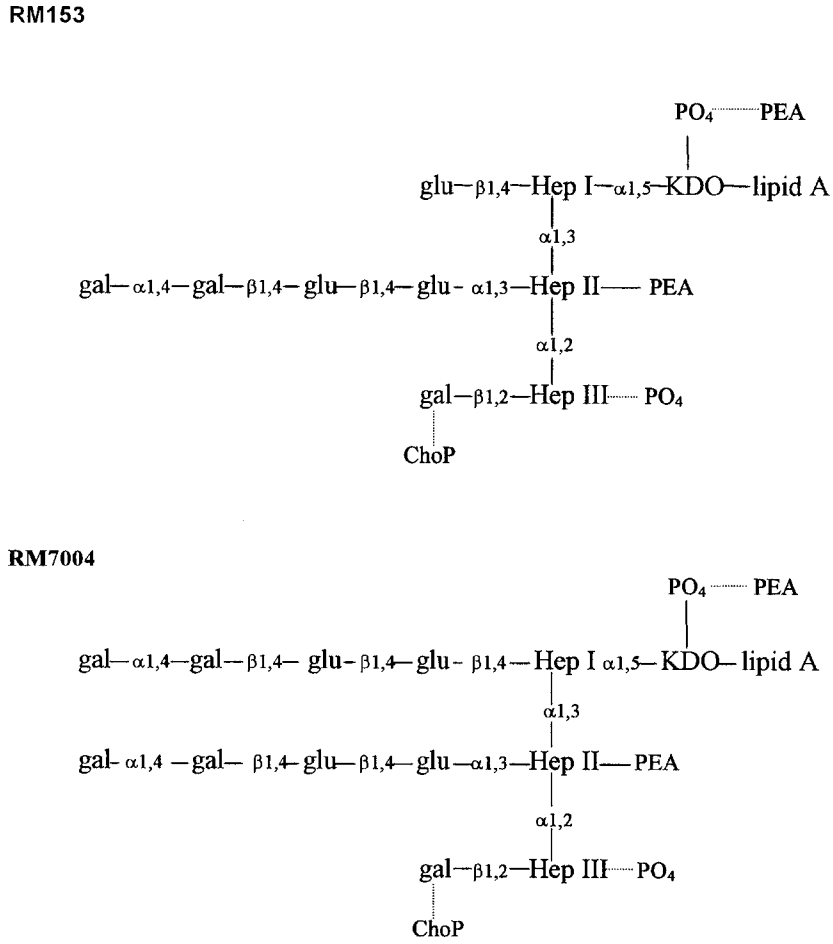

Fig. 1. Schematic representation of the structure of LPS of RM153 ***(95)***, RM7004. Heptose sugars are represented by "Hep," glucose and galactose sugars are denoted by "glu" and "gal," respectively. Phosphate groups are shown in red, and a hashed line indicates those that are variably present. Phosphorylcholine and phosphoethanolamine are abbreviated to ChoP and PEA, respectively (Richards, personal communication).

relationship between numbers of white blood cells in the CSF and the degree of BBB permeability. The toxicity of LPS was shown to be attributed mainly to the activity of the lipid A portion, since the deleterious effect of LPS was significantly inhibited by prior treatment with polymyxin B (which binds to the lipid A domain) or by deacylating LPS (to remove nonhydroxylated fatty acyl chains from the lipid A). Since lipid A is embedded in the outer membrane of the organism, its endotoxic activity is exerted mostly when the organism is

lysed. In order for LPS to strongly activate host cells, LPS must bind a plasma LPS-binding protein, LBP ***(104)***. The LPS–LBP complex binds membrane CD14 (mCD14), present mainly on myeloid cells ***(105)*** and soluble CD14 (sCD14), a secreted form that circulates in the plasma ***(106,107)***. CD14 then interacts with Toll-like receptor proteins (TLR) ***(108–110)*** culminating in the transduction of a cytoplasmic signal ***(110,111)***. Through the activation of a complex cascade of events, the production of cytokines is triggered. The activity of cytokines and complement components may lead to septic shock.

Specific components of the polysaccharide portion of LPS have been shown to be important at different stages of pathogenesis. For example, the expression of phosphorylcholine has been shown to be important for colonization of the nasopharynx ***(101)***, while expression of a specific digalactoside (α-D-galactose[1–4]-β-D-galactose) ***(101,112–114)*** and sialic acid ***(43)*** are important during systemic stages of infection, permitting resistance against immune-mediated clearance.

Several loci involved in the assembly of the polysaccharide domain of LPS were identified by classical genetics. Four of these loci were shown to contain tetranucleotide repeats near the 5' end. It is thought that during DNA replication homologous strands mis-pair in the repeat region, culminating in the loss or gain of one or more repeats. This places the downstream coding sequence in or out of frame with the upstream initiation codon. Thus a translational switch mechanism is generated. The availability of the whole genome sequence of *H. influenzae* (strain Rd) permitted the identification of another tetranucleotide repeat-containing LPS gene along with up to 30 more non-repeat-containing candidate LPS genes. Importantly, the high-frequency on–off switching of phase-variable loci permits the generation of a plethora of LIPS glycoforms. The most appropriate form may then be selected at each stage of pathogenesis.

3.5. Capsule

Polysaccharide capsules are considered important determinants of pathogenicity in several species of bacteria ***(115)***. *H. influenzae* may express one of six antigenically and chemically distinct capsular polysaccharides, designated a–f ***(2)*** **(Fig. 2)**. Type a and b polysaccharides differ from types c, d, e, and f in that they contain the five-carbon sugar, ribitol ***(116)***. The type a capsule consists of a polymer of glucose-ribitol phosphate, while type b consists of poly-ribose ribitol phosphate (PRP). Types c and f contain 2-acetamido-2-deoxyhexose and are O-deacylated ***(117,118)***. Type d and e polysaccharides contain 2-aceta mide-2-deoxy-D-mannose uronic acid ***(119,120)***.

3.5.1. Contribution of Capsule to Pathogenesis

A strong correlation was established between expression of capsule by *H. influenzae* and invasive disease in humans ***(4)***. Interestingly, it was reported

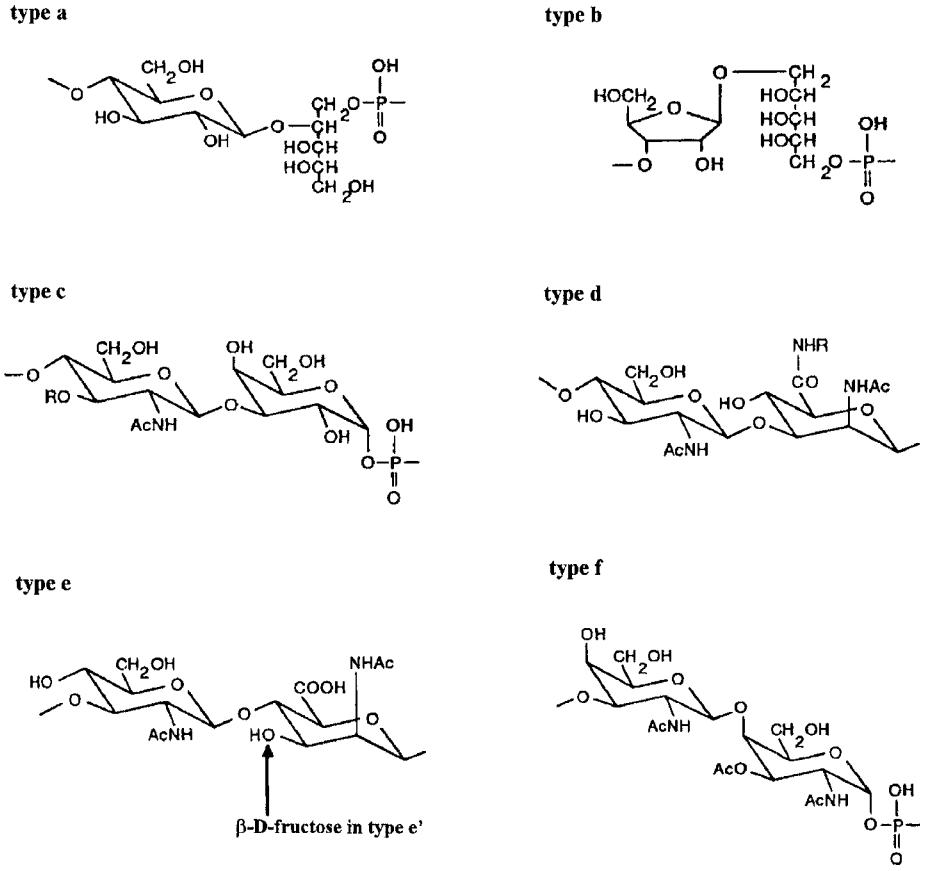

Fig. 2. Structure of the capsular polysaccharides of *H. influenzae* (serotypes a–f).

that whereas each serotype could successfully colonize the nasopharynx, type b strains accounted for more than 95% of systemic disease in humans ***(4,121)***. Similar findings were reported in the infant rat model of infection ***(48)***. After intraperitoneal (ip) inoculation, all encapsulated strains possessed the potential for systemic infection, but type b strains were found to be the most virulent. Noncapsulated strains were noninvasive. Furthermore, after intravenous (iv) inoculation, only type b strains caused persistent bacteremia. This investigation was extended by the construction of a series of capsular transformants representing all six serotypes of *H. influenzae* which, other than capsule, were identical with respect to OMP profiles and LPS ET ***(99)***. After intranasal inoculation, all strains colonized the nasopharynx, but bacteremia was generally detected only in serotype a and b strains. After ip administration, the type b strain was

found to be significantly more virulent than each of the other transformants as assessed by the magnitude of bacteremia 48 h after inoculation.

3.5.2. Genetics of Capsule Expression

A single clone of *H. influenzae* can synthesize only one capsular serotype, which does not show antigenic variation. However, the quantity of capsule expressed by an individual bacterium can vary. This has been shown in serotype b strains of phylogenetic lineage 1, which are the majority of strains that cause invasive infection ***(122,123)***. Capsule production depends on a cluster of genes in an 18-kb chromosomal locus termed *cap*. The *cap* locus may be divided into three regions. Region 1 contains the bex genes *(bexA-D)*, of which *bexA*, at least, is essential for the capsule export to the cell surface. Region 2 contains four genes involved in polysaccharide biosynthesis. This region is capsule-type specific. Region 3 contains two open reading frames (ORF)s that are thought to be involved in polysaccharide export. It is reported that in about 98% of type b strains there is a deletion of part of one copy of *bexA* in an otherwise duplicated cap locus, that is flanked by direct repeats of the insertion sequence IS1016 ***(124,125)***. The type b *cap* locus in lineage I strains exists mostly in duplicate form, one copy of which has a deletion in *bexA*. As a result of the duplication, recombinatorial loss of one copy of *cap* occurs, leaving the copy with the *bexA* deletion. Irreversible loss of capsule expression occurs ***(126)***. These "class 1" mutants are generated at a frequency of approx 20% during late exponential liquid culture growth. Secondary mutations arise that alleviate potentially lethal consequences of the buildup of PRP within the cytoplasm ***(126)***. The presence of the insertion element also facilitates amplification of the copy number of *cap* and up to five copies have been detected in clinical isolates ***(123)***. The quantity of expressed capsule is increased in a gene-dose manner, which may be crucial to serotype b pathogenesis. Organisms producing more capsule may have a selective advantage in the respiratory tract, for example, where the hydrophilic capsule may provide a physical barrier that protects them from desiccation and promotes resistance against nonspecific attack by neutrophils and macrophages ***(127)***. Organisms that lose the ability to express capsule may have a selective advantage in invasion of host cells. Several research groups have provided evidence that capsule-deficient mutants show enhanced adherence to and invasion of human epithelial cells compared with wild-type strains ***(128–130)***. This has also been demonstrated for endothelial cells. Virji and co-workers investigated interactions of capsulate (b+) and capsule deficient (b−) Hib with HUVECs ***(46)***. The presence of type b capsule resulted in decreased bacterial association with endothelial cells. More b− bacteria were internalized by the HUVIECs compared with b+.

3.5.3. Development of Capsule-Based Vaccines

Antibodies to the capsular polysaccharide of Hib mediate protective immunity. Adults make highly effective thymic (T)-independent responses to these polysaccharide antigens, including IgM and IgG2 responses, but T-cell-independent responses are weak in the immature immune system of infants until after 118–24 mo old ***(131–134)***. Young infants are therefore more susceptible to infection. To achieve immunoprophylactic control of Hib meningitis caused by Hib, attempts to generate a more effective vaccine were developed that stimulated the production of T-dependent antibodies. As T-cell help is recruited in response to protein antigens, chemically linking the capsular polysaccharide to a protein carrier induces T-cell responses such that B cells proliferate and produce antibodies to the polysaccharide. During the 1980s, several conjugate vaccines were developed for use against disease caused by Hib. These consisted of PRP conjugated to T-dependent immunogens such as diphtheria toxin (PRP-D) ***(135,136)***, an OMP of *N. meningitidis* (PRP-OMP) ***(137)*** and tetanus toxoid (PRP-T) ***(138)***. The immunogenicity of these vaccines was modified by adjusting the structure and length of the polysaccharide, the ratio of protein to carbohydrate, as well as the method of coupling the protein with the polysaccharide. Each of these vaccines was found to induce an enhanced antibody response to PRP and a vigorous booster response when administered to young children ***(139,140)***.

Since 1989 in North America and 1992 in the United Kingdom, the Hib vaccine has been administered to infants, typically PRP-T at 2, 3, and 4 mo of age in the UK. Booy and colleagues studied the efficacy of this vaccine over a 3-yr period (1992–1995) in the UK and reported it to be overall greater than 98% effective in preventing *H. influenzae* type b disease ***(141)***.

Despite the success in preventing Hib diseases, there is still a need to develop vaccines that are effective against nontypeable *H. influenzae*. LPS and OMPs have been investigated in this capacity ***(142,143)***.

References

1 . Pfeiffer, R. (1892) Vorlaufige meitteilungen uber die erreger der *influenza*. *Dtsch. Med. Wochenschr.* **18,** 28–34.
2. Pittman, M. (1931) Variation and type specificity in the bacterial species of *Haemophilus influenzae*. *J. Exp. Med.* **53,** 471–492.
3. Alexander, H. E. (1965) The *Haemophilus* group, in *Bacterial and Mycotic Infections of Man* (Dabos, R. J. and Hirsch, J. G., eds.), Pitman Medical Publishing Co., Ltd., London.
4. Turk, D. C. and May, J. R. (1967) *Haemophilus influenzae, Its Clinical Importance.* The English Universities Press, Lts., London.
5. Fothergill, L. D. and Wright, J. (1933) Influenzal meningitis. The relation of age incidence to the bactericidal power of blood against the casual organism. *J. Immunol.* **24,** 273–284.

6. Taylor, H. G., Mills, E. L., Ciampi, A., et al. (1990) The sequelae of *Haemophilus influenzae* meningitis in school-age children. *N. Engl. J. Med.* **323,** 1657–1663.
7. Haggerty, R. J. and Ziai, M. (1964) Bacterial meningitis in infancy and childhood, in *Advances in Pediatrics* (Levine S. Z., ed.), Year Book Medical Publishers, Chicago.
8. Crook, W. G., Clanton, B. R., and Hodes, H. L. (1949). Haemphilus influenzae meningitis: observations on the treatment of 110 cases. *Pediatrics* **4,** 643–659.
9. Katz, S. L. (1966) The prevention of mental retardation through control of infectious diseases. *U. S. Public Health Service Publication 1692,* 318.
10. Sell, S. H. (1970) The clinical importance of *Haemophilus influenzae* infections in children. *Pediatr. Clin. N. Am.* **17,** 415–426.
11. Sproles, E. T., Azerrad, J., Williamson, C., and Merrill, R. E. (1969) Meningitis due to *Haemophilus influenzae*: long-term sequelae. *J. Pediatr.* **75,** 782–788.
12. American Academy of Pediatrics, Committee on Infectious Diseases. (1975) *Ampicillin-resistant strains of* Haemophilus influenzae type B. *Pediatrics,* **55,** 145–146.
13. Booy, R. and Moxon, E. R. (1991) Immunisation of infants against *Haemophilus influenzae* type b in the UK. *Arch Dis Child.* **66,** 1251–1254.
14. Jenner, B. M., Williamson, G., and Luppino, M. (1990) Fatal meningitis caused by chloramphenicol-resistant *Haemophilus influenzae*. *Med. J. Austral.* **152,** 335–336.
15. Fleischmann, R. D., Adams, M. D., White, O., et al. (1995) Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. *Science.* **269,** 496–512.
16. Davis, B. D., Dulbecco, R., Eisen, H. N., Ginsberg, H. S., and Wood, W. B., Jr. (1973) *The Haemophilus-Bordetella group. Microbiology,* Harper & Row Publishers, Inc., New York, pp. 792–800.
17. Barenkamp, S. J, Munson, R. S., Jr., and Granoff, D. M. (1981) Subtyping isolates of *Haemophilus influenzae* type b by outer-membrane protein profiles. *J. Infect Dis.* **143,** 668–676.
18. Loeb, M. R. and Smith, D. H. (1980) Outer membrane protein composition in disease isolates of *Haemophilus influenzae*: pathogenic and epidemiological implications. *Infect. Immun.* **30,** 709–717.
19. van Alphen, L., Riemens, T., Poolman, J., and Zanen, H. C. (1983) Characteristics of major outer membrane proteins of *Haemophilus influenzae. J. Bacteriol.* **155,** 878–885.
20. Inzana, T. J. (1983) Electrophoretic heterogeneity and interstrain variation of the lipopolysaccharide of *Haemophilus influenzae*. *J. Infect. Dis.* **148,** 492–499.
21. Inzana, T. J. and Pichichero, M. E. (1984) Lipopolysaccharide subtypes of *Haemophilus influenzae* type b from an outbreak of invasive disease. *J. Clin. Microbiol.* **20,** 145–150.
22. Musser, J. M., Granoff, D. M., Pattison, P. E., and Selander, R. K. (1985) A population genetic framework for the study of invasive diseases caused by serotype b strains of *Haemophilus influenzae*. *Proc. Nat. Acad. Sci. USA* **82,** 5078–5082.
23. Musser, J. M., Kroll, J. S., Granoff, D. M., et al. (1990) Global genetic structure and molecular epidemiology of encapsulated *Haemophilus influenzae*. *Rev. Infect. Dis.* **12,** 75–111.

24. Urwin, G., Musser, J. M. and Yuan, M. F. (1995) Clonal analysis of *Haemophilus influenzae* type b isolates in the United Kingdom. *J. Med. Microbiol.* **43,** 45–49.
25. Caugant, D. A. (1998) Population genetics and molecular epidemiology of *Neisseria meningitidis*. *APMIS* **106,** 505–525.
26. Jones, G. R., Christodoulides, M., Brooks, J. L., Miller, A. R., Cartwright, K. A., and Heckels, J. E. (1998) Dynamics of carriage of *Neisseria meningitidis* in a group of military recruits: subtype stability and specificity of the immune response following colonisation. *J. Infect. Dis.* **178,** 451–459.
27. Gilsdorf, J. R., McCrea, K. W., and Marrs, C. F. (1997) Role of pili in *Haemophilus influenzae* adherence and colonization. *Infect. Immun.* **65,** 2997–3002.
28. Turk, D. C. (1984) The pathogenicity of *Haemophilus influenzae*. *J. Med. Microbiol.* **18,** 1–16.
29. Farley, M. M. and Stephens, D. S. (1992) Pathogenic events during *Haemophilus influenzae* type b infection of human nasopharyngeal mucosa. *J. Infect. Dis.* **165 (Suppl 1),** S109–110.
30. Reddy, M. S., Bernstein, J. M., Murphy, T. F., and Faden, H. S. (1996) Binding between outer membrane proteins of nontypeable *Haemophilus influenzae* and human nasopharyngeal mucin. *Infect. Immun.* **64,** 1477–1479.
31. Davies, J., Carlstedt, I., Nilsson, A. K., Hakansson, A., et al. (1995) Binding of *Haemophilus influenzae* to purified mucins from the human respiratory tract. *Infect. Immun.* **63,** 2485–2492.
32. Guerina, N. G., Langermann, S., Clegg, H. W., Kessler, T. W., Goldman, D. A., and Gilsdorf, J. R. (1982) Adherence of piliated *Haemophilus influenzae* type b to human oropharyngeal cells. *J. Infect. Dis.* **146,** 564.
33. Pichichero, M. E., Loeb, M., Anderson, P., and Smith, D. H. (1982) Do pili play a role in pathogenicity of *Haemophilus influenzae* type B? *Lancet* **2,** 960–962.
34. Farley, M. M., Stephens, D. S., Kaplan, S. L., and Mason, E. O., Jr. (1990) Pilus- and non-pilus-mediated interactions of *Haemophilus influenzae* type b with human erythrocytes and human nasopharyngeal mucosa. *J. Infect. Dis.* **161,** 274–280.
35. St. Geme, J. W., III and Falkow, S. (1990) *Haemophilus influenzae* adheres to and enters cultured human epithelial cells. *Infect. Immun.* **58,** 4036–4044.
36. Fakih, M. G., Murphy, T. F, Pattoli, M. A., and Berenson, C. S. (1997) Specific binding of *Haemophilus influenzae* to minor gangliosides of human respiratory epithelial cells. *Infect. Immun.* **65,** 1695–1700.
37. Sundberg, L., Cederberg, A., Eden, T., and Emston, S. (1984) The effect of erythromycin on the nasopharyngeal pathogens in children with secretory otitis media. *Acta. Otolaryngol.* **97,** 379–383.
38. Williams, A. E., Maskell, D. J., and Moxon, E. R. (1991) Relationship between intracellular survival in macrophages and virulence of *Haemophilus influenzae* type b. *J. Infect. Dis.* **163,** 1366–1369.
39. Noel, G. J., Barenkamp, S. J., St. Geme, J. W.,III, Haining, W. N., and Mosser, D. M. (1994) High-molecular-weight surface-exposed proteins of *Haemophilus influenzae* mediate binding to macrophages. *J. Infect. Dis.* **169,** 425–429.

40. Johnson, A. P. and Inzana, T. J. (1986) Loss of ciliary activity in organ cultures of rat trachea treated with lipo-oligosaccharide isolated from *Haemophilus influenzae*. *J. Med. Microbiol.* **22,** 265–268.
41. Read, R. C., Wilson, R., Rutman, A., et al. (1991) Interaction of nontypable *Haemophilus influenzae* with human respiratory mucosa *in vitro. J. Infect. Dis.* **163,** 549–558.
42. Jackson, A. D., Cole, P. J,. and Wilson, R. (1996) Comparison of *Haemophilus influenzae* type b interaction with respiratory mucosa organ cultures maintained with an air interface or immersed in medium. *Infect. Immun.* **64,** 2353–2355.
43. Hood, D. W., Makepeace, K, Deadman, M. E., et al. (1999) Sialic acid in the lipopolysaccharide of *Haemophilus influenzae*: strain distribution, influence on serum resistance and structural characterization. *Mol. Microbiol.* **33,** 679–692.
44. Moxon, E. R. and Murphy, P. A. (1978) *Haemophilus influenzae* bacteremia and meningitis resulting from survival of a single organism. *Proc. Natl. Acad. Sci. USA* **75,** 1534–1536.
45. Moxon, E. R. and Ostrow, P. T. (1977) *Haemophilus influenzae* meningitis in infant rats: role of bacteremia in pathogenesis of age-dependent inflammatory responses in cerebrospinal fluid. *J. Infect. Dis.* **135,** 303–307.
46. Virji, M., Kayhty, H., Ferguson, D. J., Alexandrescu, C., and Moxon, E. R. (1991) Interactions of *Haemophilus influenzae* with cultured human endothelial cells. *Microb. Pathog.* **10,** 231–245.
47. Rubin, L. G. and Moxon, E. R. (1983) Pathogenesis of bloodstream invasion with *Haemophilus influenzae* type b. *Infect. Immun.* **41,** 280–284.
48. Moxon, E. R. and Vaughn, K. A. (1981) The type b capsular polysaccharide as a virulence determinant of *Haemophilus influenzae*: studies using clinical isolates and laboratory transformants. *J. Infect. Dis.* **143,** 517–524.
49. Noel, G. J., Mosser, D. M., and Edelson, P. J. (1990) Role of complement in mouse macrophage binding of *Haemophilus influenzae* type b. *J. Clin. Invest.* **85,** 208–218.
50. Betz, A. L. and Goldstein, G. W. (1986) Specialized properties and solute transport in brain capillaries. *Annu. Rev. Physiol.* **48,** 241–250.
51. Patrick, D., Betts, J., Frey, E. A., Prameya, R., Dorovini-Zis, K., and Finlay, B. B. (1992) *Haemophilus influenzae* lipopolysaccharide disrupts confluent monolayers of bovine brain endothelial cells via a serum-dependent cytotoxic pathway. *J. Infect. Dis.* **165,** 865–872.
52. Quagliarello, V. J., Long, W. J., and Scheld, W. M. (1986) Morphologic alterations of the blood-brain barrier with experimental meningitis in the rat. Temporal sequence and role of encapsulation. *J. Clin. Invest.* **77,** 1084–1095.
53. Wispelwey, B., Lesse, A. J, Hansen, E. J., and Scheld, W. M. (1988) *Haemophilus influenzae* lipopolysaccharide-induced blood brain barrier permeability during experimental meningitis in the rat. *J. Clin. Invest.* **82,** 1339–1346.
54. Kimura, A., Gulig, P. A., McCracken, G. H. Jr., Loftus, T. A., and Hansen, E. J. (1985) A minor high-molecular-weight outer membrane protein of *Haemophilus influenzae* type b is a protective antigen. *Infect. Immun.* **47,** 253–259.

55. Green, B. A., Metcalf, B. J., Quinn-Dey, T., Kirkley, D. H., Quataert, S. A., and Deich, R. A. (1990) A recombinant non-fatty acylated form of the Hi-PAL (P6) protein of *Haemophilus influenzae* elicits biologically active antibody against both nontypeable and type b *H. influenzae*. *Infect. Immun.* **58,** 3272–3278.
56. Erwin, A. L. and Kenny, G. E. (1984) *Haemophilus influenzae* type b isolates show antigenic variation in a major outer membrane protein. *Infect. Immun.* **46,** 570–577.
57. Munsen, R. S. Jr. and Granoff, D. M. (1985) Purification and partial characterization of outer membrane proteins P5 and P6 from *Haemophilus influenzae* type b. *Infect. Immun.* **49,** 544–549.
58. Nelson, M. B., Munson, R. S., Jr., Apicella, M. A., Sikkema, D. J., Molleston, J. P., and Murphy, T. F. (1991) Molecular conservation of the P6 outer membrane protein among strains of *Haemophilus influenzae*: analysis of antigenic determinants, gene sequences, and restriction fragment length polymorphisms. *Infect. Immun.* **59,** 2658–2663.
59. Murphy, T. F, Dudas, K. C., Mylotte, J. M., and Apicellia, M. A. (1983) A subtyping system for nontypable *Haemophilus influenzae* based on outer-membrane proteins. *J. Infect. Dis.* **147,** 838–846.
60. Loeb, M. R., Zachary, A. L., and Smith, D. H. (1981) Isolation and partial characterization of outer and inner membranes from encapsulated *Haemophilus influenzae* type b. *J. Bacteriol.* **145,** 596–604.
61. Coulton, J. W. and Wan, D. T. (1983) The outer membrane of *Haemophilus influenzae* type b: cell envelope associations of major proteins. *Can. J. Microbiol.* **29,** 280–287.
62. Cope, L. D., Yogev, R., Mertsola, J., Argyle, J. C., McCracken, G. H. Jr., and Hansen, E. J. (1990) Effect of mutations in lipooligosaccharide biosynthesis genes on virulence of *Haemophilus influenzae* type b. *Infect. Immun.* **58,** 2343–2351.
63. Gulig, P. A. and Hansen, E. J. (1985) Coprecipitation of lipopolysaccharide and the 39,000-molecular-weight major outer membrane protein of *Haemophilus influenzae* type b by lipopolysaccharide-directed monoclonal antibody. *Infect. Immun.* **49,** 819–827.
64. Chanyangam, M., Smith, A. L., Moseley, S. L., Kuehn, M., and Jenny, P. (1991) Contribution of a 28-kilodalton membrane protein to the virulence of *Haemophilus influenzae*. *Infect. Immun.* **59,** 600–608.
65. Murphy, T. F, Bartos, L. C., Campagnari, A. A., Nelson, M. B., and Apicella, M. A. (1986) Antigenic characterization of the P6 protein of nontypable *Haemophilus influenzae*. *Infect. Immun.* **54,** 774–779.
66. Stull, T. L., Mendelman, P. M., Haas, J. E., Schoenborn, M. A., Mack, K. D., and Smith, A. L. (1984) Characterization of *Haemophilus influenzae* type b fimbriae. *Infect. Immun.* **46,** 787–796.
67. St. Geme, J. W., Pinkner, J. S., 3rd, Krasan, G. P., Heuser, J., Bullitt, E., Smith, A. L., and Hultgren, S. J. (1996) *Haemophilus influenzae* pili are composite structures assembled via the HifB chaperone. *Proc. Natl. Acad. Sci. USA* **93,** 11913–11918.
68. Mason, E. O. Jr., Kaplan, S. L., Wiedermann, B. L., Norrod, E. R., and Stenback, W. A. (1985) Frequency and properties of naturally occurring adherent piliated strains of *Haemophilus influenzae* type b. *Infect. Immun.* **49,** 98–103.

69. Anderson, P. W., Pichichero, M. E., and Connor, E. M. (1985) Enhanced nasopharyngeal colonization of rats by piliated *Haemophilus influenzae* type b. *Infect. Immun.* **48,** 565– 568.
70. Guttierrez, M. K, Joffe, L. S., Forney, L. J., and Glode, M. P. (1990). Programs and abstracts of the 30th Interscience Conference. *Am. Soc. Microbiol.*
71. Tosi, M. F., Anderson, D. C., Barrish, J., Mason, E. O. Jr., and Kaplan, S. L. (1985) Effect of piliation on interactions of *Haemophilus influenzae* type b with human polymorphonuclear leukocytes. *Infect. Immun.* **47,** 780–785.
72. Krogfelt, K. A. (1991) Bacterial adhesion: genetics, biogenesis, and role in pathogenesis of fimbrial adhesins of *Escherichia Coli. Rev. Infect. Dis.* **13,** 721–735.
73. van-Ham, S. M., van Alphen, L., Mooi, F. R., and van Putten, J. P. (1993) Phase vatiation of *H. influenzae* fimbriae transcriptional control of two divergent genes through a variable combined promoter region. *Cell.* **73,** 1187–1196.
74. Mhlanga-Mutangadura, T., Morlin, G., Smith, A. L., Eisenstark, A., and Golomb, M. (1998) Evolution of the major pilus gene cluster of *Haemophilus influenzae*. *J. Bacteriol.* **180,** 4693–4703.
75. Pohlner, J., Halter, R., and Meyer, T. F. (1987) *Neisseria gonorrhoeae* IgA protease. Secretion and implications for pathogenesis. *Antonie Van Leeuwenhoek* **53,** 479–484.
76. Poulsen, K., Brandt, J., Hjorth, J. P., Thogersen, H. C., and Kilian, M. (1989) Cloning and sequencing of the immunoglobulin A1 protease gene (iga) of *Haemophilus influenzae* serotype b. *Infect. Immun.* **57,** 3097–3105.
77. Klauser, T., Pohner, J., and Meyer, T. F. (1993) The secretion pathway of IgA protease-type proteins in gram-negative bacteria. *Bioessays* **15,** 799–805.
78. Kilian, M., Reinholdt, J., Lomholt, H., Poulsen, K., and Frandsen, E. V. (1996) Biological significance of IgA1 proteases in bacterial colonization and pathogenesis: critical evaluation of experimental evidence. *APMIS* **104,** 321–338.
79. Plaut, A. G. (1983) The IgA1 proteases of pathogenic bacteria. *Annu. Rev. Microbiol.* **37,** 603–622.
80. Kilian, M., Mestecky, J., and Russell, M. W. (1988) Defense mechanisms involving Fc-dependent functions of immunoglobulin A and their subversion by bacterial immunoglobulin A proteases. *Microbiol. Rev.* **52,** 296–303.
81. Bricker, J., Mulks, M. H., Plaut, A. G., Moxon, E. R., and Wright, A. (1983) IgA1 proteases of *Haemophilus influenzae*: cloning and characterization in *Escherichia coli* K-12. *Proc Natl Acad Sci USA* **80,** 2681–2685.
82. Bricker, J., Mulks, M., Moxon, E. R., Plaut, A. G., and Wright, A. (1985) Physical and genetic analysis of DNA regions encoding the immunoglobulin A proteases of different specificities produced by *Haemophilus influenzae*. *Infect. Immun.* **47,** 370–374.
83. Grundy, F. J, Plaut, A. G., and Wright, A. (1990) Localization of the cleavage site specificity determinant of *Haemophilus influenzae* immunoglobulin A1 protease genes. *Infect. Immun.* **58,** 320–331.
84. Lomholt, H., van Alphen, L., and Kilian, M. (1993) Antigenic variation of immunoglobulin A1 proteases among sequential isolates of *Haemophilus*

influenzae from healthy children and patients with chronic obstructive pulmonary disease. *Infect. Immun.* **61,** 4575–4581.

85. Lomholt, H., Poulsen, K., and Kilian, M. (1995) Antigenic and genetic heterogeneity among *Haemophilus influenzae* and Neisseria IgA1 proteases. *Adv. Exp. Med. Biol.* **371A,** 599–603.
86. Zamze, S. E., and Moxon, E. R. (1987) Composition of the lipopolysaccharide from different capsular serotype strains of *Haemophilus influenzae*. *J. Gen. Microbiol.* **133,** 1443–1451.
87. Flesher, A. R., and Insel, R. A. (1978) Characterization of lipopolysaccharide of *Haemophilus influenzae*. *J. Infect Dis.* **138,** 719–730.
88. Helander, I. M., Lindner, B., Brade, H., Altmann, K., Lindberg, A. A., Rietschel, E. T., and Zahringer, U. (1988) Chemical structure of the lipopolysaccharide of *Haemophilus influenzae* strain I-69 Rd-/b+. Description of a novel deep-rough chemotype. *Eur. J Biochem.* **177,** 483–492.
89. Raetz, C. R. (1990) Biochemistry of endotoxins. *Annu. Rev. Biochem.* **59,** 129–170.
90. Raetz, C. R. (1993) Bacterial endotoxins: extraordinary lipids that activate eucaryotic signal transduction. *J. Bacteriol.* **175,** 5745–5753.
91. Apicella, M. A., Dudas, K. C., Campagnari, A., Rice, P., Mylotte, J. M., and Murphy, T. F. (1985) Antigenic heterogeneity of lipid A of *Haemophilus influenzae*. *Infect. Immun.* **50,** 9–14.
92. Gibson, B. W., Melaugh, W., Phillips, N. J, Apicella, M. A., Campagnari, A. A., and Griffiss, J. M. (1993) Investigation of the structural heterogeneity of lipooligosaccharides from pathogenic *Haemophilus* and *Neisseria* species and of R-type lipopolysaccharides from *Salmonella typhimurium* by electrospray mass spectrometry. *J. Bacteriol.* **175,** 2702–2712.
93. Phillips, N. J., Apicella, M. A., Griffiss, J. M., and Gibson, B. W. (1993) Structural studies of the lipooligosaccharides from *Haemophilus influenzae* type b strain A2. *Biochemistry.* **32,** 2003–2012.
94. Phillips, N. J., McLaughlin, R., Miller, T. J., Apicella, M. A., and Gibson, B. W. (1996) Characterization of two transposon mutants from *Haemophilus influenzae* type b with altered lipooligosaccharide biosynthesis. *Biochemistry.* **35,** 5937–5947.
95. Masoud, H., Moxon, E. R., Martin, A., Krajcarski, D., and Richards, J. C. (1997). Structure of the variable and conserved lipopolysaccharide oligosaccharide epitopes expressed by *Haemophilus influenzae* serotype b strain Eagan. *Biochemistry.* **36,** 2091–2103.
96. Risberg, A., Masoud, H., Martin, A., Richards, J. C., Moxon, E. R., and Schweda, E. K. (1999) Structural analysis of the lipopolysaccharide oligosaccharide epitopes expressed by a capsule-deficient strain of *Haemophilus influenzae* Rd. *Eur. J. Biochem.* **261** 171–180.
97. Zwahlen, A., Rubin, L. G., Connelly, C. J., Inzana, T. J., and Moxon, E. R. (1985) Alteration of the cell wall of *Haemophilus influenzae* type b by transformation with cloned DNA: association with attenuated virulence. *J. Infect. Dis.* **152,** 485–492.
98. Zwahlen, A., Rubin, L. G,. and Moxon, E. R. (1986) Contribution of lipopolysaccharide to pathogenicity of *Haemophilus influenzae*: comparative virulence of genetically-related strains in rats. *Microb. Pathog.* **1,** 465–473.

99. Zwahlen, A., Kroll, J. S., Rubin, L. G., and Moxon, E. R. (1989) The molecular basis of pathogenicity in *Haemophilus influenzae*: comparative virulence of genetically-related capsular transformants and correlation with changes at the capsulation locus cap. *Microbiol. Pathog.* **7,** 225–235.
100. Kimura, A. and Hansen, E. J. (1986) Antigenic and phenotypic variations of *Haemophilus influenzae* type b lipopolysaccharide and their relationship to virulence. *Infect. Immun.* **51,** 69–79.
101. Weiser, J. N. and Pan, N. (1998). Adaptation of *Haemophilius influenzae* to acquired and innate humoral immunity based on phase variation of lipopolysaccharide. *Mol. Microbiol.* **30,** 767–775.
102. Hood, D. W., Deadman, M. E., Allen, T., et al. (1996) Use of the complete genome sequence information of *Haemophilus influenzae* strain Rd to investigate lipopolysaccharide biosynthesis. *Mol. Microbiol.* **22,** 951–965.
103. Syrogiannopoulos, G. A., Hansen, E. J., Erwin, A. L., et al. (1988) *Haemophilus influenzae* type b lipooligosaccharide induces meningeal inflammation. *J. Infect. Dis.* **157,** 237–244.
104. Tobias, P. S., Soidau, K., and Ulevitch, R. J. (1989). Identification of a lipid A binding site in the acute phase reactant lipopolysaccharide binding protein. *J. Biol. Chem.* **264,** 10867–10871.
105. Goyert, S. M., Ferrero, E. M., Seremetis, S. V., Winchester, R. J., Silver, J., and Mattison, A. C. (1986) Biochemistry and expression of myelomonocytic antigens. *J. Immunol.* **137,** 3909–3914.
106. Kruger, C., Schutt, C., Obertacke, U., et al. (1991) Serum CD14 levels in polytraumatized and severely burned patients. *Clin. Exp. Immunol.* **85,** 297–301.
107. Haziot, A., Rong, G. W., Silver, J., and Goyert, S. M. (1993) Recombinant soluble CD14 mediates the activation of endothelial cells by lipopolysaccharide. *J. Immunol.* **151,** 1500–1507.
108. Kirschning, C. J., Wesche, H., Merrill-Ayres, T., and Rothe, M. (1998) Human toll-like receptor-2 confers reponsiveness to bacterial lipopolysaccharide. *J. Exp. Med.* **188,** 2091–2097.
109. Yang, R. B., Mark, M. R., Gray, A., et al. (1998) Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signalling. *Nature* **395,** 284–288.
110. Chow, J. C., Young, D. W., Golenbock, D. T., Christ, W. J., and Gusovsky, F. (1999) Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. *J. Biol. Chem.* **274,** 10689–10692.
111. Medzhitov, R., Preston-Hurlburt, P., and Janeway, C. A. Jr. (1997) A human homologue to the Drosophilia Toll protein signals activation of adaptive immunity. *Nature* **388,** 394–397.
112. Virji, M., Weiser, J. N., Lindberg, A. A., and Moxon, E. R. (1990) Antigenic similarities in lipopolysaccharides of Haemophilus and Neisseria and expression of a digalactoside structure also present on human cells. *Microbiol. Patho.* **9,** 441–450.
113. Maskell, D. J., Szabo, M. J., Deadman, M. E., and Moxon, E. R. (1992) The gal locus from *Haemophilus influenzae*: cloning, sequencing and the use of gal mutants to study lipopolysaccharide. *Mol. Microbiol.* **6,** 3051–3063.

114. Hood, D. W., Deadman, M. E., Jennings, M. P., Bisercic, M., Fleischmann, R. D., Venter, J. C., and Moxon, E. R. (1996) DNA repeats identify novel virulence genes in *Haemophilus influenzae*. *Proc. Natl. Acad. Sci. USA* **93,** 11,121–11,125.
115. Robbins, J. B. (1978) Vaccines for the prevention of encapsulated bacterial diseases: current status, problems and prospects for the future. *Immunochemistry* **15,** 839–854.
116. Crisel, R. W., Baker, R. S., and Dorman, D. E. (1975) Capsular polymer of *Haemophilus influenzae*, type b. I. Structural characterization of the capsular polymer of strain Eagan. *J. Biol. Chem.* **250,** 4926–4930.
117. Egan, W., Tsui, F. P., Climenson, P. A., and Schneerson, R. (1980) Structural and immunological studies of the *Haemophilius influenzae* type c capsular polysaccharide. *Carbohyd. Res.* **80,** 305–316.
118. Egan, W., Tsui, F. P., and Schneerson, R. (1980) Structural studies of the *Haemophilus influenzae* type f capsular polysaccharide. *Carbohyd. Res.* **79,** 271–277.
119. Tsui, F. P., Schneerson, R., Boykins, R. A., Karpas, A. B., and Eagan, W. (1981) Structural and immunological studies of the *Haemophilus influenzae* type d capsular polysaccharide. *Carbohyd. Res.* **97,** 293–306.
120. Tsui, F. P., Schneerson, R., and Egan, W. (1981) Structural studies of the *Haemophilus influenzae* type e capsular polysaccharide. *Carbohyd. Res.* **88,** 85–92.
121. Weller, P. F., Smith, A. L., Anderson, P., and Smith, D. H. (1977) The role of encapsulation and host age in the clearance of *Haemophilus influenzae* bacteremia. *J. Infect. Dis.* **135,** 34–41.
122. Brophy, L. N., Kroll, J. S., Ferguson, D. J., and Moxon, E. R. (1991) Capsulation gene loss and 'rescue' mutations during the Cap+ to Cap− transition in *Haemophilus influenzae* type b. *J. Gen. Microbiol.* **137,** 2571–2576.
123. Corn, P. G., Anders, J., Takala, A. K., Kayhty, H., and Hoiseth, S. K. (1993) Genes involved in *Haemophilus influenzae* type b capsule expression are frequently amplified. *J. Infect. Dis.* **167,** 356–364.
124. Kroll, J. S., Loynds, B. M., and Moxon, E. R. (1991) The *Haemophilus influenzae* capsulation gene cluster: a compound transposon. *Mol. Microbiol.* **5,** 1549–1560.
125. Kroll, J. S., Moxon, E. R., and Loynds, B. M. (1993) An ancestral mutation enhancing the fitness and increasing the virulence of *Haemophilus influenzae* type b. *J. Infect. Dis.* **168,** 172–176.
126. Brophy, L. N., Kroll, J. S., Ferguson, D. J., and Moxon, E. R. (1991) Capsulation gene loss and 'rescue' mutations during the Cap+ to Cap− transition in *Haemophilus influenzae* type b. *J. Gen. Microbiol.* **137,** 2571–2576.
127. Roche, R. J. and Moxon, E. R. (1995) Phenotypic variation of carbohydrate surface antigens and the pathogenesis of *Haemophilus influenzae* infections. *Trends Microbiol.* **3,** 304–309.
128. Lampe, R. M., Mason, E. O. Jr., Kaplan, S. L., Umstead, C. L., Yow, M. D., and Feigin, R. D. (1982) Adherence of *Haemophilus influenzae* to buccal epithelial cells. *Infect. Immun.* **35,**166–172.
129. Pichichero, M. E. (1984) Adherence of *Haemophilus influenzae* to human buccal and pharyngeal epithelial cells: relationship to pilation. *J. Med. Microbiol.* **18,** 107–116.

130. St. Geme, J. W., III and Falkow, S. (1991) Loss of capsule expression by *Haemophilus influenzae* type b results in enhanced adherence to and invasion of human cells. *Infect. Immun.* **59,** 1325–1333.
131. Anderson, P., Smith, D. H., Ingram, D. L., Wilkins, J, Wehrle, P. F., and Howie, V. M. (1977) Antibody of polyribophate of *Haemophilus influenzae* type b in infants and children: effect of immunization with polyribophosphate. *J. Infect. Dis.* **136 (Suppl),** S57–S62.
132. Makela, P. H., Peltola, H., Kayhty, H., et al. (1977) Polysaccharide vaccines of group A Neisseria meningtitidis and *Haemophilus influenzae* type b: a field trial in Finland. *J. Infect. Dis.* **136 (Suppl),** S43–50.
133. Parke, J. C. Jr., Schneerson, R., Robbins, J. B., and Schlesselman, J. J. (1977). Interim report of a controlled field trial of immunization with capsular polysaccharides of *Haemophilus influenzae* type b and Group C *Neisseria meningitidis* in Mecklenburg county, North Carolina March 1974–March 1976. *J. Infect. Dis.* **136 (Suppl),** S51–56.
134. Peltola, H., Kayhty, H., Sivonen, A., and Makela, P. H. (1977) *Haemophilus influenzae* type b capsular polysaccharide vaccine in children: a double-blind field study of 100,000 vaccinees 3 months to 5 years of age in Finland. *Pediatrics* **60,** 730–737.
135. Anderson, P. (1983) Antibody responses to *Haemophilus influenzae* type b and diphtheria toxin induced by conjugates of oligosaccharides of the type b capsule with the nontoxic protein CRM197. *Infect. Immun.* **39,** 233–238.
136. Gordon, L. K. (1986) Studies on the combined administration of *Haemophilus influenzae* type B-diphtheria toxoid conjugate vaccine (PRP-D) and DTP. *Dev. Biol. Stand.* **65,** 113–121.
137. Einhorn, M. S., Weinberg, G. A., Anderson, E. L., Granoff, P. D., and Granoff, D. M. (1986) Immunogenicity in infants of *Haemophilus influenzae* type B polysaccharide in a conjugate vaccine with *Neisseria meningitidis* outer-membrane protein. *Lancet* **2,** 299–302.
138. Claesson, B. A., Schneerson, R., Robbins, J. B., et al. (1989) Protective levels of serum antibodies stimulated in infants by two injections of *Haemophilus influenzae* type b capsular polysaccharide- tetanus toxoid conjugate. *J. Pediatr.* **114,** 97–100.
139. Decker, M. D., Edwards, K. M., Bradley, R. and Palmer, P. (1992) Comparative trial in infants of four conjugate *Haemophilus influenzae* type b vaccines. *J. Pediatr.* **120,** 184–189.
140. Granoff, D. M. Anderson, E. L., Osterholm, M. T.,et al. (1992) Differences in the immunogenicity of three *Haemophilus influenzae* type b conjugate vaccines in infants. *J. Pediatr.* **121,** 187–194.
141. Booy, R., Heath, P. T., Slack, M. P., Begg, N., and Moxon, E. R. (1997) Vaccine failures after primary immunisation with *Haemophilus influenzae* type-b conjugate vaccine without booster. *Lancet.* **349,** 1197–1202.
142. Moxon, E. R., Hood, D., and Richards, J. (1998) Bacterial lipopolysaccharides: candidate vaccines to prevent Neisseria meningitidis and *Haemophilus influenzae* infections. *Adv. Exp. Med. Biol.* **435,** 237–243.
143. Wu, T. H. and Gu, X. X. (1999) Outer membrane proteins as a carrier for detoxified lipooligosaccharide conjugate vaccines for nontypeable *Haemophilus influenzae*. *Infect. Immun.* **67,** 5508–5513.

3

General Methods for Culturing *Haemophilus influenzae*

Grant Poje and Rosemary J. Redfield

1. Introduction

1.1. Nutritional Requirements

Haemophilus influenzae is differentiated from other *Haemophilus* species primarily by its growth requirements for both hemin (called factor X in the old literature) and nicotinamide adenine dinucleotide (NAD or factor V). *H. influenzae* strains normally grow well in rich media such as brain heart infusion (BHI), suitably supplemented with hemin and NAD (sBHI), and on sBHI or chocolate agar plates. Many isolates classified as *H. influenzae* differ in their other nutritional requirements, for a purine and for specific amino acids ***(1,2)***. Thus, although a number of defined media have been described, their use often leads to frustration. The medium MMB, described by Klein and Luginbuhl ***(2)***, is simplest to prepare; when supplemented with a small amount of casamino acids it reproducibly gives good growth.

1.2. Growth and Viability

Standard laboratory strains of *H. influenzae* grow in rich medium with a doubling time of about 30 min, to a maximum density of 5×10^9–1×10^{10} cells/mL. Cells are not well adapted to survival under conditions that do not permit growth; viability of cells in liquid will decline continuously once the maximum density is reached. Colonies on solid medium will continue to contain viable cells for a week or more at room temperature or at 4°C, but the number of viable cells will decrease.

1.3. Storage

Cultures are best stored frozen in glycerol (*see* **Subheading 3.3.**).

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1.4. Recognizing Contaminants

1. *H. influenzae* colonies are grayish-buff and slightly translucent, with little odor. Colonies that are opaque, white, pigmented, or strongly smelling are contaminants.
2. If suspected contaminants produce colonies on a Luria Bertani (LB) plate or other rich medium lacking hemin and NAD, the cells are not *H. influenzae*.
3. When examined microscopically, *H. influenzae* cells are very small rods, much smaller than *Escherichia coli*.

2. Materials

2.1. Media

1. Brain heart infusion broth: 37 g/L Difco brain heart infusion (BHI) in distilled H_2O. Autoclave, store at room temperature. For plates, add 12–15g Bacto or MBI agar before autoclaving (*see* **Note 1**).
2. Hemin (1mg/mL): Place 4 mL triethanolamine, 96 mL dH_2O, and 100 mg hemin (Equine, Difco) in a glass bottle. Cap loosely and heat at 65°C for 30 min (do not autoclave). The hemin will not dissolve, but will form a stable suspension upon heating. This stock is sterile and stable for many mo at 4°C. If preparing more than one bottle of hemin stock, put the weighed hemin into each bottle separately, then add 100 mL of 4% triethanolamine and heat.
3. Nicotinamide adenine dinucleotide (NAD) stock: 10 mg/mL in dH_2O. Filter sterilize. Hemin stock is stable for many months at −20°C, and for at least several weeks at 4°C.
4. Supplemented BHI (sBHI): Add 10 mL hemin stock and 200 µL NAD stock per liter of BHI. Prepare as needed; use within 24 h.
5. cMMB (MMB supplemented with casamino acids): Dissolve in dH_2O (1L): L-arginine (300 mg), glutamic acid (1.3 g), glutathione (200 mg), uracil (100 mg), inosine (2.0 g), casamino acids (5 g), K_2HPO_4 (3.5 g), KH_2PO_4 (2.7 g), NaCl (5.8 g), $MgCl_2$ (430 mg) and $CaCl_2$ (22 mg). Autoclave and when cool, add hemin (10 mL of 1-mg/mL stock), NAD (200 µL of 10-mg/mL stock), thiamine (400 µL of 10-mg/mL stock; filter-sterilized; stored at −20°C); pantothenic acid (400 µL of 10-mg/mL stock; filter-sterilized; store at −20°C).
6. Antibiotic stocks: Standard concentrations of antibiotics used for genetic manipulations in *H. influenzae* are given in **Table 1**. Unless otherwise indicated, prepare in dH_2O, filter-sterilize, and store in 1-mL aliquots at −20°C. Stocks prepared in ethanol need not be sterilized.

2.2. Other

1. 80% glycerol stock: Combine 80 mL glycerol with 20 mL dH_2O. Mix well and autoclave. Store at room temperature. Keeps indefinitely.
2. Phosphate-buffered saline (PBS): 0.3 g KH_2PO_4, 1.1 g $Na_2HPO_4{\cdot}7H_2O$, 8.5 g NaCl, and dH_2O to 1 L. Mix and autoclave. Store at room temperature. Keeps indefinitely (*see* **Note 2**).
3. Dilution solution: PBS supplemented with 5–10% BHI or sBHI.

Table 3
Recommended antibiotic concentrations for *H. influenzae*

Antibiotic	Working concentration	Stock
Kanamycin (*see* **Note 6**)	7 µg/mL	70 mg/mL (10,000 ×)
Novobiocin	2.5 µg/mL	25 mg/mL (10,000 ×)
Spectinomycin	20 µg/mL	20 mg/mL (1000 ×)
Naladixic acid	3 µg/mL	30 mg/mL (10,000 ×)
Chloramphenicol (*see* **Note 7**)	1–2 µg/mL	20 mg/mL in ethanol (10,000 ×)
Tetracycline	5 µg/mL	10 mg/mL in ethanol (2000 ×)
Streptomycin	250 µg/mL	100 mg/mL (400 ×)
Ampicillin	5 µg/mL	50 mg/mL in 50% ethanol (10,000 ×)

2.3. Chromosomal DNA Preparation (Miniprep Method)

1. Cell resuspension solution: 50 m*M* Tris HCl, pH 7.4; 50m*M* EDTA.
2. 10% sodium dodecyl sulfate (SDS).
3. Phenol/chloroform (50/50 ratio).
4. 5 *M* NaCl.
5. 95% ethanol.
6. Flame-sealed Pasteur pipets for DNA preparations.
7. 70% ethanol.
8. TE buffer: 10 m*M* Tris-HCl, pH 7.4, 1 m*M* EDTA.

2.4. Lysis Reagents for Colony PCR

1. 0.5 *M* NaOH.
2. 0.1 *M* Tris-HCl, pH 7.4.

2.5. Cloning Vectors

Many *E. coli* cloning vectors cannot replicate in *H. influenzae* (e.g,. pGEM, pUC). The most convenient cloning vectors for genetic manipulation in *H. influenzae* are the pSU series (pSU8, pSU9, pSU18, pSU19, pSU20, pSU21, pSU23, pSU24) ***(3)***. They are based on pACYC184, confer chloramphenicol resistance, have useful multi-cloning sites, and provide moderate copy number in both *E. coli* and *H. influenzae*.

2.6. Strains

1. Obtaining strains: The American Type Culture Collection has an extensive collection of *H. influenzae* isolates. Strains containing specific mutations are usually readily obtained from their creators.

2. Shipping strains: Cells may be grown on agar prepared in a plastic tube suitable for mailing, or a sterile spatula may be used to transfer colonies and agar from a fresh plate into a suitable sterile tube. Most strains will survive a week or more at ambient temperature, and so may be sent by mail if economy is an issue.

2.7. Equipment

1. Roller wheel or shaker at 37°C for small-volume liquid cultures in tubes (≤ 10 mL).
2. Air or waterbath shaker at 37°C for larger volumes of liquid cultures.
3. Glass beads for spreading cells on plates (3 mm diameter, autoclaved in glass screw-capped tubes) or alcohol-sterilized spreader.
4. Sterile loops or pipets for streaking cells on plates and transferring colonies.
5. 37°C incubator for growing cells on solid media.

3. Methods

3.1. Liquid Culture

1. Small-volume cultures: Prepare a test tube containing 5 mL of sBHI and any needed antibiotics. Inoculate with either a loopful of cells from a fresh, well-isolated colony, a drop of a previous liquid culture, or scrapings from a frozen culture. Cap loosely and incubate at 37°C on a roller wheel or shaker overnight or until desired culture density is reached.
2. Moderate-volume cultures: Instead of a test tube use a flask having at least five times the volume of the culture (e.g., 20 mL of culture in a 100 mL flask). Shake at 200 rpm at 37°C.
3. Large-volume cultures: Inoculate from a fresh overnight culture if rapid initial growth is desired. Do not overfill flasks; inadequate aeration is often the cause of low yields of cells or plasmids. Shake at 200 rpm at 37°C.
4. Monitoring growth: Read optical density (OD) at a wavelength of 600 nm. Use sBHI as a blank (*see* **Note 3**). In dense cultures, erroneously low readings are caused by light scattering, so if the OD_{600} of the sample is higher than 0.3, dilute the sample five- or 10-fold with sBHI to get an accurate reading.
5. Diluting cultures for plating: Cultures may be diluted in medium or in phosphate-buffered saline (PBS) that has been supplemented with 5–10% BHI. Cells diluted in plain PBS rapidly lose viability.

3.2. Agar Culture

1. Prepare BHI agar. Let agar cool to pouring temperature (approx 50°C) before adding hemin, NAD, and any antibiotics (*see* **Note 4**). Standard concentrations of antibiotics used for genetic manipulations are given in **Table 1**. Allow surfaces of freshly poured plates to dry before streaking or spreading cells, to prevent subsequent 'weeping' of the agar surface. For noncritical experiments, plates may be dried uncovered in a forced-air incubator or at 37°C for 1 h; for critical experiments, use a laminar flow-hood.
2. To spread cells for colony counting, liquid may be spread on plates using a sterile spreader or sterile glass beads. To use glass beads, sprinkle 3–5 beads on each

plate. Add liquid and shake gently to roll beads around, spreading the liquid. Once liquid is absorbed, tip beads into a container of alcohol or disinfectant for later washing and reuse.
3. To streak cells for isolation of single colonies, use a flamed loop or a sterile pipet.
4. Incubate plates at 37°C for 18–24 h. Colonies are smaller than those of *E. coli*. They will grow slightly larger if left for 48 h, but will not continue to grow beyond that time.

3.3. Freezing Cultures

Stock cultures of noncompetent cells are prepared by inoculating cells into sBHI and growing to an OD_{600} of 0.3– 0.4. Before freezing, add 0.25 mL of 80% glycerol to 1 mL of culture, and mix well. Store at −70°C to −80°C. Competent cells in M-IV competence medium may also be frozen this way for later use.

3.4. Preparation of DNA

1. In general, the methods developed for isolation of plasmids and chromosomal DNA from *E. coli* work well. Because the *H. influenzae* peptidoglycan layer is weak, pretreatment with lysozyme is usually unnecessary. For plasmid preparation, use the standard protocols developed for *E. coli* ***(4)***.
2. Rapid small-scale preparation of chromosomal DNA: Pellet 1.5–3.0 mL of a fresh overnight culture. Resuspend in 450 µL of cell resuspension solution (*see* **Subheading 2.3.1.**) in a microfuge tube. Add 50 µL of 10% SDS and mix until cells lyse and suspension becomes viscous. The suspension may be heated to 50°C to speed lysis. Extract twice with phenol/chloroform, each time transferring the supernatant to a fresh tube. Add sufficient 5 *M* NaCl to give a final concentration of 0.15 *M* (usually 12 µL), then add two vol of 95% ethanol (usually 0.8 mL). Mix gently until a fibrous clump of DNA becomes visible, and retrieve the DNA by winding it onto the sealed tip of a Pasteur pipet (*see* **Note 5**). Rinse the spooled DNA by gently dribbling 1.0 mL of 70% ethanol down the pipette tip, held over a clean container. Store the pipet in an inverted position for at least 30 min to allow the DNA to air dry. Resuspend the DNA by swirling the pipet tip in 200 µL of TE buffer (*see* **Subheading 2.1.**), and then allow several hours for the DNA to fully disperse, with occasional gentle vortexing or pipetting. The yield is about 20 µg of DNA, in 50–200kb fragments.
3. Single-colony DNA for PCR ***(5)***: Transfer a large colony from an sBHI plate into 25 µL 0.5 *M* NaOH in a microcentrifuge tube. Completely suspend the cells by vigorous vortexing, and leave at room temperature for 30 min. Then add 25 µL of 1 *M* Tris buffer, pH 7.4 and 450 µL of dH_2O. These lysates can be frozen at −20°C until needed. For use as a PCR template, 5–10 µL of the lysate is added to a 100 µL PCR reaction mixture.

4. Notes

1. We have not experienced any problems attributable to different sources of water. All deionized and distilled waters have worked well. However, attempts to econo-

mize by using less expensive brands of BHI and agar have been unsuccessful. Although their initial performance was often satisfactory, all eventually caused problems with growth or plating that ruined expensive experiments, wiping out the original cost savings.

2. When large volumes of PBS will be needed, it is convenient to prepare the PBS as a 10× stock, and dilute with sterile water as needed.
3. OD_{600} readings for different batches of sBHI often differ substantially. Always use as a blank sBHI from the same bottle as is being used for the culture.
4. Fresh hemin must be added to plates older than 24 h. Spread $1/100^{th}$ the agar volume of hemin stock on the plate (0.3 mL on a 100-mm plate). Be sure that the hemin has soaked in and the agar surface is dry before using the plate.
5. DNA collected by spooling in this way is much cleaner than DNA collected by centrifugation.
   Most *H. influenzae* strains contain one or more restriction modification systems, and DNA extracted from them will carry the corresponding methylation and thus cannot be digested by these enzymes. *Hin*dII and *Hin*dIII will not digest DNA from the Rd strain.
6. Most kanamycin-resistance plasmids contain a Tn5-derived kan® gene, which functions poorly in *H. influenzae*. Most kanamycin-resistance transposons contain the Tn903-derived Kan gene, which functions well.
7. Cells grow poorly in the presence of chloramphenicol, and 48-h incubation may be required for sufficient growth of colonies. Do not assume that an experiment has failed because no chloramphenicol-resistant colonies are visible after 24 h of incubation; wait another day.

References

1. Holt, L.B. (1961) The growth-factor requirements of *Haemophilus influenzae*. *J. Gen. Microbiol.* **27,** 317–322.
2. Klein, R.D. and Luginbuhl, G. H. (1979) Simplified media for the growth of *Haemophilus influenzae* from clinical and normal flora sources. *J. Gen. Microbiol.* **113**, 409–411.
3. Bartolome, B., Jubete, Y., Martinez, E., and de la Cruz, F. (1991) Construction and properties of a family of pACYC184-derived cloning vectors compatible with pBR322 and its derivatives. *Gene.* **102,** 75–78.
4. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) in *Molecular Cloning, A Laboratory Manual,* 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
5. Lampel, K. A., Keasler, S. P., and Hanes, D. E. (1996) Specific detection of *Salmonella enterica* serotype Enteritidis using the polymerase chain reaction. *Epidemiol. Infect.* **116,** 137–145.

4

Transformation of *Haemophilus influenzae*

Grant Poje and Rosemary J. Redfield

1. Introduction

1.1. Conjugation

Although *Haemophilus influenzae* genes may be transferred by plasmid-mediated conjugation, this process is not discussed further below, as it has received little attention and is rarely used for strain construction. Conjugative plasmids are common in *H. influenzae*, as in other bacteria ***(1–3)***. The F plasmid of *Escherichia coli* can conjugate into *H. influenzae* cells ***(4)*** (and into many other cell types), and can then transfer by conjugation from one *H. influenzae* strain to another. As in *E. coli*, efficient transfer of chromosomal genes requires a region of homology between the plasmid and the chromosome. A method for F-mediated conjugation into *H. influenzae* is described by Deich and Green ***(4)***.

1.2. Transformation

H. influenzae has an efficient natural competence system, which also serves as the primary method of strain construction. Cells are described as competent when they are able to take up DNA from the environment, and transformation occurs when this DNA uptake leads to a changed genotype, either by recombination or by establishment of a plasmid. In the laboratory, transformation is used to transfer chromosomal alleles into different backgrounds, to introduce plasmids into cells, and to create new mutants by introducing into the chromosome mutations created in *E. coli* or in vitro.

Natural competence and transformation are also areas of ongoing research in their own right. There is extensive interest in mechanisms by which DNA is transferred across the cell envelope, in the regulatory processes that control competence, and in the evolutionary function and consequences of DNA uptake and recombination. *H. influenzae* cells develop competence spontaneously

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when appropriate physiological conditions induce expression of the genes responsible for DNA uptake, and they then efficiently take up and recombine linear DNA fragments. In this they differ from *E. coli* and many other bacteria, which become competent only after artificial cell-permeablizing treatments and which can only be transformed with self-replicating plasmids. Before describing the laboratory methods used to transform *H. influenzae*, we will consider how competence is induced and how DNA is taken up and recombined.

There are no recent reviews of competence or transformation in *Haemophilus*; however, several old reviews contain useful information ***(5–7)***. Recent reviews on DNA uptake and on the regulation of competence in diverse bacteria are those by Dubnau ***(8)*** and by Solomon ***(9)***; both include discussions of *H. influenzae*. For a discussion of the evolution of competence, see Redfield ***(10)***.

Not all strains of *H. influenzae* can be easily transformed with all genes. Derivatives of the completely sequenced laboratory strain Rd (also called KW20) are readily transformed, as are the standard serotype b strains. However, some clinical strains do not develop competence at all, at least under laboratory conditions. In such cases, electroporation can be used to introduce plasmids ***(11)***. Efficiency of transformation can also be limited by sequence differences between donor and recipient DNA.

1.3. Regulation of Competence

Many bacteria can develop natural competence, but regulation appears to be quite idiosyncratic, with different systems controlling competence development in different bacteria. In *H. influenzae*, competence development requires expression of the regulatory genes *sxy* (also called *tfoX*) ***(12)*** and *crp* ***(13)***, and an elevated intracellular concentration of cyclic AMP (cAMP) ***(14)***. These regulators act to induce expression of a number of genes, many under the transcriptional control of a promoter sequence called the competence regulatory element or CRE box ***(15)***.

The relationship between culture growth and development of competence is shown in **Fig. 1**. When *H. influenzae* cells are growing exponentially in a rich medium such as supplemented brain heart infusion (sBHI), competence is undetectable until their density reaches about 10^9 cells/mL, at which time growth begins to slow and the first competent cells can be detected by transformation assays. However, under these "spontaneous competence" conditions, only about 1% of the cells in the culture become competent, and transformation frequencies with chromosomal markers rarely exceed 10^{-4}. A similar level of competence is rapidly induced when 1 m*M* cAMP is added to exponentially growing cells ***(16)***. Several mutants are known whose spontaneous competence is greatly elevated ***(17,18)***; although their mode of action is not understood, they may provide useful tools for certain experiments.

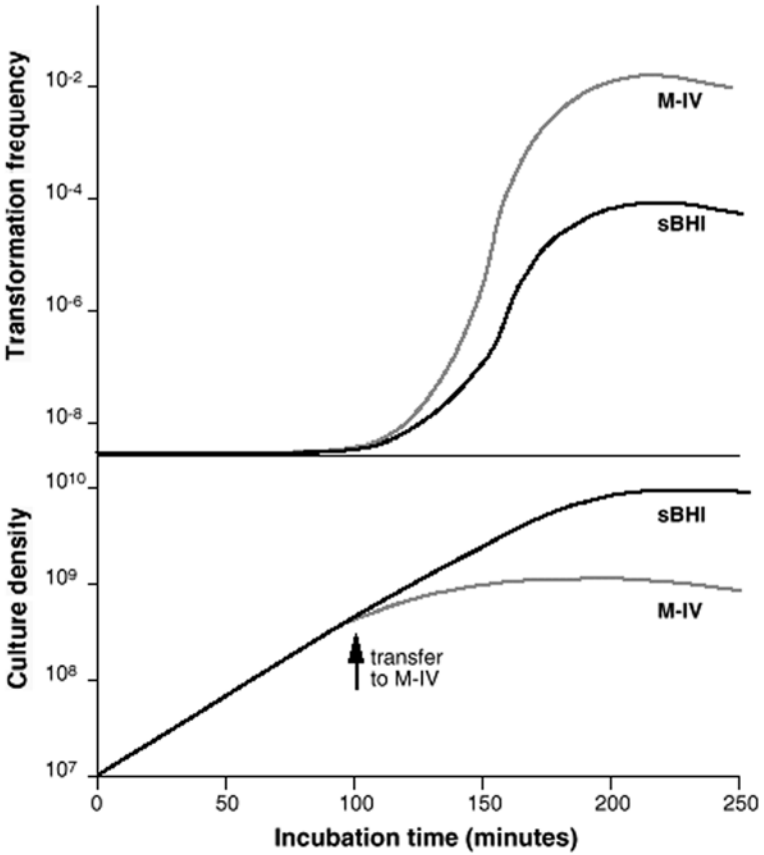

Fig. 1. *H. influenzae* growth and competence development in sBHI and M-IV. Black lines: cells growing in sBHI medium. Gray lines: cells in M-IV competence-induction medium.

Somewhat higher competence can be induced by temporarily shifting a rapidly growing, well-aerated culture to anaerobic growth conditions. However, the usual procedure for inducing competence is to transfer exponentially growing cells to a synthetic medium called M-IV, which lacks a number of components necessary for sustained growth, including sugars, nucleotides, and cofactors. In this medium, cell division quickly ceases but synthesis of new proteins continues. Incubation in M-IV for 100 min causes most of the cells in the culture to develop competence, giving transformation frequencies that average about 5×10^{-3} (range 10^{-3} to 2×10^{-2}).

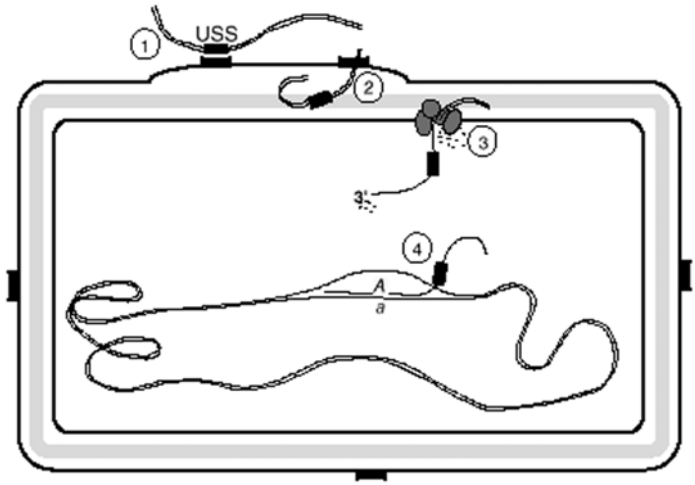

Fig. 2. Schematic drawing of the transformation process of *H. influenzae*. *Step 1*: Receptor binds DNA containing a USS. *Step 2*: Movement of DNA across outer membrane into periplasmic space. DNA becomes resistant to external and internal nucleases. *Step 3*: Translocation of DNA across inner membrane into the cytoplasm. The 5' leading strand is completely degraded whereas the 3' leading strand is degraded slowly. *Step 4*: Integration of the 3'-leading donor strand (with allele *A*) into the homologous region of the recipient chromosome (allele *a*).

1.4. DNA Uptake and Transformation

During DNA uptake in *H. influenzae* (**Fig. 2**), DNA is transported across the outer membrane and into the periplasmic space as intact duplex molecules ***(7)***. Once in the periplasm, DNA crosses the thin peptidoglycan layer and is then moved across the inner membrane into the cytoplasm. Only linear molecules can be transported into the cytoplasm; circular molecules remain in the periplasm, suggesting that that DNA must be threaded through a translocation complex in the inner membrane ***(19)***. Transport of DNA across the inner membrane is thought to be concomitant with degradation of the strand whose 5' end first enters the cytoplasm.

Competent *H. influenzae* cells are very proficient at recombination, and after DNA enters the cytoplasm, homologous strands are often integrated into the recipient chromosome. If donor and recipient DNA sequences differ slightly, heteroduplex regions may be corrected in either direction by mismatch repair enzymes, or may give rise to homoduplex chromosomes by DNA replication. However, recombination is very inefficient if sequences differ by more than a few percent.

1.5. Practical Issues

1.5.1. Amount of DNA Taken Up

Each competent cell can take up about 200 kb of DNA, of which about 20 kb will recombine with the chromosome, if homology permits. The rest is normally degraded before homologous sequences are found. Linkage studies indicate that single fragments as long as 100 kb may be taken up by competent cells. Usually cells take up multiple fragments (8–12 long fragments, or a larger number of shorter fragments). Uptake is usually saturated by a DNA concentration of 200 ng/mL; adding additional DNA will not increase the transformation frequency. This is not a kinetic saturation of the rate of uptake but an absolute saturation: once cells have taken up a certain amount of DNA, they do not take up any more. In experiments designed to measure competence, an excess of chromosomal DNA (1 μg/mL) is standard. Because cells usually take up multiple fragments, they may acquire two independent alterations if both are present in the DNA fragments provided. This can cause problems if the recipient gains an unwanted allele from donor DNA, but can also be exploited to create doubly altered strains in a single transformation.

1.5.2. Measuring Competence

The most sensitive measure of the competence of a culture is transformation frequency—the fraction of cells transformed when given a saturating amount of genetically marked DNA. This provides a very sensitive assay of competence; frequencies as low as 10^{-8} can be detected. Sensitivity may be limited by "bald patches" on plates, caused by very high backgrounds of nontransformed cells (*see* **Note 1**). A convenient DNA for competence assays is that of strain MAP7, which carries seven different chromosomal point mutations conferring antibiotic resistance. Competence may also be quantitated by uptake of radiolabeled DNA; this is less sensitive than transformation but provides a measure of competence that is independent of recombination. In vivo DNA labeling is inefficient, and it is usually easier to label DNA in vitro, either by nick translation of chromosomal DNA or by end labeling of short fragments. Labeling with ^{33}P provides a good compromise between high specific activity and long half-life. When the goal is to carry out a time-course analysis of the development of competence, DNA uptake should be stopped by addition of DNase I after 15 min; a shorter time may be used if greater precision is needed.

1.5.3. Transformation with Linear DNA Fragments

The issues to be addressed depend on the purpose of the transformation experiment. If the goal is to introduce a different allele into the chromosome, transformation may use either chromosomal DNA from a different strain, or a

linear DNA fragment produced by cloning or PCR. Transformation with chromosomal DNA is usually trouble-free. The presence of insertions or deletions of 10 kb or more does not normally reduce the transformation frequency by more than 10-fold, presumably because in chromosomal DNA, these heterologies usually occur in large fragments with extensive flanking homologous DNA. However, cloned or PCR product fragments present several problems. Transformation with short fragments (smaller than 1 kb) is usually quite inefficient, as these often are completely degraded before homology can be found. Short fragments carrying insertions or deletions will recombine poorly unless there is at least 1 kb of homologous sequence on each side of the heterology.

Another potential problem arises when transforming with a saturating amount of a pure DNA fragment (a plasmid insert or a PCR product), when cells may take up multiple copies of the same fragment. This often leads to integration of compound fragments and tandem repeats, presumably formed by an unidentified periplasmic ligase acting on the incoming fragments. To prevent this, it is best to use a limiting amount of transforming DNA in such experiments. The simplest procedure is to transform with several different DNA concentrations in parallel (e.g., 100 ng/mL, 10 ng/mL and 1 ng/mL), and to retain only colonies from the lowest concentration that produces transformants. For important constructions, it is also wise to check the transformants by Southern blotting or polymerase chain reaction (PCR), to ensure that they have the desired genotype.

1.5.4. Plasmid Transformation

Different issues apply if the goal is to introduce an autonomously replicating plasmid into a *H. influenzae* strain. The strategy used depends on whether the plasmid contains or lacks a segment homologous to the cell's chromosome, and on whether recombination with the chromosome is to be promoted or prevented. If the goal is to introduce a plasmid-borne allele into the chromosome, transform with the isolated insert or with plasmid linearized by cleavage in the vector, rather than with intact plasmid.

1.5.4.1. Plasmids with No Chromosomal Homology

Because the DNA uptake machinery cannot transport circular molecules across the inner membrane, the second step of DNA uptake must be circumvented by an osmotic shock using glycerol, which promotes passage of the intact plasmid into the cytoplasm. Plasmid molecules that have been linearized can be taken up, but they rarely yield plasmid-bearing transformants because of the exonucleolytic degradation that accompanies transport across the inner membrane. However, if a cell takes up two or more broken plasmid molecules, strands from these may sometimes recombine within the cell and regenerate an intact plasmid.

1.5.4.2. Transformation by Plasmids with Chromosomal Homology

Homologous recombination between incoming DNA and the chromosome is of little consequence if the plasmid and chromosome carry the same allele. However, if they carry different alleles, this recombination may either be exploited to transfer a chromosomal allele to the plasmid or a plasmid allele to the chromosome, or prevented to maintain the original alleles on the plasmid and chromosome. Plasmid-chromosome recombination can be promoted by cutting the plasmid in the region of DNA homology, by using no glycerol in the transformation, and by selecting for transformants expressing the antibiotic resistance encoded by the vector (not an antibiotic resistance in the insert). Although the transformation frequency will have been reduced by the cutting, a substantial fraction of the plasmids in the successful transformants will have acquired the chromosomal allele by recombinational repair. On the other hand, recombination between plasmid and host can be prevented by using the *rec-1* mutant strain DB117, which lacks the *H. influenzae* homolog of RecA and thus cannot carry out homologous recombination. If the *rec-1* mutation will interfere with subsequent manipulations, recombination can instead be limited by using only supercoiled DNA and glycerol-promoted transformation. However, the resulting transformants will need to be carefully screened to ensure that the plasmid retains its original allele.

Plasmids not grown in *H. influenzae* will lack *H. influenzae*-specific modification and thus be sensitive to intracellular cleavage by *H. influenzae's* restriction enzymes (*Hin*dII and *Hin*dIII in the Rd strain, others in other strains). This need not prevent successful transformation, but may reduce its efficiency. Cloned fragments and PCR products also lack appropriate modification but usually transform efficiently, presumably because linear DNA enters the cytoplasm as single strands, which are not substrates for restriction enzymes.

Transformation can also be used to integrate into the chromosome a plasmid that cannot replicate in *H. influenzae*. This is useful when constructing strains carrying null alleles and gene fusions, as it allows retention of a wild-type allele ***(20)***. To promote recombination between chromosomal and insert sequences, the plasmid may be linearized by restriction cleavage somewhere within the insert. However, Southern blotting should be used to check the structure of the resulting transformants.

1.5.4.3. Sequence-Specificity of DNA Uptake (Especially Its Relevance to Transformation)

Competent *H. influenzae* cells preferentially bind and take up DNA fragments containing the uptake signal sequence AAGTGCGGT (the USS, which is abundant in *H. influenzae* genomic DNA). The bias may be as strong as 100-fold for some fragments. However, the absence of USS from particular

genes or cloned fragments does not normally limit strain-construction experiments, for two reasons. First, fragments of genomic DNA are large enough that they usually carry many USS. Second, cells will readily take up purified fragments and plasmids with no USS, provided there are no competing fragments carrying it.

2. Materials

2.1. Preparation of Competent H. influenzae

1. Exponentially growing culture of the cells to be transformed, at a density of $OD_{600} \leq 0.3$. A 35-mL culture in a 500-mL flask at OD_{600} 0.3 is traditional, but larger or smaller volumes may be used, and $OD_{600} \leq 0.25$ is optimal. Keep the flask size large to promote aeration.
2. Vacuum filtration apparatus with sterile filter funnel (0.2µm pore size) (*see* **Note 2**).
3. M-IV medium, usually prepared fresh before each use, from the stock solutions outlined in **Table 1**. To 50 mL of solution 21 (or 5 mL of 10 × solution 21 and 45 mL dH_2O), add 0.5 mL each of solutions 22, 23, 24, and 40.
4. 250-mL flask.
5. Sterilized forceps (wiping with ethanol is sufficient).
6. Sterile 80% glycerol and sterile freezer tubes if cells are to be frozen.

2.2. Transformation

1. Chromosomal or plasmid DNA (100 µg/mL stock in Tris-EDTA (TE) buffer (10 m*M* Tris-HCl, pH 7.4, 1m*M* EDTA) (a simple miniprep method for chromosomal DNA is given in Chapter 3). If the DNA or the competent cells have not been tested previously, include MAP7 or other genetically marked DNA of known quality, as a positive control.
2. 1 mL competent cells for each transformation, and for the no-DNA control.
3. DNase I (100 µg/mL). Stock is prepared aseptically in sterile 50% glycerol and stored at −20°C.
4. Dilution solution (*see* Chapter 3).
5. Sterile culture tubes for transformations and dilutions.
6. sBHI agar plates with and without appropriate antibiotics.
7. Sterile 3-mm glass beads or spreader.

2.3. DNA Uptake Assays

2.3.1. Preparation of Radiolabeled DNA

1. End-labeling of short fragments: DNA (equivalent to 1–50 pmol of 5' termini), T4 polynucleotide kinase, T4 polynucleotide kinase buffer (10×), 50 pmol gamma-^{33}P ATP, 2500 Ci/mmol, 0.5 *M* EDTA, phenol-chloroform for extraction, and a Microspin G-50 column (Amersham).
2. Nick-translation of long fragment: DNA (0.25 µg), DNase I (10 ng/mL; 10^{-4} dilution of the DNase I stock used for transformations). Mix of 3 dNTPs (0.5 m*M* each of dCTP, dGTP, and dTTP), α-^{33}P dATP (or other α-labeled

Table 1
M-IV Competence Medium

Solution 21	
L-Aspartic acid	4.0 g
L-Glutamic acid	0.2 g
Furmaric Acid	1.0 g
NaCl	4.7 g
K_2HPO_4	0.87 g
KH_2PO_4	0.67 g
Tween-80	0.2 mL
Distilled water	850 mL

Adjust pH to 7.4 with 4 N NaOH. Add distilled water to 1 L. Dispense 100 mL per bottle; autoclave. Solution may appear cloudy after autoclaving but clears upon cooling.

Solution 22	
L-Cystine	0.04 g
L-Tyrosine	0.1 g
Dissolve in 10 mL of 1 N HCl at 37°C. Bring to 100 mL with distilled water and add:	
L-Citruline	0.06 g
L-Phenylalanine	0.2 g
L-Serine	0.3 g
L-Alanine	0.2 g
Filter-sterilize	

Solution 23	
CaCl2	0.1 *M* solution, autoclave.

Solution 24	
MgSO4	0.1 *M* solution, autoclave.

Solution 40
5% (w/v) vitamin-free casamino acids (Difco) in distilled water, autoclave.

deoxynucleotide; change the dNTP mix accordingly), *E. coli* DNA polymerase I (not Klenow fragment), *E. coli* DNA polymerase I buffer (10×) and EDTA, phenol-chloroform, and a G-50 column as above.

2.3.2. DNA Uptake Assay

For each sample of competent cells, 200 ng of ^{33}P-labelled DNA is required. Specific activity should be about 10^5 cpm/μg. DNA with higher specific activity may be diluted with unlabeled DNA. The following components are

required: competent cells, DNase I (1 mg/mL in 50% glycerol; note that this is 10-fold more concentrated than the stock used for transformations), cold 5 *M* NaCl, cold M-IV supplemented with 1 *M* NaCl (20 mL M-IV + 5 mL of 5 *M* NaCl), room-temperature M-IV, and scintillation vials, fluid, and counter.

3. Methods

3.1. Induction of Competence

1. Place 10 mL of room temperature M-IV into a 250-mL flask and set aside.
2. When the culture to be made competent reaches OD_{600} 0.20–0.25, remove 10 mL to the filter funnel and collect cells by filtering under gentle vacuum (*see* **Note 3**).
3. As soon as the sBHI has been drawn through the filter, replace it with 10 mL M-IV and continue filtration.
4. Release vacuum and remove top of filter housing. Lift the filter from the apparatus with sterilized forceps and place it in the flask of M-IV. The cells will quickly resuspend from the filter, which may remain in the flask.
5. Shake culture at 100 rpm 37°C, for 100 min. At this time the culture will have achieved the maximum level of competence and will maintain it for at least 1 h at 37°C.
6. If cells are to be frozen, add 1 mL of 80% glycerol for each 4 mL of cells and store at −80°C. Aliquots of 1.0 mL are convenient.

3.2. Transformation

1. If using previously frozen competent cells, first pellet the cells and remove the M-IV and glycerol by aspiration. Resuspend the pellet in an equal volume of fresh M-IV (*see* **Note 4**).
2. To 1 mL of competent cells add 1 μg of chromosomal DNA or a smaller amount of a cloned DNA and mix gently. Incubate cells with DNA at 37°C for 15 min. Modifications:
   a. If transforming with closed-circular plasmid, extend the incubation to 30 min. Then add sterile 80% glycerol to a final concentration of 30–32%, mix, and leave at room temperature for 10 min. Dilute and plate as usual.
   b. If carrying out a time-course of competence, terminate DNA uptake by adding 10 μL of DNase (100 mg/mL) and incubating for an additional 5 min at 37°C before plating.
   c. Resistance to certain antibiotics requires expression time. Cells can be plated immediately onto kanamycin or novobiocin plates. For other antibiotics, add two volumes of sBHI to the transformation mixture and incubate at 37°C for the time indicated in **Table 2** before plating.
3. Plating: When transforming with chromosomal DNA, expect a transformation frequency of about 10^{-3}–10^{-2}. Cloned and PCR-product DNA may give higher or lower transformation frequencies, depending on the molecule and the concentration used. If using chromosomal DNA and standard conditions, plate 100 μL of 10^{-5} and 10^{-6} dilutions on sBHI plates without antibiotic, and 100 μL of 10^{-2}, 10^{-3}, and 10^{-4} dilutions on antibiotic plates.

Table 2
Expression Times Needed for Development of Antibiotic Resistances

Antibiotic	Concentration	Expression time (min)
Kanamycin	7μg/mL	0
Novobiocin	2.5 μg/mL	0
Spectinomycin	20 μg/mL	70
Naladixic acid	3 μg/mL	80
Chloramphenicol	2 μg/mL	90
Tetracycline	5 μg/mL	90
Streptomycin	2.5 μg/mL	100

4. Controls: Transform cells with 1 μg of MAP7 DNA and select for resistance to the same antibiotic, or to novobiocin. If using a non-Rd strain, transform the DNA into M-IV competent Rd cells (strain KW20). As a negative control, plate cells that have not been incubated with DNA on the antibiotic plates.
5. Incubate plates at 37°C for 16–24 h (48 h if selecting for chloramphenicol resistance).
6. The degree of competence of the cells is usually measured as the transformation frequency obtained with an excess of chromosomal DNA. This is calculated by dividing the number of transformants per milliliter (calculated from colony numbers on antibiotic plates) by the total number of cells per milliliter (calculated from colony numbers on plain plates). If the transforming ability of the DNA is in question, a nonsaturating concentration should be used, ideally less than one molecule per cell if a pure fragment is being evaluated. The DNA's transformation efficiency can then be calculated by dividing the number of transformants per milliliter (calculated from colony numbers on antibiotic plates) by the amount of DNA used to transform this volume of cells.

3.3. DNA Uptake Measurement—End-Labeling Short DNA Fragments

1. Combine DNA (1–50 pmol of 5' ends), 20 U T4 polynucleotide kinase, 50 pmol gamma ^{33}P-ATP, buffer and H_2O to a final volume of 50 μL.
2. Incubate at 37°C for 30 min.
3. Stop reaction by adding 1 μL of 0.5 *M* EDTA and 50 μL of dH_2O. Extract once with phenol-chloroform.
4. To remove unincorporated nucleotides, load onto a G-50 column and collect flow-through.

3.4. DNA Uptake Measurement—Nick Translation

1. Combine 0.25 μg DNA, 1 μL DNase I, 2.5 μL dNTP mix, buffer, 1 μL DNA polymerase (5–15 units), and dH_2O to a final volume of 25 μL.
2. Incubate at 12–14°C for 15–45 min.
3. Stop reaction and remove unincorporated nucleotides as above.

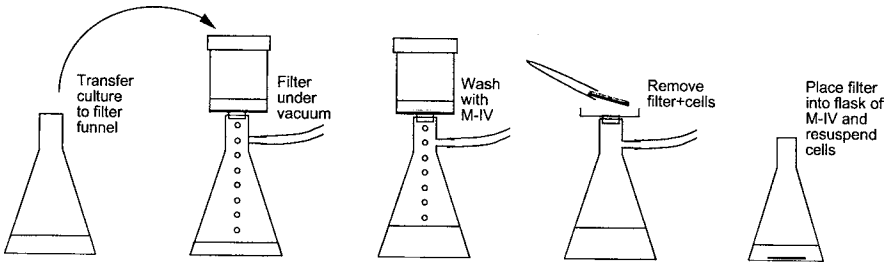

Fig. 3. Preparation of competent cells by filtration.

3.5. Measuring DNA Uptake

1. Place 200 ng of labeled DNA and 1 mL of cells to be tested in a microfuge tube (assays should be done in duplicate). Include as negative controls noncompetent cells and tubes with no DNA.
2. Roll or shake gently for 10 min at 37°C.
3. Stop uptake by adding 50 µL of DNase I and placing on ice for 5 min.
4. Add 100 µL of 5 *M* NaCl, vortex, and pellet cells at 15,000*g* for 1 min.
5. Discard supernatant and resuspend cells in M-IV + NaCl.
6. Pellet cells again, discard supernatant, and resuspend cells in 200 µL of room temperature M-IV.
7. Transfer cells to a scintillation vial and add 1 mL scintillation fluid.
8. Calculate DNA uptake by first correcting uptake for background counts in the negative control tubes, then multiply corrected counts taken up by the specific activity of the DNA.

4. Notes

1. Bald patches can interfere with competence assays. These are areas of small or missing colonies, often centrally located on sBHI agar plates containing antibiotic. They are commonly seen when large numbers of antibiotic-sensitive cells have been plated to detect a small number of resistant cells. In some cases no colonies at all are seen on a plate that should contain thousands of colonies. Bald patches are most troublesome when using kanamycin selection. The biological cause of these patches is unknown, but they interfere with colony counts and thus can limit the ability to measure transformation frequencies of moderately competent cultures. Use of MBI agar appears to reduce the problem.
2. The disposable filter funnels from Nalgene (illustrated in **Fig. 3**) are very convenient, especially if several cultures are to be made competent. A non-disposable filtration apparatus may also be used; this is less convenient, as it must be presterilized with the filter in place. Use only filters with 0.2-µm pores, because *H. influenzae* cells will pass through 0.45 µm pores. If the volume to be filtered is too large or the culture is too dense, the cells will clog the filter. If 0.2-µm filters are not available, or if large or very small volumes of competent cells are needed,

exponentially growing cells can instead be collected and washed by centrifugation at 2000*g* for 5 min at room temperature.

3. Cells must be in exponential growth when they are filtered. Do not let the culture sit for even a few min before filtering; if the cells are ready but you are not, dilute the culture with additional sBHI to keep it in exponential growth below OD_{600} 0.3. A larger volume of cells at a lower density may be used if more convenient.
4. The transformation frequency of competent cells may decrease slightly upon freezing. This does not usually cause problems, but freshly prepared cells should be used if highly competent cells are needed.

References

1. Thorne, G. M. and Farrar, W. E. Jr. (1975) Transfer of ampicillin resistance between strains of *Haemophilus influenzae* type B. *J. Infect. Dis.* **132,** 276–281.
2. Stuy, J. H. (1979) Plasmid transfer in *Haemophilus influenzae*. *J. Bacteriol.* **139,** 520–529.
3. Dimopoulou, I. D., Jordens, J. Z., Legakis, N. J., and Crook, D. W. (1997) A molecular analysis of Greek and UK *Haemophilus influenzae* conjugative resistance plasmids. *J. Antimicrob. Chemother.* **39,** 303–307.
4. Deich, R.A. and Green, B. A. (1987) Mobilization of *Haemophilus influenzae* chromosomal markers by an Escherichia coli F' factor. *J. Bacteriol.* **169,** 1905–1910.
5. Kahn, M. E. and Smith, H. O. (1984) Transformation in Haemophilus: a problem in membrane biology. *J. Membrane Biol.* **81,** 89–103.
6. Goodgal, S. H. (1982) DNA uptake in Haemophilus transformation. *Ann. Rev. Genet.* **16,** 169–192.
7. Smith, H. O., Danner, D. B., and Deich, R. A. (1981) Genetic transformation. *Ann. Rev. Biochem.* **50,** 41–68.
8. Dubnau, D. (1999) DNA uptake in bacteria. *Ann. Rev. Microbiol.* **53,** 217–244.
9. Solomon, J. M. and Grossman, A. D. (1996) Who's competent when: regulation of natural genetic competence in bacteria. *Trends Genet.* **12,** 150–155.
10. Redfield, R.J. (1993) Genes for breakfast: the have your cake and eat it too of transformation. *J. Hered.* **84,** 400–404.
11. Mitchell, M. A., Skowronek, K., Kauc, L., and Goodgal, S. H. (1991) Electroporation of *Haemophilus influenzae* is effective for transformation of plasmid but not chromosomal DNA. *Nucleic Acids Res.* **19,** 3625–3628.
12. Williams, P. M., Bannister, L. A., and Redfield, R. J. (1994) The *Haemophilus influenzae sxy-1* mutation is in a newly identified gene essential for competence. *J. Bacteriol.* **176,** 6789–6794.
13. Chandler, M. S. (1992) The gene encoding cyclic AMP receptor protein is required for competence development in *Haemophilus influenzae* Rd. *Proc. Natl. Acad. Sci. USA* **89,** 1626–1630.
14. Dorocicz, I., Williams, P., and Redfield, R. J. (1993) The *Haemophilus influenzae* adenylate cyclase gene: cloning, sequence and essential role in competence. *J. Bacteriol.* **175,** 7142–7149.
15. Karudapuram, S. and Barcak, G. J. (1997) The *Haemophilus influenzae dprABC* genes constitute a competence- inducible operon that requires the product of the *tfoX* (*sxy*) gene for transcriptional activation. *J. Bacteriol.* **179,** 4815–4820.

16. Wise, E. M., Alexander, S., and Powers, M. (1973) Adenosine 3':5'-cyclic monophosphate as a regulator of bacterial transformation. *Proc. Natl. Acad. Sci. USA* **70,** 471–474.
17. Redfield, R. J. (1991) *sxy-1*, a *Haemophilus influenzae* mutation causing greatly enhanced competence. *J. Bacteriol.* **173,** 5612–5618.
18. Ma, C. and Redfield, R. J. (2000) Point mutations in a peptidoglycan biosynthesis gene cause competence induction in *Haemophilus influenzae*. *J Bacteriol.* **182,** 3323–3330.
19. Barany, F., Kahn, M. E., and Smith, H. O. (1983) Directional transport and integration of donor DNA in *Haemophilus influenzae* transformation. *Proc. Natl. Acad. Sci. USA* **80,** 7274–7278.
20. Gwinn, M. L., Stellwagen, A. E., Craig, N. L., Tomb, J. F., and Smith, H. O. (1997) In vitro Tn7 mutagenesis of *Haemophilus influenzae* Rd and characterization of the role of *atpA* in transformation. *J. Bacteriol.* **179,** 7315–7320.

5

Diagnosis of Infection

Alex van Belkum and Loek van Alphen

1. Introduction

With the advent of effective vaccination strategies, life-threatening *Haemophilus influenzae* infections are now rare in developed countries ***(1)***. However, *H. influenzae* can still cause infection at a variety of anatomical sites, especially the upper and the lower airway. For this reason, adequate diagnosis of *H. influenzae* infections remains important. Classical microbiological diagnosis relies on growth on blood-based agar media, including chocolate agar or brain heart infusion (BHI) agar supplemented with Levinthals blood, hemoglobin, or hemin. In addition, *H. influenzae* can be distinguished from its closest relatives because it is incapable of producing porphyrin ***(2)***. Modern developments allow for testing without a cultivation step. The most direct and simple way of detection and identification without culture is provided by commercially available latex agglutination tests ***(2)***. Antibody-coated latex particles are mixed directly with clinical samples and agglutinate if *H. influenzae* is present. Sophisticated means for direct *H. influenzae* detection and identification are now available in the medical microbiology laboratory, including immunological and molecular methods. They include specific DNA probing and ultrasensitive nucleic acid amplification. This chapter surveys several of the more frequently applied culture-based, immunological and molecular approaches to diagnosis.

2. Materials

2.1. Classical Microbiology

1. Clinical sample.
2. Gram staining reagents: Crystal violet solution, Grams Iodine and 0.1% basic fuchsin (Becton-Dickinson, Sparks, MD).

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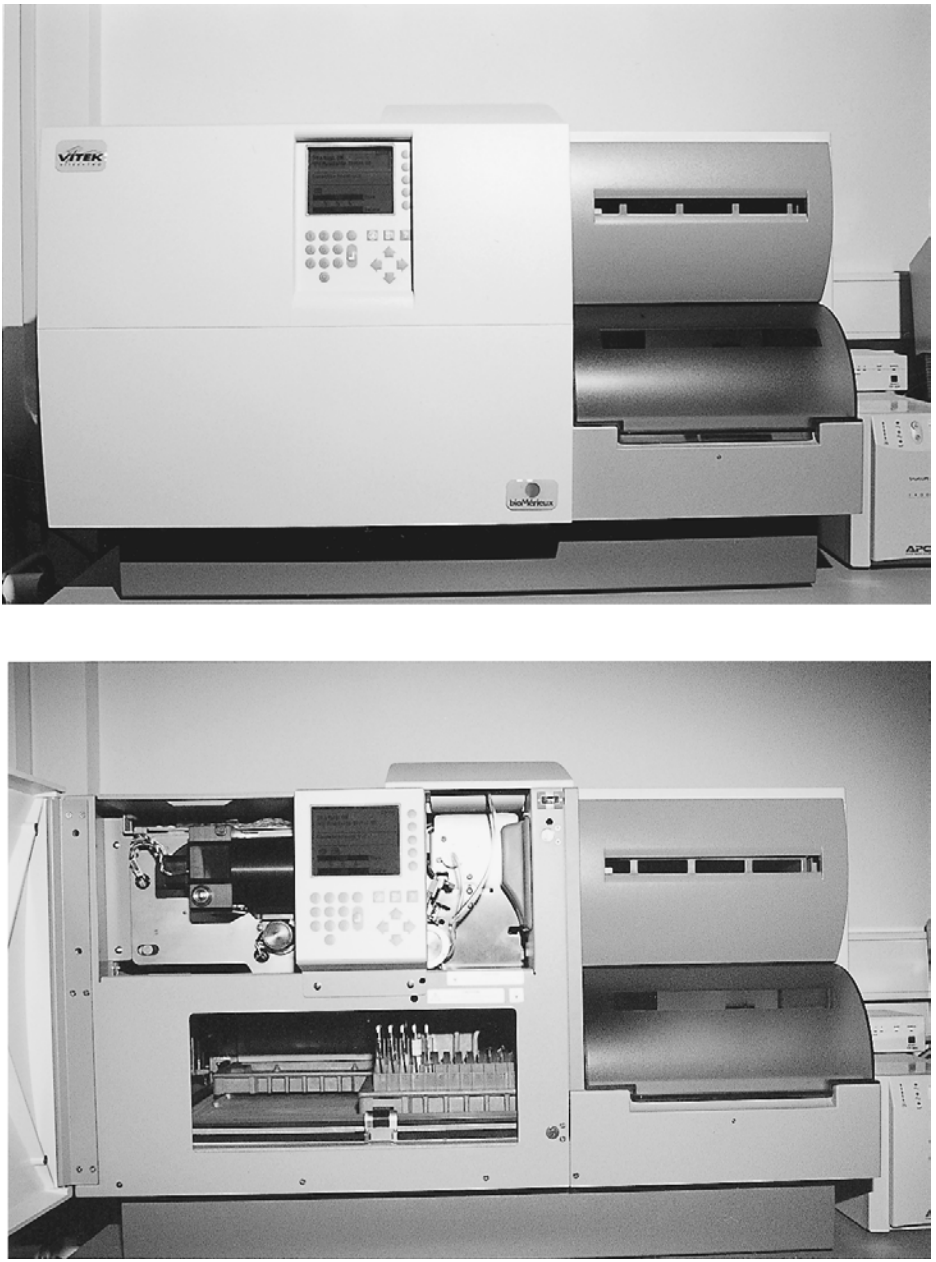

Fig. 1. A Vitek 2 machine. The upper picture shows the exterior features of the machine, including the personal computer for data storage and interpretation. The right-hand side of the machine consists of the loading bay (dark plastic lid), with a carousel above. In this carousel the cartridges containing bacteria for susceptibility

3. Blood agar medium (Becton Dickinson).
4. Chocolate agar medium (Becton Dickinson).
5. V-factor (NAD), X-factor (hemin), and X/V factor disks (Becton Dickinson).
6. Physiological salt solution (0.9% NaCl in distilled water).
7. ISO agar: 36.8 g of Balanced Sensitivity Test Medium (BSTM, Difco, Detroit, MI, USA) dissolved in 1 L dH_2O and autoclaved for 15 min at 121°C. After cooling down to 50°C, 9 mL plates are poured which can be stored in the dark at 4°C for approx 1 mo.

2.2. Selective Enrichment Culture

1. N-Acetyl L-cystine–sodium citrate buffer pH.
2. Chocolate agar containing 300 µg bacitracin /mL (CHOC-BAC medium).
3. NAG medium: Blood agar base no. 2 (40 g/L; Oxoid, Basingstoke, Hampshire, UK) including 0.1% N-acetyl-D-glucosamine (Centrafarm BV, Etten-Leur, The Netherlands), 30-mg/L bacitracin (Centrafarm BV), 10 mg/L hemin (Sigma Chemicals, St. Louis, MO) and 10-mg/L NAD (Sigma Chemicals).
4. Cefsulodin disk (30 µg, Rosco Diagnostics).
5. Cefsulodin chocolate blood agar: Blood agar base no. 2 (8 g/200 mL; CM27 Oxoid) is suspended in water and autoclaved for 15 min at 121°C, allowed to cool to 75°C, when 14 mL defibrinated horse blood is added. The mixture is incubated at 75°C until chocolating has taken place. After cooling down to 50°C, cefsulodin is added at an amount of 8 mg/L.

2.3. Automated Procedures in Microbiology

1. Vitek I machine (bioMerieux, Lyon, France; *see* **Fig. 1**).
2. 0.45% NaCl solution.
3. Spot indole reagent: *p*-dimethylaminocinnamaldehyde (1%) in 10% HCl.
4. Catalase reagent: 3% hydrogen peroxide (store away from light).
5. Vitek *Neisseria Haemophilus* identification (NHI) cards (bioMerieux).

2.4. Classical Serotyping

1. *H. influenzae* antisera: Lyophilized polyclonal rabbit antisera, either polyvalent or specific for types a–f, containing 0.02% thiomersal as preservative (Difco, Becton-Dickinson, Sparks, MD).
2. Agglutination slides.
3. Applicator sticks.
4. Sterile water.
5. Sterile 0.85% NaCl.

Fig. 1. *(continued)* testing or species determination are analyzed spectrophotmetrically every 15 min. to monitor for growth or fluorescence. The left and largest part of the machine is essentially a pipeting station. Cartridges, five of which are shown, are inoculated and prepared for analysis. The machine covers approximately 1 m of bench space.

2.5. Monoclonal Antibodies

1. Phosphate-buffered saline (PBS), pH 7.2.
2. Methanol (Sigma).
3. Monoclonal antibody 8BD9 against the outer membrane protein P6 ***(3)***.
4. PBS-BSA-Tw: Phosphate buffered saline with 0.5% bovine serum albumin (BSA; Sigma) and 0.05% Tween-20.
5. Peroxidase conjugated rabbit anti-mouse antibodies (Dakopatt, Glostrup, Denmark).
6. Substrate: 4 mg of 3-amino-9-ethylcarbazole dissolved in 0.5 mL N,N-dimethylformamide (DMF) to which 9.5 mL H_2O needs to be added. After adjustment of the pH to 5.0, add 100 µL of 1% H_2O_2.
7. 0.5% methylene blue in distilled water.

2.6. DNA Isolation

1. Lysis buffer I: Add 120 g guanidine isothiocyanate and 2.6 g Triton X-100 to 100 mL of 0.1 *M* Tris-HCl, pH 6.4, and 22 mL of 0.2 *M* EDTA.
2. Lysis buffer II: Add 120 g guanidine isothiocyanate to 100 mL of 0.1 *M* Tris-HCl, pH 6.4.
3. Celite suspension: Suspend 1 g of Celite (Acros, Geel, Belgium) in 5 mL of dH_2O and add 50 µL 37% HCl.
4. Acetone (Merck).
5. 10 m*M* Tris-HCl, pH 8.0.

2.7. DNA Probe Testing

1. Bacterial monoculture.
2. Accuprobe *H. influenzae* (art. nr. 2825, DPC, The Netherlands).
3. Accuprobe universal reagent 1 (art. nr. 2800, DPC).
4. Accuprobe universal reagent 2 (art. nr. 2805, DPC).
5. GenProbe detection reagent (art. nr. 1791,DPC).
6. Leader™ 50 GenProbe luminometer.

2.8. Specific DNA Amplification Assays

1. PCR buffer: 10 m*M* Tris-HCl, pH 8.3; 50 m*M* KCl, 2 m*M* $MgCl_2$, gelatin (to a final concentration of 0.001%), deoxyribonucleotide triphosphate (dNTP; 0.2 m*M* of each), primers (see pairs described below; 0.35 µ*M* of each) and AmpliTaq Gold polymerase (2.5 U; PE Biosystems).
2. PCR primers and probes:
   a. Set 1
      HI-I: 5'-CGTTTGTATGATGTTGATCCAGACT-3'
      HI-II: 5'-TGTCCATGTCTTCAAAATGATG-3'
      HI-III (Probe): 5'-GTGATTGCAGTAG-GGGATTCGCGCTTTGCAG-3'.
   b. Set 2
      HI-IV: 5'-ACTTTTGGCGGTTACTCTGT-3'
      HI-V: 5'-TGTGCCTAATTTACCAGCAT-3'

HI-VI (Probe): 5'-GCATATTTAAAT-GCAACACCAGCTGCT-3'.
All primers are commercially synthesized. For a list of alternative primers and probes, *see* **Table 1**.

3. Thermal cycler: BioMed model 60 (BioMed, Germany).
4. Electrophoresis supply: Pharmacia (Roosendaal, The Netherlands).
5. Nylon filters: ZetaProbe (BioRad, Veenendaal, The Netherlands).
6. Hybridization buffer and probe labeling: 90 m*M* sodium citrate, pH 7.8, and 0.9 *M* NaCl (6 × SSC), 0.5% sodium dodecyl sulphate (SDS), 0.1% Ficoll-polyvinylpyrrolidone-bovine serum albumin (5 × Denhardts solution), sheared and denatured salmon sperm DNA (100 µg/mL), and labeled probe (approx 20 pmol per 10 mL hybridization buffer). Probes can be conveniently labeled using the ECL™ 3'-oligonucleotide labeling system according to the manufacturer's instructions (RPN2130, Amersham Pharmacia Biotech, Buckinghamshire, UK). Oligonucleotides are labeled by 3'-terminal incorporation of fluorescein-11-dUTP catalyzed by terminal transferase. Labeled probe can be detected by incubation with anti-fluorescein–horseradish peroxidase conjugate and subsequent staining with substrates as described in **Subheading 2.5.6.** Chemiluminescence assays are available as well.
7. Washing buffer: 30 m*M* sodium citrate, pH 7.8, 0.3 *M* NaCl (2x SSC), 0.1% SDS.

2.9. General Primer Mediated PCR Approaches

1. PCR ingredients: *see* **Subheadings 2.7.1., 2.7.2.,** and **2.7.4.**
2. PCR primers:
   DG74: 5'-AGGAGGTGATCCAACCGCA-3';
   RDR080: 5'-AACTGGAG-GAGGAAGGTGGGAC-3'.
3. Probes:
   H. influenzae: 5'-TACCAGAATAGATAGCT-3';
   Staphylococcus aureus: 5'-GCCGGTGGAGTAACCTTTTAGGGAC-3';
   Pseudomonas aeruginosa: 5'-CGTGAATCAGAATGTCA-3'
   See **Table 2** for additional examples of general primer pairs.
4. Cloning requirements: TA Cloning Kit, version E (InVitrogen, Leek, The Netherlands) including plasmid pCR1 and competent *Eschericia coli* JM109 cells; DNA ligase (Boehringer Manheim, Germany); LB agar containing 50 µg ampicillin/mL and IPTG and X-Gal.
5. Nylon filters: Hybond N^+ (Amersham, Buckinghamshire, UK).
6. Hybridization and washing buffers: *see* **Subheadings 2.7.7.** and **2.7.8.**
7. DNA sequencing: Prism Ready Reaction Dye Di-deoxy terminator cycle sequencing kit (Amersham, Gouda, The Netherlands); ABI 373 DNA sequencer (PE Biosystems, Gouda, The Netherlands); Basic Local Alignment Search Tool (BLAST) software ***(4)***.

3. Methods

The detection of *H. influenzae* can be based on direct staining of bacterial cells present in clinical material, but definitive identification usually requires

Table 1
Molecular Probes Used for the Identification of *Haemophilus* spp.

Probe type	Sequence motif	Procedure	Specificity	Ref.
5-Kbp DNA fragment	PBP genes	Dotblot	*Hinf*	***(17)***
		Colony lift	*Haeg*	***(18)***
Total genomic DNA	Capsulation genes	*In situ* hybridization	*Hinf, Hpar Hhae*	***(19)***
PUO38 plasmid	Partial 16S rRNA sequence	Southern, RFLP	*Hinf* serotype b	***(22)***
Oligonucleotide	HMW-1/2	Liquid chemiluminescence	*Hinf*	***(15)***
Adhesin clones[a]	Hia gene	Southern	*Hinf*	***(20)***
Oligonucleotide	Partial 16S rRNA sequence	Fluorescent *In situ* hybridization	*Hinf*	***(21)***

Note: Since the description of the whole genome sequence for *H. influenzae* Rd, several other probes have been suggested ***(43–47)***.

[a]These clones are mutually exclusive: if a strain is positive for one, it will be negative for the other probe; approx 5% of all strains will be double negative. *Hinf*: *H. influenzae*; *Haeg*: *H. aegyptius*; *Hpar*: *H. parainfluenzae*; *Hhae*: *H. haemolyticus*.

Table 2
Survey of PCR Primers and Probes Used for Specific Detection and Identification of DNA from *Haemophilus influenzae* and Some of its Clinically Relevant Characteristics

Target gene	Primer code (p: probe)	Primer /probe sequence5'→ 3'	Length of primer or product	Length of PCR product	Specificity[a]	Year	Ref.
Encapsulation-Associated Targets							
*Bex*A (capsule protein)	HI-I	CGTTTGTATGATGTTGATCCAGACT	25	343	Hinf (exc. non-typeables)	1990	***(9)***
	HI-II	TGTCCATGTCTTCAAAATGATG	22				
	p: HI-III	GTGATTGCAGTAGGGGATTCGCGCTTTGCAG	31	associated			
Capsule genes[b]	a1	CTACTCATTGCAGCATTTGC	20	250	*Hinf* serotype a	1994	***(32)***
	a2	GAATATGACCTGATCTTCTG	20				
	p: a3	AGTGGACTATTCCTGTTACAC	21				***(29)***
	b1	GCGAAAGTGAACTCTTATCTCTC	23	480	*Hinf* serotype b		
	b2	GCTTACGCTTCTATCTCGGTGAA	23				
	p: b3	ACCATGAGAAAGTGTTAGCG	20				
	c1	TCTGTGTAGATGATGGTTCA	20	250	*Hinf* serotype c		
	c2	CAGAGGCAAGCTATTAGTGA	20				
	p: c3	TGGCAGCGTAAATATCCTAA	20				
	d1	TGATGACCGATACAACCTGT	20	150	*Hinf* serotype d		
	d2	TCCACTCTTCAAACCATTCT	20				
	p: d3	CTCTTCTTAGTGCTGAATTA	20				
	e1	GGTAACGAATGTAGTGGTAG	20	1350	*Hinf* serotype e		
	e2	GCTTTACTGTATAAGTCTAG	20				
	p: e3	CAGCTATGAACAAGATAACG	20				
	f1	GCTACTATCAAGTCCAAATC	20	450	*Hinf* serotype f		
	f2	CGCAATTATGGAAGAAAGCT	20				
	p: f3	AATGCTGGAGTATCTGGTTC	20				

(Continued)

Table 2 *(Continued)*

Target gene	Primer code (p: probe)	Primer /probe sequence5'→ 3'	Length of primer or product	Length of PCR product	Specificity[a]	Year	Ref.
Outer-Membrane Protein Gene							
Outer membrane	HI-IV	ACTTTTGGCGGTTACTCTGT	20	273	*Hinf*, *Hpar*	1990	***(9)***
Protein P6 gene	HI-V	TGTGCCTAATTTACCAGCAT	20		*Hhae, Haeg*		
	p: HI-VI	GCATATTTAAATGCAACACCAGCTGCT	27				
Outer membrane	F1	AACTTTTGGCGGTTACTCTG	20	351		1995	***(30)***
Protein P6 gene ***(31)***	R1	CTAACACTGCACGACGGTTT	20				
	p: HI[c]	TAAATATGACATTACTGGTG	20		*Hinf*		
	p: HP[c]	CAAATTCAACATCGAAGG	18		*Hpar*		
Ribosomal Gene Targets[d]							
16S rDNA	DG74	AGGAGGTGATCCAACCGCA	19	370	*Hae* spp.	1994	***(38)***
	RDR080	AACTGGAGGAAGGTGGGGAC	20				
	p:RDR125[e]	GGAGTGGGTTGTACCAGAAGTAGAT	25				
	p: Hinfspec[e]	TACCAGAATAGATAGCT	17		*Hinf*	2000	***(37)***
16S rDNA	pm27	GGATTAGATACCCTGGTAGTCC	22	608	*Hinf*	1999	***(39)***
	pm8	CAAGGCCCGGGAACGTATTC	20				
	p: pm37	ACTCTGGCACCCGTAGCTAACGTGA	25				
16S rDNA	common	CTACGCATTTCACCGCTACAC	21	490	*Hinf*	1999	***(36)***
	*Hinf*spec	CGTATTATCGGAAGATGAAAGTGC	24			2000	***(35)***
23S rDNA	FOR6	GCGATTTCYGAAYGGGGRAACCC	23	330	*Hinf*	2000	***(42)***
	REV10	TTCGCCTTTCCCTCACGGTACT	22				
	p: P	GTGAGGAGAATGTGTTGGGAAG	22				
Virulence Gene Targets[f]							
LgtC	Hi 4-1a	TCATTGTCTGACTGACAGTC	20	138	*Hinf* and others	1997	***(46)***
	Hi 4-1b	CGTTAATCAGAGATAATTTC	20				
	p: Hi 4-1c	GTCTGTCTGTCTGTCTGTCT	20				

Table 2 *(Continued)*

Target gene	Primer code (p: probe)	Primer /probe sequence5'→ 3'	Length of primer or product	Length of PCR product	Specificity[a]	Year	Ref.
Lic3	Hi 4-2a	ATTACCTGCAATAATGACAG	20	178	*Hinf* and others		
	Hi 4-2b	TATTCAATGAACGGTAGAAT	20				
	p: Hi 4-2c	CAATCAATCAATCAATCAAT	20				
Lic2	Hi 4-3a	CCTCTTATATTATGTAATAT	20	142	*Hinf*		
	Hi 4-3b	TTTAGTTTCTTTAATGCGTA	20				
	p: Hi 4-3c	CAATCAATCAATCAATCAAT	20				
Hemoglobin receptor	Hi 4-4a	CTAGTTGTTCAGAAACATTA	20	134	*Hinf* and others		
	Hi 4-4b	TAAATGCAAGCATAGCCTAT	20				
	p: Hi 4-4c	TTGGTTGGTTGGTTGGTTGG	20				
Hemoglobin receptor	Hi 4-5a	CTAGTTGTTCAGAAACATTA	20	130	*Hinf*		
	Hi 4-5b	GGCAGGTGTTGCTTATGCAG	20				
	p: Hi 4-5c	TTGGTTGGTTGGTTGGTTGG	20				
Bacillus homolog	Hi 4-6a	ATAATTGGTGAACCTAAAAT	20	74	*Hinf* and others		
	Hi 4-6b	TTTCCAGTCATAAAAATACC	20				
	p: Hi 4-6c	TTTATTTATTTATTTATTTA	20				
Hemoglobin receptor	Hi 4-7a	CTAATTGTTCAGAAACATTA	20	198	*Hinf* and others		
	Hi 4-7b	TAAATGCAAGCACAGTCTAT	20				
	p: Hi 4-7c	TTGGTTGGTTGGTTGGTTGG	20				
Methyltransferase	Hi 4-8a	TTTCGGGTTAATTTGGTGT	20	130	*Hinf* and others		
	Hi 4-8b	AATTTATTTTAAACGCAATA	20				
	p: Hi 4-8c	TGACTGACTGACTGACTGAC	20				
Unknown	Hi 4-9a	AAAATGAAAAGGATCTATAC	20	114	*Hinf* and others		
	Hi 4-9b	ACTACCGCAACGGTTTTATT	20				
	p: Hi 4-9c	TTGGTTGGTTGGTTGGTTGG	20				

(Continued)

Table 2 *(Continued)*

Target gene	Primer code (p: probe)	Primer /probe sequence5'→ 3'	Length of primer or product	Length of PCR product	Specificity[a]	Year	Ref.
YadA homolog	Hi 4-10a	GACAGATGAAAAGAAAAGAT	20	150	*Hinf*		
	Hi 4-10b	TATAATATGTTTTATTACAA	20				
	p: Hi 4-10c	TTGCTTGCTTGCTTGCTTGC	20				
Lic1	Hi 4-11a	TAAAAATGAATACAAAAATG	20	118	*Hinf*		
	Hi 4-11b	AAGTTTTAACAAATCCTACA	20				
	p: Hi 4-11c	CAATCAATCAATCAATCAAT	20				
Hemoglobin receptor	Hi 4-12a	AACGGCAAGTGTTGCTTATG	20	126	*Hinf*		
	Hi 4-12b	CTAGTTGTTCAGAAGCATTA	20				
	p:Hi 4-12c	TTGGTTGGTTGGTTGGTTGG	20				
Antimicrobial-Resistance Gene Targets							
Ampicillin-resistance genes[g]	TEM(321)	TGGGTGCACGAGTGGGTTAC	20	526	TEM β-lact	1994	***(27)***
	TEM(846)	TTATCCGCCTCCATCCAGTC	20				
	ROB(419)	ATCAGCCACACAAGCCACCT	20	692	ROB β-lact		
	ROB(1110)	GTTTGCGATTTGGTATGCGA	20				
	p:	subfragment of cloned β-lactamase gene		±400			

[a]The specificity of the test determines which *Haemophilus* species is recognized; the PCRs specific for one of the capsular serotypes enabled discrimination of strains belonging to the species *H. influenzae*.

[b]The probes described can be used in hybridization testing, but also as primers in hemi-nested PCR tests. The amplicons can be further identified by restriction fragment length polymorphism (RFLP) analysis using restriction enzymes such as *Taq*I, *Eco*RI or *Ms*eI.

[c]The probe used determines which species of *Haemophilus* will be detected: *H. influenzae* or *H. parainfluenzae*.

[d]Assays described here aim specifically at either the small subunit ribosomal RNA gene (16S rDNA) or the large subunit ribosomal RNA gene (23S rDNA). Mostly, general primers are used, although sometimes one or two species-specific primers are used. Additional general primer mediated rDNA tests have been described in the literature. These tests could eventually be used in combination with species-specific *Haemophilus* probes as, for instance, described in **refs. *35*, *37*, *39,*** and ***41***.

[e]The probe used determines whether all *Haemophilus* spp. amplicons are detected or the *H. influenzae* amplicon specifically. The DG74/RDR080 PCR amplifies ribosomal DNA from all eubacteria.

[f]The virulence gene targets presented here are deduced from the whole genome sequence of *H. influenzae* Rd ***(43,47)*** and were identified on the basis of the presence of repetitive DNA motifs. These regions comprising arrays of variable numbers of four nucleotide long repeat units are highly characteristic for *H. influenzae* virulence factors. Twelve such loci were identified, and PCRs aiming at each individual locus were developed. The tests provide species-specific information (Hi 4–3, 4–5, 4–10, 4–11, and 4–12 are species specific), but also describe the virulence status of a bacterial isolate and provide a molecular marker for epidemiological typing studies ***(46)***. The size of the PCR product stated here is based on the whole genome sequence for strain Rd. Note that the sizes may vary significantly among various strains. Probes give the repeat motif for every independent locus.

[g]This test should be performed on a pure culture only and could be combined with one of the species-specific tests described in this table.

Abbreviations: *Haeg: Haemophilus aegyptius; Hhae: Haemophilus haemolyticus; Hinf: Haemophilus influenzae; Hpar: Haemophilus parainfluenzae: Hae* spp.: *Haemophilus* species; *Lgt*C, *Lic*1-3: genes involved in lipopolysaccharide biosynthesis; R: adenosine/guanine; Ref: literature reference; Y: cytosine/thymidine; *Yad*A: *Yersinia enterocolitica* adhesin A.

analysis of cultured bacteria. Bacterial speciation can be determined by automated biochemical analysis, by monoclonal antibody studies, or by DNA-based tests. Several of these strategies will be explained in detail in the following sections.

3.1. Classical Microbiology

1. Gram staining is performed on smears of clinical samples that have been heat- and ethanol-fixed. A glass slide containing the denatured smear is immersed in crystal violet solution (1 min), rinsed in water, immersed in Gram's Iodine (2×30 s) and twice in 96% ethanol (30 s). After rinsing in water, fuchsin solution is applied as a counterstain (1 min). Light pink-stained Gram-negative rods are visible by light microscopy.
2. Blood agar and chocolate agar are also inoculated with the clinical material and incubated at 37°C in an aerobic atmosphere. If after 24 h no growth is observed on the blood agar, whereas colonies are observed on the chocolate agar, the diagnosis *Haemophilus* spp. is entertained.
3. In order to define the bacterial species, additional testing is necessary. The requirement for factors X and V is tested. Suspect colonies are suspended in physiological salt solution at a density of 0.5–1.0 McFarland. An ISO agar plate is inoculated with this suspension and three different disks (X, V, and X/V) are placed on the agar. Plates are incubated at 37°C overnight in an atmosphere containing 5% CO_2. The next day the plate is judged for bacterial growth in the vicinity of the disks (*see* **Note 1**).

3.2. Selective Enrichment Cultures

1. Three improved culture media have been described in the recent literature for selective enrichment of *H. influenzae* ***(5–7)***.
2. Respiratory-derived secretions are diluted in N-acetyl-L-cystine sodium citrate buffer in a 1:1 ratio, especially sputum derived from cystic fibrosis patients ***(5)***. Samples are vortexed, left to stand at room temperature for 15 min, then revortexed. The suspension is inoculated directly onto CHOC-BAC plates, incubated at 35°C in 5–7% CO_2, and examined daily for 3 days for growth.
3. In the protocol developed by Müller et al. ***(6)***, clinical material is streaked directly onto NAG medium. Two disks containing cefsulodin are placed at a distance of 3 cm of the second set of streaks in order to suppress the growth of *Pseudomonas* spp. The use of these surface disks generates a gradient of antibiotic concentrations. This is to certify that also the most sensitive *H. influenzae* strains (MIC usually between 8 and 128 µg/mL) will be able to grow. Plates are incubated overnight in a completely anaerobic environment at 37°C.
4. Plates made according to Smith and Baker ***(7)*** are inoculated directly with clinical material and incubated for 18 h at 37°C in air enriched with 5% CO_2 (*see* **Note 2**).

3.3. Automated Procedures

1. Manual tests are necessary for the preliminary identification of *H. influenzae*.

a. The indole spot test is simple: A cotton swab is coated with *H. influenzae* and then saturated with spot indole reagent. Development of a blue color within 2 min is considered a positive reaction.
b. A positive catalase test is characterized by spontaneous bubbling when a colony of the test organism is suspended in catalase reagent.
c. The outcome of these two assays needs to be imported into a Vitek machine prior to automated analysis of the bacterial strain.
d. A suspension of bacterial cells is made in 0.45% NaCl. The density is adjusted to 3 McFarland with the specialized Vitek colorimeter. The suspension and the NHI card are inserted into the Vitek I cassette and loaded into the machine. Relevant data are entered into an integrated Smart System computer. The suspension is dispensed into 30 NHI wells containing 15 separate biochemical substrates and growth and biochemical characteristics are recorded. After 4 h of off-line incubation at 35°C, the wells are read by the Vitek machine. The output data are used to generate a species identification on the basis of a probabilistic similarity score established by comparison of the strain score with the Vitek computer database. It is advisable to include type strains as controls in all tests. These presumptive identifications can be downloaded to hospital registration systems (*see* **Note 3**).

3.4. Classical Serotyping

1. Antisera are hydrated in 1 mL of sterile water. Prior to testing, all reagents should be equilibrated at room temperature.
2. A loopfull of bacteria is transferred from chocolate agar to a drop of 0.85% saline and emulsified. The emulsion needs to be checked for autoagglutination over 1 min. If there is no detectable autoagglutination, the procedure is repeated with a drop of antiserum.
3. After 1 min, agglutination should be visible. Include known positive and negative control strains for each batch of isolates being tested (*see* **Note 4**).

3.5. Monoclonal Antibody Serotyping

1. Wash sputum samples twice in PBS, smear onto a glass slide, and dry.
2. Immerse the slide in methanol for 5 min for fixation.
3. Incubate at 37°C for 30 min in the presence of a 1:500 dilution of 8BD9 in PBS with 0.5% bovine serum albumin (BSA) and 0.05% Tween-20.
4. Wash twice for 5 min in PBS and 0.5% Tween-20.
5. Initiate detection by 30 min incubation with peroxidase conjugated rabbit anti-mouse antibody.
6. Wash twice for 5 min in PBS and 0.5% Tween-20.
7. Incubate with substrate solution for 20 min at room temperature.
8. Rinse slides for 10 min in running tap water.
9. Counterstain with methylene blue.
10. The number of positively stained bacteria is assessed in at least 30 separate light microscope fields (×1000, oil immersion) per slide (*see* **Note 5**).

3.6. DNA Isolation

1. Suspend bacteria in 100 µL of 10 m*M* Tris-HCl buffer. Clinical material can also be used, but in some cases mucolytic pretreatment of sputum is necessary. For general PCR approaches (*see* **Subheading 3.9.**), it is advisable to wash the sputum sample excessively in order to remove contaminating throat flora.
2. Add lysis buffer I (1 mL) and vortex vigorously ***(8)***.
3. Add 50 µL Celite suspension, vortex the suspension, and let stand at room temperature for 15 min.
4. Pellet by microcentrifugation and decant the supernatant.
5. Wash the pellet twice in lysis buffer II, once in 70% ethanol, and once in acetone, then dry in a desiccator and resuspend in 100 µL 10 m*M* Tris-HCl, pH 8.0.
6. After incubation at 56°C for 10 min the sample is microcentrifuged at 9000*g* for 30 s.
7. Transfer the supernatant containing the eluted DNA to a clean tube, and store at 4°C or −20°C. The protocol described here provides the researcher with DNA preparations that are of sufficient quality to perform all the DNA-based identification procedures that are described in this chapter.

3.7. DNA Probe Testing

1. Genprobe tests represent the first generation of routinely applicable nucleic acid-mediated identification tests in clinical microbiology. The DNA probes employed are species-specific and are directed at nucleotide motifs in the small subunit (ssu, 16S) ribosomal RNA and DNA. These probes are labeled with acridinium esters that can be hydrolyzed when not hybridized to target molecules, the basis of the hybridization protection assay. Only hybridization to the target spares the probe from chemical breakdown, and nondegraded probe is subsequently identified by luminometrics.
2. Test each bacterial isolate in a separate GenProbe test tube, and include positive and negative controls.
3. Suspend 1–4 bacterial colonies in 50 µL universal lysis buffer.
4. Heat reagents 2 to 60°C, then add 50 µL to the bacterial suspension, briefly vortex, and incubate at 60°C for 15 min.
5. Add 300 µL of detection reagent and incubate at 60°C for another 5 min.
6. Leave samples at room temperature for at least 5 min, after which measure luminescence (relative luminescence units, RLU) with a luminometer, such as the Leader 50. A positive result is defined by an RLU of greater than 50,000. An RLU of less than 20,000 indicates that the isolate is not *H. influenzae*. Intermediate results should be confirmed with a second assay. Control data should be interpreted similarly; in case of deviating controls, the entire set of samples tested should be rerun.
7. In addition to the commercially available oligonucleotide hybridization assays, many in-house tests have been developed in laboratories studying *H. influenzae*. **Table 1** surveys some of the research DNA probes that are described in the current literature (*see* **Note 6** for background information and further details).

3.8. Specific PCR Assays

1. Set up PCR with a 100 µL volume containing 1× PCR buffer, primers (50–200 pmol), Taq DNA polymerase (0.1–2 units, depending on the brand of enzyme), and approx 10 ng of DNA isolated from samples such as cerebrospinal fluid (CSF) or sputum (for DNA isolation *see* **Subheadings 2.5.** and **3.5.**).
2. Set the thermocycler for 40 cycles of 1 min at 95°C, 1 min at 55°C, 2 min at 72°C; followed by 8 min at 72°C to complete the synthesis of full-length PCR products according to Van Ketel et al ***(9)***.
3. Analyze 10 µL by electrophoresis through 2% agarose gels at 100 mA for 90 min. PCR products are visualized by ethidium bromide staining and UV irradiation of the gel.
4. Southern blot the gel to improve the sensitivity and specificity of the PCR. The gel is blotted by capillary action onto nylon filters under alkaline conditions (0.4 *N* NaOH, 1.5 *M* NaCl).
5. Neutralize the filter in 5× SSC and hybridize with probe HI-III or HI-VI, depending on the primer pair used during PCR, for 18 h at 55°C. During the labeling of the oligonucleotide probe, care has to be taken with the terminal transferase enzyme, which is thermo-unstable. Labeled probe can be stored at −20°C without apparent loss of activity.
6. After hybridization, wash the filter twice at room temperature washing buffer for 10 min and twice at 55°C for 30 min.
7. Expose the membrane to x-ray film ***(9)*** adjusting the exposure time for activity and type of label used.
8. Over the past few years, multiple additional tests have been developed 'in-house' and are well described in the literature. Some of these diagnostic tests are outlined in **Table 2** (for further technical detail, *see* **Note 7**).

3.9. General Primer Mediated PCR

1. DNA isolated from clinical material is amplified by PCR using primers DG74 and RDR080 as described in **Subheading 3.7.**
2. If a characteristic 350-bp fragment is visualized after gel electrophoresis, the amplicon is cloned into pCR2.1 according to the guidelines of the TA cloning kit.
3. Recombinants are plated on to LB-ampicillin plates and resulting colonies are screened for the presence of the insert in the plasmid by standard M13/T7 primer-mediated PCR.
4. Clones containing an insert can be maintained in numbered arrays on selective agar plates, and stored in BHI/glycerol long-term at −80°C. The arrays can be transferred to Hybond N^+ membranes by colony blotting. Bacterial DNA is liberated by lysis and the DNA fixed by UV light exposure. The colony blots are hybridized as described in **Subheading 3.7.** with probes specific for those microorganisms that frequently co-occur with *H. influenzae*, thereby minimizing the number of clones derived from unknown pathogens residing in the same anatomical niches.

5. Clones that cannot be identified through the hybridization strategy are sequenced and the results BLAST-searched for identification of the underlying bacterial species, assuming that the homologous sequence has been deposited in one of the data libraries before (for further detail, *see* **Note 8**).

4. Notes

1. X and V factor disks discriminate between *H. influenzae* and *H. parainfluenzae*. *H. influenzae* requires both X and V, whereas *H. parainfluenzae* grows on ISO agar with factor V alone. Where growth is observed around X factor only, the isolate belongs to an unidentified species of *Haemophilus*. Not all *Haemophilus* spp. require X and/or V factor, so exceptions to these rules can occasionally be noted in the clinical laboratory. It should also be realized that *Haemophilus* die readily from desiccation and occasionally from short periods at 0–4°C.
2. CHOC-BAC plates have excellent sensitivity for *H. influenzae* isolation ***(2)***, especially from clinical specimens such as sputum from cystic fibrosis patients. Cefsulodin is successful at suppressing growth of non-*Haemophilus* bacteria from sputum. Inclusion of this antibiotic in the culture medium produces a significant increase in the number of strains that can be isolated ***(6,7)***. More than 10% additional positive cultures may be identified.
3. The Vitek NHI card is intended for identification of *Neisseria gonorrhoeae*, *N. meningitidis*, *N. lactamica*, *Moraxella* spp, *Kingella* spp., and various *Haemophilus* spp. A specific β-lactamase test is included. It is advised to perform gram staining, catalase testing, and a spot indole test prior to the Vitek analysis, because these tests provide additional power to the biochemical analysis. NHI cards must be stored at 4–8°C, organisms to be identified must be pure and fresh (less than 48 h in age), and a specialized Vitek colorimeter is advised for preparation of the bacterial cell suspension. When used correctly, the machine will provide identifications varying from "excellent" (highly typical biopattern) to "unidentified organism" (no close match in biopattern to permit identification). Potentially mixed inocula or insufficient bacterial density are identified by the test.
4. Serotyping is a widely used procedure for the identification of the *H. influenzae* type. Since the introduction of vaccines, the role of serotyping has been considerably reduced. Successful serotyping depends on the purity of the culture, evasion of excessive heat during the procedure, adequate control for autoagglutination, and the use of undiluted bacterial cultures.
5. Immunochemical staining is clearly superior to isolation by culture when sensitivities are compared. Antibody testing identified *H. influenzae* in 73% of cystic fibrosis patients' sputum whereas the organism was cultured from only 52% of the same patients ***(10)***. Immunochemical staining can be used for the detection of *H. influenzae* in cerebrospinal fluid from patients suspected for meningitis. Cross-reactivity that is sometimes observed against *Staphylococcus aureus* may be prevented by introducing a blocking step with normal human serum. The irregular dissemination of *H. influenzae* in complex samples such as sputum may be the rare cause of a false negative result. Recently, monoclonal antibodies were

described that are suited for identification of interstrain differences in lipooligosaccharide construction ***(11)***. These monoclonals allow for discrimination among strains from within the species. We have not discussed in detail the commercially available antisera for serotyping strains of *H. influenzae*. For more information, the reader is referred to the manufacturer's manual ***(12)*** and two selected references ***(13,14)***.

6. The AccuProbe test is an automated version of assays that were developed and validated over a decade ago. The test detects specific ribosomal gene sequences and has been used in a large variety of clinical trials ***(15,16)***. Oligonucleotides and acrydinium dyes give rise to fluorescence upon intercalation in the probe–target complex and allow for direct detection of a positive hybridization reaction. GenProbe was one of the first commercially available microbial identification assays. This and other assays are still widely used and have been adapted over time into semiamplification tests as well. It is important to note that strains to be tested should not be older than 48 h because the rRNA molecules that are identified have limited half-lives. Contamination can occur when bacterial material is transferred between tubes; avoiding this problem necessitates strict laboratory discipline.

   A large number of alternative DNA probe-based tests are described in the *H. influenzae* literature. Probes used vary from long genomic DNA fragments encoding penicillin-binding proteins ***(17,18)***, the use of which is explained in detail by Terpstra et al. ***(19)***, to DNA probes useful for assessing the presence of genes encoding specific bacterial virulence factors ***(20)***. The tests employ dot blot, Southern, or *in situ* hybridization methods. *In situ* hybridization allows detection of *H. influenzae* directly, without culture or DNA amplification, in a variety of clinical materials ***(21)***.

   Probes that assess capsular type at the molecular level are worth a separate mention. Probe pUO38, used in Southern hybridization, gives rise to banding patterns that are distinct for each *H. influenzae* capsular serotype ***(22)***; capsule-deficient type b strains are also clearly identifiable, whereas nontypeable strains have no banding pattern. Cap gene probing is useful for confirmation of data obtained by the sometimes hard-to-interpret serological agglutination tests. Radiolabeled probe assays can be adapted into chemiluminescence tests quite easily ***(23)***.

7. PCR testing for the presence of microbial DNA is now commonplace, with a wide diversity of reagents and equipment commercially available. It therefore has to be emphasized that the protocol described here is just a single example. The primer sets given here amplify genes encoding the Bex A protein involved in capsulation (set 1 and probe III) or the P6 outer membrane protein (set 2 and probe VI). The Bex A PCR produces an amplicon from all the encapsulated strains, but not from nontypeable *H. influenzae*. The P6 PCR produces an amplicon from all *H. influenzae*, but also from some *H. parainfluenzae* and all *H. haemolyticus* and *H. aegyptius*. These latter species are closely related to *H. influenzae* ***(24–26)***. Both PCR assays have adequate sensitivity and excellent specificity for differentiating non-*Haemophilus* bacterial species. PCR has been

employed frequently over the years, and has been adapted to identify the presence of ampicillin-resistance genes ***(27)*** or genes involved in capsulation ***(28)***. Some of the tests allow for a detailed assessment of the serotype on the basis of a set of serotype-specific PCR assays ***(29)***. P6 PCR has been used to study the etiological role of *H. influenzae* in samples of nasopharyngeal secretion, middle ear effusion, and otitis media ***(30,31)***. Of particular interest are those PCR tests specific for *H. influenzae* that can be combined with species specific PCRs aimed at other bacteria. So-called multiplex PCRs have been published that allow for the single-tube detection of potential or suggested pathogens such as *Moraxella catarrhalis*, *Streptococcus pneumoniae* and *Alloiococcus otitidis* together with *H. influenzae* ***(32–35)***. A strategy in which a general broad-spectrum PCR was followed by a nested PCR with specificity for a single bacterial species only was recently published ***(36)***. Similar approaches are described in **Subheadings 2.8.** and **3.8.**

8. The method given in detail here ***(37)*** is especially suited for the identification of microbial organisms in samples where disease etiology is unknown. The PCR primers are designed on the basis of ribosomal consensus motifs enabling amplification of a 350-bp DNA fragment from virtually all eubacteria ***(38)***. The use of probes is advised when known pathogens may be present in bulk: DNA probing of individual clones enables separation of bulk from speciality in a short-term fashion. A large number of DNA oligonucleotide probes that can be used in combination with various general primer-mediated PCR tests are available, which may reduce the need to clone and sequence PCR products ***(39,40)***. An alternative approach for identification of PCR products or clones takes advantage of specific electrophoretic conditions in which a fragment's mobility depends on its DNA sequence. Denaturing gradient gel electrophoresis (DGGE) and single strand conformation polymorphism (SSCP) analysis are two examples ***(41)***. The most recent developments use immobilized DNA oligonucleotide probes ordered on membranes ***(42)*** or in microarrays, termed DNA chips (*see* **Subheading 5.**).

5. Future Developments and Closing Remarks

Recently, the complete genome sequence for a strain of *H. influenzae* was elucidated ***(43)***. This provides insight into the genetic makeup of the species, in the sense that a complete catalog of all essential genes is now available. Based on this sequence, species-specific DNA elements can be traced. Motifs vary from ubiquitous and frequent oligonucleotide sequences ***(44)*** to larger genetic elements encoding essential genes. Moreover, several genes that show clinically and epidemiologically important antigenic diversity may be used for molecular identification below the species level ***(45)***. This detailed microbial gene cataloging is currently commonplace: many bacterial chromosomes have been sequenced in full. Comparative studies will identify not only species-specific nucleic acid regions, but also those regions that may be informative at another taxonomic level, such as at the genus or family level. On the other hand, data as detailed as currently are available may also help in the identifica-

tion of distinct strains or even distinct primary clinical isolates. Together with developments in the fields of DNA chip technology, nucleic acid characterization of strains of *H. influenzae* may be performed in unprecedented detail in the near future, thereby surpassing the current repertoire of classical and molecular identification methods in frequency of application and clinical importance.

References

1. Urwin, G., Krohn, J. A., Deaver-Robinson, K., Wenger, J. D., Farley, M. M., and the *Haemophilus influenzae* Study Group. (1996) Invasive disease due to *Haemophilus influenzae* serotype f: clinical and epidemiological characteristics in the *H. influenzae* serotype b vaccine era. *Clin. Infect. Dis.* **22,** 1069–1076.
2. Isenberg, H.D. (1998) *Essential procedures for clinical microbiology,* American Society for Microbiology, Washington, DC.
3. Groeneveld, K., van Alphen, L., van Ketel, R. J., Geelen van den Broek, N. J., Eijk, P. P., and Zanen, H. C. (1989) Nonculture detection of *Haemophilus influenzae* in sputum with monoclonal antibodies specific for outer-membrane lipoprotein P6. *J. Clin. Microbiol.* **27,** 2263–2267.
4. Altschul, S., Gish, W., Miller, W., Meyers, E. W., and Lipman.D. J. (1990) Basic local alignment search tool. *J. Mol. Biol.* **215,** 403–410.
5. Doern, G., and Brogden-Torres, B. (1992) Optimum use of selective plated media in primary processing of respiratory tract specimens from patients with cystic fibrosis. *J. Clin. Microbiol.* **30,** 2740–2742.
6. Möller, L.V.M., van Alphen, L., Grasselier, H., and Dankert, J. (1993) N-Acetyl-D-glucosamine medium improves recovery of *Haemophilus influenzae* from sputa of patients with cystic fibrosis. *J. Clin. Microbiol.* **31,** 1952–1954.
7. Smith, A., and Baker, M. 1997. Cefsulodin chocolate agar: a selective medium for the recovery of *Haemophilus influenzae* from the respiratory secretions of patients with cystic fibrosis. *J. Med. Microbiol.* **46,** 883–885.
8. Boom, R., Sol, C. J. A., Salimans, M. M. M., Jansen, C. L., Wertheim-van Dillen, P. M. E., and van der Noordaa, J. (1990) Rapid and simple method for purification of nucleic acids. *J. Clin. Microbiol.* **28,** 495–503.
9. Van Ketel, R.J., de Wever, B., and van Alphen, L. (1990) Detection of *Haemophilus influenzae* in cerebrospinal fluids by polymerase chain reaction DNA amplification. *J. Med. Microbiol.* **33,** 271–276.
10. Möller, L.V.M., Ruijs,G. J., Heijerman, H. G. M., Dankert, J., and van Alphen, L. (1992) *Haemophilus influenzae* is frequently detected with monoclonal antibody 8BD9 in sputum samples from patients with cystic fibrosis. *J. Clin. Microbiol.* **30,** 2495–2497.
11. Ueyama, T., Gu, X. X., Tsai, C. M., Karpas, A. B., and Lim, D. J. (1999) Identification of common lipooligosaccharide types in isolates from patients with otitis media by monoclonal antibodies against nontypeable *Haemophilus influenzae* 9274. *Clin. Diagn. Lab. Immunol.* **6,** 96–100.
12. Becton Dickinson (2000) Difco manual: *Haemophilus influenzae* antisera, pp. 646–648.

13. Hamel, J., Brodeur, B. R., Belmaaza, A., Montplaisir, S., Musser, J. M., and Selander, R. K. (1987) Identification of *Haemophilus influenzae* type b by a monoclonal antibody coagglutination assay. *J. Clin. Microbiol.* **25,** 2434–2436.
14. Landgraf, I.M. ,and Vieira, M. F. P. (1993) Biotypes and serotypes of *Haemophilus influenzae* from patients with meningitis in the city of Sao Paulo, Brazil. *J. Clin. Microbiol.* **31,** 743–745.
15. Daly, J.A., Clifton, N. L., Seskin, K. C., and Gooch, W. M. (1991) Use of rapid non-radioactive DNA probes in culture confirmation tests to detect *Streptococcus agalactiae*, *Haemophilus influenzae*, and *Enterococcus* spp. from paediatric patients with significant infections. *J. Clin. Microbiol.* **29,** 80–82.
16. Davis, T.E., and Fuller, D. D. (1991) Direct identification of bacterial isolates in blood cultures by using a DNA probe. *J. Clin. Microbiol.* **29,** 2193–2196.
17. Malouin, F., and Bryan, L. E. (1987) DNA probe technology for detection of *Haemophilus influenzae*. *Mol. Cell. Probes* **1,** 221–232.
18. Malouin, F., Bryan, L. E., Shewciw, P., Douglas, J., Li, D., van den Elzen, H., and Lapointe, J. R. (1988) DNA probe technology for rapid detection of *Haemophilus influenzae* in clinical specimens. *J. Clin. Microbiol.* **26,** 2132–2138.
19. Terpstra, W.J., ter Schegget, J., and Schoone, G. J. (1990) Detection of *Leptospira, Haemophilus* and *Campylobacter* using DNA probes, in: *Gene Probes for Bacteria,* (Macario, A. J. L, and Conway de Macario, E. eds.), Academic, New York, pp. 295–322.
20. St. Geme, J.W., Kumar, V. V., Cutter, D., and Barenkamp, S. J. (1998) Prevalence and distribution of the hmw and hia genes and the HMW and Hia adhesins among genetically diverse strains of nontypeable *Haemophilus influenzae*. *Infect. Immun.* **66,** 364–368.
21. Hogardt, M., Trebesius, K., Geiger, A. M., Hornef, M., Rosenecker, J., and Heeseman, J. (2000) Specific and rapid detection by fluorescent *in situ* hybridization of bacteria in clinical samples obtained from cystic fibrosis patients. *J. Clin. Microbiol.* **38,** 818–825.
22. Kroll, J. S., Ely, S., and Moxon, E. R. (1991) Capsular genotyping of *Haemophilus influenzae* with a DNA probe. *Mol. Cell. Probes.* **5,** 375–379.
23. Muehlemann, K., Balz, M., Aebi, S., and Schopfer, K. (1996) Molecular characteristics of *Haemophilus influenzae* causing invasive disease during the period of vaccination in Switzerland: analysis of strains isolated between 1986 and 1993. *J. Clin. Microbiol.* **34,** 560–563.
24. Casin, I., Grimont, F., and Grimont, P. A. (1986) Deoxyribonucleic acid relatedness between Haemophilus aegyptius and *Haemophilus influenzae*. *Ann. Inst. Pasteur Microbiol.* **137B,** 155–163.
25. Dewhirst, F. E., Paster, B. J., Olsen, I., and Fraser, G. J. (1992) Phylogeny of 54 representative strains of species in the family Pasteurellaceae as determined by comparison of 16S rRNA sequences. *J. Bacteriol.* **174,** 2002–2013.
26. Quentin, R., Ruimy, R., Rosenau, A., Musser, J. M., and Christen, R. (1996) Genetic identification of cryptic genospecies of Haemophilus causing urogenital and neonatal infections by PCR using specific primers targeting genes coding for 16S rRNA. *J. Clin. Microbiol.* **34,**1380–1385.

27. Tenover, F. C., Huang, M. B., Rasheed, J. K., and Persing, D. H. (1994) Development of PCR assays to detect ampicillin resistance genes in cerebrospinal fluid samples containing *Haemophilus influenzae*. *J. Clin. Microbiol.* **32,** 2729–2737.
28. Hassan-King, M., Adegbola, R., Baldeh, I., Mulholland, K., Omosigho, C., Oparaugo, A., et al. (1998) A polymerase chain reaction for the diagnosis of *Haemophilus influenzae* type b disease in children and its evaluation during a vaccine trial. *Pediatr. Infect. Dis.* **17,** 309–312.
29. Falla, T. J., Crook, D. W. M., Brophy, L. N., Maskell, D., Kroll, J. S., and Moxon, E. R. (1994) PCR for capsular typing of *Haemophilus influenzae*. *J. Clin. Microbiol.* **32,** 2382–2386.
30. Ueyama, T., Kurono, Y., Shirabe, K., Takeshita, M., and Mogi, G. 1995. High incidence of *Haemophilus influenzae* in nasopharyngeal secretions and middle ear effusions as detected by PCR. *J. Clin. Microbiol.* **33,** 1835–1838.
31. Sakamoto, N., Kurono, Y., Ueyama, T., and Mori, G. (1996) Detection of *Haemophilus influenzae* in adenoids and nasopharyngeal secretions by polymerase reaction. *Acta Otolaryngol. Suppl.* **523,** 145–146.
32. Hassan-King, M., Baldeh, I., Adegbola, R., Omosigho, C., Usen, S. O., Oparaugo, A., and Greenwood, B. M. (1996) Detection of *Haemophilus influenzae* and Streptococcus pneumoniae DNA in blood cultures by a single PCR assay. *J. Clin. Microbiol.* **34,** 2030–2032.
33. Post, J.C., White, G. J., Aul, J. J., Zavoral, T., Zadowsky, R. M., Zhang, Y., et al. (1996). Development and validation of a multiplex PCR-based assay for the upper respiratory tract bacterial pathogens *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Moraxella catarrhalis. Mol. Diagn.* **1,** 29–39.
34. Bakaletz, L.O., White, G. I., Post, J. C., and Ehrlich, G. D. (1998) Blinded multiplex PCR analyses of middle ear and nasopharyngeal fluids from chinchilla models of single and mixed-pathogen-induced otitis media. *Clin. Diagn. Lab. Immunol.* **5,** 219–224.
35. Hendolin, P.H., Paulin, L., and Ylikoski, J. (2000) Clinically applicable multiplex PCR for four middle ear pathogens.*J. Clin. Microbiol.* **38,** 125–132.
36. Backman, A., Lantz, P., Radstrom, P., and Olcen, P. (1999) Evaluation of an extended diagnostic PCR assay for detection and verification of the common causes of bacterial meningitis in CSF and other biological samples. *Mol. Cell. Probes* **13,** 49–60.
37. Van Belkum, A., Renders, N. H. M., Smith, S., Overbeek, S. E., and Verbrugh, H. A. (2000) Comparison of conventional and molecular methods for the detection of bacterial pathogens in sputum samples from cystic fibrosis patients. *FEMS Immunol. Med. Microbiol.* **27,** 51–57.
38. Greisen, K., Loeffelholz, M., Purohit, A., and Leong, D. (1994) PCR primers and probes for the 16S rRNA gene of most species of pathogenic bacteria including bacteria found in cerebrospinal fluid. *J. Clin. Microbiol.* **32,** 335–351.
39. Dicuonzo, G., Lorino, G., Lilli, D., et al. (1999) Use of oligoprobes on amplified DNA in the diagnosis of bacterial meningitis. *Eur. J. Clin. Microbiol. Infect. Dis.* **18,** 352–357.

40. Rantakokko-Jalava, K., Nikkari, S., Jalava, J., et al. (2000) Direct amplification of rRNA genes in diagnosis of bacterial infections. *J. Clin. Microbiol.* **38,** 32–39.
41. Turenne, C.Y., Witwicki, E., Hoban, D. J., Karlowsky, J. A., and Kabani, A. M. (2000) Rapid identification of bacteria from positive blood cultures by fluorescence-based PCR-single -strand conformation polymorphism analysis of the 16S rRNA gene. *J. Clin. Microbiol.* **38,** 513–520.
42. Anthony, R.M., Brown, T. J., and French, G. L. (2000) Rapid diagnosis of bacteremia by universal amplification of 23S ribosomal DNA followed by hybridization to an oligonucleotide array. *J. Clin. Microbiol.* **38,** 781–788.
43. Fleischmann, R. D., Adams, M. D., White, O., et al. (1995). Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. *Science* **269,** 496–512.
44. Karlin, S., Mrazek, J., and Campbell, A. M. (1996) Frequent oligonucleotides and peptides of the *Haemophilus influenzae* genome. *Nucleic Acids Res.* **21,** 4263–4272.
45. Gilsdorf, J. R. (1998) Antigenic diversity and gene polymorphisms in *Haemophilus influenzae*. *Infect. Immun.* **66,** 5053–5059.
46. van Belkum, A., Scherer, S., van Leeuwen, W., Willemse, D., van Alphen, L., and Verbrugh, H. (1997) Variable number of tandem repeats in clinical strains of *Haemophilus influenzae*. *Infect. Immun.* **65,** 5017-5027.
47. Hood, D.W., Deadman, M. E., Jennings, M. P., Bisercic, M., Fleischmann, R. D., Venter, J. C., and Moxon, E. R. (1996) DNA repeats identify novel virulence genes in *Haemophilus influenzae*. *Proc. Natl. Acad. Sci. USA* **93,** 11,121–11,125.

6

Characterization of Plasmids

Ioanna D. Dimopoulou, Mohd-Zain Zaini, and Derrick W. Crook

1. Introduction

Antibiotic resistant strains of *Haemophilus influenzae* appeared in the early 1970s and studies on these strains led to the detection of large 40–50 kb conjugative plasmids ***(1–3)***. Early work was hampered by the failure to easily detect extrachromosomal plasmid in clinical isolates. However, closed circular plasmid was readily detected in exconjugants following conjugal transfer of plasmid ***(4)***. Early, it was recognized that small, approx 5-kb, nonconjugative plasmid was associated with a proportion of strains exhibiting β-lactamase-positive ampicillin resistance ***(1,2)***. We concentrate here on characterization of large conjugative plasmids.

Subsequent investigations of large conjugative plasmids have shown that these resistance plasmids share sequence homology and, therefore, constitute a family of plasmids that are widely distributed in the nasopharyngeal haemophili (both *H. influenzae* and V-dependent haemophili) worldwide ***(5–7)***. These plasmids encode a wide range of resistances, including β-lactamase positive ampicillin resistance, tetracycline, chloramphenicol, erythromycin, and aminoglycoside resistance. Trimethoprim or sulfonamide resistance is not linked to these plasmids ***(2)***. This plasmid has also been detected in fully susceptible strains, suggesting that there is a core 'cryptic' plasmid that constitutes the natural reservoir, which under antibiotic-selective pressure acquire resistance genes ***(7,8)***. These genes are located on transposons, and *Tn*10 and *Tn*s3 account for tetracycline and ampicillin resistance, respectively, in the few strains studied ***(3)***. The location of other resistance genes has not been studied.

In primary isolates, these plasmids are largely integrated site-specifically ***(7)*** with chromosomal $tRNA^{leu}$ (D. Crook, personal communication). Following conjugal transfer, plasmid occurs largely extrachromosomally in

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Table 1
PCR Primers for Detecting Plasmid Specific Sequences

Primer	Nucleotide Sequence
F1	5'-TTATCGTAATGTTTGTATCAGCCT-3'
F2	5'-CGATCGTAGTGTATTGGCTAGGTAC-3'
R1	5'-CCGGGAGCTTCTTCAGTTAAAGC-3'
R2	5'-GTACCTAGCCAATACACTACGATCG-3'

exconjugants and can be isolated and purified ***(4)***. However, excised extrachromosomal closed circular plasmid is detectable by PCR in very low copy in primary isolates, while co-integrated plasmid is also detectable by PCR in very low copy in exconjugants. As the plasmid copy number even in exconjugants is low (approx 2–3 copies per cell) ***(9)***, the yield on isolation of closed circular plasmid is low. In those strains containing cryptic plasmid, there is no ready phenotype for detecting strains with plasmid; also, isolation of plasmid has not been achieved other than in a single case where such a plasmid occurred extrachromosomally in the primary isolate ***(8)***. A polymerase chain reaction (PCR) based method for detecting such strains targeted at sequences close to the point of excision and recircularization is available ***(7)*** (*see* **Table 1** and **Fig. 1**). Currently, the whole plasmid sequence of one of the well-characterized plasmids, p1056, is nearing completion, and based on this data a sequence-based typing scheme analogous to multi-locus sequence typing for bacteria is being developed (D. Crook, personal communication).

2. Materials

2.1. Bacteria and Media

1. As recipients, *H. influenzae* strains Eagan, Rd, and a rec deficient Rd *H. influenzae* strain (made by J. K. Setlow) are all efficient recipient strains in mating experiments. For mating, streptomycin-resistant mutants (MIC $>$ 100 µg/mL) of each of these strains was obtained following serial passage in streptomycin containing medium.
2. For liquid medium, Columbia (10 µg; Oxoid) and yeast extract (5 g; Oxoid) are added to 1 L dH_2O and autoclaved for 15 min at 121°C. Once the medium has cooled to approx 50°C, filter-sterilized X and V factors are added to a final concentration of 10 µg/mL.
3. For solid medium, 1.5 g agar is added to 1 L of Columbia and Yeast extract as in **step 2**, thereafter, it is processed as in **step 2**.
4. For antibiotic selections in matings or growth of antibiotic-resistant strains in liquid medium, the desired filter-sterilized antibiotic is added to the medium at the same time as X and V factors. The final concentration of antibiotic is as follows: ampicillin, 2 µg/mL; tetracycline, 4 µg/mL; chloramphenicol, 4 µg/mL, and streptomycin, 100 µg/mL.

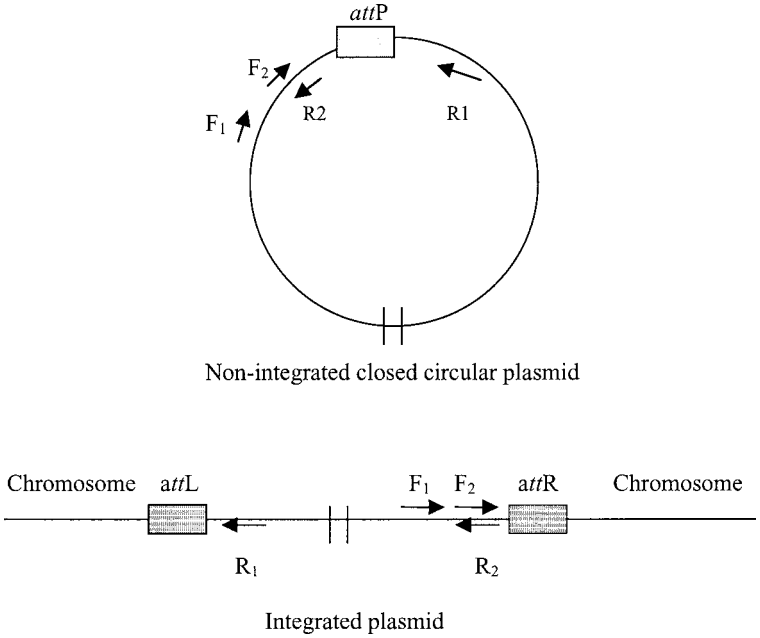

Fig. 1. The orientation of primers for amplification of plasmid specific sequences flanking *att* B are represented by arrows and are labeled F1, F2, R1, or R2.

3. Methods

3.1. Isolation and Purification of Small β-lactamase-Positive, Ampicillin-Resistant Plasmid

Standard alkaline lysis methods for the isolation and purification of plasmid can be used for small plasmids. Kit-based column purification of plasmids in common use will be adequate for most purposes.

3.2. Characterization of Large Conjugative Plasmid

3.2.1. Conjugation

For filter paper mating, 0.45-µm millipore filters are used. Donor and recipient strains are grown to mid-log and a 1-mL vol of each is gently deposited onto the membrane by suction. The membrane is incubated overnight, approx 18 h, on solid medium without antibiotic ***(4)***.

For colony mating, equal concentrations of mid-log-phase donor and recipient organisms are spotted onto solid medium and incubated overnight (approx 18 h) ***(10)***.

The bacterial growth is suspended from the filter by vigorous vortexing in 10 mL of liquid medium, or the growth of the colony is scraped off the agar

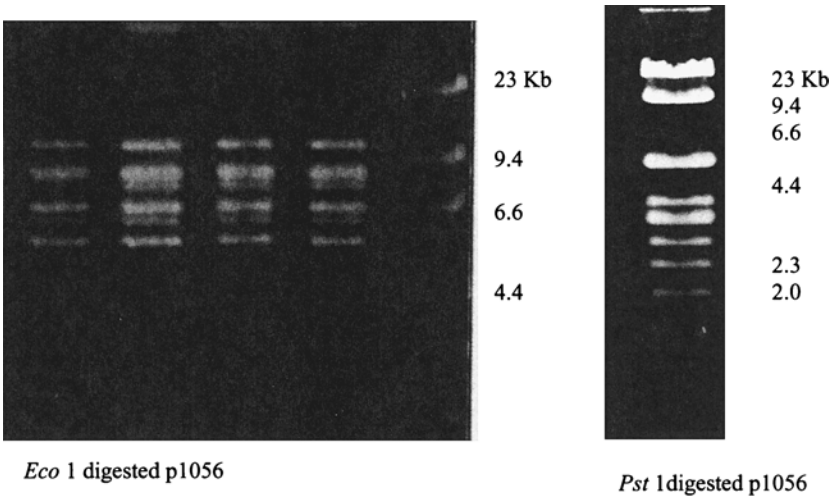

Fig. 2. Photographs of purified plasmid p1056 digested with *Eco*RI and *Pst*I and stained with ethidium bromide.

and dispersed through 10 mL of liquid medium by vortexing. Viable counts of donor, recipient, and exconjugants are detected by culture on solid medium containing the appropriate selective antibiotic(s) and using appropriate dilutions of inoculum. The frequency of conjugation is calculated by dividing the number of exconjugants by the number of donor cells.

A representative sample of the exconjugants is purified on solid medium containing the selective antibiotic(s) and the presence of plasmid confirmed either using PCR or Southern blotting and probing using whole plasmid as described below.

3.2.2. PCR Detection of Plasmid-Specific Sequences

1. The following three primer sets are plasmid specific and are directed at sequences adjacent to and spanning *att*P *(7)*. Set 1: F1 and R2 yields an amplicon of 370 bp; set 2: F2 and R1 yields a product of 410 bp; and set 3: F1 and R1 yields a product of 780 bp (*see* **Fig. 1**). These primers are derived from a sequence deposited with Genebank accession number U68467.
2. Total cellular DNA for PCR is prepared by boiling 2 colonies suspended in 100 µL dH_2O for 10 min.
3. Each PCR reaction (25 µl) 0.5 U *Taq* polymerase, 10 m*M* Tris-HCl, pH 8.0; 50 m*M* KCl, 2.5 m*M* $MgCl_2$, 0.01% (w/v) gelatin, 250 µM of each dNTP and 1 µM of each oligonucleotide primer.
4. Detection of PCR products is by electrophoresis in 1% agarose gels and staining with ethidium bromide.

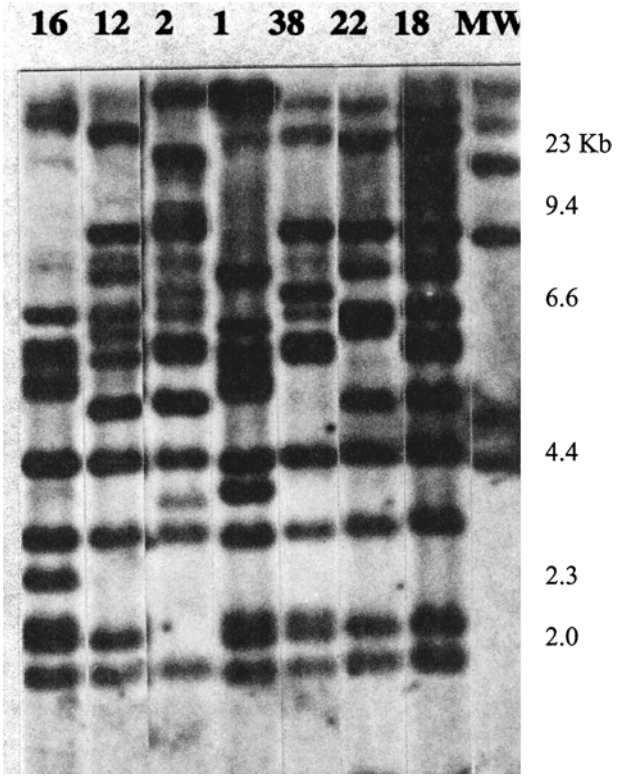

Fig. 3. An autoradiograph of a Southern blot of *Pst*I-digested whole cellular DNA from a range of ampicillin-resistant β-lactamase-positive strains probed with p1056.

3.2.3. Purification of Plasmid or Total Cellular DNA

1. Alkaline lysis and cesium chloride ethidium bromide purification by centrifugation of these plasmids has been superceded by purification using commercial kits based on columns containing resins.
2. Plasmid is only detectable and available for isolation and purification in exconjugants. The cells are cultured in antibiotic-containing liquid medium (antibiotic included in the medium will depend on the resistances encoded by the plasmid) until late log phase, which is achieved by overnight culture in air with orbital shaking at 150 rpm.
3. A volume of 500 mL to 1 L is used, as the plasmid occurs in low copy number. Care is taken to reduce shearing of DNA during the isolation procedure, to minimize the contamination of purified plasmid with chromosomal DNA. Improved purification of closed circular plasmid is achieved by using kits designed specifically for purification of BAC DNA (Macherey-Nagel or Qiagen) and contaminating chromosomal fragments are removed by digestion with an ATP-dependent

exonuclease (as described by kits provided by Macherey-Nagel or Qiagen). Alternatively, a mixture of Exonuclease 1 and Lambda exonuclease can be used in place of ATP-dependent exonuclease to digest linear fragments of contaminating DNA.
4. Whole cellular DNA is extracted using standard kits such as those manufactured by Macherey-Nagel or Qiagen.

3.2.4. Typing of Plasmids Using Restriction Enzymes and Future Techniques

1. *Pst*1 digestion gives the most readily interpretable results. Whole plasmid DNA is digested with *Pst*1 and separated in 0.7% agarose gels and the banding patterns visualized by UV transillumination and staining with ethidium bromide (*see* **Fig. 2**).
2. Banding patterns useful in typing plasmid can be obtained by Southern blotting *Pst*1 digested total cellular DNA (either of the natural isolate containing plasmid or an exconjugant) and probing using whole plasmid (*see* **Fig. 3**).
3. In the future it is possible that sequence-based typing methods will be available based on sequence of 'core plasmid' genes analogous to multilocus sequence typing of bacteria. This technique is under development and is benefiting from the data accumulated from the whole plasmid sequence of p1056. The whole plasmid sequence of p1056 will be deposited with Genebank.

References

1. Brunton, J., Clare, D., and Meier, M. A. (1986) Molecular epidemiology of antibiotic resistance plasmids of *Haemophilus* species and *Neisseria gonorrhoeae. Rev. Infect. Dis.* **8,** 713–724.
2. Powell, M. (1988) Antimicrobial resistance in *Haemophilus influenzae*. *J. Med. Microbiol.* **27,** 81–87.
3. Elwell, L. (1994) R plasmids and antibiotic resistances, in *Molecular Genetics of Bacterial Pathogenesis,* (Miller, V., Kaper, J., Portnoy, D., and Isberg, R., eds.), pp. 17–41.
4. Dimopoulou, I., Kraak, W. A. G. , Anderson, E.C. , Nichols, W. W., Slack, M. P. E., and Crook, D. W. M. (1992) Molecular Epidemiology of unrelated clusters of multi-resistant strains of *Haemophilus influenzae*. *J. Infect. Dis.* **165,** 1069–1075.
5. Laufs, R. and Kaulfers, P.M. (1977) Molecular characterization of a plasmid specifying ampicillin resistance and its relationship to other R factors from *Haemophilus influenzae*. *J. Gen. Microbiol.* **103,** 277–286.
6. Dimopoulou, I., Jordens, J.Z., Legakis, N. J., and Crook, D. W. M. (1997) A molecular analysis of Greek and UK *Haemophilus influenzae* conjugative resistance plasmids. *J. Antimicrob. Chem.* **39,** 303–307.
7. Leaves, N. I., Dimopoulou, I., Hayes, I., et al. (2000) Epidemiological studies of large resistance plasmids in *Haemophilus. J. Antimicrob. Chemo.* **45,** 599–604.
8. Laufs, R., Reiss, F.C., Jahn, G., Fock, R., and Kaulfers, P.M. (1981) Origin of *Haemophilus influenzae* R factors. *J. Bacteriol.* **147,** 563–568.

9. de Graaff, J., Elwell, L. P., and Falkow, S. (1976) Molecular nature of two beta-lactamase-specifying plasmids isolated from *Haemophilus influenzae* type b. *J. Bacteriol.* **126,** 439–446.
10. Stuy, J. H. (1985) Transfer of Genetic Information within a colony of *Haemophilus influenzae*. *J. Bacteriol.* **162,** 1–4.

7

Protein Characterization by Two-Dimensional Gel Electrophoresis

Phillip Cash and J. Simon Kroll

1. Introduction

The bacterial proteome is the total protein complement expressed from the genome under defined conditions of growth. Unlike the genome, the proteome is not a fixed characteristic, but varies with the conditions of growth of the organisms from which proteins are harvested. Proteomics gives both qualitative data on the physical characteristics of the proteins and quantitative data on the synthesis of the individual proteins. These data provide a global overview of the variability of proteins among bacterial isolates as well as allowing global changes in protein synthesis (reflecting changes in gene expression) to be monitored in response to environmental stimuli. The proteomes for a number of bacteria are currently under investigation, in each case the studies benefiting from the extensive genome sequence data currently available. Several researchers have also published extended studies on the analysis of the *Haemophilus influenzae* proteome ***(1–5)***.

Proteomics has arisen through technical developments in two areas—the separation of individual components from complex protein mixtures, normally using two-dimensional electrophoresis (2DE), and the ability to identify by sequencing the resolved proteins at sensitivities compatible with the amounts recovered from 2DE. 2DE separates proteins on the basis of their native charge in the first dimension, followed by a molecular-weight (mol-wt) separation in the second dimension. A theoretical analysis of the isoelectric points and molecular-weights of the proteins predicted from the *H. influenzae* strain Rd genome sequence shows that the majority of *H. influenzae* proteins are predicted to migrate within the pH range 4.0–7.0 and with molecular weights of approx 10,000–150,000. There appears to be a bimodal distribution with an

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absence of proteins migrating in the pH range 7.5 to 7.6 *(4)*. This chapter considers the analysis of the *H. influenzae* proteome using 2DE, with a brief description of the postelectrophoretic analysis of 2D protein profiles and the identification of proteins resolved by 2DE.

2. Materials

2.1. Standards and Safety

1. All chemicals used for 2DE must be of the highest quality; suitable grades are Aristar, Analar, and Electran (Merck). All buffers and acrylamide solutions are prepared using MilliQ water. For the colloidal Coomassie blue staining procedure, GPR-grade chemicals can be used.
2. Acrylamide is a neurotoxin, and the solutions must be prepared and handled with care. A facemask and gloves should be worn when weighing the acrylamide monomer powders. Gloves should also be worn when handling the solutions and freshly prepared gels.

2.2. Growth of Bacteria and Protein Preparation

1. *Haempohilus* test (HT) media: 5 g Mueller Hinton Broth (Oxoid) and 1 g Yeast extract (Oxoid) in 500 mL dH_2O. Autoclave at 121°C for 15 min. Immediately before use, add X and V factors (*Haempohilus* test medium supplement SR158E; Oxoid), reconstituted in 2 mL of sterile dH_2O to 500 mL of medium.
2. PBS-A: 17 m*M* NaCl, 3.3 m*M* KCl, 10 m*M* Na_2HPO_4, and 2 m*M* NaH_2PO_4. Once the PBS-A has been prepared it is sterilized by autoclaving in volumes of 100 mL and stored at room temperature.
3. 2D Lysis Buffer: 0.01 *M*, Tris-HCl, pH 7.4, 1 m*M* EDTA , 8 *M* urea, 0.05 *M* dithiothreitol (DTT), 10%, (v/v) glycerol, 5% (v/v) NP40, and 6% (w/v) ampholytes. Store the 2D lysis buffer in 1 mL aliquots at −70°C and minimize repeated cycles of freeze-thaw.

2.3. First Dimension Electrophoresis

1. Isoelectric focusing (IEF) acrylamide stock solution: 5% (w/v) acrylamide, 0.25% (w/v) methylenebisacrylamide, 2% (v/v) NP40, 8 *M* urea. Deionize this stock solution by stirring overnight with approx 20 g amberlite MB-1 resin (Sigma) that has been washed with MilliQ water followed by IEF acrylamide solution. Following deionization, the acrylamide solution is removed from the resin and stored in 10-mL aliquots at 4°C. The IEF acrylamide solution is normally stable for up to 4 wk.
2. Carrier ampholytes: The protocol described below was developed using 40% (w/v) carrier ampholytes (stock solution Resolyte™; Merck). Ampholytes are also available from other commercial sources. Although various pH ranges for carrier ampholytes can be obtained, the most commonly used are pH 4.0–8.0 for the first dimension separation in 2DE and broad-range pH 3.0–10.0 carrier ampholytes for preparing the 2D lysis buffer. Stock solutions of carrier ampholytes should be stored at 4°C and kept sterile.

3. Gel polymerization catalysts: The same catalysts are used for preparing the first and second dimension gels—TEMED and 10% (w/v) ammonium persulfate. The latter solution is prepared fresh when required.
4. First dimension electrode buffers. Catholyte 0.1 *M* NaOH, Anolyte: 25 m*M* H_3PO_4, prepared immediately before use.
5. Tracking dye: Add bromophenol blue to MilliQ water at a concentration sufficient to provide a blue color. Store this solution at room temperature.
6. Carrier ampholyte (CA) equilibration buffer: 60 m*M* Tris-HCl, pH 6.8, 2.3% (w/v) sodium dodecyl sulfate (SDS), 2.5 m*M* DTT. Prepare the CA equilibration buffer when required; 10 mL is normally sufficient to process a batch of 10 small format 2D gels.
7. 1% agarose: 1% (w/v) agarose, 0.125 *M* Tris-HCl, pH 6.8, 1% (w/v) SDS. Dissolve the agarose in a boiling-water bath. Add bromophenol blue powder to provide blue color.
8. IPG re-swelling buffer: 7 *M* urea, 2 *M* thiourea, 4% (w/v) CHAPS, 0.3% (w/v) DTT. Add bromophenol blue powder to the re-swelling buffer to provide pale blue color. Store at −20°C in aliquots of convenient volumes to avoid repeated cycles of freeze-thaw.
9. IPG equilibration buffer: 50 m*M* Tris-HCl, pH 8.8, 6 *M* urea, 30% (v/v) glycerol, 2%, (w/v) SDS. Add bromophenol blue powder to provide a pale blue color. The solution can be prepared as a 200 mL volumes and then stored at −20°C in 20-mL aliquots.

2.4. Second Dimension Electrophoresis

1. Second Dimension acrylamide solution: 30% (w/v) acrylamide, 0.8% (w/v) methylenebisacrylamide. Store at 4°C, protected from light. It is normally stable for up to 3 mo under these conditions.
2. Second Dimension electrophoresis running buffer: 0.4% (w/v) glycine, 0.05 *M* Trizma base, 1% (w/v) SDS. Prepare when required.

2.5. Colloidal Coomassie Brilliant Blue Staining

1. Gel Fixer: 50 (v/v) ethanol, 2% (v/v) phosphoric acid.
2. Stain equilibration buffer: 34% (v/v) methanol, 17% (w/v) ammonium sulfate, 2% (v/v) phosphoric acid. Freshly prepared as required.

3. Methods

3.1. Growth of Bacteria and Preparation of Bacterial Proteins

Alterations in the conditions of growth of *H. influenzae* can lead to distinctive patterns of protein synthesis and thus changes in the proteome. Specific growth conditions are selected depending on the purpose of the study, and it is essential that they are maintained and controlled throughout. The following sample preparation protocols describe the recovery of bacteria grown on agar plates and in broth culture.

3.2. Recovery of Bacteria from Agar Plates

1. Grow *H. influenzae* on chocolate agar in 5% CO_2 at 37°C. Typically, a single plate containing a confluent growth of *H. influenzae* provides sufficient material for subsequent analysis by 2DE. Cell lysates prepared as described below are stable at −70°C for up to 12 mo for the abundant proteins, although if repeated analyses are to be carried out, then the sample should be frozen in small volumes to minimize potentially destructive cycles of freeze–thaw.
2. After incubation, use a plating loop to remove the bacteria from the surface of the plate. Dislodge the bacteria into 1 mL of PBS-A in a 1.5-mL Eppendorf centrifuge tube. The bacteria are recovered from the suspension by centrifugation at 11,000*g* for 5 min.
3. Discard the supernatant, taking care to remove all of the PBS-A, and resuspend the bacterial pellet in 0.5 mL of 2D lysis buffer. Leave the bacterial suspension on ice for 5 min to lyse and then centrifuge at 11,000*g* for 5 min. Remove the supernatant and store at −70°C for subsequent analysis. (*see* **Note 1**).

3.3. Recovery of Bacteria from Broth Culture

1. Grow *H. influenzae* in rich or minimal liquid culture media. The following protocol has been used to examine bacteria grown in *Haempohilus* test (HT) medium. Bacteria previously grown on agar plates should be grown overnight in 10 mL of liquid medium prior to large-scale culture.
2. Inoculate 25 mL of HT medium with 1 mL of the overnight broth culture. Incubate at 37°C in a shaking water bath and harvest the bacteria when the optical density is OD_{A605} 0.6. Alternative inoculation and growth conditions can be determined empirically.
3. Harvest the bacteria from the broth culture by centrifugation at 2000*g* for 15 min. Resuspend the bacterial pellet in 1 mL of PBS-A and transfer to a clean 1.5-mL Eppendorf tube. Pellet the bacteria by centrifugation at 11,000*g* for 5 min and discard the supernatant. Wash once in PBS-A and remove all traces of the PBS-A before resuspending the pellet in 0.3 mL of 2D lysis buffer. Leave the suspension on ice for 5 min before removing insoluble cell debris by centrifugation at 11,000*g* for 5 min. Store the supernatant at −70°C for later analysis.

3.4. Analysis of Proteins by 2DE

1. Typically, 2DE uses isoelectric focusing in the first dimension and SDS-PAGE in the second dimension for the separation of complex protein mixtures. Two methods can be employed for the first dimension charge separation: the use of carrier ampholytes, or immobilized pH gradients (IPG). The second dimension electrophoresis is the same regardless of which first dimension separation protocol is used. By varying the conditions of separation in the first and second dimensions, it is possible to optimize the separation of proteins with specific isoelectric points and molecular weights. **Figure 1** shows the effect of using either carrier ampholyte containing gels or broad-range IPG gels for the first dimension separation.

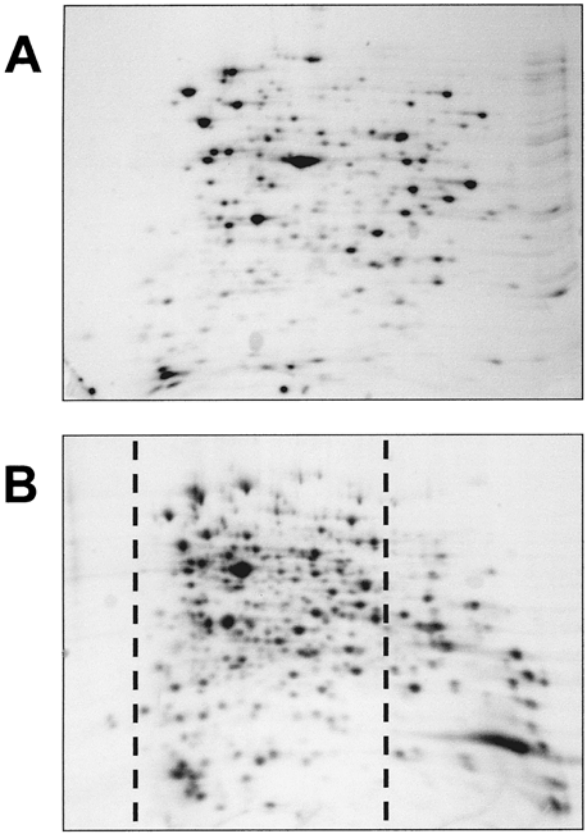

Fig. 1. Analysis of cellular proteins from *H. influenzae* strain Rd by 2DE. Bacterial proteins were prepared as described in the text and analyzed using either pH 4.0–8.0 carrier ampholytes **(A)** or pH 3.0–10.0 (nonlinear) immobilized pH gradients **(B)** for the first dimension separation. The second dimension separation used 10–15% gradient polyacrylamide gels. Proteins were detected using colloidal Coomassie brilliant blue G250 staining. The vertical dotted lines in panel **B** indicate the approximate pH range covered by the carrier ampholytes in panel **B**.

2. The protocols described below use 2D minigels, which are simple and rapid to use, although they have limitations in their resolution and the amount of protein that can be loaded before distortion of the protein pattern becomes apparent.

3.4.1. First Dimension Separation Using Carrier Ampholytes

The following protocol uses a Mini-PROTEAN II tube gel apparatus (Bio-Rad) for the first-dimension gels.

1. The first dimension tube gels are prepared in disposable glass capillary tubes (length 75 mm and internal diameter 1 mm) held within a larger glass casting tube (length 85 mm and internal diameter 10 mm) that is sealed at the bottom with a double layer of Parafilm. The first-dimension acrylamide gel solution is prepared as follows: 9.4 mL IEF acrylamide solution, pH 4.0–8.0; 0.6 mL carrier ampholytes, 15 µL TEMED, 15 µL 10% ammonium persulfate.
2. The catalysts (TEMED and 10% ammonium persulfate) are added immediately before casting the tube gels. The gel solution is added to the casting tube to a level 3–5 mm above the top of the capillary tubes, taking care not to introduce air bubbles. The gel solution is overlaid with water-saturated butanol and left to polymerize overnight. Twelve tube gels are cast simultaneously using this method.
3. Following polymerization, the acrylamide plug in which the capillary tubes have become embedded is expelled from the casting tube using gentle pressure from a 25-mL pipet bulb. The capillary tubes are separated from the surrounding acrylamide and cleaned by gentle washing with MilliQ water.
4. Capillary tubes are assembled in the Mini-PROTEAN II tube gel module using the sample wells and connecting tubes supplied. The tube gel assembly is lowered into the lower buffer tank containing the anolyte and the upper buffer chamber is filled with the catholyte. Care is required to prevent air bubbles from being trapped at the tops of the capillary gels during the addition of the catholyte and the sample itself. Remove trapped air bubbles using a disposable 1-mL syringe fitted with a 21-gage needle.
5. Samples prepared as described in the previous section are mixed with the tracking dye solution (ca. 2 µL dye solution to 30 µL sample), clarified by centrifugation at 11,000*g* for 5 min and loaded on to the gels. The volume of sample loaded should be no more than 10 µL, as lower volumes produce better-quality protein profiles. Increasing the amount of protein loaded on the gels can cause smearing of the proteins in the first dimension (*see* **Note 2**).
6. The first dimension gels are focused using the following conditions: 500 V (constant voltage) for 15 min and 1500 V (constant voltage) for 2 h. Extended periods of focusing can result in streaking, particularly of those proteins with basic isoelectric points (*see* **Note 3**).
7. Following electrophoresis, expel the gels from the capillary tubes using hydrostatic pressure with a gel ejector (Bio-Rad) connected to a 1-mL syringe filled with CA equilibration buffer. Lay the gels along the tops of the second dimension slab gels and flood with equilibration solution. During the equilibration (7 min at room temperature), lay the second dimension slab gels horizontally to produce a natural trough in which to hold the tube gel and equilibration buffer. Then remove the equilibration buffer and attach the tube gels to the top of the slab gels using 1% agarose. Avoid using excess agarose for this stage, since it can interfere with protein resolution in the second dimension.

3.4.2. First Dimension Separation Using Immobilized pH Gradient Gels

Many of the problems encountered with carrier ampholytes can be overcome using immobilized pH gradient (IPG) gels for separation in the first

dimension. IPG gels provide more stable gradients; they can be prepared with defined broad or narrow pH gradients; they show high gel-to-gel reproducibility; and they will accept high protein loads suitable for preparative gels. The following protocol uses 7-cm IPG gels from Amersham-Pharmacia Biotech which are available commercially in pH ranges of 4.0–7.0 and 3.0–10.0.

1. The IPG gels produced commercially are available in a dehydrated form and are rehydrated in IPG reswelling buffer. Carrier ampholytes (final concentration 2%, w/v) are added to the IPG reswelling buffer. The pH range of the carrier ampholytes should be the same as that covered by the IPG gel. Immobilized pH gradient gels from Amersham-Pharmacia Biotech have a protective plastic film over the gel surface, and this must be removed before rehydration. The most convenient method for gel rehydration is to use a DryStrip Reswelling Tray (Amersham-Pharmacia Biotech). The required volume of reswelling buffer is added to a trough in the tray and the IPG strip is placed gel side down into the buffer and left overnight at room temperature. This method has the advantage that the sample can be loaded on to the IPG gel during the rehydration phase by adding it to the reswelling buffer. Thus the sample is distributed throughout the gel and efficient entry of the sample to the gel is obtained. During rehydration, the strips are overlaid with 2–3 mL of DryStrip cover fluid (Amersham-Pharmacia Biotech).
2. It is important to reswell the IPG strips in the correct volume of buffer. For 7 cm IPG gels, the final volume of sample and reswelling buffer is 125 µL. Up to 60 µL of protein sample prepared as described in the previous sections can be loaded on to a single IPG gel strip. To achieve higher protein loads for preparative studies with 7 cm IPG gels, the bacteria are lysed in smaller volumes than those stated above. Small sample volumes must be loaded if they have been prepared in buffers containing neither urea nor nonionic detergent, in order to minimize the dilution of the urea and detergent in the reswelling buffer.
3. Following their rehydration, remove the IPG gels from the tray and drain excess DryStrip cover fluid. The IPG gels are focused using a Multiphor II apparatus fitted with an Immobiline DryStrip kit (Amersham-Pharmacia, Biotech). Briefly, place the IPG gel strips in the DryStrip tray of the Multiphor II with the ends of the gels aligned. Place filter paper strips moistened in MilliQ water over the extreme ends of the gels and place the electrodes over the filter paper strips. Once assembled, flood the gel tray with DryStrip cover fluid.
4. The following running conditions are suitable for 7 cm IPG gels with pH ranges of pH 4–8 and pH 3–10 (linear or nonlinear gradients): stage 1, 200 V for 1 min; stage 2, 3500 V for 1 h 30 min; stage 3, 3500 V for 1 h 5 min (all stages at 2 mA and 5 W). The isoelectric focusing is carried out using a programmable EPS 3500XL Power Pack (Amersham-Pharmacia, Biotech) with a gradient voltage increase between the steps (*see* **Notes 3** and **4**).
5. Following electrophoresis, the DryStrip cover fluid is drained from the strips, which are then placed in 11-mL conical screw-capped centrifuge tubes. The IPG

gels are equilibrated in two stages prior to the second dimension electrophoresis. The first equilibration—in IPG equilibration buffer containing 1% (w/v) DTT for 35 min—is followed by a second 35-min equilibration in IPG equilibration buffer now containing 2.5% (w/v) iodoacetamide. Care should be taken when handling iodoacetamide and solutions containing this compound, since it is toxic and highly irritant to mucous membranes. During the equilibration stages the tubes should be vigorously agitated.

6. Lay the equilibrated IPG gels into the trough formed at the top of the second dimension slab gel. Flood the trough with second dimension electrophoresis running buffer to prevent air bubbles from being trapped below the strip. Add a small amount of tracking dye to provide a visible bromophenol blue dye front during the second dimension electrophoresis.

3.4.3. Separation in the Second Dimension

The following protocol for the second dimension electrophoresis uses the SE250 slab gel apparatus from Amersham-Pharmacia Biotech.

1. The second dimension electrophoresis is carried out using linear gradient polyacrylamide gels, which provide better resolution than single-concentration acrylamide gels. The compositions of the acrylamide gel solutions used for the second-dimension gels are given in **Table 1**. *H. influenzae* proteins can be resolved using either 7–15% or 10–15% polyacrylamide gradients.
2. Prepare the second dimension acrylamide gels in batches of up to 12 to optimize the reproducibility among them. Individual gel sandwiches for the SE250 gel apparatus are each made with a plain glass and a notched aluminum plate separated with plastic spacers of either 0.75-mm or 1-mm thickness for first dimension tube gels or IPG gels, respectively. Assemble the gel sandwiches in the multiple gel-casting unit (Amersham-Pharmacia, Biotech), separated from each other by Parafilm strips. Excess space in the casting unit is filled using a combination of glass plates and plastic sheets. The gel sandwiches should fit closely into the casting unit but not so tightly that the pressure distorts them.
3. The acrylamide gradient is formed using a standard linear gradient maker. The casting units are filled from the bottom and all solutions are pumped into the casting unit at a flow rate of 2.5 mL/min. MilliQ water is first used to fill the space below the gel sandwiches and also to act as an overlay for the upper gel surface. Immediately after the water has been pumped from the chamber, and before air enters the connection tubing, the gel solutions are added using the volumes stated in **Table 2**; the low density (7% or 10%) acrylamide gel solution is placed in the mixing chamber of the gradient maker. Once all of the gel solution has been pumped from the gradient maker, and again before air enters the connection tubing, 75% glycerol is added to the gradient maker and pumped into the casting unit to displace the acrylamide solution from the space below the gel sandwiches. The glycerol–acrylamide interface should be just below the base of the gel plates.

Table 1
Composition of Second Dimension Acrylamide Gel Solutions for Gradient Gels

Stock solution	Final acrylamide solution 7%	10%	15%
30% Acrylamide (mL)	11.2	16.0	24.0
2M Tris-HCl, pH 8.8 (mL)	9.0	9.0	9.0
75% Glycerol (mL)	1.4	1.4	14.5
10% (w/v) SDS (mL)	0.5	0.5	0.5
MilliQ water (ml)	25.9	21.4	–
		Catalysts	
TEMED (μL)	10	10	10
10% (w/v) Ammonium persulfate (μL)	100	100	30

The solution compositions are suitable for the preparation of 7–15% and 10–15% polyacrylamide gradient gels. The catalysts are added immediately before adding the solutions to the gradient maker.

Table 2
Gel Capacity of Casting Units Supplied by Amersham-Pharmacia Biotech

	Large casting unit		Small casting unit	
	0.75-mm gels	1.0-mm gels	0.75-mm gels	1.0-mm gels
Number of gels	12	9	5	4
Volume of 7% or 10% acrylamide solution	43	39	19	18
Volume of 15% acrylamide solution	43	39	19	18

4. The slab gels are allowed to polymerize overnight. The individual gel sandwiches are separated and the first-dimension tube or IPG gels placed on the surface of the gels as described above. When using carrier ampholyte tube gels, remove excess polymerized gel using a scalpel from the slab gels to the level of the notch in the aluminum plate. Alternatively, if the level of the gel surface is below the level of the notch, fill the space with 1% agarose. For analyses using IPG gels in the first dimension, a trough approx 3–4 mm deep is required at the top of the slab gels, into which the strips are placed.
5. Assemble the second-dimension slab gels into the running units. Fill the upper and lower buffer chambers with second-dimension electrophoresis running buffer and perform electrophoresis at 75 V (constant voltage) for 60 min, followed by

150 V (constant voltage) for 160 min, by which time the bromophenol blue dye front has reached the end of the slab gel. The gels are cooled with running tap water during electrophoresis.

3.5. Detection of Proteins after Electrophoretic Separation

Most protein detection systems compatible with 1D SDS-PAGE can also be used for 2DE. Total protein profiles are normally detected using either Coomassie brilliant blue or silver staining. Silver staining is the more sensitive of the two methods but, unlike Coomassie brilliant blue, interferes with subsequent protein identification using peptide mass mapping and MALDI-TOF mass spectrometry (*see* **Note 5**). Silver staining can routinely be carried out using a commercially available kit following the recommendations of the manufacturer; our laboratory uses the Plus-One Protein staining kit (Amersham-Pharmacia, Biotech), which can be used with a programmable autostainer. The Coomassie brilliant blue staining procedure described below is fairly sensitive, convenient for batch processing of slab gels, and suitable for subsequent protein identification by peptide mass mapping.

3.5.1. Colloidal Coomassie Blue G250 Staining

1. The following Coomassie blue staining protocol has been modified from that described by Anderson et al. *(6)* for use with the small gel format *(2)*. The volumes given are suitable for processing ten 8 × 7 cm slab gels as a single batch. Volumes should be adjusted proportionately for different numbers and sizes of gels. Carry out the staining in a plastic sandwich box large enough to allow the gels to float freely. The gels are continuously agitated throughout the staining procedure.
2. Fix the gels overnight in 200 mL of gel fixer and then wash three times in excess (ca. 300 mL) tap water for 30 min each.
3. Soak the gels in 200 mL stain equilibration buffer for 1 h before adding 0.2 g Coomassie brilliant blue G250 to the solution. Although protein spots can be detected after a few hours of staining, optimal staining is normally achieved after 4 d. No destaining is required, and following the staining period the gels are washed with water to remove stain particles.
4. For long-term storage, dry the stained gels between cellophane sheets in a hot-air dryer.

3.5.2. Determination of Isoelectric Points and Molecular Weights

Estimates of the isoelectric points and molecular weights of the bacterial proteins can be obtained by extrapolation from known standard proteins co-electrophoresed with the protein samples. Commercial software packages (**Table 3**) available for the analysis of 2D protein profiles contain routines for this purpose. Carbamylated creatine phosphokinase (Merck) can be used to determine the isoelectric points in the range pH 4.95–7.0 and standard protein

Table 3
Commercial 2D Gel Analysis Packages

Software name	Operating system	Supplier
BioImage 2D Software	UNIX	Genomic Solutions
Melanie II	UNIX, MS Windows, Power Macintosh	Bio-Rad
PDQuest	MS Windows, Power Macintosh	Bio-Rad
Phoretix 2D	MS Windows	Phoretix International

mixtures (e.g., low-mol.-wt. standards from Amersham-Pharmacia Biotech) can be used for mol. wt. determination. The standards are mixed with the sample before electrophoresis. The ratio of standard to sample is determined empirically, depending on the staining procedure adopted, to allow detection of the marker and sample proteins simultaneously. In addition, once some of the proteins within the 2D protein profile have been identified, their theoretical isoelectric points and molecular weights can be used as internal standards against which to calibrate the remaining proteins within the sample.

3.5.3. Computer Analysis

At least four analytical software packages, working under different operating systems, are now available commercially for the analysis of 2D protein profiles (**Table 3**). Digitized images of the gels can be produced by a variety of input devices, including high-quality cameras containing a light-sensitive charge-coupled device (CCD), densitometers, and phosphoimagers to generate 8-, 12-, or 16-bit images. Low-cost document scanners can also be used to generate 8-bit images suitable for analysis. Although the specific underlying routines used by the packages vary, the software permits the detection of protein spots on the digitized image, the matching of the protein profiles across a series of gels, and the production of quantitative data on protein abundance. Each of these processes is partially automated, but operator input is also required to optimize some of the steps. Subsequent analyses (e.g., statistical analysis of the data) may be part of the analytical package itself, or the data can be exported from the 2D package to an external statistical software program. No matter which analytical 2D software package is finally selected for use, it is essential that the raw data, i.e., the 2D gels, be of the highest quality.

3.5.4 . Protein Identification

Since the initial description of 2DE 25 yr ago, various methods have been used to identify the resolved protein spots and link them to the bacterial genes

open reading frames (ORFs) encoding them (reviewed by Cash *[7]*). The most generally applicable method for protein identification is peptide mass mapping, using mass spectrometry to size tryptic peptide fragments. Detailed experimental protocols for protein identification using mass spectrometry are beyond the scope of this chapter, and interested readers as referred to the article by Jensen et al. *(8)*. Peptide mass mapping is ideal for working with bacterial species for which the genome sequence has been determined in at least one representative isolate. Briefly, cut out gel fragments containing single protein spots, remove the stain, and then digest the proteins *in situ* using trypsin (*see* **Note 6**). The peptides are extracted and sized using a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer. These peptide masses can then be used to interrogate databases in which predicted peptide profiles have been generated from known protein and gene sequences.

3.5.5. Application of 2DE to H. influenzae Studies

Three representative applications of 2DE in the study of *H. influenzae* are presented here to highlight the specific practical points made in the text.

3.5.5.1. Comparison of *H. influenzae* Isolates

Variability at the protein level among clinical isolates of *H. influenzae* can be readily investigated using 2DE. This can be used in qualitative studies in which the identities of the proteins showing electrophoretic differences are unknown. However, once protein identities are assigned to the 2D protein profiles, then more value can be added to the data and the extent of variation in specific sets of genes can be annotated. To analyze electrophoretic variability of individual proteins, it is more reliable to compare samples from different bacterial isolates using co-electrophoresis rather than running separate gels for each sample. An example of co-electrophoresis, comparing proteins from a clinical isolate with an extract of the sequenced Rd strain of *H. influenzae*, is presented in **Fig. 2**. The protein products for three genes are indicated, which show differences in electrophoretic mobilities between the two isolates. In each case, the alteration affects the protein's charge only, suggesting an amino acid substitution.

For cross comparisons of a large number of isolates, it is impractical to compare the bacteria in every combination by co-electrophoresis. The most convenient approach is to select one isolate as a reference to which each of the other isolates is compared.

3.5.5.2. Induced Changes in Protein Synthesis

The proteome is a very sensitive indicator of the environment in which the organism exists. Alterations to the proteome consisting primarily of quantita-

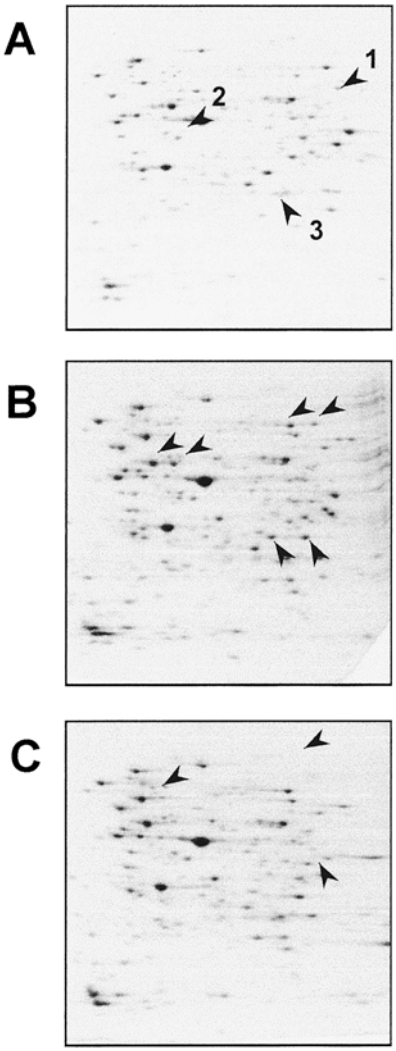

Fig. 2. Comparison of the cellular proteins of *H. influenzae* strains Rd and HI64443 by 2DE. The bacterial proteins from each isolate were resolved by 2DE (pH 4.0–8.0 carrier ampholytes) either individually or by co-electrophoresis. Proteins were detected using colloidal Coomassie brilliant blue G250 staining. The arrows in the panels indicate three proteins identified by peptide mass mapping that show differences in their isoelectric points between the two isolates. The proteins are numbered in panel **A** as follows: (1) 2',3'-cyclic-nucleotide 2'-phophodiesterase precursor; (2) aspartate ammonia lyase; (3) hypothetical protein HI0146. The 2D protein profiles are as follows: **(A)** strain Rd; **(B)** co-electrophoresis of Rd and HI64443; **(C)** strain HI64443.

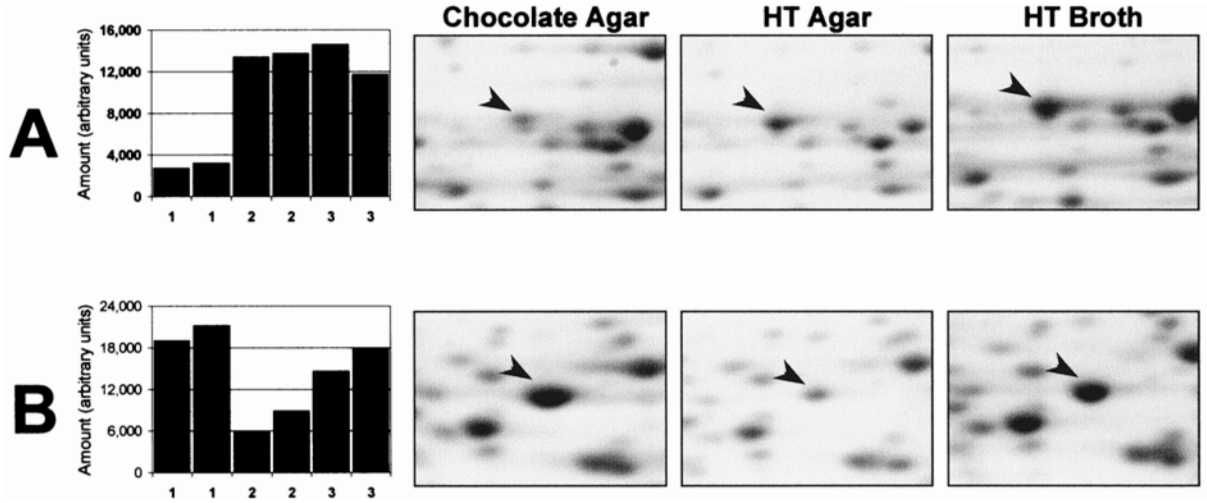

Fig. 3. Effect of growth conditions on *H. influenzae* protein synthesis. *H. influenzae* strain Rd was grown under the three growth conditions indicated and the proteins analyzed by 2DE; only a limited region of the 2D protein profile is presented for each gel. Phoretix 2D was used to quantify protein synthesis, and the normalized amounts of protein synthesized under each condition are given in the graphs in arbitrary units. Replicate analyses for each determination are presented in the graphs: (1) chocolate agar; (2) HT agar; (3) HT broth. The selected proteins were identified by peptide mass mapping as follows: (**A**) heme binding protein A precursor; (**B**) hypothetical protein HI0146. (HT, *Haemophilus* test medium—*see* **Subheading 2.2.** for composition).

tive changes in protein abundance can be induced by differences in the growth conditions of the organism—for example, by changing nutrient levels, the method of growth (e.g., solid vs broth media), or altering the phase of the growth cycle at which the bacteria are harvested (**Fig. 3**). Although these changes are significant in their own right, they should be considered in comparative studies. Care should be taken in interpreting differences in the protein profiles, which may be due to differences in growth conditions.

Although the detection of such changes may be the goal of an investigation, it is important that the sensitivity of the proteome to growth conditions be fully appreciated when it comes to interpreting the differences in protein profiles between strains in comparative studies. These differences may simply be due to differences in growth conditions.

3.5.5.3. Characterization of Genetically Engineered Mutants

The characterization of genetically engineered mutants using 2DE is an attractive approach to the elucidation of complex networks of interaction between gene products. **Figure 4** shows the comparison of proteins synthesized by *H. influenzae* strain Eagan with those from an otherwise isogenic *sodA* knockout mutant *(9)*. Comparative 2DE of the total cellular proteins prepared from each strain identified the spot corresponding to SodA in the wild type (absent in the mutant, and its identity subsequently confirmed by N-terminal amino acid sequencing). However, two further protein spots (labeled 1 and 2 in **Fig. 4**) showed quantitative differences in their abundance between the wild type and mutant bacterial strains. Peptide mass mapping of these protein spots revealed that the ORF HI0572 encoded both, their differing positions perhaps representing posttranslationally processed variants of the protein.

4. Notes

1. The standard cell lysis conditions described in **Subheading 3.1.** are suitable for the majority of *H. influenzae* isolates. For bacterial isolates possessing capsules, sonication can be used to increase the disruption of the bacterial cells. Sonication is most efficient using a probe sonicator immersed in the bacterial suspension. Bursts (ca. 30 s) of sonication are used with the sample held on ice between the cycles of sonication.
2. The presence of the detergent in the 2D lysis buffer restricts the use of the majority of standard protein assays for quantifying the protein concentration of the samples, necessary as a guide for subsequent loading of the 2D gels. A useful approach to assess loading volumes for 2D gels is to analyze a standard volume of sample by 1D-PAGE on minigels and stain the gels using the Coomassie brilliant blue protocol described in this chapter. It is a relatively quick process to establish a correlation between the loading of 1D and 2D gels to provide suitable loading volumes. In practice, however, using standard growth conditions and

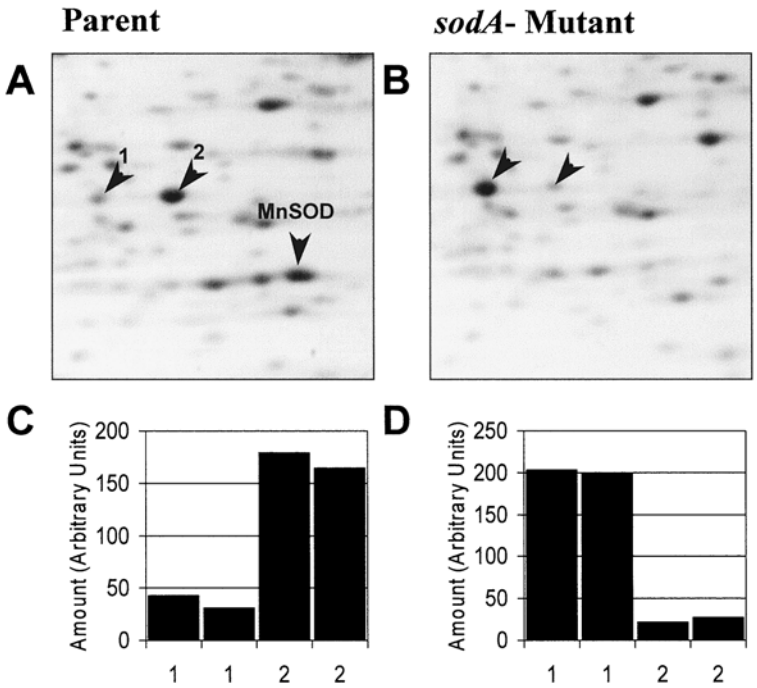

Fig. 4. Characterization of protein synthesis in a *sodA* knockout mutant. The cellular proteins from *H. influenzae* strain Eagan and from a *sodA* knockout mutant were analyzed by 2DE; only a limited region of the 2D protein profile is presented for each gel. The protein product of *sodA* (MnSOD) was identified on the basis of its absence in the mutant and by N-terminal sequencing of the protein spot. Quantitative differences were demonstrated by Phoretix 2D for two protein spots (labeled 1 and 2 in **A** and identified as hypothetical protein HI0572 by peptide mass mapping), and these are represented in arbitrary units in the graphs, which show replicate determinations.

sample preparation protocols, reproducible and consistent protein recoveries are normally achieved over time.

3. The times indicated for the first dimension electrophoresis with either carrier ampholytes (*see* **Subheading 3.2.**) or IPG gels (*see* **Subheading 3.2.**) are suitable for analyzing a wide range of samples. However, before embarking on extended studies it is wise to optimize the time for the first dimension separation. This is done by removing first dimension gels at intervals during electrophoresis and processing them for the second dimension electrophoresis. The time for the first dimension electrophoresis should be such that there is clear resolution of the proteins and minimal streaking of basic proteins that can occur during extended electrofocusing, particularly when using carrier ampholytes.
4. The first dimension electrophoresis conditions may require further modification if the sample contains high concentrations of salt, which can be a problem with

the first dimension separation, particularly when using carrier ampholytes. IPG gels are less sensitive to the presence of salt in the sample, and further improvements can sometimes be achieved by modifying the run conditions to the following: stage 1, 100 V for 1 min; stage 2, 100 V for 2 h; stage 3, 200 V for1 min; stage 4, 3500 V for 1 h 5 min; stage 5, 3500 V for1 h 5 min (all stages at 2 mA and 5 W).

5. Sypro Ruby has a reported sensitivity equivalent to silver staining and is compatible with mass spectrometry ***(10)***. After staining with Sypro Ruby, the proteins are detected using a fluorescent transilluminator.
6. Great care must be taken to minimize keratin contamination of the samples, which in extreme situations may swamp the peptide signal of the protein being analyzed. The major sources of keratin contamination are the solutions used for electrophoresis, and of course the skin and clothing of the operator. Precautions should routinely be taken to maintain the purity of stock solutions: gloves should be worn at all times for gel preparation and processing. In addition, the handling of the gels after electrophoresis should be kept to the absolute minimum.

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References

1. Cash, P., Argo, E., and Bruce, K. I. (1995) Characterisation of *Haempohilus influenzae* proteins by 2-dimensional gel electrophoresis. *Electrophoresis* **16,** 135–148.
2. Cash, P., Argo, E., Langford, P. R., and Kroll, J. S. (1997) Development of an *Haempohilus* two-dimensional protein database. *Electrophoresis* **18,**1472–1482.
3. Langen, H., Gray, C., Roder, D., Juranville, J. F., Takacs, B., and Fountoulakis, M. (1997) From genome to proteome: Protein map of *Haempohilus influenzae*. *Electrophoresis* **18,** 1184–1192.
4. Link, A. J., Hays, L. G., Carmack, E. B., and Yates, J. R. (1997) Identifying the major proteome components of *Haempohilus influenzae* type-strain NCTC 8143. *Electrophoresis* **18,** 1314–1334.
5. Fountoulakis, N., Takacs, B., and Langen, H. (1998) Two-dimensional map of basic proteins of *Haempohilus influenzae*. *Electrophoresis* **19,** 761–766.
6. Anderson, N. L., Esquer-Blasco, R., Hofinann, J. P., and Anderson, N. G. (1991) A two-dimensional gel database of rat liver proteins useful in gene regulation and drug effects studies. *Electrophoresis* **12,** 907–930.
7. Cash, P. (1998) Characterisation of bacterial proteomes by two-dimensional electrophoresis. *Analytica. Chirnica. Acta.* **372,** 121–146.

8. Jensen, O. X., Wilin, M., Shevchenko, A., and Mann, M. (1999) Sample preparation methods for mass spectrometric peptide mapping directly from 2-DE gels, in *2-D Proteome Analysis Protocols* (Link, M., ed.), Humana Press, Totowa, NJ, pp. 513–530.
9. D'Mello, R. A., Langford, P. R., and Kroll, J. S. (1997) Role of bacterial Mn-cofactored superoxide dismutase in oxidative stress responses, nasopharyngeal colonization, and sustained bacteremia caused by *Haempohilus influenzae* type b. *Infect. Immun.* **65,** 2700–2706.
10. Steinberg, T. R., Haugland, R. P., and Singer, V. L. (1996) Applications of SYPRO orange and SYPRO red protein gel stains. *Anal. Biochem.* **239,** 238–245.

8

Monitoring Gene Expression Using DNA Arrays

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1. Introduction

A single phenotypic change observed during an organism's growth or adaptation to its environment is usually the result of a coordinated expression of genes ranging from a few to many. Various techniques are in use today for quantifying gene expression. These include indirect methods, such as protein reporter fusions (e.g., β-galactosidase, chloramphenicol acetyltransferase, green fluorescent protein, and luciferase), and more direct techniques such as Northern blotting. Nevertheless, these techniques suffer from the drawbacks of being time-consuming, labor-intensive and allow only small numbers of genes to be studied at any given moment. DNA array technology now enables the analysis of a larger number of genes, including whole genomes, in a single experiment ***(1)***. In general, DNA samples of genes of interest are spotted onto a solid support surface in an ordered manner. RNA is isolated from both the control and experimental bacterial samples and reverse-transcribed while simultaneously incorporating marker molecules (radioactive or Cy3 and/or Cy5 nucleotides), resulting in labeled cDNA. The degree of hybridization of the labeled cDNA probes to the arrays is then detected using appropriate systems. A comparison is made of the intensities of the probe between the control and experimental samples and any differences observed reflect either up- or down-regulation of particular genes.

Two sizes of array are in use, macro or micro. Macroarrays can be easily constructed in the laboratory by hand using a multichannel pipet or dot-blot equipment. Whereas microarrays are the preserve of robotics due to the small size of the sample spots generated (ca. hundreds of micrometers in diameter). Microarrays can themselves be split into two categories depending on the manufacturing technique. One approach is to spot DNA samples, generally

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PCR products or oligonucleotides, using a microdispensing robot to apply the DNA directly to either glass slides or membranes *(2)*. The other approach is to synthesize oligonucleotides directly on a silicon surface using a photolithographic process reminiscent of the microprocessor industry *(3)*.

In this chapter we describe the procedures involved in the construction of a macroarray. *Haemophilus influenzae* Rd genes of interest can be identified from the genome sequence (*4*, www.tigr.org).

2. Materials

RNA is very labile, and extreme care must be taken not to introduce ribonucleases (RNase) inadvertently. To minimize any degradation, all procedures should be carried out on ice unless otherwise indicated, and most important, gloves must be worn. It is also vital that all glassware and plasticware is RNase free, and where possible, sterile disposable plasticware is used. Ensure that all reagents are of molecular biology grade and used specifically for RNA-related work. It is also advisable to use validated DNase/RNase-free water sold specifically for RNA work.

2.1. Preparation of the Array—Isolation of Genomic DNA

1. Supplemented brain heart infusion (sBHI) broth: BHI supplemented with protoporphyrin IX (Sigma P5889) and NAD (Roche 127981). Make up the BHI broth according to the manufacturer's instructions (Difco) and autoclave at 120°C for 15 min. Add protoporphyrin (10 µg/mL) and NAD (2 µg/mL) immediately before use.
2. TSE buffer: 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA, 100 m*M* NaCl.
3. TE buffer: 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA.
4. 20-mg/mL Lysosyme, (Sigma L6876). Make up in 1-mL aliquots in water and store at −20°C.
5. Lysis buffer: 400 m*M* Tris-HCl, pH 8.0, 60 m*M* EDTA, 150 m*M* NaCl, 1% sodium dodecyl sulfate (SDS).
6. 10-mg/mL RNaseA, (Sigma R5503).
7. 20-mg/mL Proteinase K, (Sigma P8044). Make up in 1-mL aliquots in water and store at −20°C.
8. Phenol/chloroform/isoamyl alcohol (25/24/1); obtained ready-mixed from commercial sources.
9. Chloroform.
10. Ethanol.
11. 3 *M* Sodium acetate, pH 4.6.

2.2. PCR Products for the Arrays

1. Genomic DNA.
2. 50 pmol gene-specific oligonucleotides.
3. 5-U/µL Taq DNA polymerase.
4. 10× reaction buffer (supplied with the Taq polymerase).

5. 25 m*M* Mg^{2+} (supplied with the Taq polymerase).
6. Pre-mixed dNTPs (10 m*M* of each dATP, dCTP, dGTP and dTTP).
7. Sterile dH_2O.

2.3. Examination of PCR Products by Gel Electrophoresis

1. Agarose.
2. 50× TAE buffer: 242 g Tris-base, 100 mL of 0.5 *M* EDTA, pH 8.0, 57.1 mL glacial acetic acid per liter.
3. 10-mg/mL ethidium bromide.
4. Minigel electrophoresis casting tray and tank.
5. DNA molecular-weight markers (1-kb ladder, Life Technologies, 156150-16).
6. 6× gel loading buffer: 0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll, Type 400, Amersham Pharmacia Biotech (AP Biotech) in water.
7. UV transilluminator and gel documentation system.
8. Power supply.

2.4. Purification and Quantification of PCR Products—Small Number of Samples

1. 3 *M* Sodium acetate, pH 4.6.
2. Ethanol.
3. TE buffer.

2.5. Precipitation of PCR Products in a 96-Well Format Plate

1. Isopropanol.
2. 3 *M* Sodium acetate, pH 4.6.
3. 20-µg/µL Glycogen (Life Technologies 10814-010).
4. 70% ethanol.
5. TE buffer.

2.6. Array Construction

1. 20-ng/µL PCR products.
2. Charged nylon membrane (Hybond-N, AP Biotech RPN303N).
3. Absorbent paper (Whatman 3MM).
4. Square Petri dishes (15 × 15 cm).
5. Dot-blot equipment.
6. Vacuum pump or other source of vacuum.
7. Denaturation solution: 0.5 *M* NaOH, 1.5 *M* NaCl. Freshly prepared.
8. Neutralization solution: 1 *M* Tris-HCl, pH 7.4, 1.5 *M* NaCl.
9. UV crosslinker (e.g., Stratagene UV Stratalinker 2400).

2.7. Preparation of cDNA Probe

2.7.1. RNA Isolation

1. *H. influenzae*.
2. RNA isolation kit (Qiagen RNAeasy kit).
3. RNase/DNase-free water (Merck 443847D).

4. *Escherichia coli* 16s + 23s ribosomal RNA (Sigma R8510).
5. DNase I (Life Technologies 18068–015).
6. 10× DNase I buffer (supplied with the DNase I).
7. 25 m*M* EDTA, (supplied with the DNase I).
8. Heating block.
9. UV spectrophotometer.

2.7.2. Preparation of cDNA Probe

1. RNA.
2. Reverse transcriptase (Superscript II, Life Technologies 18064–022).
3. Labeling primers (reverse PCR set—0.05 pmol final concentration).
4. dATP, dGTP, and dTTP nucleotides (Life Technologies).
6. Nuclease-free water.
7. [α-^{32}P]dCTP nucleotide (AP Biotech AA0005).
8. Heating block.

2.7.3. Purification of cDNA Probe

Sephadex G50 columns (MicroSpin G-50 columns, AP Biotech 27-5330-01).

2.7.4. Denaturation of Probe

1. Heating block.
2. Ice.

2.8. Probing and Detection

2.8.1. Hybridization Solutions

1. 20× SSC: 3 *M* NaCl, 0.3 *M* Na citrate (adjusted to pH 7.0 with 10 *M* NaOH)
2. 50× Denhardt's solution: 1% (w/v) Ficoll, 1% (w/v) polyvinylpyrrolidone, 1% (w/v) bovine serum albumin. Stored at −20°C in 3 mL aliquots.
3. 10% (w/v) SDS.
4. Sheared salmon sperm DNA (Sigma D9156).
5. Formamide (Merck 444475W).

2.8.2. Wash Solutions

1. 20× SSC.
2. 10% SDS

Prepared in advance and kept at the appropriate temperatures until required.

2.8.3. Stripping Solution

A boiling solution of 10% SDS.

3. Methods

3.1. Array Construction

3.1.1. Isolation of Genomic DNA

Extreme care must be taken when working with phenol and chloroform.

1. Set up an overnight culture of *H. influenzae* in 20 mL of sBHI at 37°C shaking at 160 rpm.
2. The following day, centrifuge the culture at 2500*g* for 15 min at 20°C and discard the supernatant.
3. Resuspend the pelleted cells in 25 mL of TSE buffer.
4. Centrifuge the resuspended cells at 2500*g* for 15 min at 20°C.
5. Resuspend the cells in 2 mL of TE buffer.
6. Add 2 µL of lysozyme and incubate the cells at 20°C for 10 min.
7. Add 400 µL of lysis buffer, 5 µL of RNase and 12 µL of proteinase K. Mix thoroughly and incubate at 50°C for at least 30 min.
8. Aliquot the lysed cells into four microcentrifuge (1.5-mL) tubes.
9. Add an equal volume of the phenol/chloroform/isoamyl alcohol and mix by repeatedly inverting the microcentrifuge tubes for at least 5 min (an emulsion should form).
10. Spin at 12,000*g* in a microcentrifuge for 10 min.
11. Remove the upper aqueous phase, carefully avoiding the denatured protein at the interface, and place into new microcentrifuge tube.
12. Repeat **steps 9–11** twice more.
13. Add an equal volume of chloroform and repeatedly invert for 1 min.
14. Spin at 12,000*g* in a microcentrifuge for 5 min.
15. Remove and pool the upper aqueous layer into a 10-mL polypropylene tube.
16. Add 1/10th vol of 3 *M* sodium acetate and 2 vol of ethanol. The DNA should precipitate out at this stage.
17. Spool the DNA into a microcentrifuge tube containing 1 mL of 70% (v/v) ethanol.
18. Invert the tube a few times and briefly spin (use the pulse button) to just pellet the DNA.
19. Remove the ethanol and briefly air-dry (*see* **Note 1**).
20. Add 200 µL of TE and leave overnight at 4°C. If the DNA has not dissolved, then heat to 65°C for 20 min.
21. Determine the concentration and purity of the chromosomal DNA by taking OD_{A260} and OD_{A280} readings in a spectrophotometer. The ratio of OD_{A260}/OD_{A280} should be at least 1.8 (*see* **Note 2**).

3.1.2. PCR Products for the Arrays

1. The PCR reactions are performed in volumes of 100 µL, although 50 µL volumes can also be used if there are a large number of reactions, thus saving on reagents. The use of machines without heated lids necessitates the use of mineral oil to prevent evaporation of the reaction mixture.

2. Set up the PCR as follows: genomic DNA (100 ng), dNTP's (10 m*M* of each), reaction buffer (×10), $MgCl_2$ (25 m*M*; *see* **Note 3**), forward and reverse primers (50 pmol of each; *see* **Notes 4** and **5**), Taq polymerase (2.5 U; *see* **Note 6**), and dH_2O to a total volume of 100 µL.
3. The amplification conditions depend on a variety of factors, but in general the following should work in most cases (*see* **Note 7**): Initial denaturing at 95°C for 5 min; then 30 cycles of 95°C for 30 s, 50°C for 30 s, 72°C for 60 s; and a final extension at 72°C for 10 min.

3.1.3. Examination of PCR Products by Gel Electrophoresis

Care should be taken with ethidium bromide, since it is highly mutagenic and toxic. After microwaving the agarose will become superheated, so thermal gloves should be worn.

1. Weigh out enough agarose to make a 1% (w/v) solution with 1× TAE in a flask.
2. Microwave the flask on a high setting until the solution just starts to boil. Carefully remove the flask and gently swirl to ensure that the agarose has dissolved; if it has not, microwave slightly more.
3. Allow the agarose solution to cool on the bench until it is hand-hot (ca. 55–60°C).
4. Add ethidium bromide to a final concentration of 0.5 µg/mL and mix by swirling the solution gently, avoiding the formation of bubbles.
5. Gently pour the solution into a gel casting tray with an appropriate gel comb and allow to cool on the bench until the agarose has solidified.
6. Place the cast gel into its tank and remove the comb. Add enough 1 × TAE buffer until the gel is just submerged.
7. Remove 10 µL of the PCR reaction and to it add 2 µL of gel loading buffer. Mix well.
8. Load the mixture into the gel wells. Load DNA marker into one of the outside wells.
9. Replace the lid of the gel tank and apply a voltage of 70 V.
10. Once the bromophenol blue dye front has migrated to two-thirds of the way down the gel, remove the gel, visualize on a UV transilluminator, and photograph.
11. Examine the photograph for amplified DNA migrating at the size estimated from the sequence data.
12. In the case of PCRs performed in 96-well plates, gel systems are available that can accommodate all 96 samples on a single gel.

3.1.4. Purification and Quantification of PCR Products

1. A number of methods are available for the purification of PCR products, either standard ethanol or polyethylene glycol (PEG) precipitation, depending on the number of samples to be purified, and commercial kits are also available.
2. If mineral oil has been layered on the PCR reactions, then it is necessary to remove it to minimize carryover in the purification step. This can be achieved by freezing the PCR reaction at −20°C at which temperature the mineral oil remains liquid and so can be easily aspirated with a fine pipet tip.

3.1.4.1. If a Small Number of Samples is to be Purified

1. Add 1/10th vol of 3 *M* sodium acetate and 2.5 vol of ethanol.
2. Mix the solution and place at −70°C for 15 min to allow the DNA to precipitate.
3. Spin the microfuge tubes at maximum speed (12,000*g*) for 15 min. At this stage a small white pellet may be seen; if not, work carefully. Discard the supernatant.
4. Add 1 mL of 70% ethanol and mix by inverting gently a few times.
5. Spin the tubes again at 12,000*g* for 5 min.
6. Carefully remove the 70% ethanol.
7. Air-dry the DNA pellet briefly and dissolve in 100 µL of TE.

3.1.4.2. Samples to be Purified in a 96-Well Plate

1. Mix 1 mL of sodium acetate with 2.5 µL of glycogen.
2. Add 10 µL of the glycogen/sodium acetate mixture to each of the wells.
3. Add 88 µL of isopropanol to each of the wells.
4. Replace the lid tightly and vortex.
5. Centrifuge the plate at 500*g* for 1 min.
6. Incubate the plate at 20°C for 15 min.
7. Centrifuge the plate at 2500*g* for 45 min.
8. Immediately after the centrifugation step, open the lid and invert the plate onto tissues to drain the liquid.
9. Add 150 µL of 70% ethanol to each well.
10. Centrifuge the plate at 2500*g* for 10 min.
11. Quickly drain the liquid onto tissues and spin the plate for 1 min at 500*g* while the plate is still inverted on the tissues.
12. Add 100 µL of sterile water into each well to dissolve the DNA pellets. Quantify the amount of purified PCR product by measuring the OD_{260} (*see* **Note 8**). Dilute a small sample of each purified PCR product to 20 ng/mL (*see* **Note 9**).

3.1.5. Array Construction

Arraying of the PCR products can be achieved by either spotting them by hand or by the use of dot-blot equipment.

3.1.5.1. By Hand (See Note 10)

1. Cut two sheets of the absorbent paper such that they fit within the square Petri dish and that there is 2–3 mm space between the edge of the paper and the edges of the dish (the paper will expand when soaked).
2. With a pencil, draw a grid on the top sheet of cut paper such that the intersection of the lines is approx 7–10 mm apart. The intersecting lines can be used as a target for the DNA to be spotted. The size of the grid will also depend on the number of samples to be spotted.
3. Soak the absorbent paper with the denaturation solution. Gently layer the nylon membrane on top of the wet paper, ensuring that there are no trapped bubbles. The pencil grid should be visible through the wet membrane.

4. Carefully, with a fine pipet, spot onto the membrane the PCR samples using the intersecting lines as a target. Spot the samples in duplicate.
5. Using clean forceps, transfer the membrane to a square Petri dish containing neutralization solution. After 5 min, remove the membrane and place onto dry absorbent paper.
6. While the membrane is still slightly damp, immobilize the DNA by UV crosslinking (e.g., Stratalinker, 120 mJ/cm^2).

3.1.5.2. Using a Dot-Blot/Vacuum Pump

1. Assemble the dot-blot equipment according to the manufacturer's instructions (*see* **Note 11**).
2. Switch on the vacuum source (*see* **Note 11**).
3. Carefully avoiding the sides of the wells, add the DNA samples to the center of each of the wells.
4. Once all the samples have been applied, switch off the vacuum source.
5. Using clean forceps, remove the membrane and place into a shallow dish containing denaturation solution for 5 min.
6. Transfer the membrane into a shallow dish containing neutralization solution for 5 min.
7. Remove the membrane and place on to absorbent paper and then leave to air-dry until slightly damp. Immobilize the DNA by UV crosslinking (Stratalinker, 120 mJ/cm^2).

3.2. Preparation of cDNA Probe

Traditionally, the "hot phenol" method has been used to extract RNA; for safety reasons and ease of use, it is advisable to use RNA extraction kits. All solutions are guaranteed to be RNase-free. Various types of kits are available based on either phenol/chloroform/guanidium chloride, or columns. Columns may have the disadvantage of not binding the smaller RNA species (*see* **Note 12**), but this may not be a problem, depending on the target genes on the array.

3.2.1. RNA Isolation

1. Grow *H. influenzae* under the culture conditions required.
2. Isolate the RNA according to the procedure chosen.
3. To remove contaminating DNA, a DNase I reaction is performed: RNA (× µL), 10× DNase I reaction buffer (10 µL), DNase I (10 µL), water (RNase free; to give a total volume of 100 µL). Incubate the reaction mixture at 20°C for 15 min. Then add 10 µL of 25 m*M* EDTA, (pH 8.0) to the reaction and heat at 65°C for 10 min. Purify the RNA using the same method as above (*see* **Note 13**).

3.2.2. Quantification and Quality of RNA

Make a 10-fold dilution of the RNA in RNase-free water, and measure the OD_{260} (1 is equivalent to 40 µg/mL of RNA). Remove 1 µg of RNA for gel analysis and store the remainder at −70°C. Since this is only a qualitative procedure, we have found that a normal agarose gel is as effective as specific

formaldehyde RNA gels at indicating the extent of RNA integrity. To ensure that any degradation observed in the gel is due to initial isolation, rather than being introduced during subsequent analyses, equipment and solutions have to be RNase-free. Soaking and washing all gel tanks, gel trays, and combs with 5% (v/v) Decon (Merck 56022), then giving all items a final rinse in RNase-free water appears to be effective.

1. Prepare an agarose gel as described in **Subheading 3.1.3.**
2. Add the appropriate amount of gel loading buffer to the RNA samples.
3. Load the samples into the wells of the agarose gel and load the RNA markers into adjacent wells.
4. Electrophorese the samples at 70 V.
5. Once the dye front migrates halfway down the gel, visualize the RNA on a UV transilluminator.

Strong RNA bands should be seen co-migrating with the 16s and 23s RNA markers (*see* **Note 12**). RNA degradation is indicated by a ladder effect or smearing, regardless of the intensity.

3.2.3. Preparation of a cDNA Probe

The construction of the cDNA probe is performed in two stages, the initial denaturation of any secondary structures formed within the RNA and annealing of the oligonucleotides, followed by the incorporation of the label during the cDNA synthesis.

1. Thaw all reagents on ice and keep on ice while setting up the following reaction: 5× reverse transcriptase buffer (6.4 µL), dATP, dTTP, dGTP (10 mM each; 3 mL); labeling primers (0.5 µM; 4 µL) (*see* **Note 14**), RNA (1 µg; × µL) (*see* **Note 15**), 100 m*M* DTT (1 m*M* final; 0.32 µL), water (RNase free; to a total of 25 mL).
2. Heat the mixture to 80°C for 5 min and then cool to 42°C over a period of 20 min. Microfuge at 12,000*g* for 1 min.
3. Immediately add [α-^{32}P]dCTP (50 µCi; 5 µL) (*see* **Note 16**) and reverse transcriptase (2 µL) and incubate at 42°C for 2 h.

3.2.4. Purification of a cDNA Probe

A Sephadex microspin column is used to remove the unincorporated radioactive nucleotides. To monitor the labeling efficiency, it is recommended that the amount of radioactivity left in the column and that of the purified probe be compared (*see* **Note 17**).

3.2.5. Denaturation of Probe

Heat the purified probe to 95°C for 5 min and then immediately quench on ice. Once cool add to the hybridization solution.

3.3. Probing and Detection

Prehybridization and hybridization are best performed in glass tubes, since these are easier to handle than plastic bags. The solutions used for both the prehybridization and the hybridization (minus the probe) are the same. It is more convenient to make a bulk preparation and then split the volumes into two.

3.3.1. Prehybridization and Hybridization

Make the solution as follows: 3 mL of 20× SSC, 5 mL of formamide, 1 mL 50× Denhardt's solution, 0.25 mL of 10% SDS, sheared salmon sperm (*see* **Subheading 2.8.1.**), water (0.65 mL to a total of 9.9 mL).

3.3.1.1. Prehybridization

To 4.95 mL of the hybridization solution add 50 µL of sheared salmon sperm DNA that has been heated to 100°C for 5 min and then immediately cooled on ice (*see* **Note 18**). Add this prehybridization solution to the hybridization bottles containing the membrane, with the DNA facing into the center of the tube. Prehybridize the membrane at 42°C for 2 h.

3.3.1.2. Hybridization

To the remaining 4.95 mL of hybridization solution add 50 µL of sheared salmon sperm DNA that has been denatured as above. Drain the prehybridization solution from the bottles and add fresh solution containing the denatured cDNA probe, avoiding touching the membrane. After a brief mix by swirling, hybridize the membrane at 42°C for 48 h.

3.3.2. Washes

1. Washing of the membranes can be done in the same glass bottles as the hybridization or in large plastic sandwich boxes.
    a. Wash solution 1 (0.5 × SSC 0.1% SDS): 20 × SSC (5 mL), 10% SDS (2 mL), water (197 mL).
    b. Wash solution 2 (0.1 × SSC + 0.1% SDS): 20 × SSC (1 mL), 10% SDS (2 mL), water (197 mL).
2. Wash the membrane three times for 10 min each at 20°C with wash solution 1, and then once with wash solution 2 for 20 min at 65°C (*see* **Note 19**).
3. After washing, the membrane is transferred to absorbent Whatman 3MM paper (to remove excess liquid) and then completely covered in two sheets of Saran wrap, ensuring that creases and bubbles are kept to a minimum.

3.3.3. Detection

X-ray (Hyperfilm, AP Biotech) or phosphorimaging can be used to detect signals. It is important to note that membranes should be wrapped with Saran

wrap so that there are no wrinkles or air bubbles, otherwise out-of-focus spots may be observed in the final image. Careful rewrapping of the blot with a fresh piece of Saran wrap typically resolves the issue. It is preferable that the image is scanned at 50 µm density (phosphorimaging), since the spots tend to be of a significantly lower resolution when scanned at 100 or 200 µm. The drawback of using X-ray film, in contrast to phosphoimaging, is the nonlinear nature of the signal intensity produced over time (*see* **Note 20**). Since the amount of probe specific for a particular target sequence is very small, the time period for which the membrane has to be exposed can be as long as a week.

3.3.4. Stripping Solution

To probe the nylon membrane with another sample of labeled cDNA, the existing membrane has to be stripped. This can be achieved by placing the membrane into an appropriately sized container with a boiling solution of 10% SDS poured over it. Sufficient liquid to fill half the container should be used. The solution is left to cool to 20°C on the bench. Rinse the membrane in 2× SSC and dry between two sheets of Whatman 3MM. The membrane is now ready for further probing (*see* **Note 21**).

3.4. Analysis

3.4.1. Analysis of Array Data

Quantification of the gene expression signals is best determined from phosphorimager-generated files. The image files may be analyzed using a number of software packages, including ImageQuant (Molecular Dynamics), Quantity One (BioRad Laboratories), OptiQuant (Packard Instrument Company), or MacBas (Fuji). Array Vision (Imaging Research) can read image files generated on the Molecular Dynamics Storm, Bio-Rad Quantity, or Fuji MacBas instruments and is designed to analyze all the spots on a high-density array at one time.

3.4.2. Analysis Using ImageQuant

Full details are given in the ImageQuant manual. In brief, an image file is opened, an ellipse laid down on top of each individual spot and, after background correction and analysis (Volume Report), the results are exported as Microsoft Excel format files. This enables the user to further manipulate, sort, and represent the data in a desired manner.

It is possible to determine the fold induction or fold reduction in expression by comparison of corresponding spots from different blots probed with experimental and control samples. To compare spot intensities, the values are expressed as either percent of control spots such as genomic DNA or total pixel values from all genes in each array. The expression levels in experimental and control conditions are compared by determining the ratio of the corre-

sponding averaged percent intensities of each pair of gene-specific spots on the two blots (each gene-specific spot is normally present in duplicate). The resulting ratio of positive and negative values is considered as induction and reduction of the gene in the experimental model relative to the control. For identification of significant gene changes, the arithmetic or log ratio should be ≥2. Statistically, the significant changes are the ratio higher than 2 standard deviations (SD) of all ratios (confidence=95%) or 2.5 SD (confidence=99%). However, researchers should be aware that some genes may be induced to a small degree but still may be responsible for significant physiological changes in the organism under certain conditions.

An example of phosphorimages obtained after hybridization of an *H. influenzae* DNA array with cDNA from the organism grown under two different conditions is shown in **Fig. 1**, and their subsequent analysis using ImageQuant software is shown **Fig. 2**.

3.5. Applications

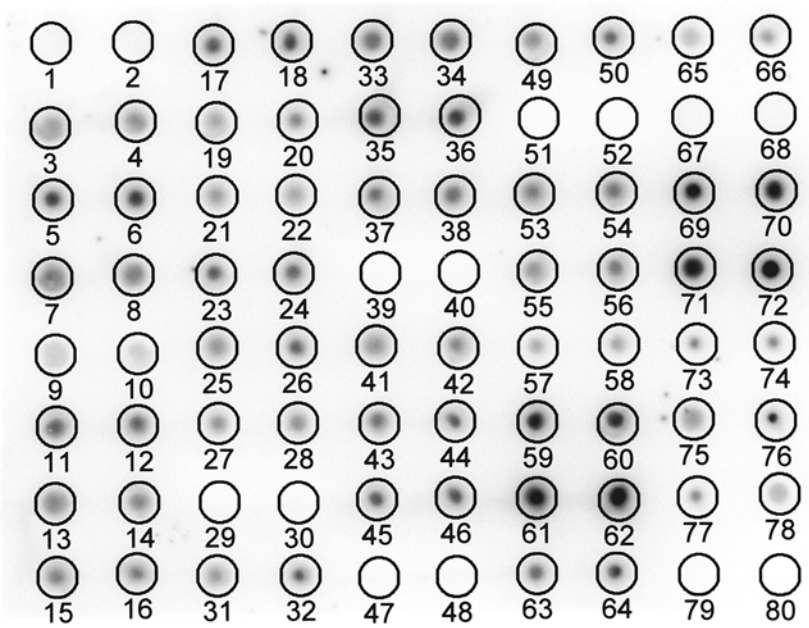
DNA arrays are typically used to analyze the gene expression of organisms grown in different environments, thus giving insights into metabolic and regulatory pathways ***(1)***. They can also be used to indicate the mode of action of inhibitory agents ***(5,6)***, comparative genomic studies between strains ***(7)*** and identification of organisms ***(2)***. Additionally, comparison of wild-type and mutant strains can be used to identify regulons and stimulons ***(8)***. Comparison of the gene expression of bacteria grown in vitro to those in vivo (or their surrogates, i.e., tissue culture models) will lead to the identification of genes differentially expressed during infection. Such genes have implications for the design of future therapeutic intervention strategies ***(9)***.

4. Notes

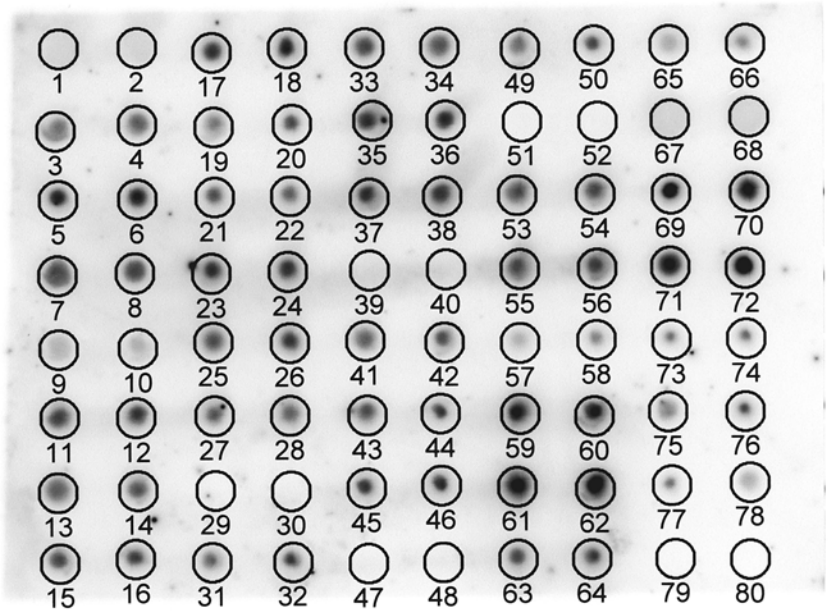
1. Do not overdry DNA as it becomes very difficult to dissolve.
2. Purity is crucial, since any inhibitors may interfere with PCR.
3. Taq can be obtained either with Mg^{2+} in the enzyme buffer or separately. Although it may be convenient to use an all-in-one buffer, separate Mg^{2+} is more useful when the templates are difficult to amplify, as using different Mg^{2+} concentrations can result in successful amplifications.
4. Generally when designing primers for mass PCR, try to ensure that the annealing temperatures of the primers are very similar, i.e., within 2–3°C of each other.

Fig. 1. *(opposite page)* Phosphorimages of a single DNA macroarray of *H. influenzae* genes. The genes were probed with radiolabeled first-strand cDNA obtained from the organism grown under different conditions (**A** and **B**). The images were processed with ImageQuant, the ellipses (ELPS) over each spot defining the areas that were analyzed. Each gene was spotted in duplicate (1–2, 3–4, 5–6, etc.).

A


1 2 17 18 33 34 49 50 65 66
3 4 19 20 35 36 51 52 67 68
5 6 21 22 37 38 53 54 69 70
7 8 23 24 39 40 55 56 71 72
9 10 25 26 41 42 57 58 73 74
11 12 27 28 43 44 59 60 75 76
13 14 29 30 45 46 61 62 77 78
15 16 31 32 47 48 63 64 79 80

B


1 2 17 18 33 34 49 50 65 66
3 4 19 20 35 36 51 52 67 68
5 6 21 22 37 38 53 54 69 70
7 8 23 24 39 40 55 56 71 72
9 10 25 26 41 42 57 58 73 74
11 12 27 28 43 44 59 60 75 76
13 14 29 30 45 46 61 62 77 78
15 16 31 32 47 48 63 64 79 80

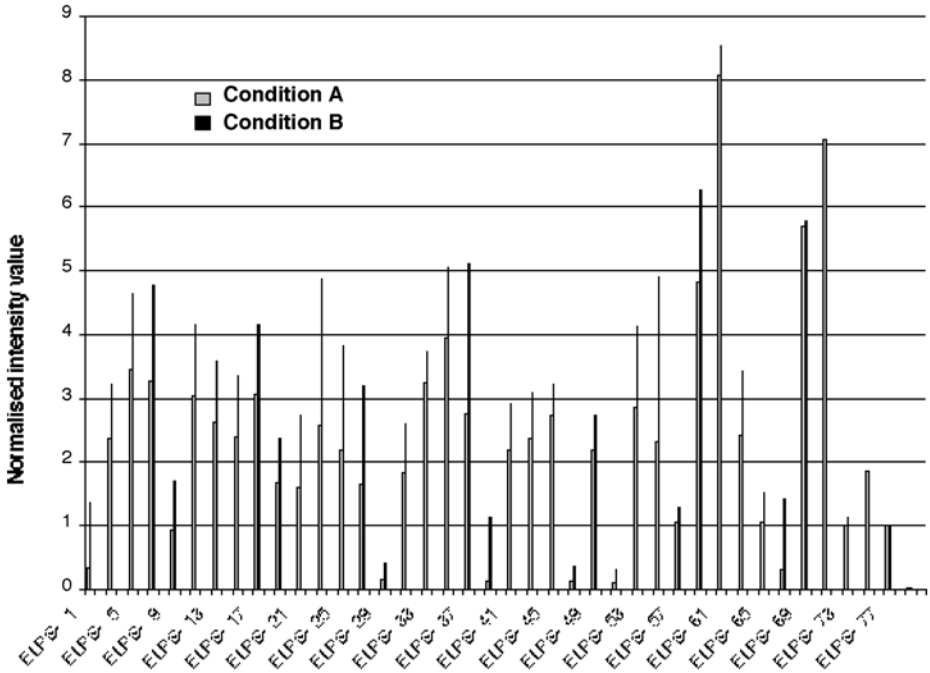


Fig. 2. Comparison of *H. influenzae* gene expression under two different growth conditions. The phosphorimages shown in **Fig. 1A, B** (representing growth conditions A and B, respectively) were analyzed with ImageQuant. The data show the comparison of mean normalized values of spot intensity obtained for individual genes under growth conditions A (left columns) and B (right columns). It is possible to calculate comparative gene expression from this data. For example, for gene (ELPS) 55 there is approximately twice the gene expression under condition B compared to condition A.

5. We have found that HotStar Taq (Qiagen) is a reliable, robust polymerase.
6. Quite a lot of time can be saved and possible errors due to repetitive pipetting can be avoided if a master mix is made containing all the reagents except for the primers.
7. The conditions given are for starting only; these will have to be modified depending on the annealing temperatures of the primers. Also, in minor cases alterations will have to be made due to differences in the G+C content between genes.
8. If a large number of PCR samples has to be quantified, a convenient fluorescence method is available that uses Picogreen (Molecular Probes). This can be performed in 96-well plates using a fluorometer to measure the excitation of the bound dye to DNA. A curve has to be plotted using known standards against which values are measured.

9. If a strong PCR product is obtained, as judged by gel electrophoresis, then quantification can be a problem. However, semiquantification is possible by measuring accurately the concentration of one PCR sample and then comparing the intensity in the gel picture with other samples. This is usually of sufficient accuracy to prepare arrays where workers do not have access to UV spectrophotometers or fluorimeters.
10. Try experimenting with different mixtures of TE buffer with gel loading dye, to see how well formed the spots are. Spreading can be minimized by using sample volumes not larger than 1 μL.
11. When using a dot-blot system it is advisable to practice as in **Note 10** to get the combination of vacuum and the pressure of the lid right to prevent significant leakage around the wells. The Minifold I 96-spot blot system (Schleicher & Schuell) has rubber rings around each of the wells, forming a very tight seal against the membrane. The company also manufactures a 96-well format "spot blot" accessory that has well dimensions of 1×2 mm. This can be used to make arrays of 96 samples on the same membrane as well as providing enough space to make duplicates by moving the membrane slightly. If this system is to be used, then adding sample dye to the DNA will ensure that there is no overlap of the wells after moving the membrane.
12. If the Qiagen RNAeasy kit has been used to purify the RNA, then the 5s RNA band will be missing.
13. Generally, phenol/chloroform extraction is performed, but if possible, the use of commercial kits is recommended due to their ease of use.
14. The PCR reverse set of labeling primers can be substituted for random hexamers (need to use 1 μg).
15. If the random hexamers are to be used for the cDNA synthesis, this will entail the use of much larger quantities of RNA (approx 40 μg) to achieve the same kind of labeling efficiency of mRNA as specific primers, since mRNA is only 5% of total RNA.
16. Use radioactivity that is very close to its activity date (within 3–4 d) to ensure a good signal.
17. Similar readings using a calibrated Geiger counter should be obtained when comparing the column with the purified probe, since this indicates the probe has labeled efficiently. If the probe count is low compared to that of the column, then the labeling reaction is best repeated. If this is not possible, then the exposure time will have to be much longer to obtain a good signal.
18. Prepare the prehybridization solution without the sheared salmon sperm DNA beforehand and warm at 42°C. Immediately before use, add the denatured salmon sperm DNA and then add to the hybridization bottles containing the membrane.
19. Make the wash solutions in advance and keep at the appropriate temperature until ready for use.
20. If X-ray film is to be used, then different exposures will have to be taken to ensure that the values fall within the linear range of the film.
21. It is advisable not to strip the membrane more than four times, since the membrane deteriorates.

References

1. Tao, H. (1999) Functional genomics: expression analysis of *Escherichia coli* growing on minimal and rich media. *J. Bacteriol.* **181,** 6425–6440.
2. Wilson, M. (1999) Exploring drug-induced alterations in gene expression in *Mycobacterium tuberculosis* by microarray hybridization. *Proc. Natl. Acad. Sci. USA* **96,** 12,833–12,838.
3. Cho, R. J. (1998) Parallel analysis of genetic selections using whole genome oligonucleotide arrays. *Proc. Natl. Acad. Sci. USA* **95,** 3752–3757.
4. Fleischmann, R. D. (1995) Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. *Science* **269,** 496–512.
5. Marton, M. J. (1998) Drug target validation and identification of secondary drug target effects using DNA microarrays. *Nat. Med.* **4,** 293–301.
6. Troesch, A. (1999) *Mycobacterium* species identification and rifampin resistance testing with high-density DNA probe arrays. *J. Clin. Microbiol.* **37,** 49–55.
7. Behr, M. A. (1999) Comparative genomics of BCG vaccines by whole-genome DNA microarray. *Science* **284,** 1520–1523.
8. Hughes, T. R. (2000) Widespread aneuploidy revealed by DNA microarray expression profiling. *Nat. Genet.* **25,** 333–337.
9. McKenney, D. (1999) Broadly protective vaccine for *Staphylococcus aureus* based on an *in vivo*-expressed antigen. *Science* **284,** 1523–1527.

9

Gene Expression Technology

Xavier de Bolle and Christopher D. Bayliss

1. Introduction

Reporter genes are widely used in molecular biology. In mammalian cells, expression of a gene encoding luciferase allows the monitoring of promoter activity through the measurement of light produced during catalysis. In yeast and procaryotic cells, the *lacZ* gene of *Escherichia coli* (encoding a β-galactosidase enzyme) is the most frequently used reporter gene. In the last decade, the use of genes encoding fluorescent proteins, such as the *Aequorea* green fluorescent protein (GFP), has become widespread. In *Haemophilus influenzae*, gene expression has been monitored successfully with both *lacZ* and *gfp* genes.

1.1. The lacZ and gfp Reporter Genes

The *lacZ* gene is part of the *lacZYA* operon in *E. coli*. The *lacY* gene encodes a lactose permease (LacY), which transports lactose into the cytoplasm. Lactose is the natural substrate of the cytoplasmic enzyme β-galactosidase (LacZ), the product of the *lacZ* gene. LacZ cleaves lactose into glucose and galactose. LacZ also catalyzes the synthesis from lactose of allolactose, this compound is bound by the Lac repressor (LacI) and inhibits binding of this repressor to the Lac operator, which is located upstream of the *lacZYA* operon. Thus, in *E. coli*, a basal level of β-galactosidase causes derepression of the *lacZYA* operon in the presence of lactose. β-galactosidase is a large protein, with 1,023 amino acids. The active enzyme is a tetramer, whose structure has been solved ***(1)***.

The activity of β-galactosidase forms the basis of the α-complementation test, a technique routinely used in molecular biology. Briefly, the chromosome or an episome of an *E. coli* strain carries a copy of the *lacZ* gene that has a deletion in its N-terminal portion (*lacZΔM15*). Bacteriophages of the M13mp series, plasmids of the pUC, series and phagemids such as pGEm or pBluescript

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have a shortened version of the *lacZ* gene (*lacZ'*) that comprises only the N-terminal portion of the enzyme (also named the α peptide). The two truncated forms of the *lacZ* product can associate into an active enzyme. The enzymatic activity can be monitored by plating colonies on a medium containing the 5-bromo-4-chloro-3-indolyl-β-D-galactoside chromophore, often abbreviated to X-Gal. Since X-Gal is an analog of lactose, it is recognized and cleaved by β-galactosidase to give galactose and 5-bromo-4-chloro-indole; the latter is a blue-colored compound, whereas X-Gal is not colored. The *lacZ* gene has proved to be a versatile reporter gene because the cleavage of X-Gal produces a phenotype (white/blue test) that is easy to detect during a screening procedure and also because the β-galactosidase enzyme is tolerant to some modifications of its N terminus.

β-galactosidase activity can be measured with a simple colorimetric test. This test relies on the ability of β-galactosidase to cleave ortho-nitrophenyl-β-D-galactopyranoside (ONPG) into ortho-nitrophenol (ONP) and β-D-galactose. ONP has a peak of absorbance at a wavelength of 420 nm, whereas ONPG does not absorb at this wavelength.

GFP (for a review, *see* **ref. 2**) is a small protein compared to β-galatosidase, with a monomer molecular weight of 27 kDa. GFP was first extracted from *Aquorea* species. The structure of GFP is a "β-can," i.e., a barrel structure with a major antiparallel β-sheet. The fluorophore, arising from an intramolecular reaction, is buried into the structure, and is not accessible to potential fluorescence quenchers. There are various versions of the *gfp* coding sequence, some being adapted to use with laboratory equipment such as fluorescence-activated cell sorter (FACS) systems. The use of GFP in *Haemophilus ducreyi* ***(3)*** suggests that it could be an interesting tool to trace *H. influenzae* in ex vivo or animal models of infection, as reported for the interaction of *Neisseria gonorrhoeae* with human endometrial cells ***(4)***. However, expression of GFP from a single copy of the gene incorporated into the chromosome of *H. influenzae* may not produce enough fluorescence for routine, reproducible detection of gene expression (Bayliss, unpublished data).

1.2. Transcriptional Fusions

One principal use of reporter genes is to monitor the transcription level of a particular gene. This is achieved by fusing the promoter of the gene of interest with the coding sequence of a reporter gene. This technology is extremely powerful for the detailed analysis of the transcriptional activity of a promoter. Indeed, the major usage of promoter/reporter fusions is for measuring the activity of numerous variants of a promoter, such as small deletions in regions of the promoter that are suspected to play a role in regulation of transcription initiation. It should be noted, however, that only the initiation of transcription

is monitored using these types of fusions. The effects of any mechanisms that act after initiation of transcription, such as pausing of the RNA polymerase during elongation of the transcript, are missed.

For the monitoring of the transcriptional activity of several promoters simultaneously, DNA microarray technology is preferable to the use of reporter genes. DNA microarrays and their use for the study of the transcriptome of *Haemophilus* are described in Chapter 8. Reporter gene technology is more suited to the study of small perturbations in promoters and to the discovery of bacterial promoters that are specifically induced in particular conditions ***(5)***. Differential fluorescence induction (DFI) ***(6)*** is an example of a method that uses a GFP reporter gene to find promoters that are induced in specific conditions—for example, during the infection of cultured macrophages ***(7)***.

1.3. Translational Fusions

In *H. influenzae*, as well as in other pathogenic bacteria such as *Neisseria meningitidis* ***(8)*** or *Helicobacter pylori* ***(9)***, several genes have tandem repeats or microsatellites in their coding sequences. In *H. influenzae*, these repeats are usually tetranucleotides and the genes that contain such repeat tracts are often surface-exposed molecules or involved in biosynthesis of such molecules—for example, proteins involved in the acquisition of iron or in the biosynthesis of lipopolysaccharide (LPS) ***(10)***. In the latter case, variant bacteria with changes in the number of repeats in the gene, which occurs at a high rate, can be detected using monoclonal antibodies directed to specific epitopes of LPS. The molecular mechanism responsible for changes in the number of repeats is still to be determined, but slip-strand mispairing (polymerase slippage) during a DNA replication event is one of the most commonly cited mechanisms. In order to further characterize the molecular mechanism in *H. influenzae*, a reporter gene system was set up to quantify the slippage rate at a particular locus ***(11)***. In this chapter we describe the experimental procedures used to construct and analyze translational fusions between a *lacZ* reporter gene and a gene containing tandem repeats. These procedures can easily be adapted for investigating promoters or non-repeat-containing genes.

2. Materials

2.1. Construction of lacZ Reporter Gene Fusions

1. PCR apparatus (Perkin-Elmer). *Taq* polymerase (Roche).
2. Cloning vector: pGEM-T (Promega) or pCR2.1 (Invitrogen). Competent cells (e.g., DH5α) are transformed and plated on LB medium containing 40 µg/mL X-Gal (Roche). The stock solution of X-Gal is made by dissolving X-Gal in N,N'-dimethyl formamide (DMF), as a 1000-fold stock (40 mg/mL). The solution is light-sensitive and can be kept at −20°C for up to 6 mo.

3. The pGEM11Zf(+) vector (Promega).
4. The pCH110 vector (Amersham-Pharmacia) (*see* **Note 1**). This plasmid contains *lacZ* fused to a eukaryotic promoter that has a high background activity in *E. coli*. This *lacZ* gene has a *Kpn*I site (*see* **Note 2**) that is ideally located for insertion of foreign DNA.
5. The pUC4K vector (Amersham-Pharmacia).

2.2. Analysis of Reporter Expression

2.2.1. H. influenzae *Transformation*

1. BHI medium, used to cultivate *H. influenzae* strains, is made with brain heart broth (Merck). Plates are made with this media and bacteriological agar (Oxoid), 14.8 and 6 g, respectively, are added to 400 mL of distilled water and then autoclaved. Levinthal blood preparation ($1/10^{th}$ v/v) is added to the molten BHI medium just before pouring plates. Levinthal blood preparation is made as follows: 500 mL of defibrinated horse blood (Tissue Culture Services HB035) is added to 1 L of sterile liquid BHI (Roche) in a 2-L flask. The mix is heated in a waterbath at 95°C for 40 min, with occasional shaking. It is then allowed to cool down to below 50°C. The mix is centrifuged at 4500*g* for 25 min at 4°C. The supernatant is collected and 1 g of NAD is added. The Levinthal blood preparation may be aliquoted and stored at −20°C for mo. BHI plates are stable for up to 2 wk at 4°C.
2. For liquid cultures, NAD and hemin are added to the BHI broth. Two stock solutions of NAD (1-mg/mL sterile water, stable for months at −20°C) and hemin (1-mg/mL sterile water, stable for months at +4°C) are prepared. The NAD solution is added at 1/500 and hemin is added at 1/100 just before adding the bacteria to the BHI broth. A spectrophotometer able to measure OD_{A650}.
3. Kanamycin is added to media for plates or liquid cultures at a final concentration of 10 µg/mL from a 50-mg/mL stock solution in sterile water (stored at −20°C).
4. The MIV competence medium is composed of 100 mL of solution *21*, 1 mL of solution *22*, 1 mL of sterile 0.1 *M* $CaCl_2$, 1 mL of sterile 0.05 *M* $MgSO_4$, and 1mL of 5% vitamin-free Casamino acids (sterilized by filtration and stored at −20°C). One liter of solution *21* is made up of L-asparte (4 g), L-glutamate (0.2 g), fumarate (1 g), NaCl (4.7 g), $K_2HPO_4 \cdot 3H_2O$ (0.87 g), KH_2PO_4 (0.67 g) and Tween 80 (0.2 mL). The solution is adjusted to pH 7.0 with NaOH, sterilized by autoclaving and stored in 100-mL aliquots at 4°C. This solution must be heated to 37°C before use in the *H. influenzae* transformation procedure. Solution *22* is made by first dissolving L-cystine (40 mg) and L-tyrosine (100 mg) in 10 mL of 1 *M* HCl at 37°C and then adding sterile water for a total volume of 100 mL, followed by L-citrulline (60 mg), L-phenylalanine (200 mg), L-serine (300 mg) and L-alanine (200 mg). The solution is sterilized by filtration and stored at −20°C.
5. Centrifuge allowing 4000*g* for tubes of 50 mL.
6. Shaker at 90 rpm.
7. A 37°C waterbath.

2.2.2. Preparation of Genomic DNA

1. 0.145 *M* NaCl solution, sterilized by autoclaving.
2. TNE: 100 m*M* NaCl, 10 m*M* Tris-HCl, pH 8.0, 100 m*M* EDTA, sterilized by autoclaving.
3. 10% sodium dodecyl sulfate (SDS) solution.
4. A 65°C water bath.
5. Proteinase K solution (Roche), 14 mg/mL.
6. A water bath at 37°C.
7. Phenol saturated with TE pH8 (Sigma).
8. 3 *M* sodium acetate adjusted to pH 5.4 with acetic acid, sterilized by autoclaving.
9. TE/RNase: 10 m*M* Tris, 1 m*M* EDTA, pH 7.5 (sterilized by autoclaving), and 50 µg/mL RNaseA.

2.2.4. Analysis of lacZ Fusions

1. Centrifuge allowing 4000*g* for tubes of 50 mL. The Z buffer, used to test β-galactosidase activity, is made by dissolving $Na_2HPO_4 \cdot 7H_2O$ (16.1 g), $NaH_2PO_4 \cdot H_2$ (5.5 g), KCl (0.75 g), $MgSO_4 \cdot 7H_20$ (0.246 g), and β-mercaptoethanol (2.7 mL) in 1 L of sterile water.
2. Toluene solution. A Pasteur pipet. A Vortex shaker.
3. A 37°C incubator and a shaker.
4. 4-mg/mL ONPG solution in sterile water.
5. A 28°C water bath.
6. 1.67 *M* Na_2CO_3 solution.
7. Spectrophotometer able to measure OD_{A420}.

3. Methods

3.1. Construction of lacZ Reporter Gene Fusions

In this section we describe the construction of a vector in which a given gene (containing a repeat tract) is replaced by a translational fusion (also containing the repeat tract) with a *lacZ* reporter gene. The outline of the whole process is illustrated in **Fig. 1**. Other constructs can be made (by using different polymerase chain reactions [PCR] primers) in which the repeat tract is not present, such that the *lacZ* gene is fused "in frame" or "out of frame" with the initiation codon of the gene of interest. These constructs can serve as controls, either to measure background β-galactosidase activity (an "out-of-frame" fusion) or to evaluate the frequency of mutations leading to a loss of the β-galatosidase activity (an-"in-frame" fusion). A similar strategy might also be followed to construct other translational or transcriptional fusions.

In this protocol, PCR products are inserted into classical plasmid or phagemid vectors. Each DNA fragment amplified by PCR and inserted into a plasmid should be checked by sequencing. The primers used for each PCR are designed to contain restriction sites compatible with those located in the *lacZ* gene of the pCH110 vector or the cloning vector. Because *lacZ* is very long,

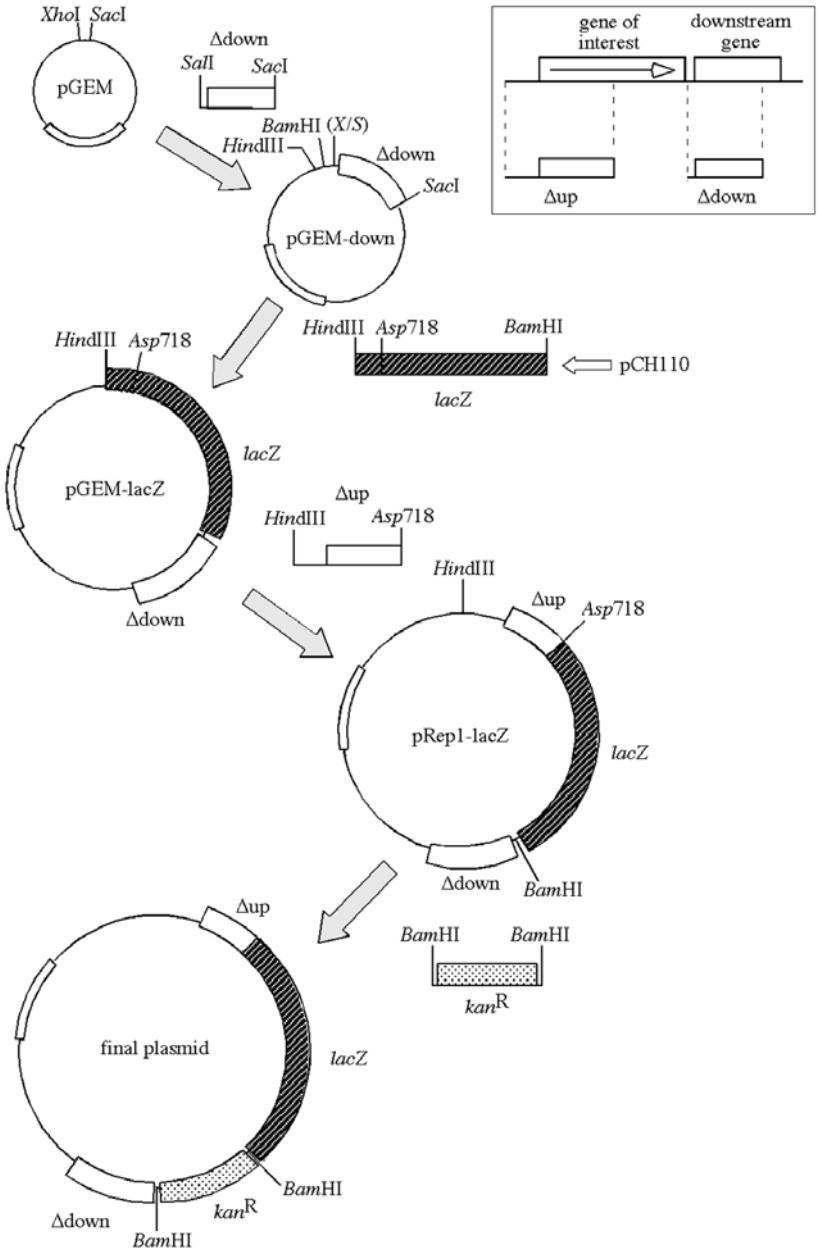

Fig. 1. Development of a *lacZ* reporter construct. Construction of the plasmids allowing integration of the *lacZ* reporter gene at a specific locus. The position of the PCR products "Δup" and "Δdown" compared to the position of the gene of interest is presented in the top right corner. The pGEM plasmid is the pGEM11Zf(+) vector.

there are numerous commonly used restriction sites in its coding sequence. Therefore, the restriction-ligation strategy, as the one described below and illustrated in **Fig. 1**, must be set up carefully in advance. The restriction sites used in this example may be adapted to the possible presence of some sites in the genes of interest.

Several variants of the gene of interest with different repeat tract lengths may be obtained by amplifying the gene from numerous, diverse strains of *H. influenzae* with primers located close to each side of the repeat tract. The strain with the longest repeat tract is selected for further experiments and the repeat tract is sequenced. The region containing the repeats and the promoter (located upstream of the coding sequence) is amplified from genomic DNA of this strain by PCR (*see* **Fig. 1**) with primers that hybridize upstream of the promoter or after the repeats, these primers have, respectively, a *Hin*dIII or an *Asp*718 site (*see* **Note 2**) at their 5' ends. It is also possible to generate several variants with different repeat tract lengths by using a primer that hybridizes within the repeat tract ***(11)*** **(***see* **Note 2**).

1. A first vector (here named pGEM-down) is constructed by amplifying a DNA fragment that consists of sequences located downstream of the gene of interest using primers containing *Sac*I and *Sal*I sites. This PCR product is digested with the relevant enzymes and cloned into the *Sac*I-*Xho*I sites of the pGEM-Zf11 vector (Promega).
2. A second vector (pGEM-lacZ) is constructed by inserting a *Hin*dIII-*Bam*HI fragment of pCH110, which contains most of the *lacZ* coding sequence, into the corresponding sites of the pGEM-down vector.
3. The PCR product containing the promoter and the first part of the coding sequence (including the repeats) is inserted into the *Hin*dIII and *Asp*718 sites of pGEM-lacZ, producing the plasmid, pRep1-lacZ. At this step, another fragment, for example, the same part of the gene but without the repeats, can be inserted upstream of the *lacZ* coding sequence.
4. The *kan*^{R} gene is excised from the pUC4K vector (Phamacia) using *Bam*HI and cloned into the *Bam*HI site of the pRep1-lacZ. Clones should be obtained for both possible orientations of the *kan* gene relative to the *lacZ* gene, and both should be used in subsequent steps, as it appears that the orientation of the antibiotic cassette effects the frequency of recombination into the chromosomal DNA (X. De Bolle, and C. Bayliss, unpublished results) and interferes with frequency of switching; *see* **Note 4**.

3.2. Analysis of Reporter Expression

3.2.1. H. influenzae *Transformation*

In order to monitor the transcriptional or translational activity of *lacZ* fusions, the constructs must first be inserted into the genome of a *H. influenzae* strain.

Several strains can be used in parallel, depending on the hypotheses that need to be tested (for example, strains lacking one of the few transcriptional regulators). Strain Rd is frequently used because it has a high transformation frequency and its genome has been completely sequenced. The most commonly used transformation method for *H. influenzae* is indicated here.

1. *H. influenzae* strain is grown on a BHI plate overnight at 37°C.
2. A whole colony (or a stretch of small colonies) is added to 20 mL of BHI broth, with NAD and hemin, and this culture is grown until it reach an OD_{A650} between 0.2 and 0.4.
3. Cells are harvested by centrifugation (10 min at 4000*g*) and resuspended in an equal volume of MIV (the MIV competence medium is described in **Subheading 2.**).
4. After harvesting by centrifugation, the cells are resuspended in 5 mL of MIV and incubated for 100 min at 37°C, with slow (90 rpm) shaking. The resulting cells are competent.
5. A mix of 250 µL of competent cells with 3–4 µg of linearized DNA is incubated for 30 min at 37°C. The plasmid to be transformed into *H. influenzae* is linearized by cutting it with a restriction enzyme that recognizes a site located outside the region comprising the gene of interest, the *lacZ* reporter, and the *kan* marker. With the constructs described in **Fig. 1**, the final plasmid is digested with *Sca*I, which cuts only once, in the amp^R gene.
6. One milliliter of BHI broth with NAD and hemin is then added to the DNA/competent cells mix, and the cells are incubated for 30 min at 37°C. Three dilutions of the cells are plated on BHI plates containing kanamycin (10 µg/mL), and the plates are incubated overnight at 37°C.

3.2.2. Preparation of Genomic DNA

The genotypes of several candidate transformants are analyzed by Southern blotting in order to check that the reporter gene has been inserted at the right locus (i.e., the gene of interest).

1. Transformants are restreaked on BHI containing kanamycin and then several colonies from this plate are used to inoculate 4 mL of BHI with kanamycin (10 µg/mL), NAD and hemin. After overnight incubation at 37°C, the cells are harvested from 3 mL of this culture by centrifugation and then washed with sterile 0.145 *M* NaCl. (A stock culture can be made at this point by adding 0.2 mL of sterile 80% glycerol to 0.8 mL of the overnight culture. This glycerol stock can be stored at −80°C, indefinitely).
2. Cells are resuspended in 250 µL TNE and then 25 µL of 10% SDS is added
3. The mix is incubated for 10 min at 65°C.
4. After a short cooling period, 15 µL of proteinase K (14 mg/mL, Roche) is added, and the mix is incubated for 4–5 h at 37°C.
5. The solution is extracted twice with an equal volume of TE-saturated phenol.
6. 10 µL of 3 *M* sodium acetate, pH 5.4, and 1 mL of ice-cold ethanol are added to precipitate the genomic DNA.

7. After centrifugation in a microcentrifuge (for 15 min), the pellet is gently washed with 70% ethanol and allowed to dry. The pellet is resuspended in 200 µL of TE /RNaseA and stored at 4°C .
8. The insertion of one copy of the reporter gene at the right place in the genome is then checked by a standard Southern blotting procedure. For example, genomic DNA could be digested with *Eco*RI, transferred to nitrocellulose, and then hybridized with a *kan* probe or a probe containing the gene of interest.

3.2.3. Analysis of the Mutation Rate of lacZ *Fusions*

Fusions should first be examined by spreading dilutions of transformed *H. influenzae* colonies onto BHI containing 40 µg/mL X-Gal. The X-Gal, if it is imported into the cytoplasm, will be cleaved into galactose and 5-bromo-4-chloro-indole, a blue compound (*see* **Note 3**). If the colonies are obviously blue (this assumes that the construct has an in-frame number of repeats), then it will be possible to measure the rate of phase variation. A change in the number of repeats located between the initiator codon and the *lacZ* coding sequence will put the *lacZ* open reading frame (ORF) either *in*-or-*out* of frame with the initiator codon. An *in*-to-*out*-of-frame transition will be an *on*-to-*off* phenotype with regard to the blue/white phenotypes. A protocol for determining the rate of phase variation is detailed in this section, and conditions that may interfere with the frequency of phenotype transition are given in **Note 4**.

1. The *H. influenzae lacZ* fusion construct is streaked on to a BHI plate containing 10 µg/mL kanamycin and 40 µg/mL X-Gal and incubated overnight at 37°C.
2. Single colonies are picked from this plate and resuspended in 500 µL of BHI medium. Ten fold dilutions of this colony are made in BHI broth and then 50 µL of the 10^{-3}, 10^{-4}, and 10^{-5} dilutions are plated on BHI plates containing 40 µg/mL X-Gal and incubated overnight.
3. Sixteen single colonies are picked from these plates and dilutions are made of each colony, as above. Two 50 µL aliquots of the 10^{-3} and one 50 µL aliquot of the 10^{-4} and 10^{-5} dilutions are plated on BHI plates containing 40 µg/mL X-Gal and incubated overnight (*see* **Note 5**).
4. The number of colonies on the 10^{-4} and 10^{-5} plates and the number of revertants on the 10^{-3} plates are counted (*see* **Note 6**). The frequency of phase variants for each of the 16 colonies is estimated by dividing the total number of variants by the total number of bacteria.
5. The mutation rate (µ) can be determined using the equation of Drake ***(12)***, i.e.,

$$\mu = 50.4 \quad \mu = 0.4343f/\log(N\mu)$$

where f is the median frequency of phase variants and is the average of the middle two values determined for the 16 colonies. N is the average population size and is determined by taking the average of the total numbers of bacteria in each colony: µ is then determined using these values and by reiteratively solving the equation using arbitrary values of µ, using Microsoft Excel.

3.2.4. Analysis of the Transcriptional Activity of lacZ Fusions

The relative level of transcription of the gene of interest can be assessed by quantifying β-galactosidase activity in the fusion constructs. This is especially useful for analysis of promoters.

1. A given volume of cells (e.g., 10 mL), depending on the transcriptional level, is centrifuged for 10 min at 3000*g* and the cells are resuspended in 1 mL of Z buffer.
2. One drop of toluene is added to the suspension with a Pasteur pipet, and the mix is shaken vigorously for 10 s.
3. Incubate the tubes in a shaker at 37°C for 40 min, with the top open in order to allow the toluene to evaporate.
4. The mix is cooled to 28°C and a volume of 0.2 mL of ONPG (4 mg/mL) is added to each sample.
5. The mix is incubated 10–15 min at 28°C.
6. For each sample, the reaction is stopped by the addition of 0.3 mL of 1.67 *M* Na_2CO_3. The OD_{A420} is measured and compared to a standard curve made using known concentrations of ortho-nitrophenol (ONP). One unit of activity is the amount of enzyme necessary to hydrolyze 1 nmol of ONPG into ONP per min. This β-galactosidase activity must be correlated with the concentration of protein in the lysate, which can be measured by a standard method such as the Lowry method ***(13)***.

4. Notes

1. The *lacZ* gene is found in many commercially available plasmids, and a similar strategy may be followed to construct alternative integrative cassettes.
2. The restriction enzyme *Asp*718 is an isoschizemer of *Kpn*I and may be used instead of *Kpn*I, which often cleaves DNA nonspecifically. Generating versions of the gene with varying numbers of repeats may be done by a *slip PCR* protocol. Briefly, a PCR product containing all the repeats is used as template for a second PCR (the *slip PCR*). One primer (the *up* primer) hybridizes upstream of the promoter of the gene and a small primer (the *slip* primer) containing a restriction site (e.g., *Asp*718) followed by two tetranucleotide repeats at its 3' end hybridizes in the repeat region and an antiparallel primer hybridizes downstream of the promoter region. These primers are used at standard concentrations (e.g., 400 n*M*) to amplify from reducing dilutions of template DNA. The second PCR is set up with various ratios of *slip* primer/*up* primer, using the lowest concentration of template that gave a detectable amplification in the first trial PCR. The DNA fragments of the correct size are gel extracted and ligated into a classical plasmid or phagemid vector.
3. In *H. influenzae*, X-Gal is imported, since dark blue phenotypes are observed if high levels of β-galactosidase are produced. A *lacZ* reporter in frame with the initiator codon of the gene of interest will give a blue phenotype on X-Gal plates only if the level of transcription of the gene of interest is sufficiently high. The reporter genes require a minimum level of expression to be useful markers of the

expression of a gene of interest, or to monitor changes in the number of repeats present in a given coding sequence. Some loci, such as the *mod* gene of *H. influenzae*, produce a dark blue phenotype when transformants with a reporter gene are spread on plates containing X-Gal. For other loci, the phenotype may be less clear, presumably due to a lower level of transcription. Counting of blue and white colonies should be performed within 24 or 48 h, otherwise phenotypes such as "bull's eye colonies" may appear. It is also important to note that the 5-bromo-4-chloro-indole (i.e., the X chromophore of X-Gal) is slightly toxic when it is present at high concentration in the cell. Indeed, colonies producing high amounts of β-galactosidase are smaller than those lacking the enzyme, if the cells have been spread on X-Gal-containing plates, whereas this difference in phenotype is not observed on BHI plates lacking X-Gal. In order to have blue/white screening in the presence of X-Gal with the *lacZ* reporter gene, there must be a system for importing X-Gal into the cell. In *E. coli*, this import is catalyzed by a lactose permease (LacY). *H. influenzae* does not have β-galactosidase (otherwise all the *H. influenzae* colonies would be blue on X-Gal plates), indicating that X-Gal enters cells of this bacteria by an unknown mechanism. It is possible, as has been suspected for some yeast strains, that one or several other permeases are sufficiently non specific to catalyze the entry of X-Gal into the cell. Our data indicate that the *lacZ* reporter gene is functional in RM118 (a close derivative of Rd strain), RM153 (also called Eagan), and RM7004 strains.

4. Selection with kanamycin increases the number of in-frame variants in colonies by approx 10-fold. We therefore recommend that antibiotics not be used when analyzing the frequency of switching between the blue and white phenotypes. Because the reporter gene is inserted into the genome, there is no obvious risk of simple excision and it is reasonable to cultivate the transformants without kanamycin selection.
5. If the in-frame fusions produce only weak or light blue color the colonies can be plated on X-Gal plates containing 80 µg/mL X-Gal.
6. Only variants that are wholly or greater than 80% of the opposite phenotype to the parental colony should be counted; i.e., sectored colonies should not be counted. Counting of variants is facilitated by marking the plate into different sections using a marker pen and by use of a binocular microscope to view the colonies.

References

1. Jacobson, R. H., Zhang, X. J., DuBose, R. F., and Matthews, B. W. (1994) Three-dimensional structure of β-galactosidase from *E. coli*. *Nature* **369,** 761–766.
2. Chalfie, M. and Kain, S. (1998) *Green fluorescent protein, properties, applications and protocols*. Wiley-Liss, New York.
3. Pagotto, F. J., Salimnia, H., Totten, P. A., and Dillon, J. R. (2000) Stable shuttle vectors for *Neisseria gonorrhoeae*, *Haemophilus* spp. and other bacteria based on a single origin of replication. *Gene* **22,** 13–19.
4. Christodoulides, M., Everson, J. S., Liu, B. L., et al. (2000) Interaction of primary human endometrial cells with *Neisseria gonorrhoeae* expressing green fluorescent protein. *Mol. Microbiol.* **35,** 32–43.

5. Dawids, S., Barenkamp, S. J., and St. Geme III, J. W. (1999) Variation in expression of the *Haemophilus influenzae* HMW adhesins: a prokaryotic system reminiscent of eukaryotes. *Proc. Natl. Acad. Sci. USA* **96,**1077–1082.
6. Valdivia, R. H. and Falkow, S. (1996) Bacterial genetics by flow cytometry: rapid isolation of *Salmonella typhimurium* acid-inducible promoters by differential fluorescence induction. *Mol. Microbiol.* **22,** 367–378.
7. Valdivia, R. H., Hromockyj, A. E., Monack, D., Ramakrishnan, L., and Falkow, S. (1996) Applications for green fluorescent protein (GFP) in the study of host-pathogen interactions. *Gene* **173,** 47–52.
8. Saunders N. J., Jeffries A. C., Peden, J. F., Hood, D. W., Tettelin, H., Rappuoli, R., Moxon, E.R. (2000) Repeat-associated phase variable genes in the complete genome sequence of *Neisseria meningitidis* strain MC58. *Mol. Microbiol.* **37,** 207–215.
9. Saunders, N. J., Peden, J. F., Hood, D. W., and Moxon, E. R. (1998) Simple sequence repeats in the *Helicobacter pylori* genome. *Mol. Microbiol.* **27,** 1091–1098.
10. Hood, D. W., Deadman, M. E., Allen, T., et al. (1996) Use of the complete genome sequence information of *Haemophilus influenzae* strain Rd to investigate lipopolysaccharide biosynthesis. *Mol. Microbiol.* **22,** 951–965.
11. de Bolle, X., Bayliss, C. D., Field, D., van de Ven, T., Saunders, N. J., Hood, D. W. and Moxon, E. R. (2000) The length of a tetranucleotide repeat tract in *Haemophilus influenzae* determines the phase variation rate of a gene with homology to type III DNA methyltransferases. *Mol. Microbiol.* **35,** 211–222.
12. Drake, J. W. (1991) A constant rate of spontaneous mutation in DNA-based microbes. *Proc. Natl. Acad. Sci. USA* **88,** 7160–7164.
13. Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Rondall, R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193,** 265–275.

10

The Genome Sequence of *Haemophilus influenzae*

Derek W. Hood

1. Introduction

Publication of the complete 1.83-Mb genome sequence of the bacterium *Haemophilus influenzae* strain Rd in 1995 ***(1)*** heralded a revolution in the study of microorganisms. The availability of a complete microbial genome sequence enormously facilitates computer-based and experimental investigations of the respective organism by providing complete lists of genes, their genetic contexts, and their predicted functions. The significance of the *H. influenzae* sequence was not just that it presented for the first time a complete genome from a free-living organism, but that it proved that complete genomes could be sequenced rapidly and effectively at a low cost. This was achieved through the novel approach taken by Venter and his colleagues at The Institute for Genomic Research (TIGR) to sequence the *H. influenzae* genome. Randomly generated small chromosomal fragments, 1–2 kb in length, were cloned into a high-copy-number plasmid vector and then both ends of the clone were sequenced using plasmid specific primers. The vast amount of DNA sequence obtained from the shotgun clones was assembled into large contiguous sequences by computers using specifically developed software. The equivalent to sequencing the entire genome 6 times was required for sufficient accuracy and coverage of the majority of the chromosome. To obtain a final contiguous sequence, gaps between contigs (tracts of contiguous sequence) were closed using homology comparisons between end sequences, the polymerase chain reaction, and sequencing of larger DNA inserts from lambda libraries. The shotgun method was rapid, as it circumvented the need for construction of a physical map of the genome, a prerequisite of the ordered approach taken to other bacterial sequencing projects.

The linear DNA sequence of a bacterial chromosome gives information not obtainable by other means, identifying all potential open reading frames

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(ORFs), intergenic sequences, and their organization within the genome. For any organism, the genome sequence gives detailed insight into what specifies one organism and its biology. Importantly for pathogenic bacteria such as *H. influenzae*, the genome contains information on the genetic basis for each determinant influencing host–microbe interactions (virulence factor), each vaccine candidate, and all potential drug and diagnostic targets.

Microbial genome sequencing is rapidly becoming a routine undertaking, and the completion of a genome sequence signals the end of some traditional strategies in microbiology, in particular for the identification and analysis of gene function. In this chapter we discuss the utility of the *H. influenzae* bacterial genome sequence and some of the ways in which the information can be used.

2. Materials

Access to the Internet at the following sites:

http://www.tigr.org/
http://www.acedb.org/
http://www.sanger.ac.uk
ftp://ncbi.nlm.nih.gov
ftp://ftp.virginia.edu/pub/fasta
http://protein.toulouse.inra.fr/prodom.html
http://www.cgr.ki.se/Pfam/
http://www.expasy.ch/prosite/
http://www.expasy.ch/

3. Methods

3.1. Identification of Genes

A majority of genes in any genome have not been experimentally investigated, so the expedient method of determining the function of encoded proteins is through inference from genes of known or proposed function, on the basis of sequence similarity. The annotation of genes in a genome is an essential step in genome analysis and constitutes a primary interface for researchers wishing to access the information. The *H. influenzae* genome has been annotated using an ORF-based method of analysis. ORFs are first identified in a sequence defined by the predicted start and stop codons. A lower size limit must be assigned to reduce the number of spurious reading frames included for analysis, although some small ORFs can be truly coding. Gene functions were assigned after homology comparisons by automated searching of ORFs from the new genome (query sequence) against databanks of sequenced and published genes. Various search algorithms were used for the *H. influenzae* genome sequence, based on the BLAST and FASTA search programs. The best matches are noted and then a judgment is made to assign a predicted function to a query gene sequence. The program currently available from the TIGR Website, GLIMMER, is an evolu-

tion of the annotation package used for the *H. influenzae* genome sequence. Some more recent genome sequencing projects, such as that for *Neisseria meningitidis* serogroup B strain MC58 ***(2)***, have used GLIMMER in conjunction with a non-ORF-based method ***(3)***. This latter method searches database sequences using large tracts of the genomic DNA and aligns homologies independent of reading frame. Start and stop codons are then overlaid on the results of the search, which are viewed via a graphical interface, e.g., ACEDB (runs on a UNIX platform, information and program available from http://www.acedb.org/), allowing the operator to select the most appropriate reading frame in the genome sequence. This method is particularly valuable for identifying translational frameshifts, often found associated with phase-variable genes and pseudogenes. Pseudogenes, genes not expressed due to the presence of stop codons or frameshifts that interrupt the translated reading frame, are found in the *H. influenzae* genome. A further program, ARTEMIS (a JAVA-based tool), which allows viewing of sequence and sequence features via a graphical interface, is publicly available and can be downloaded from the Sanger Centre, UK, WWW site (http://www.sanger.ac.uk).The gene pool contains all of the genes required for the existence of the free-living bacterium. Riley ***(4)*** proposed a system to catalog genes into broad functional groups. These 12 groups have proved useful as a framework for classifying genes, allowing researchers to access the DNA sequence information in a directed and informative manner relevant to their particular research interest. For *H. influenzae*, 1743 putative genes were identified in the 1.83 Mb genome sequence, of which 60% have been assigned to these broad functional categories. Importantly, as with the majority of bacterial genome sequences, about 40% of the *H. influenzae* ORFs at the time of annotation remained unassigned with respect to function by homology comparisons alone. ORFs that appear only within one genome and that have no sequence similarity to other sequences in the databases are termed "hypothetical" or "function unknown" (FUN), whereas those of unknown function but that are present in more than one genome are classified as "conserved hypothetical." The number of FUN genes has decreased as subsequent sequencing projects have identified homologous FUN genes in other organisms. However, the total figure for genes of unknown function remains at around 40% for most prokaryotic genome sequencing projects.

Like any genome annotation procedure, based solely on homology comparisons, some genes listed in the *H. influenzae* genome database may remain erroneous. Homology predictions do not always provide rigorous evidence for the biological function of genes and their products. Existing databank sequences may have putative, probable, or confirmed gene functions, and each will be given equal weighting by most annotation teams. Good-quality partial and lower-quality, more extensive matches may not always be distinguished but

could be crucial in deciding likely gene function. Examples are known where gene functions can be complemented between species with amino acid identity of just over 20% ***(5)***. Also, examples are known where only a very few changes to an amino acid sequence results in altered substrate or functional specificity of a protein. Several updates have been made of the *H. influenzae* genome sequence database since its completion, to improve some aspects of the annotation. This is based on more rigorous methods developed at TIGR and some external independent analysis. When analyzing genomes, useful terms for considering genes of comparable function are paralogs, homologous genes in the same organism whose products perform related but not identical functions, and orthologs, homologous gene functions from different organisms ***(6,7)***. Orthologs are likely to have the same function, whereas paralogs are more likely to have diverged in their function. Orthologs are expected to have sequences that are very similar to each other, whereas more sequence divergence is expected to have occurred between paralogs. Gene duplication and transfer can give rise to complex groups of orthologs and paralogs in different genomes. An estimate is that 35% of the genes in *H. influenzae* are paralogs, compared to half of the genes in *Escherichia coli* ***(8)***. The lower level of paralogy in *H. influenzae* correlates with its smaller genome size.

The gene list itself can give the first indication of novel findings from a genome sequence. Gene lists allow observations to be made regarding the predicted metabolic pathways present in an organism and how these might influence the niches in which it is found. For *H. influenzae*, found only in the human host, the metabolism is biased toward an anaerobic, nitrogen-rich environment, and key enzymes in the TCA cycle and electron transport chain are apparently absent. *E. coli* allocates more than 4% of its genome to regulatory genes, including 45 or more two-component regulatory systems, whereas *H. influenzae* has only four such systems. This reflects the metabolic versatility and diverse habitats of *E. coli*, being able to respond to environmental signals, and the host dependence and restricted niche of *H. influenzae*.

There is a good argument for undertaking a systematic experimental approach to mutate all reading frames to maximize the information on gene function. Such an approach was undertaken for *Saccharomyces cerevisiae* by the European Union of Yeast Genome Sequencing Network using collaborative laboratories around the world ***(9)*** and could prove especially useful to understand the high proportion of genes of unknown function present in most organisms. The role of orphan genes may be of particular interest in pathogenic bacteria such as *H. influenzae*, where their restriction to particular host compartments may not allow the function to be defined in laboratory studies.

3.2. Using the H. influenzae *Genome Sequence via the TIGR Website*

Access to the TIGR Website for the *H. influenzae* genome sequence database is through the TIGR Database Link on the TIGR Website home page (http://www.tigr.org/). This will present a menu page, Genome Home, for interface with the strain Rd (KW20) genome sequence data through a number of menus listed at the top of the page:

3.2.1. Overview

This menu allows genome information to be accessed through:

1. *DNA Molecule Information.* Lists size, A+T content, and the number of reading frames in the genome.
2. *Condensed Genome Display.* Genes displayed by functional category.
3. *Codon Usage Chart.* Lists codon usage for all genes in the genome or for each role category.
4. *Role Category Graph.* Lists number and percentage of genes in the genome assigned to each role category.
5. *Download TIGR Genomes.* Files including the complete DNA sequence, predicted reading frames, and coding regions can be downloaded directly for further analysis. Instructions are included within this opinion.

3.2.2. Genome Analysis

1. *GC Plot.* Shows a plot of %G+C for nucleotides across the entire DNA molecule.
2. *COG/TIGRFAM/PFAM.* Allows comparison of the genes encoding proteins of related function assigned as Clusters of Orthologous Groups (COG), a TIGRFAM, a PFAM, or any combination of the three.
3. *2-D Gel.* Shows a computer model of a representative two-dimensional protein for the genome.
4. A number of comparisons of the genome sequence and its complement of protein products with sequences from other genomes can be accessed through this menu in the following ways:
   Genome vs All Organisms.
   GC Comparison.
   Levels of Parology.
   For each comparison, the results can be viewed as total protein hits or best protein hits.

3.2.3. Genome Lists by Category

1. *Batch Download.* Retrieves to the user's own computer a large number of sequences.
2. *Gene Attribute Download.* Retrieves selected gene sequences to user's own computer.
3. *Gene List by Role Category.* Lists all genes in the genome by predicted role categories.

4. *TIGRFAM List.* Lists all TIGRFAMs assigned to genes by role category.
5. *RNAs.* Lists small stable RNAs, ribosomal RNAs, and transfer RNAs predicted from the genome sequence.
6. *Enzyme Commission Number.* Lists all EC#s assigned to genes in the genome.

3.2.4. Genome Searches

A number of searches for particular genes can be carried out by:

1. *Name.* Use gene common name or symbol.
2. *Locus.* Use TIGR locus or other database number.
3. *Enzyme Commission Number.*
4. *Sequence/BLAST.* Nucleotide or peptide sequences can be typed in and used to search either the complete Rd genome sequence or the predicted coding regions for matches. Searches are carried out locally using one of the forms of the BLAST search algorithm, and are presented on screen.
5. *Position Search/Segment Retrieval.* Predicted coding regions can be viewed for any given size of DNA segment defined by coordinates typed in by the user. For instance, the coding sequences in a region of DNA around a gene of interest can be viewed. By choosing the Retrieve Sequence Segment option any size of DNA sequence from the genome can be viewed. This can then be copied and inserted (pasted) into most other programs for further sequence analysis on the user's own computer.
6. *Region View.* Accesses a graphical display of a small segment of the genome between coordinates defined by the user or surrounding a chosen gene.
7. *TIGRFAMs Text Search.* Enables a search through TIGRFAMs.
8. *TIGRFAMs Sequence Search.* Enables a search through TIGRFAMs looking for a particular sequence.
9. *PubMed Search.* Enables a search through PubMed for references related to this genome.

3.2.5. Related Links

This menu allows access to other information on the TIGR Website, to PubMed, GenBank, American Type Culture Collection (ATTC), and to other genome databases and analyses.

A further useful Website is that of the NCBI Entrez facility, which provides information on genome sequences, but also hyperlinks to taxonomy tables and to some specific analyses data, such as COGs ***(7,10)*** and taxonomic distribution of homologues (TAXTABLE).

3.3. Specific Genome Comparisons

The TIGR *H. influenzae* Website home page (http://www.tigr.org/tdb/CMR/ghi/htmls/SplashPage.html) described above allows the searching of the genome sequence annotation using key words and the genome sequence by a GRASTA search algorithm. To allow more detailed analysis on the investigator's own computer system, the complete genome sequence can be downloaded using the

Download Sequences instructions on the TIGR Website. Files chosen from one of the three options will be obtained in a FASTA format comprising a plain text file where the first line is the label for the sequence that follows. The label begins with the "greater than" character (>). For a multi-FASTA format, further sequences are appended one after the other in a single file. Once downloaded, the sequence can be investigated or manipulated as required.

Two widely used software packages are freely available for sequence searching; Standalone BLAST (available from NCBI ftp server, ftp://ncbi.nlm.nih.gov) and FASTA (available from ftp://ftp.virginia.edu/pub/fasta). Both packages contain a number of programs (often called "flavors") that are used depending on the nature of the query and database sequences. Each package has user information in an associated README file. Searches with Standalone BLAST are generally quicker than those with FASTA, and the results and statistics of alignment given by the two are calculated differently. In general, protein-vs-protein sequence searches are the most desirable for homology comparison. For protein-vs-protein and DNA-vs-DNA searches, the BLASTP and BLASTN programs should be used, respectively. The slightly slower but perhaps more sensitive FASTA program SSEARCH3 can be used for protein-vs-protein sequence searches. When searching translated DNA query sequences against protein databases, the BLASTX program can be used. However, the FASTX3 and FASTY3 programs have an advantage, as they allow for frameshifts in the DNA query sequence. For searching protein query sequences against translated DNA databases, the TFASTAX3 or TFASTAY3 program can be used, again offering an advantage over the equivalent TBLASTN program in allowing for frameshifts. Prior to analysis, each genome or sequence to be searched with the BLAST programs must be converted into the correct format. This is done using the FORMATDB program from the Standalone BLAST package. Programs in the FASTA package use files in the format obtained directly from the download of the TIGR sequence(s).

3.3.1. Interpreting Search Results

The output from both BLAST- and FASTA-based searches include an expectation (E) value for each match. This is a statistical estimate of the significance of the particular comparison against that expected purely by chance and is the important information for deciding the homology of a query sequence. The smaller the value, the greater the confidence of a true match; typically, E values of 10^{-5} to 10^{-6} are considered as the threshold for homology assignment. This threshold value can be increased or decreased depending on the specificity required in the search.

A number of parameters can be changed within the BLASTA and FASTA packages to alter the sensitivity of searching. An important parameter that can

be changed is the scoring matrices, empirically calculated tables that list scores for each pairwise match of amino acid or nucleotide residues, which are used to calculate expectation values. Details of alternatives will not be described here but are given within each program package.

An alternative approach to identify distantly related sequences is to use the PSI-BLAST (Position-Specific Iterative-BLAST) program ***(11)***. This program performs a standard BLASTP search of a database, combines all matches over a user-defined threshold into a summary sequence, then uses this as the query search for a further BLASTP search. Successive iterative searches are made until no new database matches are found.

3.4. Motif Searching

The availability of complete genome sequences permits new approaches to identify genes or features of particular interest. Genomes can be searched for conserved short sequences, sequence signatures, and similarities to domains associated with particular families of proteins or functions. One example published at the same time as the genome sequence was the prevalence of a DNA uptake signal sequence (USS) in the *H. influenzae* genome, which mediates the preferred uptake of homologous DNA in an organism that is considered to be naturally competent. A total of 1465 copies of the USS, comprising a conserved 9-bp core with a larger consensus 29-bp sequence, were identified as dispersed, mainly in intergenic regions, throughout the chromosome ***(12)***. Only 9 copies of this sequence would be expected by chance in a genome of this size.

For analysis of families of proteins of related structure and function the ProDom (Protein Domain) (http://protein.toulouse.inra.fr/prodom.html), Prosite (http://www.expasy.ch/prosite/), and Pfam (Protein family) (http://www.cgr.ki.se/Pfam/) databases are extremely useful. All three databases contain proteins grouped by homologous domains using different automated searches. These databases take into account the size and common ancestry of domains and proteins. The Prosite database includes signature sequences for particular protein families and domains. TIGR includes the results from Pfam and their own analysis of protein families (TIGRFAMs) in the *H. influenzae* Website as described in **Subheading 3.3.**

When interrogating any gene sequence in the TIGR *H. influenzae* database, hyperlinks allow access to the details of the matches on which the annotation was based and also information on the domains and/or protein families that have conserved sequence. The EC number listed for any putative enzyme function can be used to find further information on function within the Expasy Website (http://www.expasy.ch/). This site locates the enzyme in a biochemical pathway and provides links to other useful information on function.

A further application of the *H. influenzae* genome sequence has been identification of simple DNA repeats that have been shown to be associated with

some virulence-related genes. The length of a tract of repeats of a DNA motif of between 1 and 6 nucleotides in length is unstable and can change by polymerase slippage during nucleic acid replication, altering gene expression. If located within an ORF, alteration in the number of repeats leads to translational frame shifting, and if in the promoter region can lead to altered transcription by affecting RNA polymerase binding. If the gene product is a cell surface-associated structure or is required for virulence, then random on–off switching of expression (phase variation) could enhance survival of the bacterium in different host compartments or microenvironments. These genes are often termed "contingency genes" ***(13)***. A search for simple DNA repeats in the *H. influenzae* genome sequence revealed 12 loci containing tetranucleotides, repeated 6 or more times, within the 5' end of ORFs ***(14)***. A role for each of these loci in the virulence of *H. influenzae* can be postulated. This general principle can be extended to screening genome sequences with any conserved motif representing gene, protein, or structural functions, or features of interest.

3.5. Beyond the Index Genome

A significant problem for every genome project is the choice of the strain of an organism as the source of DNA to be sequenced. A sequenced genome represents only the genetic complement from an isolate of a single strain of a single species, usually sampled at a single point in time. Genomes can be labile and can be added to and subtracted from by processes such as transformation with plasmid or genomic DNAs, and by transfection with bacteriophage DNA. In addition, the replicative transposition of mobile elements can give rise to rearrangements of portions of the genome such as inversions, duplications, and deletions. Such genome plasticity is believed to be an important mechanism allowing bacteria to adapt and evolve over time. Therefore, a microbial genome sequence from a particular strain should be considered as only the reference or index for that species. For example, it is known from physical mapping that the *H. influenzae* pathogenic type b strain Eagan has a genome that is approx 300 kb larger than that of the sequenced strain, Rd. For some other species, such as *E. coli*, *Mycobacterium tuberculosis*, *Helicobacter pylori* and *N. meningitidis*, the genome sequences of related strains are available to allow comparisons of genome structure and more detailed assessment of features relevant to pathogenicity. No such comparison is possible at present for *H. influenzae*.

Physical methods other than DNA sequencing can give useful information from genome comparisons, an example being subtractive hybridization. This technique has been used to compare *E. coli* and *Salmonella typhimurium* ***(15)*** and can identify sequences that are unique to each bacterium. Representation difference analysis has identified regions of the chromosome of the related

pathogens *N. meningitidis* and *N. gonorrhoeae*, which are specific to the pathogenic *Neisseria* species but not the commensal species *N. lactamica* ***(16)***.

3.6. Gene Expression Analysis

The information available from sequencing projects can be used to examine either individual or global gene function. The database of ORF sequences allows genes of interest to be identified from global gene investigations that produce short stretches of DNA or amino acid sequences. This facilitates approaches such as signature-tagged mutagenesis (STM) ***(17)***, where sequence-flanking transposon insertions can be easily identified, and in-vitro expression technology (IVET) ***(18)***. Genome sequences facilitate the use of DNA microarrays. Oligonucleotides or DNA fragments representing all genes from the index organism can be assembled as a microarray on a DNA chip ***(19)***. This can then be used to compare the complement of genes between organisms or can be used to investigate gene expression through examination of total messenger RNA ***(20–22)***.

An alternative strategy for analyzing gene expression is to investigate total cellular proteins, so-called proteomics. Reference two-Dimensional gel electrophoresis maps of bacteria integrated to the genome sequence ***(23,24)*** provide a powerful technique with which to investigate bacterial environmental responses and transitions encountered during infection. Integrated with mass spectrometry, this method can be used to directly monitor expressed protein levels within cells.

3.7. Virulence, Pathogenicity, and Foreign DNA

An obvious bias in the selection of bacteria for genome sequencing projects is driven by our need and desire to understand and control bacteria of clinical significance. The DNA sequence of *H. influenzae* and other microbial pathogens is a catalog of every gene product and therefore contains the basis for every determinant influencing the host-microbe interaction. A particular emphasis in genetic studies of microbial pathogens is placed on identifying so-called pathogenicity islands, regions of DNA normally containing clusters of virulence-related genes. These may be mobile and identified by a divergence in DNA base (%G+C) composition, and, when acquired are sometimes all that is apparently required to convert a bacterium to a virulent phenotype ***(15)***. A potentially more powerful search tool for foreign DNA is through dinucleotide composition. As well as being a string of single nucleotides, DNA can also be considered a string of longer components, including dinucleotides. Each species has a specific dinucleotide signature (DNS), based on the proportion of each possible dinucleotide in the total genome. Stretches of DNA with atypical DNS can be used to identify regions of DNA derived from another

organism ***(25)***. Apart from an integrated phage, no such islands have been identified in the *H. influenzae* genome sequence.

One particular experimental application of a genome sequence to pathogenicity studies was the analysis of the genetics of biosynthesis and the biology of lipopolysaccharide (LPS) in *H. influenzae*. LPS is a complex cell surface glycolipid and a characterized virulence determinant in *H. influenzae*. The saccharide portion of *H. influenzae* LPS is involved in host cell interaction and the lipid A portion of the molecule, the endotoxin, initiates the cytokine cascade as part of the host response to infection. Twenty-five genes potentially involved in LPS biosynthesis were identified from the genome sequence by homology comparisons with characterized genes from other organisms ***(26)***. A study of these genes facilitated analyses of LPS structure synthesis and biological function in *H. influenzae*. The significance of this work was the speed and completeness of a task that would have been virtually impossible without the genome sequence.

3.8. Vaccines and Therapeutic Targets

The complete genome sequence of an organism of clinical interest offers information on every gene product responsible for the synthesis of each vaccine candidate and potential therapeutic target. Homology searches and structural predictions can identify all surface-accessible proteins and other molecules as immunological targets. This search can be fine-tuned with information on known immunogenic structures in the same or related bacteria. An exemplar of such functional genome analysis was that undertaken to identify all potential surface-exposed proteins from the *N. meningitidis* genome sequence and to investigate these as potential vaccine candidates ***(27)***. As the number of genome sequences continues to grow, it is likely that a detailed comparison and systematic informatics approach will help focus on the more suitable candidates for vaccine development.

It is now over 30 yr since the discovery of the last significant new antibacterial agent. Undoubtedly, genome sequences offer a means to combat the emergence of antibiotic resistance in common bacterial pathogens. Structurally novel classes of antibiotics can be tested against selected bacterial targets, chosen from the genome sequence. The first stages of identifying suitable new targets, studies on its novelty, spectrum of action, and selectivity of target, can be achieved by comparative genome analyses alone.

4. Notes

The completion of the genome sequence of *H. influenzae* heralded a revolution in microbiology. The sequence provides the starting point for significant research on bacterial metabolism, host adaptation, and virulence. Studies to

answer the questions of how bacteria function and become pathogens should allow us to better understand the organisms and develop successful measures for their control.

References

1. Fleischmann, R. D., Adams, M. D., White, O., et al. (1995) Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. *Science* **269,** 496–512.
2. Tettelin, H., Saunders, N. J., Heidelberg, J., et al. (2000) Complete genome sequence of *Neisseria meningitidis* serotype B strain MC58. *Science* **287,** 1809–1815.
3. Saunders, N. J., Peden, J. F., Hood, D. W., and Moxon, E. R. (1998) Simple sequence repeats in the *Helicobacter pylori* genome. *Mol. Microbiol.* **27,** 1091–1098.
4. Riley, M. (1998) Systems for categorizing functions of gene products. *Curr. Opin. Struct. Biol.* **8,** 388–392.
5. Tatusov, R. L., Mushegian, A. R., Bork, P., et al. (1996) Metabolism and evolution of *Haemophilus influenzae* deduced from a whole-genome comparison with *Escherichia coli*. *Curr. Biol.* **6,** 279–291.
6. Koonin, E. V., Tatusov, R. L., and Rudd, K. E. (1996). Protein sequence comparison at genome scale. *Methods Enzymol.* **266,** 295–322.
7. Tatusov, R. L., Koonin, E. V., and Lipman, D. J. (1997) A genomic perspective on protein families. *Science* **278,** 631–637.
8. Hinton, J. C. (1997) The *Escherichia coli* genome sequence: the end of an era or the start of the FUN? *Mol. Microbiol.* **26,** 417–422.
9. Goffeau, A., Barrell, B., Bussey, H., et al (1996) Life with 6,000 genes. *Science* **274,** 546.
10. Tatusov, R. L., Galperin, M. Y., Natale, D. A., and Koonin, E. V. (2000) The COG database: a tool for genome-scale analyses of protein functions and evolution. *Nucleic Acids Res.* **28,** 33–36.
11. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) Basic local alignment search tool. *J. Mol. Biol.* **215,** 403–410.
12. Smith, H. O., Tomb, J.-F., Dougherty, B. A., Fleischmann, R. D., and Venter, J. C. (1995) Frequency and distribution of DNA uptake sequences in the *Haemophilus influenzae* Rd genome. *Science* **269** 538–540.
13. Moxon, E. R., Rainey, P. B., Nowak, M. A., and Lenski, R. E. (1994) Adaptive evolution of highly mutable loci in pathogenic bacteria. *Curr. Biol.* **4,** 24–33.
14. Hood, D. W., Deadman, M. E., Jennings, M. P., et al. (1996) DNA repeats identify novel virulence genes in *Haemophilus influenzae*. *Proc. Nat. Acad. Sci. USA* **93,** 11,121–11,125.
15. Groisman, E. and Ochman, H. (1996) Pathogenicity islands: bacterial evolution in quantum leaps. *Cell* **87,** 791–794.

16. Perrin, A., Nassif, X., and Tinsley, C. (1999) Identification of regions of the chromosome of *Neisseria meningitidis* and *Neisseria gonorrhoeae* which are specific to the pathogenic *Neisseria species*. *Infect. Immun*. **67,** 6119–6129.
17. Hensel, M., Shea, J. E., Gleeson, C., et al . (1995) Simultaneous identification of bacterial virulence genes by negative selection. *Science* **269,** 400–403.
18. Slauch, J. M., Mahan, M. J., and Mekalanos, J. J. (1994) In vivo expression technology for selection of bacterial genes specifically induced in host tissues. *Methods Enzymol.* **235,** z481–492.
19. Marshall, A. and Hodgson, J. (1998) DNA chips: an array of possibilities. *Nature Biotechnol.* **16,** 27–31.
20. Derisi, J. L., Iyer, V. R., and Brown, P. O. (1997) Exploring the metabolic and genetic control of gene expression on a genomic scale. *Science* **278,** 680–686.
21. Siazieu, A., Certa, U., Warrington, J., et al. (1998) Bacterial transcript imaging by hybridisation of total RNA to oligonucleotide arrays. *Nature Biotechnol.* **16,** 45–48.
22. Wilson, M., DeRisi, J., Kristensen, H.-H., et al. (1999) Exploring drug-induced alterations in gene expression in Mycobacterium tuberculosis by microarray hybridization. *Proc. Natl. Acad. Sci. USA* **96,** 12,833–12,838.
23. Cash, P., Argo, E., Langford, P. R., and Kroll, J. S. (1997) Development of a *Haemophilus* two-dimensional protein database. *Electrophoresis* **18,** 1472–1482.
24. Humphery-Smith, I., Cordwell, S. J., and Blackstock, W. P. (1997) Proteome research: Complementarity and limitations with respect to RNA and DNA worlds. *Electrophoresis* **18,** 1217–1242.
25. Karlin, S., Campbell, A. M., and Mrazek, J. (1998) Comparative DNA analysis across diverse genomes. *Annu. Rev. Genet.* **32,** 185–225.
26. Hood, D. W., Deadman, M. E., Allen, T., et al (1996) Use of the complete genome sequence information of *Haemophilus influenzae* strain Rd to investigate lipopolysaccharide biosynthesis. *Mol. Microbiol.* **22,** 951–965.
27. Pizza, M., Scarlato, V., Masignani, V., et al. (2000) Novel proteins for vaccine development from the meningococcus B genome. *Science* **287,** 1816–1820.

11

Structural Profiling of Short-Chain Lipopolysaccharides from *Haemophilus influenzae*

Elke K. H. Schweda and James C. Richards

1. Introduction

Lipopolysaccharides (LPS) are a complex class of glycolipids that can trigger a cascade of immunological responses in mammals, including endotoxic effects and serum antibody production ***(1)***. LPS have been found to exhibit a common molecular architecture consisting of at least two distinct regions: a carbohydrate containing region and a lipid moiety referred to as lipid A ***(2)***. In enteric bacteria (e.g., *Escherichia coli*, *Salmonella* spp.), the carbohydrate containing region consists of a high-molecular-mass O-specific polysaccharide that is covalently linked to a low-molecular-mass core oligosaccharide ***(3)***. *Haemophilus influenzae* produces only short-chain LPS in which the carbohydrate region typically contains mixtures of low-molecular-mass but structurally diverse oligosaccharide components. This pathogen remains a major cause of disease worldwide. Six capsular serotypes and an indeterminate number of nontypeable (i.e., acapsular) strains of *H. influenzae* are recognized. In the developed world, non-typeable (NTHi) strains are the second major cause of otitis media infections in children, while serotype b capsular strains are associated with invasive diseases, including meningitis and pneumonia ***(4)***. The carbohydrate regions of *H. influenzae* LPS molecules provide targets for recognition by host immune responses, and expression of certain oligosaccharide epitopes is known to contribute to disease pathogenesis. Molecular structural studies of LPS from a number of different *H. influenzae* strains have resulted in a structural model in which a conserved L-*glycero*-D-*manno*-heptose (Hep)-containing inner-core trisaccharide moiety is attached via a phosphorylated 3-deoxy-D-manno-octulosonic acid (Kdo) residue to the lipid A component ***(5–16)*** (*see* **Structure 1**).

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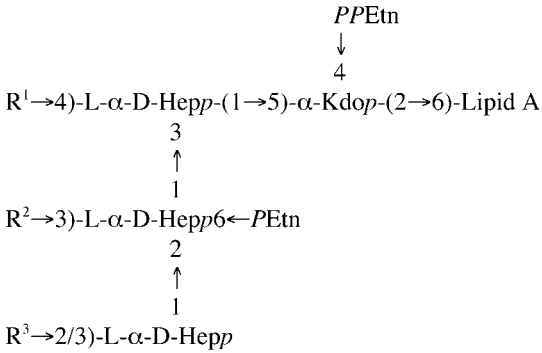

Structure 1 (R^1, R^2, R^3 = H or sugar residue)

In this structural model, each of the Hep residues within the inner core can provide a point for further oligosaccharide chain elongation. In addition, the presence of phosphate-containing substituents, including free phosphate (*P*), phosphoethanolamine (*P*Etn), pyrophosphoethanolamine (*PP*Etn) and phosphocholine (*P*Cho), as well as O-acyl groups, contribute to the structural variability of these molecules. Moreover, *H. influenzae* LPS can undergo phase variation between defined oligosaccharide structures, which creates the possibility of an extensive repertoire of oligosaccharide epitopes in a single strain ***(17,18)***. The heterogeneity and structural complexity of short-chain LPS within and between *H. influenzae* strains pose significant analytical challenges. Structural profiling of short-chain LPS involves initial delipidation to obtain water-soluble oligosaccharides that are suitable for subsequent analyses by chemical, nuclear magnetic resonance (NMR) and mass spectrometry (MS) methods. Methods involving hydrolytic removal of the lipid A ***(3)***, partial ***(19)*** or complete ***(20)*** removal of the fatty acyl groups have been described. Over the last decade, mass spectrometry has played an increasingly important role in the characterization of carbohydrate-containing regions of the short-chain LPS. For example, liquid secondary ion mass spectrometry and tandem mass spectrometry have been used to characterize oligosaccharide samples obtained following mild acid hydrolysis of LPS from *H. influenzae*, *Neisseria gonorrheae* ***(6,21)*** and *H. ducreyi* ***(22)***. More recently, electrospray ionization mass spectrometry (ES-MS) has been shown to be a robust and sensitive analytical technique for profiling the structural diversity of O-deacylated LPS samples from *H. influenzae*, either directly ***(9–16,23–25)*** or in conjunction with on-line separatory techniques ***(26–28)***. Structural profiling of LPS by ES-MS, in con-

junction with ^{1}H NMR spectroscopy, sugar and compositional analyses, has been employed to elucidate the genetic basis of LPS biosynthesis and to study the role of LPS oligosaccharide epitopes in pathogenesis ***(29–32)***.

2. Materials

2.1. Growth of Bacterial Strains

1. Chocolate agar plates from Quelab (Montreal, Canada).
2. Liquid growth medium: 3.7% (w/v) brain heart infusion (BHI), 10 mg/L haemin, 2 mg/L nicotinamide adenine dinucleotide (NAD).
3. Phosphate-buffered saline (PBS): 6.7 m*M* potassium phosphate, pH 7.4, containing 150 m*M* NaCl and 0.02% (w/v) sodium azide (NaN_3).
4. Bacterial killing solution: 0.5% (w/v) phenol in PBS.

2.2. Extraction of Cell-Wall LPS

1. General chemicals: commercial grade phenol, absolute ethanol, acetone, and light petroleum ether (40–60ºC boiling range).
2. Fume hood: water bath, ice bath, omni mixer with stainless steel container.
3. Refrigerated centrifuge, ultracentrifuge, microwave oven.

2.3. Preparation of Oligosaccharides for Structural Profiling

1. General chemicals: acetone, glacial acetic acid, borane-4-methylmorpholine complex, anhydrous hydrazine, 48% aqueous hydrofluoric acid (HF).
2. Fume hood: magnetic stirrer, oil bath, ice bath, source of dry nitrogen gas.
3. Reaction vials: hydrazinolysis, 1 mL reactivial; acid hydrolysis, 50-mL conical-bottom flask; dephosphorylation, eppendorf vial.
4. Eppendorf centrifuge.
5. Gel filtration: column, (2.5 × 80 cm) packed with Bio-Gel P-4; elution buffer, pyridinium acetate (0.1 *M*, pH 5.3); fraction collector; differential refractometer.

2.4. Sugar and Linkage Analysis

1. General chemicals and reagents: 2 *M* trifluoroacetic acid, 1 *M* ammonium hydroxide, sodium borohydride (borodeuteride), glacial acetic acid, methanol, 10% acetic acid in methanol, acetic anhydride, pyridine, ethyl acetate, 4-(dimethylamino)-pyridine, dimethyl sulfoxide (DMSO, dried over molecular sieves), butyl lithium, methyl iodide, absolute ethanol, acetonitrile, Sep-Pak C18 cartridge.
2. Fume hood: heating block with magnetic stirrer, source of dry nitrogen, ice bath.
3. Reaction vessels: sugar analysis, 13 × 100 mm screw cap vial; methylation analysis, 5-mL serum vial with rubber septum and magnetic stirring bar.
4. Gas liquid chromatograph (e.g., Hewlett-Packard 5890) equipped with a flame ionization detector; GLC-mass spectrometer (e.g., Varian Saturn II ion trap system) fitted with DB-5 fused silica capillary column (25m × 0.25 mm × 0.25 μm).

2.5. Profiling the Distribution of LPS Glycoforms by ES-MS

1. General chemicals and reagents: filtered deionized water (e.g., by using a Elga water filtration system), acetonitrile, 1% aqueous acetic acid, 1% ammonium hydroxide.
2. Triple quadrupole electrospray mass spectrometer system. We have used a VG Quattro MS system (Micromass, Manchester, U.K.) fitted with an ES interface for routine glycoform profiling.

2.6. Profiling Inner-core Substitution Patterns by ^{1}H NMR

1. General reagents: deuterium oxide (99.96% isotopic purity), deuterium oxide containing perdeutero-ethylenediamine tetraacetic acid (EDTA) (2m*M*) and perdeutero-sodium dodecyl sulfate (SDS) (10 mg/mL), acetone (spectral grade).
2. 5-mm NMR tubes.
3. High-field NMR spectrometer. We have used Varian Unity 500 or 600 MHz or JEOL ECP-500 spectrometers for profiling oligosaccharides derived from *H. influenzae* LPS.

3. Methods

3.1. Growth of Bacterial Strains (see Note 1)

Expression levels of certain oligosaccharide epitopes of *H. influenzae* LPS have been found to be dependent on growth conditions ***(10,32,36)***. The following protocol describes broth culture conditions that we have used for initial comparative structural profiling of LPS. The protocol is applicable for growth of both capsular and non-typeable strains (*see* **Note 2**).

1. Resuscitate bacterial strains from frozen stocks on chocolate agar plates and incubate overnight at 37ºC. Select colonies from plates and cultivate in 10-L batches of BHI broth supplemented with hemin and NAD at 37ºC for 20 h.
2. Harvest cells by low-speed centrifugation (5000*g*) and discard centrifugate. Resuspend cell pellet in bacterial killing solution and stir for 16 h. Collect bacterial cell mass by centrifugation (5000*g*). The wet cell mass can be stored at −20º C at this point until required.
3. The wet cell mass is dried by washing successively with ethanol (1 × 2 vol), acetone (2 × 2 vol) and light petroleum ether (2 × 2 vol), followed by air drying.

3.2. Extraction of Cell-Wall LPS

For preparative-scale extractions (from approx 20 g wet weight of bacterial cells), we have employed either the phenol/chloroform/petroleum ether method ***(34)*** as modified by Brade and Galanos ***(35)***, or the hot aqueous phenol extraction procedure ***(36)***. We have found ***(37)*** that higher yields of LPS can be achieved when the bacterial cell mass is dried before conducting the extraction procedure (*see* **Subheading 3.1., step 3**). The hot aqueous phenol extraction procedure is described here.

1. Prepare a ca. 90% phenol solution by adding 50 mL water to 500 g commercial-grade phenol. Heat on high for 3–4 min in a microwave oven to liquefy.
2. Add dry bacterial cell mass to a stainless steel container. Add 50 mL each of hot water (65–70ºC) and phenol solutions and stir vigorously by mechanical mixing for 20–30 min. Place stainless steel container in ice bucket to cool mixture to below 10ºC.
3. Separate aqueous and phenol phases by low-speed centrifugation (5000*g*) at 5ºC for 30 min. and collect the water phase. Add 50 mL warm water (65–70ºC) to phenol phase, mix, and then collect the water phase by low-speed centrifugation.
4. Dialyze the combined water phase extracts (approx 100 mL) against running tap water for 2–3 d (*see* **Note 3**). Collect LPS by lyophylisation.
5. Dissolve LPS in water to a final concentration of 1–2%, and ultracentrifuge (105,000*g*) at 4ºC for 5 h. Decant water and repeat this step 2 ×.
6. Collect purified LPS by suspending in water and lyophylization.

3.3. Preparation of Oligosaccharides for LPS Structural Profiling

H. influenzae LPS is comprised of heterogeneous populations of low-molecular-mass hydrophilic oligosaccharide components (typically in the range 1300–3000 Da) that are covalently linked to a hydrophobic lipid A moiety (*see* **Structure 1**). The ampiphilic nature of these molecules renders them poorly soluble in aqueous media as well as common organic solvents. We have employed the following procedures to obtain water-soluble oligosaccharide samples for LPS structural profiling. This involves partial or complete removal of lipid A-bound fatty acyl groups (*see* **Fig. 1**).

3.3.1. Preparation of O-deacylated LPS by Treatment with Anhydrous Hydrazine

Anhydrous hydrazine provides an effective means for O-deacylation of LPS under relatively mild conditions (*see* **Note 4**). The following protocol can be used for analytical applications (< 1-mg scale). This procedure can readily be scaled up for preparative (>100-mg) applications.

1. Suspend 0.5–1 mg of purified LPS preparation in 200 µL of anhydrous hydrazine and incubate at 37ºC for 1 h with constant stirring.
2. Cool reaction mixture in ice (0ºC) and slowly add 600 µL of cold acetone to destroy excess hydrazine. The O-deacylated LPS product precipitates and is collected by centrifugation. Wash the pellet with 600 µL of acetone (2×); and then with 500 µL of a 4/1 mixture of acetone/water. Dissolve in water and lyophylize to obtain O-deacylated LPS as a powder.

3.3.2. Preparation of Core Oligosaccharides by Treatment with Dilute Acid

This procedure takes advantage of the acid lability of the Kdo ketosidic linkage that joins the LPS oligosaccharide components to the lipid A moiety ***(3)***.

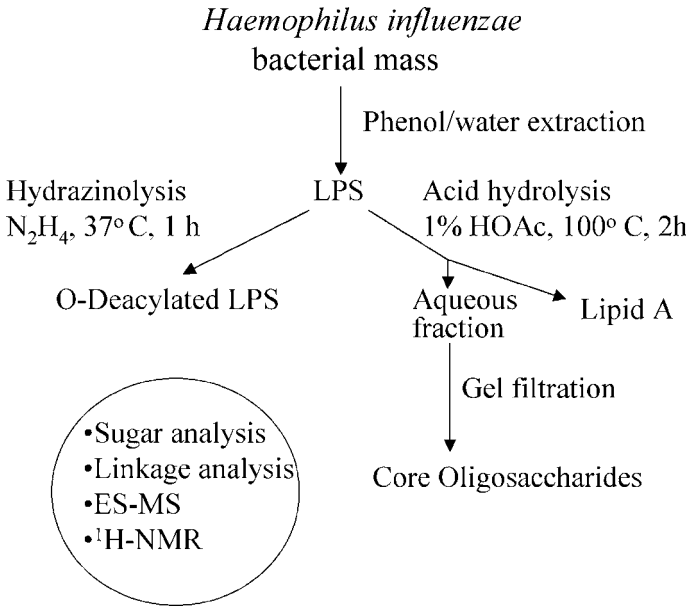

Fig. 1. Strategy for structural profiling of short-chain LPS. LPS is extracted using the aqueous phenol procedure and delipidated by treatment with anhydrous hydrazine or dilute acetic acid to obtain water soluble oligosaccharides. Subsequent sugar and linkage analysis, ES-MS, and ^{1}H NMR of O-deacylated LPS provides a structural profile of the nature and distribution of glycofroms in the LPS sample. Information on O-acyl substitution is obtained from analysis of core oligosaccharide samples.

The water soluble, delipidated material obtained is referred to as core oligosaccharide (*see* **Note 5**). The following protocol is suitable for obtaining sufficient core oligosaccharide for all applications described below. It can readily be adapted for specific microanalytical (1-mg scale) applications (without using the gel filtration step).

1. Transfer 50 mg of LPS to a conical flask (50 mL) equipped with a magnetic stirrer. Suspend in 25 mL water and, with stirring, add borane-4-methylmorpholine complex (12 mg) and adjust the pH to 3.1 with glacial acetic acid. Heat at 100°C and stir under reflux for 2 h.
2. Cool the reaction mixture and separate precipitated lipid A by centrifugation (20 min). Collect the supernatant and wash the precipitate once with water. Combine supernatant and washings which contain the core oligosaccharide fraction and lyophilize.
3. Dissolve core oligosaccharide material in a minimal volume of water and fractionate by gel filtration. For this purpose, a Biogel P4 column has proved particu-

larly useful for separating core oligosaccharides in the molecular weight range of 1000–3000 dalton ***(13,15,16)***. The products are eluted with the pyridinium acetate buffer. Column eluants are continuously monitored for changes in refractive index. Individual fractions can also be assayed for the presence of neutral glycoses by using the standard phenol/sulfuric acid procedure of Dubois et al. ***(38)***. Core oligosaccharide fractions are collected and lyophilized.

3.3.3. Preparation of Dephosphorylated Oligosaccharide

Dephosphorylation of LPS-derived oligosaccharides can provide an effective method for locating the positions of phosphate substituents by methylation analysis (*see* **Subheading 3.4.2.**). Phosphate substitutents are readily removed by treatment with cold aqueous HF. This is a relatively mild treatment and does not normally lead to hydrolysis of glycosidic linkages. The following protocol is appropriate for analytical applications (*see* **Note 6**).

1. Transfer the oligosaccharide sample (O-deacylated LPS or core oligosaccharide; approx. 1 mg) to a small polypropylene vial and add 0.1 mL chilled 48% hydrofluoric acid by using a micropipet. Seal the tube and keep it at 4°C for 48 h.
2. In a fume hood, place sample in an ice bath and evaporate to dryness under a stream of nitrogen. Dissolve the residue in 0.2 mL water and lyophilize.

3.4. Sugar and Linkage Analysis

These procedures provide information about the presence and substitution patterns of neutral and amino sugar residues in the LPS. Information on the following sugar components commonly found in *H. influenzae* LPS can be obtained: glucose (Glc), galactose (Gal), L-*glycero*-D-*manno*-heptose (LD-Hep), D-*glycero*-D-*manno*-heptose (DD-Hep), glucosamine (GlcN), and galactosamine (GalN). The method is not directly applicable for linkage analysis of sugar acids (i.e., Kdo and sialic acid). Glycoses and partially methylated glycoses are identified as their reduced alditol acetate derivatives by gas-liquid chromatography (GLC).

3.4.1. Determination of Neutral and Amino Sugar Compositions

All reactions are done in 1 mL glass reactivials to minimize sample loss and to optimize the sensitivity of the procedure. The procedure involves complete hydrolysis of the sample under acidic conditions to liberate the monosaccharide components. We typically use aqueous trifluoroacetic acid solutions, although mineral acids such as 2 *M* HCl or 2 *M* sulphuric acid have been employed (*see* for example, **ref. 39**). The hydrolysis mixture is reduced *in situ* to afford the corresponding alditols, followed by conversion to the acetylated derivatives. The acetylation step enhances the volatility of the sample rendering it amenable to analysis by GLC (*see* **Note 7**).

1. Transfer a sample (0.1–0.5 mg) of LPS, O-deacylated LPS, or core oligosaccharide to the screw-cap tube. Add 0.3 mL of 2 *M* trifluoroacetic acid and hydrolyze

for 2 h at 120°C. Remove trifluoroacetic acid by evaporation under a stream of nitrogen or compressed air in a fume hood.

2. Add 0.5 mL of 1 *M* aqueous ammonia and 10 mg sodium borohydride (or sodium borodeuteride if the sugar analysis is part of methylation analysis (*see* **Subheading 3.4.2.**) and let stand for at least 1 h at 20°C. Quench the reaction with glacial acetic acid and evaporate to dryness. This converts excess borohydride to borate with the release of hydrogen (the formation of bubbles should be observed). Excess borate is removed as volatile trimethyl borane by addition of 10% acetic acid in methanol (2× 0.5 mL) and methanol (2× 1 mL) to the residue in sequence with subsequent evaporations (under a stream of nitrogen).
3. Add 0.1 mL acetic anhydride and 0.1 mL pyridine and heat at 100°C for 20 min. Let the solution cool, then evaporate the solvent. The alditol acetate derivatives are obtained from the reaction mixture by partition between 0.5 mL ethylacetate and 0.5 mL water. The organic phase, containing the acetylated derivatives, is drawn off. The water phase is washed with ethyl acetate (2× 0.5 mL) and the combined organic extracts are concentrated to about 0.1 mL for analysis.
4. Inject sample (0.5–1.0 uL) onto the column of the GLC or GLC-MS system and separate components using a temperature gradient of 160°C (1 min) → 250°C (1 min) at 3°C/min.

3.4.2. Determination of Sugar Substitution Patterns by Methylation Analysis

Methylation analysis is a useful method for determining the positions of the glycosidic linkages within oligosaccharide samples through providing information on the degree of substitution of the monosaccharide components. The method involves formation of permethylated oligosaccharides by conversion of all free hydroxyl groups to the corresponding methyl ethers. This is achieved by employing a strong organic base such as dimsyl sodium, dimsyl potassium ***(40)*** or butyl lithium ***(41)*** in DMSO followed by reaction with methyl iodide. It is essential that all procedures are conducted under anhydrous conditions, which can be achieved by conducting the reaction steps under a flow of dry nitrogen. The procedure described involves initial peracetylation to increase the solubility of the material in DMSO. It is important to ensure that all the material is dissolved before adding the organic base, otherwise the end product will contain a high proportion of alditol acetates. Permethylated oligosaccharides obtained in this way are hydrolyzed, reduced and analyzed by GLC-MS as partially methylated alditol acetates (*see* **Notes 7** and **8**).

1. Transfer a sample of O-deacylated LPS, core oligosaccharide or dephosphorylated oligosaccharide (0.5 –1 mg) to a small serum vial containing a stirrer. Add 0.2 mL acetic anhydride and 2 mg 4-(dimethylamino)-pyridine. Seal the vial with a rubber septum and stir for 4–5 h at 20°C. Dry the sample under a stream of nitrogen, add 0.5 mL ethanol and dry again. Dissolve the sample in a minimal

volume of water and lyophilize. Dry the acetylated oligosaccharide material over phosphorous pentoxide *in vacuo* (overnight).

2. Add 0.5 mL dry dimethylsulfoxide, seal the vial, and flush gently with nitrogen which is introduced via a syringe needle. Stir for at least 2 h. Stirring for up to 2 d may be required for O-deacylated samples of highly truncated LPS. Cool vial in an ice bath and slowly add 0.2 mL buthyl lithium using a 1-mL syringe. Warm reaction mixture at 40°C in an oil bath for 1 h. Cool vial in an ice bath and add 0.25 mL methyl iodide. After stirring the mixture for at least 2 h at 20°C, remove excess methyl iodide by bubbling a stream of nitrogen through the solution. Quench the reaction mixture by adding 0.5 mL water .
3. The methylated product is purified by using a Sep-Pak C_{18} cartridge. Precondition the cartridge by rinsing it with 40 mL ethanol, acetonitrile (2 × 2 mL) and water (2 × 2 mL). Apply the quenched reaction mixture from **step 2**. Rinse the vial with 1 mL DMSO/water (1/1). Wash the Sep-Pak with water (4 × 2 mL) and acetonitrile/water (1/1) (2 × 2 mL). Elute methylated oligosaccharides with acetonitrile (2 × 1 mL) into a 13 × 100-mm screw cap vial and concentrate to dryness.
4. Methylated oligosaccharides are subjected to hydrolysis, reduction, acetylation, and analyzed by GLC-MS as described in **Subheading 3.4.1.**

3.5. Profiling the Distribution of LPS Glycoforms by ES-MS

Electrospray ionization mass spectrometry (ES-MS) is a sensitive analytical procedure that is ideally suited for profiling the structural diversity of short-chain LPS ***(9–16,23–25)***. A solution of O-deacylated LPS or core oligosaccharide (*see* **Subheading 3.3.**) can be introduced by direct infusion via the electrospray interface of the mass spectrometer or following on-line separation by liquid chromatographic or electrophoretic separation ***(26–28)***. Spectra are measured in the positive- or negative-ion mode. This is a "soft" ionization technique that generally leads to limited fragmentation, the resulting spectra being representative of the molecular species present in the sample. We routinely analyze O-deacylated LPS samples by ES-MS in the negative-ion mode by direct infusion of sample. This has proved particularly useful for providing a fingerprint of glycoform populations of LPS from *H. influenzae* strains (*see* **Note 9**). In addition, information on the presence of O-acyl substitutents can be obtained by negative-ion ES-MS of core oligosaccharide material. In the following procedure, a triple quadrupole mass spectrometer is used. This provides the opportunity to obtain MS-MS data on selected ions (*see* **Note 10**).

1. Dissolve O-deacylated LPS or core oligosaccharide samples in water/acetonitrile (1/1) to a concentration of 0.5–1 mg/mL. If necessary add methanol and 1% ammonia to enhance solubility (*see* for example, **ref. *15***).
2. Inject sample solutions via a loop into a running solvent of water:acetonitrile (1/1) at a flow rate of 5–10 µL/min. We have used a Harvard 22 syringe pump for this purpose. For optimal ionization of O-deacylated LPS, we have found it useful to include 1% acetic acid in the 50% aqueous acetonitrile running solvent ***(12)***.

3. Collect negative-ion spectra by scanning the first quadrupole (Q1).
4. Select ions for collisional activation (*see* **Note 10**) and collect spectra by scanning the third quadrupole (Q3).

3.6. Profiling Inner-Core Substitution Patterns by ^{1}H NMR

NMR studies of oligosaccharide samples obtained from mixtures of *H. influenzae* LPS can provide complete and detailed structural information. Applications involving the use of homo- and heteronuclear ^{1}H, ^{13}C and ^{31}P correlation spectroscopy are described elsewhere (*see*, for example, **refs. *10,12,15,16***). We have found one-dimensional (1D) ^{1}H NMR particularly useful for profiling small amounts (0.05–2 mg) of O-deacylated LPS or core oligosaccharide samples. This provides valuable information on the triheptosyl inner-core region, attachment of *P*Cho units, sialic acid residues (from O-deacylated LPS samples) and O-acyl substituents (from core oligosaccharide samples), while, depending on availability of sample (typically >300 µg), two-dimensional (2D) techniques can give a more detailed picture (*see* **Note 11**). Spectra are usually obtained at ambient temperature (ca. 30ºC) in deuterium oxide (D_2O) solutions. The residual water signal (HOD) usually occurs at ca. 4.565 ppm under these conditions, but can be shifted to higher field to reveal underlying peaks by increasing the temperature (60º–80ºC). This is particularly useful for 1D applications and also has the effect of giving sharper, well-resolved signals. Spectra of O-deacylated LPS in D_2O often show broad undefined peaks, even at higher temperature, due to molecular aggregation or micelle formation. We have found that a significant improvement in signal resolution can be achieved by the addition of deuterated EDTA and SDS to the sample solution ***(12)***. This is shown in **Fig. 2** for the O-deacylated LPS sample from *H. influenzae* strain Rd. The following procedure employs this approach.

1. Dissolve O-deacylated LPS or core oligosaccharide sample in D_2O (approx 0.5 mL) and lyophilize to replace exchangeable protons by deuterium (3×). Dissolve in a final volume of 0.6 mL D_2O (core oligosaccharide samples) or 0.6 mL D_2O containing perdeutero-EDTA and -SDS (O-deacylated LPS samples).
2. Place sample in standard 5-mm NMR tube, mix in acetone (10 uL) and record ^{1}H NMR spectra at 500 or 600 MHz, typically over a spectral width of 6 kHz and using a 90º pulse. The methyl ^{1}H resonance of acetone (2.225 ppm) provides an internal chemical shift standard.
3. Record 2D homonuclear chemical shift correlation (COSY, TOCSY) and nuclear Overhauser effect (NOESY) experiments using standard pulse sequences provided by the instrument manufacturer (when sufficient material is available). Typically, we use mixing times of 70 ms and 400 ms for the TOCSY and NOESY experiments, respectively. To obtain spectra in a reasonable time frame, good signal-to-noise ratio should be achievable in the 1D spectrum in 64 scans or less.

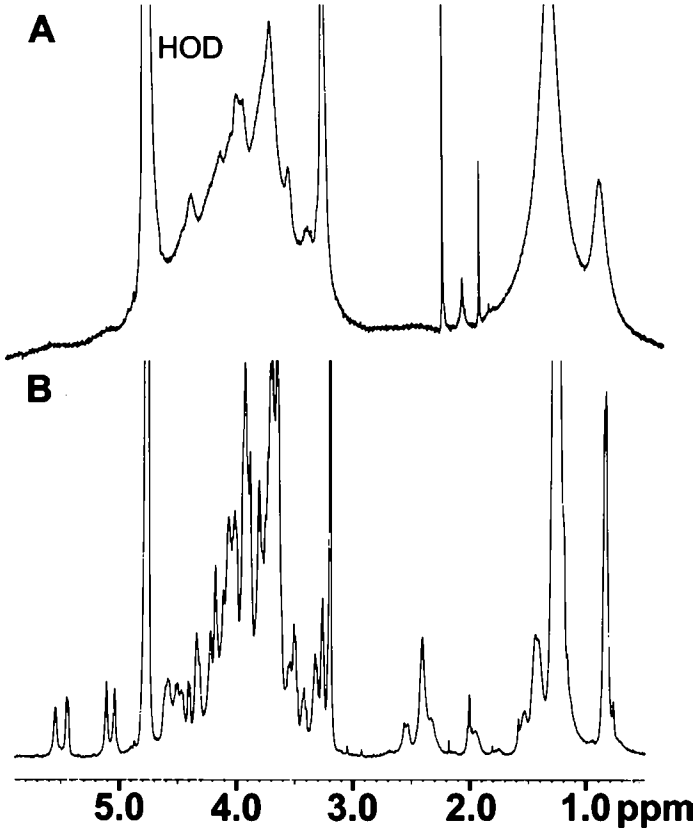

Fig. 2. 500 MHz ^{1}H NMR spectra of O-deacylated LPS from *H. influenzae* strain Rd. Spectra were obtained on ca 1 mg of material at 37°C in D_2O (**A**) or D_2O containing perdeuterio-EDTA and -SDS (**B**) (*see* **Subheading 3.6.**).

3.7. Application of Structural Profiling to H. influenzae *Strains*

The utility of this approach (*see* **Fig. 1**) is illustrated in the examples given in the Notes. In particular, the power of structural profiling is demonstrated for *H. influenzae* strains Rd (*see* **Notes 9** and **11**) and NTHi 486 (*see* **Note 11**). We have found that comparative structural profiling can provide detailed information on relatively small amounts of material (< 1 mg of LPS). For example, in strain Rd, we have used structural profiling to identify the genes that are involved in glycose additions, providing a genetic blueprint for LPS oligosaccharide biosynthesis ***(42)***. Moreover, application of this approach has facilitated a study on the effect of growth conditions on LPS expression in *H. influenzae* (*see*, for example, **ref. *32***).

4. Notes

1. It is important to carry out growth and manipulations of *H. influenzae* bacteria under level II containment to ensure proper biosafety. Once bacteria are killed by stirring cells with a phenol-containing solution (bacterial-killing solution), LPS can be extracted using the precautions normally followed in the analytical chemistry laboratory.
2. *H. influenzae* strains RM118, RM153, and the NTHi strains referred to in this chapter are from the culture collection of Professor E. R. Moxon (Oxford University, U.K.). *H. influenzae* RM118 is a capsular-deficient serotype d strain (referred to as strain Rd−), obtained from the same source as the strain used in the *Haemophilus* genome sequencing project ***(43)***. Strain Eagan is an encapsulated serotype b disease isolate from the United States ***(44)***. Strains NTHi 486 and 176 are middle ear isolates obtained as part of the Finnish Otitis Media Cohort Study ***(45)***.
3. We routinely obtain LPS from the aqueous phase following extensive dialysis against running tap water. LPS can also be obtained by precipitation with ethanol (typically by addition of 4 vol to the aqueous phase), followed by centrifugation, lyophylization, and purification by ultracentrifugation ***(10,29)***. Although significantly lower yields are obtained by the ethanol precipitation procedure (typically by an order of magnetude), these LPS preparations have been found to contain less RNA as determined by sugar analysis. Also, the latter preparations have been found to contain higher populations of LPS in which the Kdo residues are substituted by pyrophosphoethanolamine groups instead of phosphate at the 0–4 position ***(10)*** (*see* **Note 9**).
4. Using the mild O-deacylation conditions described, anhydrous hydrazine provides an excellent method for solubilizing short-chain LPS samples through the removal of ester-linked fatty acids from the lipid A region of the molecule. It is important to recognize, however, that the procedure also effects release of O-acyl substituents (e.g., O-acetyl or O-glycyl), which may be present in the core region of the molecule (*see* for example, **ref. *15***). Samples of O-deacylated LPS are amenable to analysis by ES-MS (*see* **Note 9**) since they typically carry a net negative charge due to the acidic Kdo residue and phosphate-containing substituents. This procedure is now well established for probing the heterogeneity of LPS from *H. influenzae* ***(25)***.
5. This is a well established procedure that has proved successful for obtaining detailed structural information on LPS O-polysaccharides (*see* for example, **ref. *41***). For short-chain LPS, partial acid hydrolysis with dilute aqueous acetic acid affords the insoluble lipid A and core oligosaccharides that are obtained after purification by gel filtration. The procedure often affords mixtures of oligosaccharides arising from acid-mediated modifications of the Kdo residue at the reducing terminus through loss of the 4-linked phosphate group (*see* for example, **ref. *7***). Reduction of the ketose group of the terminal Kdo moiety can limit the degree of heterogeneity and afford stability to the reaction products. This is achieved *in situ* by addition of the reducing agent, borane-4-

methylmorpholine complex, during hydrolysis. By employing this approach, valuable information can be obtained on the occurrence and location of O-acyl groups in the core region of the LPS molecules ***(13,15,16)***. Reduction of the terminal Kdo groups can also be performed with water-soluble reducing agents such as sodium borohydride in a separate step after hydrolysis, but this leads to the removal of O-acyl substituents.

6. Care should be exercised when using solutions of hydrofluoric acid. It can cause severe burns and is reactive with silicon-based glass. Latex gloves should be worn throughout. Glass vials must not be used. Standard polypropylene Eppendorf vials are ideally suited for analytical applications. All manipulations should be done in a fume hood.
7. Determinations are made by comparison of GLC retention times with those of authentic samples. Sugar identities can be confirmed by GLC-mass spectrometry (GLC-MS) using a bench top system. Relative compositions are estimated from the ratios of GLC peak areas. This is generally accurate when comparing components within, but not between, monosaccharide groups such as hexoses (e.g., Glc and Gal), heptoses (LD-Hep and DD-Hep) or hexosamines (e.g., GlcN and GalN). This is due to differences in GLC detector response factors (e.g. HexN derivatives are less than Hex derivatives). For quantitative determinations, a weighed internal standard such as *myo*-inositol is added to the sample at the beginning of the procedure. For methylation analysis, differences in the volatility of the derivatives can also be a factor. For example, certain permethylated derivatives can be lost during final evaporations, leading to low results.
8. We have found that low results are generally obtained for phosphorylated sugars (e.g., Hep-PEtn or Glc-PCho) due to incomplete dephosphorylation during the hydrolysis step. This can be overcome through prior dephosphorylation of the oligosaccharide sample with aqueous HF (*see* **Subheading 3.3.3.**). A comparison of the GLC results obtained from methylation analyses of O-deacylated LPS and the corresponding dephosphorylated analog of NTHi 176 is shown in **Fig. 3**. This *H. influenzae* strain expresses mixed populations of Hex3 and Hex4 glycoforms. Methylation analysis of the samples indicated the presence of terminal Glc, 4-substituted Glc, and terminal Gal residues in the LPS (data not shown). Hydrolysis of the permethylated O-deacylated LPS sample gave 2,6,7-trimethyl- and 2,4,6,7-tetramethyl– L,D-Hep as the major heptose-containing products as identified by GLC of the derived alditol acetate derivatives (**Fig. 3A**). This result points to the presence of HepI (3,4-Hep) and HepIII (3-Hep) in the triheptosyl inner-core moiety, respectively (*see* **Structure 1**). When the analysis was carried out on the dephosphorylated and O-deacylated LPS sample, alditol acetate derivatives of 4,6,7-trimethyl- and 3,4,6,7-tetramethyl- L,D-Hep were also detected as major components (**Fig. 3B**), corresponding to substituted (2,3-Hep) and unsubstituted (2-Hep) forms of HepII in the LPS glycoform mixture (*see* **Structure 1**). In this example, treatment of the O-deacylated LPS with aqueous HF resulted in removal of the PEtn groups from HepII, affording volatile alditol acetate derviatives for GLC analysis.

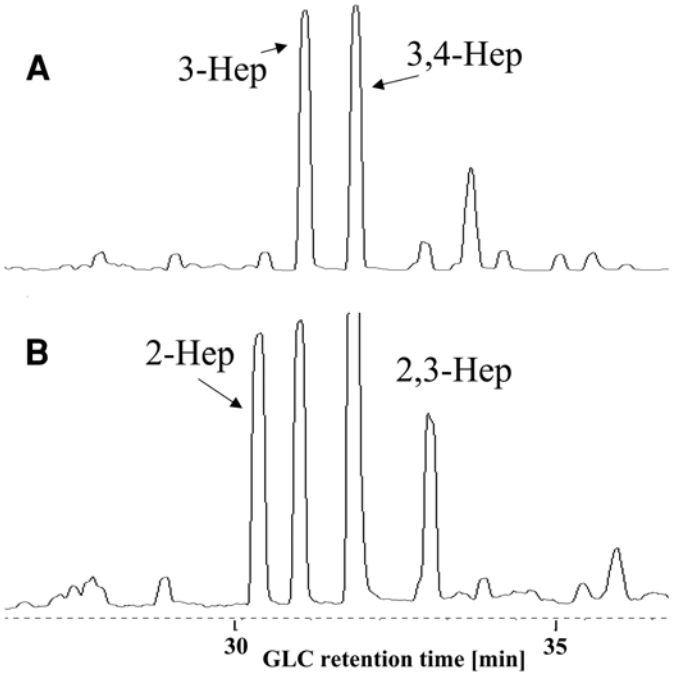

Fig. 3. GLC of partially methylated alditol acetate derivatives. The derivatives were obtained from NTHi 176 O-deacylated LPS (**A**) and dephosphorylated O-deacylated LPS (**B**) by methylation analysis (*see* **Subheading 3.4.2.**). The heptose region (28–34 min) is shown in the GLC traces which were recorded using the temperature program under **Subheading 3.4.1.** The substitution profiles of the Hep residues are indicated for each of the major peaks. 3-Hep corresponds to 1,3,5-triacetyl, 2,4,6,7-tetramethyl-heptitol, etc.

9. For rapid screening of the glycoform and isoform distributions of O-deacylated LPS samples, spectra are generally obtained in the negative-ion mode. Using a commercially available nanoelectrospray interface, it is now possible to identify LPS derived oligosaccharides at detection limits in the femtomole range. The ES spectra are generally dominated by molecular peaks corresponding to doubly, triply, or quadruply deprotonated species. The mass spectrometer measures the mass/charge ratio of the ions detected. As an example, the triply charged region of the ES-MS spectrum obtained from O-deacylated LPS samples from *H. influenzae* strain Eagan is shown in **Fig. 4** ***(10)***. In **Fig. 4**, the data indicates a series of related structures differing in the number of hexose residues, but containing the conserved PEtn-substituted inner-core moiety attached via a phosphorylated Kdo linker to the O-deacylated lipid A (*see* **Structure 1**). This is based on compositional calculation using incremental molecular mass values for Hex (162.15), Hep (192.17), Kdo (220.18), phosphate (79.98), PEtn (123.05),

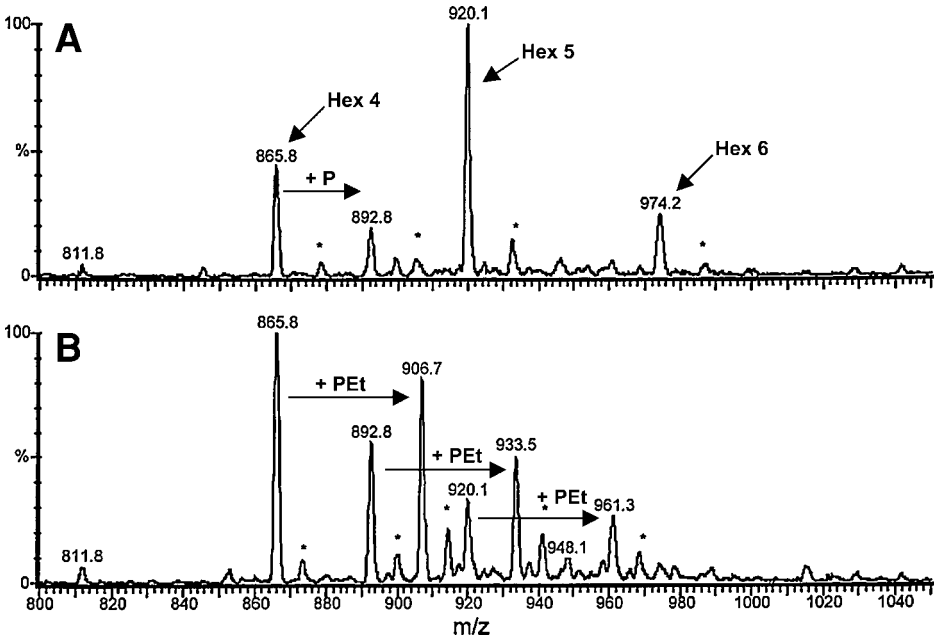

Fig. 4. Negative-ion ES-MS of O-deacylated LPS from *H. influenzae* serotype b strain Eagan. Spectra show the triply charged region for samples obtained by phenol-water extraction followed by extensive dialysis (**A**), or by ethanol precipitation (**B**) (*see* **Note 3**). Subpopulations of the major LPS glycoforms differing by the addition of P and PEtn are indicated (*see* **Note 9**).

and O-deacylated lipid A (951.03). Evidence for the inner-core structural element is readily obtained from the 1-D ^{1}H NMR spectrum (*see* **Note 11**). In **Fig. 4B**, a parallel series of ions displaced by 41 *m/z* units to higher mass in the triply charged region is attributed to subpopulations of glycoforms containing an additional PEtn group (giving an incremental addition of ca. 123 in calculated compositions). The LPS sample used for ES-MS profiling in **Fig. 4B** was obtained from strain Eagan by ethanol precipitation following phenol water extraction, whereas that used for **Fig. 4A** involved exhaustive dialysis (*see* **Note 3**). The additional PEtn group is present as a PPEtn moiety, attached to the 0–4 position of Kdo, a structural feature which was firmly established by tandem mass spectrometry (*see* **Note 10**). ES-MS has proved to be very valuable for profiling glycoform populations of O-deacylated LPS. In *H. influenzae* strain Rd, for example, three major glycoform groups containing three, four and five hexose residues can be observed ***(12)***. As shown in **Fig. 5**, each group is made up of subpopulations that contain an additional phosphate residue or are linked to the lipid A by Kdo-*P* instead of Kdo-*PP*Etn. Moreover, the major LPS glycoform

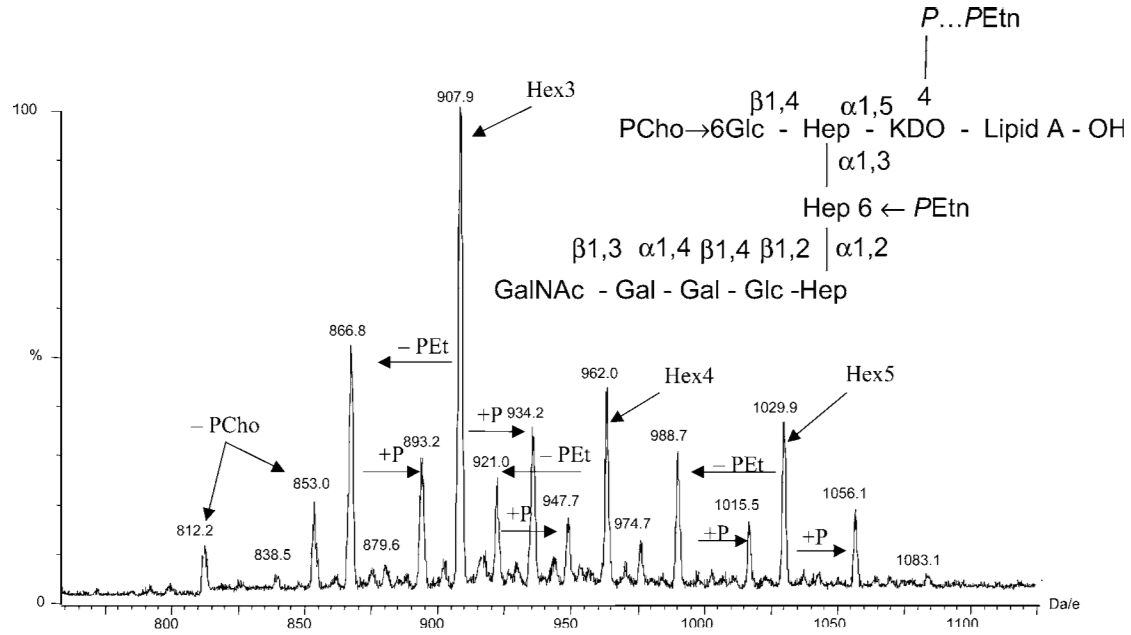

Fig. 5. Negative ion ES-MS of the triply charged molecular ion region of O-deacylated LPS from *H. influenzae* strain Rd (*see* **Note 9**). Peaks corresponding to the major glycoform groups differing in the degree of phosphorylation (P and PEtn) are indicated, and the structure of the fully extended Hex5 glycoform is shown in the inset ([M-3H$^3-$] = *m/z* 1029.9). Hex4 and Hex3 glycoforms arise from sequential deletion of GalNAc and Gal from the main oligosaccharide chain on HepIII ***(12)***.

populations in this strain contain phosphocholine (*P*Cho) residues. Expression of *P*Cho on *H. influenzae* LPS has been found to be phase variable, the extent of which can vary among different strains ***(11,12, 18,30)***. *P*Cho substitution is readily revealed from the mass of the molecular ions, since its incremental mass (165.05) differs from that of a Hex residue (162.15) by 3 dalton (1 U in the triply charged region of the spectrum). Substitution by *P*Cho groups is confirmed by ^{1}H NMR spectroscopy (*see* **Note 11**).

10. Useful structural information can be obtained for O-deacylated LPS by tandem mass spectrometry of the doubly or triply charged molecular ions. Generally, ions selected in Q1 are focused on the RF-only quadrupole (Q2), where collisional activation is induced with argon or nitrogen gas (typically at a collision energy of 75 eV in the laboratory frame of reference). Fragment ions so obtained are separated and recorded by scanning the third quadrupole (Q3) of the triple quadrupole instrument. Fragmentation in the negative-ion mode gives useful structural information on the lipid A region, because cleavage occurs primarily between the Kdo-β-D-GlcN bond to afford an O-deacylated lipid A fragment at ca. *m/z* 951 ***(27)***. Information concerning the nature of the phosphate substituent on the Kdo moiety (P or PPEtn), can also be obtained from negative-ion MS-MS experiments due to diagnostic fragment ions at *m/z* 97 or 220 ***(10)***. Similarly, terminal sialic acid groups (Neu5Ac) can be detected following loss of the negatively charged fragment, *m/z* 290 ***(28,45)***. Tandem mass spectrometry of oligosaccharides in the positive-ion mode generally produces abundant fragment ions arising from cleavage at the glycosidic bonds. The detection of positive ions from O-deacylated LPS species that are normally anionic in solution opens the door to valuable structural information not normally obtainable from their negative-ion counterparts and is described elsewhere ***(27)***.
11. 1D ^{1}H NMR spectra of LPS-derived oligosaccharide samples from *H. influenzae* can provide information about the presence of key substituents that have been linked to the virulence of the organism. For example, the presence of a strong ^{1}H methyl signal at ca. 3.24 ppm is diagnostic of the presence of *P*Cho substituents, the location of which can be determined by ^{1}H-^{31}P correlation ***(15)***. This is particularly evident for strain Rd, as can be seen in **Fig. 2B**. The H-3 methylene resonances from sialic acid residues at ca. 1.80 (a triplet) and 2.75 ppm (a double doublet) point to the presence of this residue (data not shown) in O-deacylated LPS samples (*see* for example, **ref. *32***). In the spectra of core oligosaccharides, signals in the region of 1.9–2.1 ppm indicate the presence of O-acetyl substituents, the location of which can be determined from downfield-shifted resonances relative to those in the corresponding O-deacylated sample (*see* for example, **refs. *13,15,16***). The region of the 1D ^{1}H NMR spectrum between ca. 5.0 and 6.0 ppm is particularly informative in providing information about the inner-core region. The anomeric proton resonances from the three Hep residues occur in this region. The anomeric protons of HepI and HepIII typically have similar chemical shifts, occurring between 5.0 and 5.2 ppm, whereas that from HepII invariably occurs farther downfield

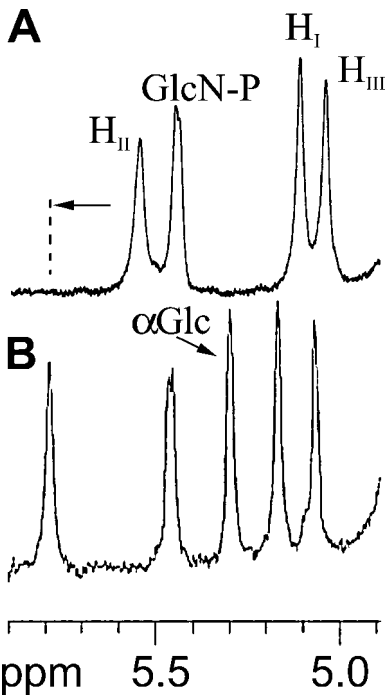

Fig. 6. Comparison of the down field anomeric region (4.8–5.8 ppm) of the ^{1}H NMR spectra of O-deacylated LPS were compared from *H. influenzae* strain Rd (**A**) and non-typeable strain 486 (**B**) recorded under the conditions given in **Fig. 2** (*see* **Note 11**).

between 5.55 and 5.80 ppm. This is evident in the spectrum of O-deacylated LPS from *H. influenzae* strain Rd shown in **Fig. 6A** ***(12)***. In addition, the anomeric ^{1}H signal from the GlcN-1-phosphate in the O-deacylated lipid A region of the molecule occurs in this region at 5.50 ppm. We have noted that the chemical-shift value of the anomeric proton of HepII can provide information about the substitution pattern at HepIII. For example, in strains Eagan and Rd in which Gal or Glc is attached to O–2 of HepIII ***(10,12)***, the anomeric resonance of HepII occurs at ca. 5.60 ppm, whereas in strains in which there is no substitution (e.g., *lpsA* mutant of stain Rd, **ref.** ***42***) or substitution occurs at O–3 (e.g., NTHi 486, **ref.** ***16***), this resonance occurs ca 0.15–0.20 ppm to lower field. A comparison of the α-anomeric regions of O-deacylated LPS from strains Rd and 486 is shown in **Fig. 6**. It is noteworthy that this region of the ^{1}H spectrum can also give information about glycose substitution at HepII (i.e., *see* **Structure 1**, R^2) since, in our experience, this is generally initiated by an α-Glc residue at O–3. This is apparent from the signal at ca. 5.3 ppm in the

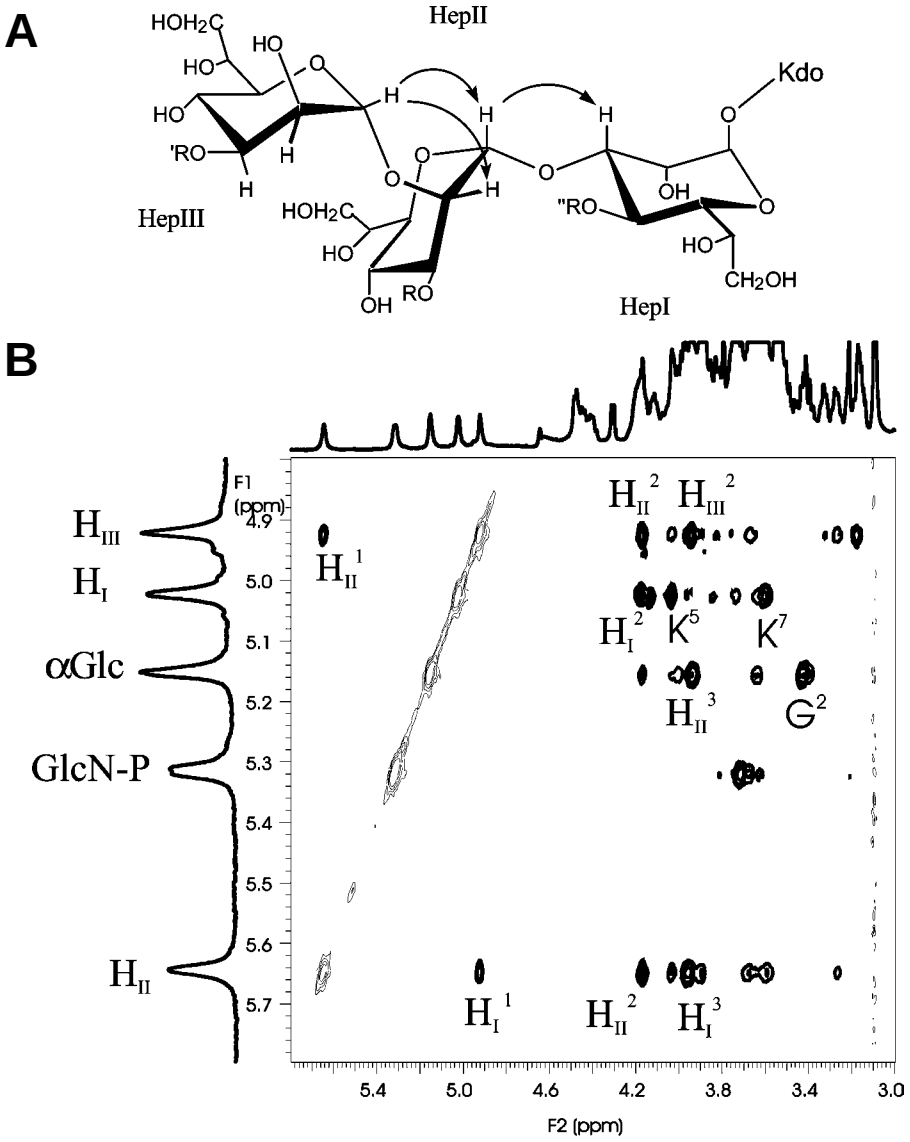

Fig. 7. Structure of the triheptosyl inner-core backbone of *H. influenzae* LPS. The network of transglycosidic NOE connectivities (**A**) observed in the 2D homonuclear NOESY spectrum of the O-deacylated LPS from NTHi 486 (**B**) is shown. The 2D spectrum was recorded at 37°C by using the conditions given in **Subheading 3.5.** A contour plot for the spectral regions F1 (4.8–5.8 ppm) and F2 (3.0–5.8) with 1D projections is shown. The anomeric resonances are indicated using the abbreviations, HI–HIII for HepI–HepIII and GlcN-P for the terminal glucosamine residue in the O-deacylated lipid A region of the molecule. The αGlc (G) residue is attached at O-3 of HepII, denoted by R in the structural model shown in **(A)** (*see* **Structure 1** and **Note 11**).

spectrum of the O-deacylated LPS from NTHi 486 (**Fig. 6B**). Definitive evidence for the triheptosyl inner-core structural element can be obtained from the network of NOE connnectivities observed in the contour plot of a 2D NOESY experiment. This is shown in **Fig. 7** for the O-deacylated LPS sample from NTHi 486. The NOE information provides a qualitative picture of short (<3 Å), through-space connectivities between anomeric and aglyconic protons on adjacient glycosydically linked residues. Of particular note, NOE connectivities are observed between the anomeric protons of HepII and HepIII indicating the preferred conformational arrangement of the triheptosyl unit (**Fig. 7**). In conjunction with these data, methylation analysis provides confirmation of the substitution patterns of the Hep residues (*see* **Note 8**).

Acknowledgments

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References

1. Rietschel, E.Th., Brade, L., Schade, U., et al. (1988) Bacterial endotoxins: properties and structure of biologically active domains, in surface structures of microorganisms and their interactions with the mammalian host, in *Proceedings Workshop Conference, Hoechst,* (Schrinner, E., Richmond, M. H., Seibert, G., and Schwarz, U., eds.), Verlag Cheme, Weinheim, Germany, pp. 1–41.
2. Raetz, C. R. H. (1990) *Annu. Rev Biochem.* **59,** 129–170.
3. Holst, O. and Brade, H. (1992) Chemical structure of the core region of lipopolysaccharides, in *Bacterial Endotoxic Lipopolysaccharides,* (Morrison, D. C. ed.), pp. 135–170.
4. Turk, D. C. (1981) Clinical importance of *Haemophilus influenzae*, in *Haemophilus influenzae Epidemiology, Immunology and Prevention of Disease*, (Sell, S. H. and Wright, P. F., eds.), Elsevier, New York, pp. 3–9.
5. Helander, I. M., Lindner, B., Brade, H., et al. (1988) Chemical structure of the lipopolysaccharide of *Haemophilus influenzae* strain I–69 Rd−/ b+, *Eur. J. Biochem.* **177,** 483–492.
6. Phillips, N. J., Apicella, M. A., Griffiss, J. M., and Gibson, B.W. (1992) Structural characterization of the cell surface lipooligosaccharides from a nontypable strain of *Haemophilus influenzae*. *Biochemistry* **31,** 4515–4526.
7. Schweda, E. K. H., Hegedus, O. E., Borrelli, S., et al. (1993) Structural studies of the saccharide part of the cell envelope lipopolysaccharide from *Haemophilus influenzae* strain AH1–3 (lic3+). *Carbohydr. Res.* **246,** 319–330.
8. Schweda, E. K. H., Jansson, P. E., Moxon, E. R., and Lindberg, A. A. (1995) Structural studies of the saccharide part of the cell envelope lipooligosaccharide from *Haemophilus influenzae* strain galEgalK. *Carbohydr Res.* **272,** 213–224.

9. Phillips, N. J., McLaughlin, R., Miller, T. J., Apicella, M. A., and Gibson, B. W. (1996) Characterization of two transposon mutants from *Haemophilus influenzae* Type b with altered lipooligosaccharide biosynthesis. *Biochemistry* **35,** 5937–5947.
10. Masoud, H., Moxon, E. R., Martin, A., Krajcarski, D., and Richards, J. C. (1997). Structure of the variable and conserved lipopolysaccharide oligosaccharide epitopes expressed by *Haemophilus influenzae* serotype b strain Eagan. *Biochemistry* **36,** 2091–2103.
11. Risberg, A., Schweda, E. K. H., and Jansson, P.-E. (1997) Structural studies of the cell-envelope oligosaccharide from the lipopolysaccharide of *Haemophilus influenzae* strain RM.118–28. *Eur. J. Biochem.* **243,** 701–707.
12. Risberg, A., Masoud, H., Martin, A., Richards, J. C., Moxon, E. R., and Schweda, E. K. H. (1999) Structural analysis of the lipopolysaccharide oligosaccharide epitopes expressed by a capsule-deficient strain of *Haemophilus influenzae* Rd. *Eur. J. Biochem.* **261,** 171–180.
13. Risberg, A., Alvelius, G., and Schweda, E. K. H. (1999) Structural analysis of the lipopolysaccharide oligosaccharide epitopes expressed by *Haemophilus influenzae* strain RM.118–26. *Eur. J. Biochem.* **265,** 1067–1074.
14. Rahman, M. M., Gu, X.-X., Tsai, C.-M., Kolli, V. S. K., and Carlson, R. W. (1999) The structural heterogeneity of the lipooligosaccharide (LOS) expressed by pathogenic non-typeable *Haemophilus influenzae* strain NTHi 9274, *Glycobiol.* **9,** 1371–1380.
15. Schweda, E. K. H., Brisson, J. -R., Alvelius, G., et al. (2000) Characterization of the phosphocholine-substituted oligosaccharide in lipopolysaccharides of type b *Haemophilus influenzae*, *Eur. J. Biochem.* **267,** 3902–3913.
16. Månsson, M., Bauer, S. H. J., Hood, D. W., Richards, J. C., Moxon, E. R., and Schweda, E. K. H. (2001) A new structural type for *Haemophilus influenzae* lipopolysaccharide, *Eur. J. Biochem.* **261,** 2148–2159.
17. Kimura, A. and Hansen, E. (1986) Antigenic and phenotypic variations of *Haemophilus influenzae* type b lipopolysaccharide and their relationship to virulence. *Infect. Immun.* **51,** 69–79.
18. Weiser, J. N., Shchepetov, M., and Chong, S. T. H. (1996) Decoration of lipopolysaccharide with phosphorylcholine: A phase-variable characteristic of *Haemophilus influenzae*. *Infect Immun.* **65,** 943–950.
19. Holst, O., Boer, W., Thomas-Oates, J. E., Mamat, U., and Brade, H. (1993) Structural analysis of two oligosaccharide biphosphates isolated from the lipopolysaccharide of a recombinant strain of *Escherichia coli* F515 (RE chemotype) expressing the genus-specific epitope of *Chlamydia* lipopolysaccharide. *Eur. J. Biochem.* **214,** 703–710.
20. Masoud, H., Altman, E., Richards, J. C., and Lam, J. S. (1994) General strategy for structural analysis of the oligosaccharide region of lipopolysaccharides. Structure of the oligosaccharide component of *Pseudomonas aeruginosa*IATS serotype 06 mutant R5 roug-type lipopolysaccharide. *Biochemistry* **33,** 10,568–10,578.
21. Phillips, N. J., John, C. M., Reinders, L. G., Griffiss, J. M., Apicella, M. A., and Gibson, B. W. (1990) Structural models for the cell surface lipooligosaccharide

(LOS) of *Neisseria gonorrhoeae* and *Haemophilus influenzae*. *Biomed. Environ. Mass Spectrom.* **19,** 731–745.

22. Melaugh, W., Phillips, N. J., Campagnari, A. A., Karalus, R., and Gibson, B. W. (1992) Partial characterization of the major lipooligosaccharide from a strain of Haemophilus ducreyi, the causative agent of chancroid, a genital ulcer disease. *Am. Soc. Biochem. Mol. Biol.* **267,** 13,434–13,439.
23. Gibson, B. W., Melaugh, W., Phillips, N. J., Apicella, M. A., Campagnari, A. A., and Griffiss, J. M. (1993) Investigation of the structural heterogeneity of lipooligosaccharides from pathogenic *Haemophilus* and *Neisseria* species and R-type lipopolysaccharides from *Salmonella typhimurium* by electrospray mass spectrometry. *J. Bacteriol.* **175,** 2702–2712.
24. Phillips, N. J., Apicella, M. A., Griffiss, J. M., and Gibson, B. W. (1993) Structural studies of the lipooligosaccharides from *Haemophilus influenzae* type b strain A2. *Biochem.* **32,** 2003–2012.
25. Gibson, B. W., Phillips, N. J., Melaugh, W., and Engstrom, J. J. (1996) Determining structures and functions of surface glycolipids in pathogenic haemophilus bacteria by electrospray ionization mass spectrometry, in *Biochemical and Biotechnological applications of electrospray ionization mass spectrometry.* (Snyder, A. P., ed.), pp. 166–184, American Chemical Society, Washington DC.
26. Li, J., Thibault, P., Martin, A., Richards, J. C., Wakarchuk, W. W., and vander Wilp, W. (1998) Development of an on-line preconcentration method for the analysis of pathogenic lipopolysaccharide using capillary electrophoresis-electrospray mass spectrometry: application to small colony isolates. *J. Chromatogr. A.* **817,** 325–336.
27. Thibault, P., Li, J., Martin, A., Richards, J. C., Hood, D. W., and Moxon, E. R. (2000) Electrophoretic and mass spectrometric strategies for the identification of lipopolysaccharides and immunodeterminants in pathogenic strains of *Haemophilus influenzae*; Application to clinical isolates, in *Mass Spectrometry in Medicine and Biology,* (Burlingame, A., Carr, S., and Bowers, M. T., eds.), Humana Press, Totowa, NJ.
28. Thibault, P. and Richards, J. C. (2000) Application of combined capillary electrophoresis electrospray mass spectrometry in the characterization of short chain lipopolysaccharides: *Haemophilus influenzae*, in *Bacterial Toxins: Methods and Protocols,* Methods in Molecular Biology, Vol. 45, (Holst, A., ed.) Humana Press, Totowa, NJ.
29. Hood, D. W., Deadman, M. E., Allen, T., et al. (1996) Use of the complete genome sequence information of *Haemophilus influenzae* strain Rd to investigate lipopolysaccharide biosynthesis. *Mol. Microbiol.* **22,** 951–965.
30. Weiser, J. N., Pan, N., McGowan, K. L., Musher, D., Martin, A., and Richards, J. C. (1998) Phosphorylcholine on the lipopolysaccharide of *Haemophilus influenzae* contributes to persistence in the respiratory tract and sensitivity to serum killing mediated by C-reactive protein. *J. Exp. Med.* **187,** 631–640.
31. Lysenko, E., Richards, J. C., Cox, A. D., Stewart, A., Martin, A., Kapoor, M., and Weiser, J. N. (2000) The position of phosphorylcholine on the lipopolysaccharide

of *Haemophilus influenzae* affects binding and sensitivity to C-reactive protein, *Mol. Microbiol.* **35,** 234–245.
32. Hood, D, W., Cox, A. D., Gilbert, M., et al. (2001) Identification of a lipopolysaccharide α-2,3-sialyltransferase from *Haemophilus influenzae*. *Mol. Microbiol.* **39,** 341–350.
33. Vimr, E., Lichtensteiger, C., and Steenbergen, S. (2000) Sialic acid metabolism's dual function in *Haemophilus influenzae*. *Mol. Microbiol.* **36,** 1113–1123.
34. Galanos, C. L., Deritz, O., and Westphal, O. (1969) A new method for extraction of R lipopolysaccharides. *Eur. J. Biochem.* **9,** 245 -249.
35. Brade, H. and Galanos, C. L., (1982) *Eur. J. Biochem.* **122,** 233–237.
36. Westphal, O., Lüderitz, O., and Bister, F. (1952) Über die Extraktion von Bakterien mit Phenol/Wasser. *Z. Naturforschg.* **7b,** 148–155.
37. Masoud, H., Perry, M. B., and Richards, J. C. (1994) Characterization of the lipopolysaccharide of *Moraxella catarrhalis.* Structural analysis of the lipid A from *M. catarrhalis* serotype A lipopolysaccharide. *Eur. J. Biochem.* **220,** 209–216.
38. Dubois, M., Gilles, K. A., Hamilton, J .K., Rebers, J. A., and Smith, F. (1956). Colorimetric method for the determination of sugars and related substances. *Anal. Chem.* **28,** 350–356.
39. Richards, J. C., Perry, M. B., and Kniskern, P. J. (1985) The structure of the specific capsular polysaccharide of *Streptococcus pneumoniae* type 11F (American type 11), *Can. J. Biochem. Cell Biol.* **63,** 953–968.
40. Hakomori, H. I. (1964) *J. Biochem.* **66,** 205–208.
41. Blakeney, A. B. and Stone, B. A. (1985) Methylation of carbohydrates with lithium methylsulphinyl carbanion. *Carbohydr. Res.* **140,** 319–324.
42. Hood, D. H., Cox, A. D., Wakarchuk, W. W., et al. (2001) Genetic basis for expression of the major globotetraose containing lipopolysaccharide from *Haemophilus influenzae* strain Rd (RM118). *Glycobiology* **11,** 957–967.
43. Fleischmann, R. D., Adams, M. D., White, O., et al. (1995) Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. *Science.* **269,** 496–498.
44. Anderson, P., Peter, G., Johnston, R. B., Wetterlow, H., and Smith, D. H. (1972) Immunization of humans with polyribophosphate, the capsular antigen of *Haemophilus influenzae* type b. *J. Clin. Invest.* **51,** 39–44.
45. Hood, D. W., Makepeace, K., Deadman, M. E., et al. (1999) Sialic acid in the lipopolysaccharide of *Haemophilus influenzae*: strain distribution, influence on serum resistance and structural characterisation. *Mol. Microbiol.* **33,** 679–692.
46. Kenne, L. and Lindberg, B. (1983) in *The Polysaccharides,* (Aspinall, G. O. ed.), Academic Press, New York, pp. 287–363.

12

Mutagenesis of *H. influenzae*

Mark A. Herbert

1. Introduction

With the advent of new techniques to explore bacterial pathogenesis, such as signature tagged mutagenesis and site-directed mutagenesis coupled with the whole genome sequence and bioinformatics, there is a need for clear descriptions of the available mutagenesis tools for individual pathogens. Here we describe those that have been applied to *Haemophilus influenzae* and discuss their advantages and limitations.

Insertional mutagenesis can be performed by nontransposon or transposon methods. Transposons are mobile genetic elements that can integrate into genomic sites independent of homologous recombination; they can generate stable mutations by insertional activation of genes. Wild-type transposons carry genes necessary for maintenance and transposition, and often encode resistance to antibiotics. The first transposon was discovered in 1971, and since then many have been identified and applied to bacterial mutagenesis ***(1)***. Transposition can occur in vivo, (it happens in the pathogen being studied), or it can occur in vitro, (it happens ex vivo). In the later case, purified transposases are provided together with the transposon and target DNA and transposition occurs outside of the living cell; the mutated DNA is then transformed into the organism and integrates by homologous recombination. Between these two poles lies shuttle mutagenesis, in which in vivo transposition of cloned DNA occurs in *Escherichia coli* and the DNA is then transformed back into the pathogen. The advantages and disadvantages of each system for *H. influenzae* are as follows:

1. In vivo mutagenesis is potentially most simple, requiring no manipulation of the host DNA before transposition, but relies on the transposon being functional in *H. influenzae*.

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2. In vitro mutagenesis is theoretically simple. A major advantage is that the transposon does not have to be active in *H. influenzae*. However, it is a new technique, only recently applied to Tn7 mutagenesis of *H. influenzae* ***(2,3)*** and Tn10 mutagenesis of *Neisseria*. The purity of the transposases, their concentrations and ratios to each other, and the transposition environment including the concentration of Mg^{2+}, all need to be carefully defined and standardized. Variation may influence target site specificity and randomness of insertion. *Haemophilus* DNA exhibits transformation bias, so that some fragments are taken up preferentially, presumably reflecting the abundance of uptake signal sequences (USS) in the fragment ***(4)***. Transformation bias, has been reported in *Neisseria meningitidis* and prevented the application of shuttle mutagenesis for signature tagged mutagenesis ***(5)***.
3. Shuttle mutagenesis is necessarily laborious because the host chromosome must be randomly digested and cloned into an *E. coli* vector and then transformed to make a library. Potentially, some DNA fragments could be lethal within *E. coli* or at least difficult to clone. Characteristically, restriction enzymes fall into this category. Cloned DNA may be modified, by methylation for instance, so that it is not easily reintroduced back into *H. influenzae*. Mutagenized DNA from *E. coli* may then not represent a random selection of the *H. influenzae* chromosome and may be affected by transformation bias when reintroduced into *H. influenzae*.

Although in vitro transposition appears promising, our current bias is towards the use of in vivo transposition. Most transposons move at low frequency and insert into many different target sites, but no one transposon behaves in a completely random fashion. For techniques such as STM, a completely random-acting, high-efficiency transposon is not required. All that is necessary is that the transposon can generate several hundred diverse mutations. Transposon mutagenesis therefore aims not to achieve saturation of all the genes in a genome and identification of every gene with an influence on virulence, but merely to identify function by screening 'random' mutants for a particular phenotype.

2. Nontransposon Mutagenesis

A chromosomal loop mutagenesis technique has been utilized by a number of *Haemophilus* investigators ***(6–9)***. Chromosomal DNA is partially digested with a high-frequency cutting enzyme, such as *Ssp*I, size-fractionated, and ligated into loops. The loops are digested with a different enzyme to linearize the DNA and an antibiotic resistance cassette is ligated in to re-create the loop. After relinearizing with the high-frequency cutting enzyme, the constructs are transformed into *H. influenzae* to create mutants by selecting onto antibiotic-containing media those that have undergone gene replacement by homologous recombination. Rearrangements, deletions, and single crossover events all may potentially occur, and the technique may be limited by transformation bias. A number of new transformation genes were identified by screening chromo-

somal loop generated mutants for transformation deficiency ***(9)***: 8000 mutants were screened and 25 transformation deficient mutants were identified. However, these contained insertions in only four regions, and only one was part of a previously identified competence operon.

Restriction enzyme-mediated integration (REMI) has been utilized for insertional mutagenesis in eukaryotic organisms and could potentially be tried in *H. influenzae*. However, no experience exists with this technique in bacteria ***(10,11)***.

3. Transposon Mutagenesis of *H. influenzae*

Transposons have become increasingly important in molecular bacteriology in part because of techniques such as STM and the Mariner strategy ***(12,13)***. Prior to the inception of these techniques, only one transposon had been described for in vivo transposition in *H. influenzae*, Tn*916*, and a few transposons had been used in shuttle mutagenesis, Tn*10* and Tn*phoA*, for instance. Recently, four other transposons have been applied to mutagenesis, Tn*1545*-Δ3 and Tn*10* in in vivo mutagenesis, and Tn7 and Mariner in in vitro mutagenesis ***(2,13,14)***. Tn10 has the advantage that it is an in vivo-acting transposon, whereas Tn7 and the Mariner transposon move in vitro and have to be introduced back into *H. influenzae* with the potential for transformation bias. Following is a discussion of the development of transposon use in *H. influenzae* and the merits of the various elements.

3.1. Tn5

Tn5 is composed of an antibiotic resistance gene, usually *aphA*II conferring resistance to kanamycin and neomycin ***(15)***, and this is flanked by two IS50 elements containing inverted repeats, each of approx 1.5 kb ***(16,17)***. Two proteins are translated from IS50R, a transposase (Tnp) and a transposase inhibitor (Inh). IS50R and IS50L differ by a single base-pair substitution producing a stop codon in IS50L, hence the IS50L-encoded proteins are inactive C-terminal truncated versions of Tnp and Inh. The base change in the IS50L does, however, create a strongly active promoter sequence that enables *aph*AII to be expressed. Upon entering a new host bacterium, Tn5 transposes at high frequency as a result of the action of the Tnp, but stability is conferred by Inh once the element has integrated ***(16,18,19)***. Numerous mini-Tn5 variants have been constructed, making this transposon a versatile molecular tool ***(20–23)*** with the broadest known host range ***(16,19)***, but unfortunately this does not appear to include members of the *Pasteurellaceae*, such as *H. influenzae*.

Tnp and Inh recognize 19 bp motifs that define the ends of IS50L and IS50R. Each end of Tn5 has an outside (O) and an inside (I) motif, and transposition of Tn5 occurs as an OO event. Transposition of either IS50 may also occur less frequently and is an OI event.

Deich attempted to introduce Tn5 into *H. influenzae* using several suicide delivery systems, which failed, and resorted finally to Tn5 shuttle mutagenesis for the purpose ***(24)***. Tn5 conjugation from *E. coli* s17-1 into *Actinobacillus pleuropneumoniae* on the suicide plasmid pJM703.1 has also previously failed ***(25)***, and conjugation of Tn5 into *A. calcoaceticus* on the plasmid pSUP:Tn5-B20 resulted in plasmid integration into the chromosome rather than transposition ***(26)***. Because we know that *E. coli* s17-1 is a viable conjugation method for other transposons and that the Tn5 antibiotic resistance genes are functional in *H. influenzae* ***(24)***, these findings imply that Tn5 transposition does not happen in this host. Non-expression of the kanamycin resistance gene of Tn5 has been reported in *A. pleuropneumoniae* ***(27)***, implying that expression may also be temperamental in *H. influenzae*. Tascon argues that the lack of transposition in *A. pleuropneumoniae* is due to a combination of low conjugation efficiency from *E. coli* s17-1 (i.e., 10^{-4}) combined with a low Tn5 transposition rate (i.e., 10^{-5} to 10^{-6}) ***(25)***. The low transposition rate is likely to be due to the wild-type Tnp having suboptimal activity. Tn10-based systems (*see* Chapter 13) have the advantage of a strong promoter under IPTG-inducible control to enhance the transposition frequency, without which Tn10 transposition does not occur.

An in vitro Tn5 transposition system has been described for *E. coli*, but not yet applied to *H. influenzae* ***(28)***. It has the advantage over Mu and Tn7 in vitro systems of utilizing only one Tnp protein. Using a variant Tnp that has increased transposition potential compared with the wild-type and that preferentially performs OO events enhances transposition. Inh is also blocked and therefore further enhances the transposition rate.

3.2. Tn7

Tn7 is a 14 kb transposon. Its attractive features are its broad host range and distinctive array of antibiotic resistance cassettes, conferring resistance to trimethoprim and aminoglycosides. The *sat* gene also produces a transacetylase that inactivates streptothricin, and the *aadA* gene produces adenylytransferase that inactivates streptomycin and spectinomycin. Tn7 transposition has been thoroughly reviewed by Craig ***(29,30)***. Tn7 with a gain of function mutation has recently been used for in vitro mutagenesis of *H. influenzae* ***(2,3)***.

Tn7 encodes five transposase genes, *tns*A–E, antibiotic resistance genes, and a recombinase of the lambda family that allows recombination of antibiotic cassettes. It moves by a cut-and-paste mechanism, excising from the donor molecule after making double-strand cuts with 3-bp overhangs. The transposon intermediate is linear, and integration into the chromosome occurs by one-step transesterification. The 3 bp overhang of the transposon is lost and the target DNA is repaired by host mismatch-repair mechanisms. The Tn7 proteins have homology to Mu, retroviral, and retrotransposon integrases and form a nucle-

oprotein complex around Tn7. TnsB recognizes direct repeats at the end of Tn7, four contiguous repeats at Tn7R, and three separated repeats at Tn7L. TnsA and B execute DNA breakage and repair of the donor molecule, TnsA cuts the 5' strand of Tn7 and TnsB cuts the 3' strand. TnsC mediates communication between the transposon ends and the target DNA and either TnsD or TnsE identify the target site, which is 25–60 bp away from the actual insertion site. Tn7 has two transposition pathways. In *E. coli*, the first pathway, requiring TnsABCD, produces transposition to a single highly preferred site in the chromosome, *att*Tn7. Whereas the second pathway, mediated by TnsABCE, has low target specificity. A speculative explanation for this duplicity is that the first pathway allows the transposon to enter and harbor in a safe haven within the chromosome, where it has no deleterious effect on the host. The second pathway allows the transposon to move away from the attTn7 site, for instance, to co-resident plasmid, so that it can be mobilized to other organisms. Both putative transposition attributes have survival advantage for the mobile element.

An in vitro Tn7 system has been developed with tnsABCD, but not yet with tnsABCE. However, a gain-of-function mutation of *tns*C means that TnsABC(gfm) can effect transposition without target site specificity and without the need for TnsD or E ***(31)***. An in vitro Tn7 system has been successful in identifying transformation genes in *H. influenzae* ***(2,3)***. Purified TnsA, TnsB, and TnsC(gfm) were mixed with a 6.1 kb donor plasmid, carrying a 1.6 kb mini-Tn7 element with a kanamycin-resistance gene, and less than a microgram of *H. influenzae* genomic DNA. The 5 bp gaps on one strand of the mutated DNA had to be filled in with Klenow and T4 ligase enzymes ***(32)***.

Tn7 contains transposase genes, but no conjugation/mobilization genes. To move between species, Tn7 is usually introduced on the back of a conjugative plasmid. No introduction of Tn7 into *H. influenzae* is required during in vitro mutagenesis, except that once mutagenesis has occurred in vitro, the DNA has to be transformed into *H. influenzae*. Although Tn7 in vitro has little insertional specificity ***(32)***, transformation bias may lead to preferential uptake of certain fragments and limit the number of attainable mutants.

Tn7 is partly autoregulated. The genes *tns*AB are co-expressed from a promotor that is placed 100 bp in from Tn7R; *tns*B represses transcription from this promoter ***(33)***. The mechanism of Tn7 activation has not been fully elucidated, but *att*Tn7 in *E. coli* is downstream of a cell-wall metabolism gene, *glm*S, and lies within that gene's transcriptional terminator. Potentially, then, activation and transposition may be coupled with cell division or growth. In common with Tn3 ***(34)*** and Mu ***(35)***, Tn7 exhibits transposition immunity. This is probably brought about after Tn7 has integrated, by high local concentrations of TnsB displacing TnsC from the target site ***(33)***. The TnsC of a second Tn7 transposon attempting to integrate in the locale of an established

Table 1
Alternative Random Mutagenesis Methods

A. Transposon Mutagenesis of Other Pasturellaceae Family Members

Transposon	Phenotypes	Species	References
Tn*916*	Deficient in capsule and prophage transduction	*Actinobacillus pleuropneumoniae* and *A. actinomycetemcomitans*	***25***
miniTn10 conjugation	Auxotrophs and non-hemolytic mutants	*A. pleuropneumoniae*	***25***
Tn10 conjugation	Auxotrophs isolated	*Pasteurella multocida*	*See* text

B. Non-transposon Random Mutagenesis of *H. influenzae*

Method	Phenotypes	References
Antibiotic resistance cassette ligated in vitro to random chromosomal fragments	Deficient in transferrin and hemoglobin binding, and in transformation; Multiple other phenotypes possible	***5–9***

transposon will be unable to find an attTn7 site that is not blocked by TnsB. Immunity occurs over at least 150 kb in the *E. coli* chromosome. Mini-Tn7 insertions introduced by the in vitro system will be stable by virtue of the absence of *tns* genes in the inserted element.

3.3. Tn10

Wild-type Tn10 is a 9.3 kb transposon conferring tetracycline resistance and whose ends are inverted repeats of IS10. Tn10 derivatives have been successfully used for mutagenesis of the *Pasteurellaceae* (*see* **Table 1**) and remnants of the transposon are found in natural isolates of *H. parainfluenzae* ***(36)***. Tn10 derivatives have been applied to shuttle and in vivo mutagenesis of *H. influenzae* ***(14,37–40)***. A summary of the Tn10 derivatives available, their applications, and delivery vehicles, is given by Kleckner ***(41)***, in a review that builds on others ***(18,42)***.

3.3.1. Structure

The left and right ends of Tn10 are IS10 elements of 1.4 kb in size. IS10R provides a functional transposase encoded by a long open reading frame that extends the entire length of IS10R. The transposase acts at the termini of Tn10, whereas IS10L is a degenerate form of IS10R. The minimal size of the Tn10 termini recognized by the transposase is just 2bp ***(42,43)***. Mini-transposons have been constructed with the 27 bp repeats facing inward and flanking an antibiotic-resistance cassette. The IS10R transposase is placed outside of the inverted repeats, under the control of the powerful, IPTG-inducible Ptac promoter. Tn10 derivatives may confer resistance to kanamycin, chloramphenicol, or ampicillin ***(41,44)***.

Reidl developed a series of three mini-Tn10 constructs delivered on adapted pACYC vectors. Plasmid pJRP207, previously used for Tn10 in vivo mutagenesis of *Vibrio cholerae*, was the source of the transposase and of Tn10*d-bla* ***(45)***, both originally derived from pZT344 ***(44)***. Tn10*d-bla* was amplified from truncated *blaM* by PCR and modified to contain flanking, inward-pointing IS10 ***(45)***. Plasmids pACYC184 and 177 ***(46,47)*** transform into *H. influenzae* at very low frequency because of the lack of USS-like elements, but when either plasmid contains *H. influenzae* cloned DNA, transformation is possible. Plasmid pAK*bla* was developed by cloning a 3.7 kb EcoRI fragment of pJRP207, containing Tn10d-*bla* and the transposase, into the *H. influenzae hel* gene cloned into the BamHI site of pACYC184, (designated pJRP4) ***(48)***. Tn10*bla* was constructed without a leader sequence in *bla* and so was unable to confer ampicillin resistance unless it fused with a surface-expressed protein.

The 900 bp *hel* gene contains one USS and confers transformability to pACYC184. A potential concern when delivering a transposon on a vector that

has a region of homology to the *H. influenzae* chromosome is that transposon integration may occur through homologous recombination rather than transposition. The gene *hel* is essential for growth under aerobic but not anaerobic conditions ***(48)***; thus, if transformation conditions are aerobic, homologous recombination cannot occur without conferring a lethal mutation.

Two larger constructs were designed in a similar fashion from pACYC177 with a 320 bp deletion in blaM, making it kan^R, amp^S. A 4.6 kb fragment of pAK*blaM* containing Tn10d-*bla* and *hel* was introduced into the adapted pACYC177 and then *cat* (1.1 kb) or *lacZ-cat* (4.3 kb) were cloned in, disrupting *bla*, to give pAK*cat* and pAK*lacZcat*. The series of constructs permitted insertional mutagenesis (pAK*bla*), and transcriptional (pAK*lacZcat*) and translational (pAK*bla*) studies of the genes into which the transposon had inserted. The individual transposons can be used individually, or *cat* can be replaced by *bla* or *lacZ-cat* by homologous recombination once an interesting mutant has been identified, to study translation and transcription secondarily. *LacZ* is promoterless and so cannot confer its chromogenic properties unless it is under the promoter control of the gene of interest ***(14)***.

Langford has developed pLOFkm to contain signature tags that were not preselected in order to perform STM in *A. pleuropneumoniae* (Langford, unpublished). As *A. pleuropneumoniae* is a member of the *Pasteurellaceae* family, the potential exists for pLOFkm to be transferable to into *H. influenzae* and donate its tagged-Tn10km. The pLOFkm contains mini-Tn10km derived from phage λ1105 ***(42)***, with the Tn10 kanamycin-resistance cassette flanked by 70 bp of the Tn10 ends containing inverted repeats recognized by the transposase ***(21,43)***.

3.3.2. Transposition

Insertions of Tn10 are preferentially into target sites consisting of 9 bp direct repeats of NGCTNAGCN ***(41)***. Transposition frequencies of the transposon constructs are 100–1000 times higher than the wild-type Tn10, and they confer a reduced frequency of transposon-promoted rearrangements. Transposition is induced by the presence of IPTG in the expression medium, during transformation of the pAK constructs, or in the conjugation agar for pLOFkm. Using USS-PCR to identify insertion sites, Reidl demonstrated that his pAK transposons gave rise to single chromosomal insertions in *H. influenzae* Rd, without obvious 'hot-spotting' of the transposon to one or just a few sites ***(14)***.

3.3.3. Transfer

Due to the presence of an USS in the *hel* gene, pAK plasmids can be transformed into *H. influenzae* Rd, but the transformation efficiency declines with increasing size of the construct and all are transformed with low efficiency into the pathogenic *H. influenzae* strain Eagan.

The pLOF vector has the RP4 oriT, thus pLOFkm can therefore be conjugally transferred from *E. coli* s17-1, as has been done in *A. pleuropneumoniae* ***(25)***.

3.3.4. Stability

The Tn10 transposase acts preferentially on nearby target sites ***(49)***, indeed it may even bind its target site before it has completely finished being translated. For the derivatives of Tn10 used in this work, the transposase is located outside the minitransposon, thus chromosomal insertions are conferred stability, provided Tn10 transposase is not provided from some nearby alternative source ***(21,50)***.

Vectors pACYC 177/184 are generally considered not to replicate in bacteria other than those of enteric origin ***(51)***, however the experiences of J. Reidl and D. W. Hood (personal communications) are that they do persist in *H. influenzae* and are not therefore suicide plasmids. The persistence of the pAK plasmids after transposition of Tn10 in *H. influenzae* mutants raises concern about instability, but this has not presented as a problem during subculture mutants deficient in iron uptake studied by Reidl (Reidl, unpublished).

The pLOF vector was developed specifically as a transposon delivery system ***(50)*** and has the advantage over pACYC-based vectors that its replication is dependent on the R6K-specified π protein of the *pir* gene ***(52,53)***, and it is therefore a suicide plasmid in *H. influenzae*. Theoretically, pLOFkm, of all the transposon constructs discussed in this work, is likely to result in the most stable chromosomal inserts.

3.4 . Transposons That Insert Preferentially into Genes Whose Products Are Surface Expressed or Secreted Proteins: Tn phoA *and Tn10* bla

A derivative of Tn5, Tn*phoA* ***(54)***, consists of the *phoA* gene of *E. coli*, lacking its signal sequence, inserted into Tn5. Where the transposon inserts into a gene, a hybrid protein may be generated that is composed of alkaline phosphatase fused to the amino-terminal sequence of the protein it is disrupting. Alkaline phosphatase activity can be detected by the highly sensitive chromogenic marker 5-bromo-4-chloro-3-indolyl phosphate. By random insertion of Tn*phoA* into the chromosome it is possible to identify new genes encoding transmembrane, periplasmic, and secreted proteins. Tn*phoA* in vivo mutagenesis has not been possible in *H. influenzae*, however, Tn*phoA* was used to study the function of genes of the capsulation chassis of Hib by *E. coli* shuttle mutagenesis ***(55, 56)***. The study indicated that the *bex*A-C encoded an ATP-driven polysaccharide export apparatus.

Tn*blaM* was an advance on Tn*phoA* ***(57)***. It is also based on Tn5, but has a signal sequence-deficient *blaM* in place of *phoA*, and has the advantage that

mutants can be isolated directly rather than via a phenotypic selection step. Use of Tn*blaM* has not been reported in *H. influenzae*. Though Tn10*bla*, based on Tn10 rather than Tn5 (*see* pAK*bla* above), was able to identify surface expressed proteins in *H. influenzae* ***(14)***.

3.5. *Tn*916 *and Tn*1545

Tn*916* and Tn*1545* belong to a family of conjugative transposons that are characterized by their ability to promote intercellular self-transfer when the donor is a Gram-positive bacterium ***(58–60)***. Conjugative transposons are promiscuous and able to transpose to a wide variety of species and genera ***(61,62)***, including *Haemophilus* spp. and *Actinobacillus* spp. ***(60,63–68)***. Recent reviews of conjugative transposons include those by Scott ***(61,69,70)***, Clewell ***(62,68,71)***, Salyers ***(72)*** and Rice ***(60)***. Tn*916* and Tn*1545* differ in their sizes, 18 kb and 25.2 kb, respectively, and in their antibiotic resistance determinants, tetracycline-minocycline (*tet*M) in Tn*916* and *tet*M, erythromycin (*erm*AM) and kanamycin (*aphA3*) in Tn*1545* ***(60,68,73,74)***. Movement and transposition has been best described for Tn*916*, but is likely to be identical or highly similar for Tn*1545*. Tn*1545*-Δ3 is a derivative of Tn*1545* that retains transposition but not conjugation properties.

3.5.1. Structure

The left end of Tn1545 is equivalent to the right end of Tn*916* and Tn*1545*-Δ3. The entire sequence of Tn*916*, encoding 24 genes, is known; from left to right are the transposase genes, *int* and *xis*, *tet*(M), genes necessary for conjugal transfer, nic sites identical to those of the IncP, Q, and R plasmids, and a putative *ori*T ***(75–78)***. Each end contains an inverted repeat (IR) and three direct repeats (DR-1 to DR-3) ***(79,80)***. Tn*1545*-Δ3 has a similar structure to Tn*916* with *int* and *xis* on the left, *aphA3* on the right, and the termini are identical to Tn*916*.

3.5.2. Transposition

The integration–excision system of conjugative transposons is similar to that of lambdoid phages ***(81,82)***; they transpose by a 'cut and paste' mechanism ***(68)***. The transposase genes, *int* and *xis*, encode Int and Xis ***(83)***, which have homology to the integrase and excisase of λ-phage and phage P22, respectively ***(80)***. Int cleaves each end of the transposon, leaving 6-bp overhangs called 'coupling sequences' ***(61)***. The nonmatching ends are covalently ligated together to form a heteroduplex, making a circular intermediate ***(84–86)***. Staggered cleavage of the target DNA permits insertion of the transposon ***(85)***, and disparities between the transposon ends and the target ends are resolved by mismatch repair.

The carboxy terminus of Int, Int-C, forms a transient covalent link with the IR repeats of the transposon (designated att-C) and recognizes and attaches to the target DNA in the host (att-T); whereas the amino terminus of the transposase, Int-N, attaches to the DR-2 repeats (att-N) ***(81)***. Int-C is characteristic of the integrase family of site-specific recombinases and is responsible for strand cleavage and exchange ***(61, 87)***. Int has two conserved amino acid domains, with Arg and His in domain I, and Arg and Tyr in domain II ***(88)***. These four amino acids are the points of contact with DNA ***(89)***. Xis attaches to a site between att-N and att-C on the left, and proximal to both att-N and att-C on the right of the transposon ***(89, 90)***.

A nucleoprotein complex composed of four Int molecules and a Xis dimer bridges the transposon ends, aligning them in an antiparallel arrangement ***(61,90)***. Xis may facilitate the action of Int either through DNA bending or though Xis-Int protein–protein interactions adjusting Int into the correct conformation so that Int-N can bind one end and Int-C the other end of the transposon ***(62,90)***. A cluster of six potential integration host factor (IHF)-binding sites occurs at the left end of Tn*916*, and IHF is produced by both *E. coli* and *H. influenzae*, and may thus form part of the nucleoprotein complex ***(71)***.

3.5.3. Transfer

Conjugative transposons are not subject to host restriction during transfer and are able to move between highly divergent species with a similar efficiency as within a species. Transfer occurs between Gram-positive bacteria by self-promoted conjugation ***(61)***. The rate-limiting step is excision in the donor bacterium, followed by formation of the nonreplicating circular intermediate, transfer genes are expressed ***(91)*** and movement of a single strand of the transposon occurs to the recipient bacterium, bound by a nucleoprotein complex that includes Int ***(92)***. The nucleoprotein complex is involved in integration into the recipient chromosome ***(93)***. Conjugation occurs in nature between gram-positive bacteria and between Gram-positive and Gram-negative bacteria ***(60,94)***.

Much of the detail of the transfer process, however, is irrelevant to the use of conjugative transposons as genetic tools in *H. influenzae* because transfer between Gram-negative bacteria has not been demonstrated ***(61)***. To use Tn*1545*-Δ3 as a tool in *H. influenzae*, it must first be introduced into the host strain on a delivery vector (*see* **Subheading 3.8.1.**) ***(67)***. When Tn*916* on plasmid pAM120 is transformed into a new host, a 'zygotic induction' occurs and the transposon excises from the plasmid and integrates into the recipient chromosome ***(71,95)***. Tn*1545*-Δ3 on plasmid pMGC20 is transferred to *H. influenzae* from *E. coli* s17-1 by RP4 conjugation, and 'zygotic induction' is presumed to initiate transposition. The plasmid is religated, leaving it in almost

its' exact original state, except that the 6-bp target sequence may have been exchanged for one carried in by the transposon.

3.5.4. Stability

Little is known about the regulation of *int* and *xis*. They are probably not regulated by a repressor, in contrast to λ-phage, as there is no immunity to the introduction of a second conjugative transposon ***(68,96)***. Three hypotheses of regulatory mechanism have been propounded, each with a little supportive evidence.

3.5.4.1. Int^s/Int Competition

Transcription/translation of *int*, in vitro and in *E. coli*, produces the full length Int and at least three other protein products with varying N-terminal truncations, here collectively called Int_s ***(61,80,83)***. Ints should be able to bind att-C and att-T; regulation may occur through competition from Ints, either through binding to the transposon-DNA junctions and preventing attachment of Int, or through the combining of Ints with Int to make inactive nucleoprotein complexes. A similar mechanism regulates Tn5 transposition ***(97)***. The Ints/Int competition model would explain well the finding of relative stability once a conjugative transposon is integrated into a site and yet a newly entering transposon (carrying its own complement of fully functional Int) is able to integrate. Where a conjugative transposon is transferred on a vector to *H. influenzae*, the transposon perhaps has a limited time to form functional nucleoprotein complexes following zygotic induction, but these may quickly become nonfunctional complexes with the production of Int_s and lead to transposon stability.

3.5.4.2. Countertranscription

Once circular intermediates are formed, expression from multiple promoters that exist near the right end of Tn*916* may lead to transcription through the coupling sequence into the left end of the transposon. This could shut down the translation of *int* and *xis* by a countertranscript effect ***(62)***. The amount of circular intermediate produced may be proportional to the frequency of transposition initially, but relate to degree of stability subsequent to movement of the transposon ***(98,99)***. In Gram-negative bacteria, such as *E. coli*, the level of circular intermediate is much higher than in gram-positive donors, such as *E. faecalis*, so that it can be detected by polymerase chain reaction (PCR) and on occasion even visualized in a DNA extract on an ethidium bromide-stained agarose gel ***(84)***. The relative concentration of circular intermediate after transposition in *H. influenzae* is unknown, but a small extrachromosomal element detected in Southern analysis by a probe directed at the transposon and not by

a probe directed to the vector is presumed to be detectable amounts of the circular intermediate.

3.5.4.3. Deletion or Rearrangement

In *N. gonorrhoeae*, Tn*916* suffers deletions that make it nontransmissible, and in many pathogens, including some natural isolates of *H. influenzae*, there is evidence of the former presence of a conjugative transposon, the *tet*M gene ***(60,100)***. Deletions or rearrangements of conjugative transposons in chromosomal sites may be a mechanism conferring stability.

Transposon stability is seen in avirulent mutants in vitro as well as in vivo during pathogenicity testing in animals. The inability of a mutant to rescue a vital attribute for survival indicates that the transposon is stable even in the face of stresses that are so harsh that the mutant will die. This fact suggests that transposons that co-transfer their integrase genes, such as Tn*1545*-Δ3, are nonetheless potentially useful for signature tagged mutagenesis.

*3.6. Tn*1545-Δ*3*

For both Tn*916* and Tn*1545*, approx 2-kb at one end of the transposon (containing *int* and *xis*) and 250 bp at the other end are the minimal requirements for transposition ***(78,80,83)***. This knowledge has been utilized in the development of the minitransposon, Tn*1545*-Δ3; the smallest of four deletion derivatives able to excise in vivo ***(80)***.

In *H. influenzae* and other species, Tn*916* and Tn*1545* show insertion preference for sites that have a static bend conferred by an A-rich region separated by any 6 bp from a T-rich region, A(A/T)AAAAAnnnnnnTTTTTT ***(89,101,102)***. Tn*916* has reduced insertion fidelity, compared with λ-phage, with potential integration into many possible AT-rich sites degenerate from the above ideal sequence; so that in *Enterococcus faecalis* and *Listeria monocytogenes* Tn*1545* exhibits no preference for a particular target sequence ***(82,103,104)***. Homology between the coupling sequence and the target may have limited influence on the choice of integration site, but the AT richness of the DNA flanking the 6-bp target is the most important attribute ***(102)***. The targets are not random ***(61)***, and some are preferred, as has been shown for Tn*916* transposition in *H. influenzae* ***(67,105)***. Sequence deviation of the actual target from this best match influences transposition frequency by up to 10,000-fold ***(98)***. In two limited studies of transposon insertion in *H. influenzae*, Tn*916* exhibited preferential insertion into the most AT-rich DNA, the noncoding regions ***(105,106)***. As these contain the regulatory elements for genes, insertions can potentially switch off downstream genes.

Tn*916* and Tn*1545* have identical ends, and the transposase genes differ by only one nucleotide, so that where Tn*916* has a lysine, Tn*1545* has an arginine

(80,83). The substitution occurs at one of the DNA-binding domains of Int ***(87)*** and could potentially alter target site specificity in a manner similar to that seen between the transposases of wild-type Tn10 and its 'ats' mutant. Tn*916* and Tn*1545*-Δ3 integrate into the genome of *H. influenzae* and *H. ducreyi* predominantly once per chromosome (*see* **Table 2**).

3.7. Other Transposons (Tn917, mγδ, Tn3, Mariner, Tn10 In Vitro)

Despite the sequential nomenclature of Tn*916* and Tn*917*, Tn*917* ***(107)*** is of the Tn3 family and not a conjugative transposon. Its ability for in vivo transposition has not been ascertained in *H. influenzae*, it has been used predominantly for mutagenesis of *Staphylococcus aureus*, but the family of transposons is thought to be highly dispersed throughout Gram-positive and Gram-negative genera ***(108)*** and functions in *E. coli* ***(109)***. Tn*917* might thus have potential for mutagenesis in *H. influenzae*. Its potential advantages are that it has been tagged ***(110,111)*** and the tagged derivative of Tn*917* carries an oriR that functions in *E. coli* permitting direct cloning of flanking chromosomal sequences ***(110,112)***.

3.7.1. Transposon mγδ-1

Transposon mγδ-1 is a 1.8k b derivative of Tn1000, a 6 kb transposon of the Tn3 family. Tn1000 has been widely used to mutagenize plasmids ***(113)***, and mγδ-1 has been utilized in shuttle mutagenesis to make pilin-deficient mutants of *H. influenzae*. The transposition of mγδ-1 is complicated, requiring a first step in *E. coli* containing a plasmid supplying a recombinase and a second step necessitating extraction of the plasmid and transformation into a host supplying resolvase function, then release of the mutagenized *H. influenzae* DNA from its vector and transformation back into *H. influenzae*. Transposon mγδ-1 has a preference for AT-rich valleys in GC-rich DNA and is likely to have preferential insertions; also, the method is shuttle mutagenesis, raising again the concern about transformation bias.

3.7.2. Tn3

Tn3 has been used only for shuttle mutagenesis of *H. influenzae* ***(114–116)*** to identify LPS mutants.

3.7.3. Mariner Strategy

Mariners are a widespread set of animal and insect transposons; that their host range is so diverse is testimony to the transposase being all that is necessary for transposition and that a single purified transposase mediates transposition in vitro ***(117)***. Little is known about the mode of transposition of these elements, but some detail can be presumed from knowledge of their closest

relatives, the *Tc1* family. Tc1 transposases have a conserved catalytic domain that is also present in Tn7 and Tn10, and a linear transposition intermediate has been recognized. The transposase has low fidelity for any specific target site; when in vitro transposition is performed in the presence of Mg^{2+}, the only requirement is the sequence AT, and if Mn^{2+} is exchanged for Mg^{2+}, only half of the insertions are into this minimal target sequence. Genomic analysis and mapping by in vitro transposition, or GAMBIT, is a technique recently developed in *H. influenzae* of in vitro mariner mutagenesis ***(13)***. The authors used genome sequencing primers to produce 10-kb PCR products that were then individually mutagenized, so that DNA flanking the transposon could be amplified with one of the same *H. influenzae*-specific primers and a transposon-directed primer. In this way the GAMBIT strategy has identified putative essential genes in five genomic sections of *H. influenzae*.

3.8. Delivery Vectors

3.8.1. pMGC20

The *Neisseria-E. coli* shuttle vector, pMGC20, contains Tn*1545*-Δ3. Its origin is a fusion of two gonococcal plasmids, which was recognized as being able to shuttle between *Neisseria* spp. and *E. coli*. Tn*1545*-Δ3 on a fragment of *S. pneumoniae* chromosomal DNA was cloned into a variant of the shuttle vector ***(118)***. Essentially, the 2.6-Mdal *N. gonorrhoeae* cryptic plasmid, pJD1, which occurs in about 96% of isolates ***(119,120)*** was fused with a 4.4-Mdal *N. gonorrhoeae* β-lactamase plasmid, pFT1, to give a large chimeric plasmid ***(121)***. A derivative with minor rearrangements was transformed into *N. gonorrhoeae* and underwent a spontaneous deletion to almost half size, and was called pGC3 ***(122)***. Both the polylinker of pUC9 and linearized pGC3 were S1 nuclease digested and blunt-end ligated to give a β-lactamase plasmid containing a polylinker, called pLES2 ***(122)***. So that pLES2 could be used in pathogenic bacteria, a 350-bp deletion in *bla* was filled in by insertion of the 1.2-kb *erm*AM gene of Tn*1545*, to give plasmid pMGC10 (renamed in this work as pHM0) ***(118)***. The 1.9-kb left end of Tn*1545* including *aph*AIII was cloned into pSC101 ***(123,124)***, and the 2.1-kb right extremity of Tn*1545* including *int* and *xis* and the terminus ***(80)*** was cloned into pUC1813 ***(125)***. Flanked by the pUC1813 polylinker, the right end of Tn*1545* was ligated with the left end of Tn*1545*, but in reverse orientation, to give Tn*1545*-Δ3 in pSC101. The right end of Tn*1545* became the left end of Tn*1545*-Δ3.

3.8.2. Suicide Vectors

Two suicide transposon delivery systems are applicable to *H. influenzae*, pAM120 and pLOFkm. pAM120 is transformed in with ease, despite no special effort to introduce USS into the vector sequence, and is unable to persist

Table 2
Transposon used for Mutagenesis of *Haemophilus* Species

Transposon and mode of delivery	Mutant phenotypes	Species	Reference
Tn*916* electroporation	Deficiencies in LPS biosynthesis, hemolysin production	*H. ducreyi*	See text
Tn*1545*-Δ3 conjugation	Deficient in LPS biosynthesis	*H. ducreyi*	See text
Tn*916* transformation	Deficiencies in transformation, transferrin binding, LPS biosynthesis; preferential insertion into intergenic regions	*H. influenzae*	***2,3,66,67,105,106***
Tn*1545*-Δ3 conjugation	Avirulent mutants	*H. influenzae*	Unpublished
Tn10 transformation	Deficiencies in outer membrane protein functions	*H. influenzae*	
Tn10km shuttle mutagenesis	Deficient in transformation	*H. influenzae*	***37,38***
miniTn10km phage delivery	Deficient in transformation	*H. influenzae*	***42***
Tn5 shuttle	Unsuccessful transposition mutagenesis	*H. influenzae*	***24***
Tn7 in vitro	Deficient in transformation	*H. influenzae*	***2,3***
Mariner	Deficient in essential genes for growth and viability	*H. influenzae*	***13***

Table 3
Vectors Used to Deliver Transposons to *H. influenzae*

Vector	Notes	Antibiotic resistance v = on vector t = on transposon	Transposon	References
pAM120	pGL101 (derivative of pBR322); a transformable suicide vector	Ampicillin (v) Tetracycline (t)	Tn*916*	***59–62***
pMGC20	Persists in *H. influenzae*	Erythromycin (v) Kanamycin (t)	Tn*1545*-Δ3	Unpublished
pAKbla	Based on pACYC184; persists in *H. influenzae*	Chloramphenicol (v) Ampicillin (t)	Tn10bla	***37,38***
pAKcat, pAKlacZcat	Based on pACYC177; persists in *H. influenzae*	Kanamycin (v) Chloramphenicol	Tn10cat, (t)Tn10lacZcat	***37,38***
pLOFkm	Conjugatable suicide vector	Ampicillin (v) Kanamycin (t)	Tn10km	***53***

once inside *H. influenzae*. pLOFkm carries a transposase adjacent to but outside the transposons; the plasmids can be mobilized by the RP4 conjugational machinery that is chromosomally located in *E. coli* s17-1, and it is only replicated in hosts producing the pi protein, courtesy of λ-phage, hence is a suicide vector in *H. influenzae*.

Other transposon delivery systems available for use with *H. influenzae* and described in this work are given in **Table 3**.

References

1. Joset, F. and Guespin-Michel, J. (1993) Prokaryotic genetics. in *Genome Organization, Transfer and Plasticity.* Blackwell Scientific Publications, Oxford, U.K.
2. Gwinn, M. L., Stellwagen, A. E., Craig, N. L., Tomb, J. F., and Smith, H.O. (1997) In vitro Tn7 mutagenesis of *Haemophilus influenzae* Rd and characterization of the role of atpA in transformation. *J. Bacteriol.* **179,** 7315–7320.
3. Gwinn, M. L., Ramanathan, R., Smith, H. O., and Tomb, J. F. (1998) A new transformation-deficient mutant of *Haemophilus influenzae* Rd with normal DNA uptake. *J. Bacteriol.* **180,** 746–748.
4. Goodgal, S. H. and Mitchell, M. A. (1990) Sequence and uptake specificity of cloned sonicated fragments of *Haemophilus influenzae* DNA. *J. Bacteriol.* **172,** 5924–5928.
5. Claus, H., Frosch, M., and Vogel, U. (1998) Identification of a hotspot for transformation of Neisseria meningitidis by shuttle mutagenesis using signature-tagged transposons. *Mol. Gen. Genet.* **259,** 363–371.
6. Mehr, I. J. and Seifert, H. S. (1997) Random shuttle mutagenesis: gonococcal mutants deficient in pilin antigenic variation. *Mol. Microbiol.* **23,** 1121–1131.
7. Whitby, P. W., Morton, D. J., and Stull, T. L. (1998) Construction of antibiotic resistance cassettes with multiple paired restriction sites for insertional mutagenesis of *Haemophilus influenzae*. *FEMS. Microbiol. Lett.* **158,** 57–60.
8. Sharetzsky, C., Edlind, T. D., Li Puma, J. J., and Stull, T. L. (1991) A novel approach to insertional mutagenesis of *Haemophilus influenzae*. *J. Bacteriol.* **173,** 1561–1564.
9. Dougherty, B. A. and Smith, H. O. (1999) Identification of *Haemophilus influenzae* Rd transformation genes using cassette mutagenesis. *Microbiol.* **145,** 401–409.
10. Schiestl, R. H. and Petes, T. D. (1991) Integration of DNA fragments by illegitimate recombination in Saccharomyces cerevisiae. *Proc. Natl. Acad. Sci. USA* **88,** 7585–7589.
11. Hensel, M. and Holden, D. W. (1996) Molecular genetic aproaches for the study of virulence in both pathogenic bacteria and fungi. *Microbiology* **142.** 1049–1058.
12. Hensel, M., Shea, J. E. , Gleeson, C., Jones, M. D., Dalton, E., and Holden, D. W. (1995) Simultaneous identification of bacterial virulence genes by negative selection. *Science* **269,** 400–403.
13. Akerley, B. J., Rubin, E. J., Camilli, A., Lampe, D. J., Robertson, H. M., and Mekalanos, J. J. (1998) Systematic identification of essential genes by in-vitro mariner mutagenesis. *Proc. Natl. Acad. Sci. USA* **95,** 8927–8932.

14. Kraiss, A., Schlor, S., and Reidl, J. (1998) In-vivo transposon mutagenesis of *Haemophilus influenzae*. *Appl. Environ. Microbiol.* **64,** 4697–4702.
15. Jorgensen, R. A., Rothstein, S. J., and Reznikoff, W. S. (1979) A restriction enzyme cleavage map of Tn5 and location of a region encoding neomycin resistance. *Mol. Gen. Genet.* **177,** 65–72.
16. Berg, D. E. (1989) Transposon Tn5. in *Mobile DNA.*, (Berg, D. E. and Howe, M. M., eds.) American Society for Microbiology,Washington, D C., pp. 185–210
17. Reznikoff, W. S. (1993) The Tn5 transposon. *Annu. Rev. Microbiol.* **47,** 945–963.
18. Berg, C. M. and Berg, D. E. (1987) Transposable element tools for microbial genetics, in *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology*, (Neidhardt , F. C., Ingraham, J. L., Magasanik, B., et al., eds.), American Society for Microbiology, Washington, D. C., p. 1071.
19. Berg, C. M., Berg, D. E. , and Groisman, E. A.(1989) Transposable elements and the genetic engineering of bacteria. in *Mobile DNA*, (Berg, D. E. and Howe, M. M., eds.), American Society for Microbiology, Washington, D. C., pp. 879–926.
20. Sasakawa, C. and Yoshikawa, M. (1987) A series of Tn5 variants with various drug-resistance markers and suicide vector for transposon mutagenesis. *Gene.* **56,** 283–288.
21. de Lorenzo, V. and Timmis, K. N. (1994) Analysis and construction of stable phenotypes in gram-negative bacteria with Tn5- and Tn10-derived minitransposons. *Methods Enzymol.* **235,** 386–405.
22. Simon, R., Quandt, J., and Klipp, W. (1989) New derivatives of transposon Tn5 suitable for mobilization of replicons, generation of operon fusions and induction of genes in gram- negative bacteria. *Gene* **80,** 161–169.
23. de Lorenzo, V., Herrero, M., Jakubzik, U., and Timmis, K. N. (1990) Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. *J. Bacteriol.* **172,** 6568–6572.
24. Deich, R. A. and Green, B. A. (1987) Mobilization of *Haemophilus influenzae* chromosomal markers by an *Escherichia coli* F' factor. *J. Bacteriol.* **169,** 1905–1910.
25. Tascon, R. I., Rodriguez-Ferri, E. F., Gutierrez-Martin, C. B., Rodriguez-Barbosa, I., Berche, P., and Vazquez-Boland, J. A. (1993) Transposon mutagenesis in *Actinobacillus pleuropneumoniae* with a Tn10 derivative. *J. Bacteriol.* **175,** 5717–5722.
26. Vosman, B., Kok, R., and Hellingwerf, K. J. (1991) Random insertional mutagenesis, in Acinetobacter, in *The Biology of Acinetobacter. Taxonomy, Clinical Importance, Molecular Biology, Physiology, Industrial Relevance*, (Towner, K. J., Bergegue-Berezin, E., and Fewson, C. A., eds.), Plenum Press, New York, pp. 183–189.
27. Wilson, P. J. (1990) *Haemophilus*, *Actinobacillus*, *Pasteurella*: mechanisms of resistance and antibiotic therapy. *Can. J. Vet. Res.* **54,** s73–s77.
28. Goryshin, I. Y. and Reznikoff, W. S. (1998) Tn5 in-vitro transposition. *J. Biol. Chem.* **273,** 7367–7374.
29. Craig, N. L. (1996) Transposon Tn7. *Curr. Top. Microbiol. Immunol.* **204,** 27–48.
30. Craig, N. L. (1991) Tn7: a target site-specific transposon. *Mol. Microbiol.* **5,** 2569–2573.

31. Stellwagen, A. E. and Craig, N. L. (1997) Gain-of-function mutations in TnsC, an ATP-dependent transposition protein that activates the bacterial transposon Tn7. *Genetics* **145,** 573–585.
32. Bainton, R. J. , Kubo, K. M. , Feng, J. N., and Craig, N.L. (19930 Tn7 transposition: target DNA recognition is mediated by multiple Tn7- encoded proteins in a purified in-vitro system. *Cell* **72,** 931–943.
33. Stellwagen, A. E. and Craig, N. L. (1997) Avoiding self: two Tn7-encoded proteins mediate target immunity in Tn7 transposition. *EMBO. J.* **16,** 6823–6834.
34. Lee, C. H., Bhagwat, A., and Heffron, F. (1983) Identification of a transposon Tn3 sequence required for transposition immunity. *Proc. Natl. Acad. Sci. USA* **80,** 6765–6769.
35. Mizuuchi, K. (1992) Transpositional recombination: mechanistic insights from studies of mu and other elements. *Annu. Rev. Biochem.* **61,** 1011–1051.
36. Levy, S. B., Buu-Hoi, A., and Marshall, B. (1984) Transposon Tn10-like tetracycline resistance determinants in Haemophilus parainfluenzae. *J. Bacteriol.* **160,** 87–94.
37. Chandler, M. S. (1992) The gene encoding cAMP receptor protein is required for competence development in *Haemophilus influenzae* Rd. *Proc. Natl. Acad. Sci. USA* **89,** 1626–1630.
38. Chandler, M. S. and Smith, R. A. (1996) Characterization of the *Haemophilus influenzae topA* locus: DNA topoisomerase I is required for genetic competence. *Gene* **169,** 25–31.
39. Tomb, J. F. , Barcak, G. J., Chandler, M. S., Redfield, R. J., and Smith, H.O. (1989) Transposon mutagenesis, characterization, and cloning of transformation genes of *Haemophilus influenzae* Rd. *J. Bacteriol.* **171,** 3796–3802.
40. Tomb, J. F., el-Hajj, H., and Smith, H. O. (1991) Nucleotide sequence of a cluster of genes involved in the transformation of *Haemophilus influenzae* Rd. *Gene* **104,** 1–10.
41. Kleckner, N. (1991) Uses of transposons with emphasis on Tn10. *Methods Enzymol.* **204,** 139–180.
42. Way, J. C. , Davis, M. A., Morisato, D., Roberts, D. E., and Kleckner, N. (1984) New Tn10 derivatives for transposon mutagenesis and for construction of lacZ operon fusions by transposition. *Gene* **32,** 369–379.
43. Way, J. C. and Kleckner, N. (1984) Essential sites at transposon Tn 10 termini. *Proc. Natl. Acad. Sci. USA* **81,** 3452–3456.
44. Elliott, T. and Roth, J. R. (1988) Characterization of Tn10d-Cam: a transposition-defective Tn10 specifying chloramphenicol resistance. *Mol. Gen. Genet.* **213,** 332–338.
45. Reidl, J. and Mekalanos, J. J. (1995) Characterization of *Vibrio cholerae* bacteriophage K139 and use of a novel mini-transposon to identify a phage-encoded virulence factor. *Mol. Microbiol.* **18,** 685–701.
46. Chang, A. C. Y. and Cohen, S. N. (1978) Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. *J. Bacteriol.* **134,** 1141–1156.
47. Rose, R. E. (1988) The nucleotide sequence of pACYC184. *Nucleic Acid Res.* **16,** 355–356.

48. Reidl, J. and Mekalanos, J. J. (1996) Lipoprotein e(P4) is essential for hemin uptake by *Haemophilus influenzae*. *J. Exp. Med.* **183,** 621–629.
49. Morisato, D., Way, J. C., Kim, H. J., and Kleckner, N. (1983) Tn10 transposase acts preferentially on nearby transposon ends in-vivo. *Cell* **32,** 799–807.
50. Herrero, M., de Lorenzo, V., and Timmis, K. N. (1990) Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in Gram-negative bacteria. *J. Bacteriol.* **172,** 6557–6567.
51. Simon, R., Priefer, U., and Puhler, A. (1983) A broad host range mobilization system for in-vivo genetic engineering: transposon mutagensis in Gram-negative bacteria. *BioTechnology* 784–791.
52. Metcalf, W. W., Jiang, W., Daniels, L. L., Kim, S. K., Haldimann, A., and Wanner, B. L. (1996) Conditionally replicative and conjugative plasmids carrying *lac*Z alpha for cloning, mutagenesis, and allele replacement in bacteria. *Plasmid* **35,** 1–13.
53. Miller, V. L. and Mekalanos, J. J. (1988) A novel suicide vector and its use in constructions of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in Vibrio cholerae requires toxR. *J. Bacteriol.* **170,** 2575–2583.
54. Manoil, C. and Beckwith, J. (1985) TnphoA: a transposon probe for protein export signals. *Proc. Natl. Acad. Sci. USA* **82,** 8129–8133.
55. Kroll, J. S., Loynds, B., Brophy, L. N., and Moxon, E. R. (1990) The *bex* locus in encapsulated *Haemophilus influenzae*: a chromosomal region involved in capsule polysaccharide export. *Mol. Microbiol.* **4,** 1853–1862.
56. Kroll, J. S. and Moxon, E. R. (1990b) Capsulation in distantly related strains of *Haemophilus influenzae* type b: genetic drift and gene transfer at the capsulation locus. *J. Bacteriol.* **172,** 1374–1379.
57. Tadayyon, M. and Broome-Smith, J. K. (1992) TnblaM: a transposon for directly tagging bacterial genes encoding cell envelope and secreted proteins. *Gene* **111,** 21–26.
58. Franke, A. E. and Clewell, D. B. (1981) Evidence for conjugal transfer of a *Streptococcus faecalis* transposon (Tn916) from a chromosomal site in the absence of plasmid DNA. *Cold Spring Harb. Symp. Quant. Biol.* **45(1),** 77–80.
59. Gawron-Burke, C. and Clewell, D. B. (1984) Regeneration of insertionally inactivated streptococcal DNA fragments after excision of transposon Tn916 in *Escherichia coli*: strategy for targeting and cloning of genes from Gram-positive bacteria. *J. Bacteriol.* **159,** 214–221.
60. Rice, L. B. (1998) Tn916 family conjugative transposons and dissemination of antimicrobial resistance determinants. *Antimicrob. Agents Chemother.* **42,** 1871–1877.
61. Scott, J. R. and Churchward, G. G. (1995). Conjugative transposition. *Annu. Rev. Microbiol.* **49,** 367–397.
62. Clewell, D. B., Flannagan, S. E., and Jaworski, D. D. (1995) Unconstrained bacterial promiscuity: the Tn916-Tn1545 family of conjugative transposons. *Trends Microbiol.* **3,** 229–236.
63. Yamaguchi, N., Kawasaki, M., Yamashita, Y., Nakashima, K., and Koga, T. (1995) Role of the capsular polysaccharide-like serotype-specific antigen in

resistance of Actinobacillus actinomycetemcomitans to phagocytosis by human polymorphonuclear leukocytes. *Infect. Immun.* **63,** 4589–4594.
64. Sato, S., Takamatsu, N., Okahashi, N., Matsunoshita, N., Inoue, M., Takehara, T., et al. (1992) Construction of mutants of Actinobacillus actinom cetemcomitans defective in serotype b-specific polysaccharide antigen by insertion of transposon Tn916. *J. Gen. Microbiol.* **138,** 1203–1209.
65. Willi, K., Sandmeier, H., Kulik, E. M., and Meyer, J. (1997) Transduction of antibiotic resistance markers among Actinobacillus actinomycetemcomitans strains by temperate bacteriophages Aa phi 23. *Cell. Mol. Life Sci.* **53,** 904–910.
66. Kauc, L. and Goodgal, S. H. (1989) Introduction of transposon Tn916 DNA into *Haemophilus influenzae* and *Haemophilus parainfluenzae*. *J. Bacteriol.* **171,** 6625–6628.
67. Holland, J., Towner, K. J., and Williams, P. (1992) Tn916 insertion mutagenesis in *Escherichia coli* and *Haemophilus influenzae* type b following conjugative transfer. *J. Gen. Microbiol.* **138,** 509–515.
68. Clewell, D. B., Flannagan, S. E., Zitzow, L. A., Su, Y. A., He, P., Senghas, E., et al. (1991) Properties of conjugative transposon Tn916, in *Genetics and Molecular Biology of Streptococci, Lactococci, and Enterococci*, (Dunny, G. M., Cleary, P. P., and McKay, L. L., eds.), American Society for Microbiology, Washington, D.C., pp. 39–44.
69. Scott, J. R. (1992) Sex and the single circle: conjugative transposition. *J. Bacteriol.* **174,** 6005–6010.
70. Scott, J. R. (1993) Conjugative transposons, in *Bacillus subtilis* and Other Gram-positive Bacteria, (Sonenshein, A. L., Hoch, J. A., and Losick, R., eds.), *Am. Soc. Microbiol.*, Washington, D.C., pp. 597–614.
71. Clewell, D. B. and Flannagan, S, E. (1993) The conjugative transposons of Gram-positive bacteria, in Bacterial Conjugation, (Clewell, D. B.,ed.), k: Plenum Press, New York, pp. 369–393.
72. Salyers, A. A., Shoemaker, N. B., Stevens, A. M., and Li, L. Y. (1995) Conjugative transposons: an unusual and diverse set of integrated gene transfer elements. *Microbiol. Rev.* **59,** 579–590.
73. Courvalin, P. and Carlier, C. (1987) Tn1545: a conjugative shuttle transposon. *Mol. Gen. Genet.* **206,** 259–264.
74. Courvalin, P. and Carlier, C. (1986) Transposable multiple antibiotic resistance in Streptococcus pneumoniae. *Mol. Gen. Genet.* **205,** 291–297.
75. Jaworski, D. D. and Clewell, D. B. (1995) A functional origin of transfer (*ori*T) on the conjugative transposon Tn916. *J. Bacteriol.* **177,** 6644–6651.
76. Jaworski, D. D., Flannagan, S. E., and Clewell, D. B. (1996) Analyses of traA, int-Tn, and xis-Tn mutations in the conjugative transposon Tn916 in *Enterococcus faecalis*. *Plasmid* **36,** 201–208.
77. Flannagan, S. E., Zitzow, L. A., Su , Y. A., and Clewell, D. B. (1994) Nucleotide sequence of the 18-kb conjugative transposon Tn916 from *Enterococcus faecalis*. *Plasmid* **32,** 350–354.
78. Senghas, E., Jones, J. M., Yamamoto, M., Gawron-Burke, C., and Clewell, D. B. (1988) Genetic organization of the bacterial conjugative transposon Tn916. *J. Bacteriol.* **170,** 245–249.

79. Clewell, D. B., Flannagan, S. E., Ike, Y., Jones, J. M., and Gawron-Burke, C. (1988) Sequence analysis of termini of conjugative transposon Tn916. *J. Bacteriol.* **170,** 3046–3052.
80. Poyart-Salmeron, C., Trieu-Cuot, P., Carlier, C., and Courvalin, P. (1989) Molecular characterization of two proteins involved in the excision of the conjugative transposon Tn1545: homologies with other site-specific recombinases. *EMB. J.* **8,** 2425–2433.
81. Lu, F. and Churchward, G. (1994) Conjugative transposition: Tn916 integrase contains two independent DNA binding domains that recognize different DNA sequences. *EMBO J.* **13,** 1541–1548.
82. Poyart-Salmeron, C,. Trieu-Cuot, P., Carlie,r C., and Courvalin, P. (1990) The integration-excision system of the conjugative transposon Tn 1545 is structurally and functionally related to those of lambdoid phages. *Mol. Microbiol.* **4,** 1513–1521.
83. Su, Y. A. and Clewell, D. B. (1993) Characterization of the left 4 kb of conjugative transposon Tn916: determinants involved in excision. *Plasmid.* **30,** 234–250.
84. Scott, J. R., Kirchman, P. A., and Caparon, M. G. (1988) An intermediate in transposition of the conjugative transposon Tn916. *Proc. Natl. Acad. Sci. USA* **85,** 4809–4813.
85. Caparon, M. G. and Scott, J. R. (1989) Excision and insertion of the conjugative transposon Tn916 involves a novel recombination mechanism. *Cell* **59,**1027–1034.
86. Marra, D. and Scott, J. R. (1999) Regulation of excision of the conjugative transposon Tn916. *Mol. Microbiol.* **31,** 609–621.
87. Taylor, K. L. and Churchward, G. (1997) Specific DNA cleavage mediated by the integrase of conjugative transposon Tn916. *J. Bacteriol.* **179,** 1117–1125.
88. Argos, P., Landy, A., Abremski, K,. Egan, J. B., Haggard-Ljungquist, E. , Hoess, R. H., et al. (1986) The integrase family of site-specific recombinases: regional similarities and global diversity. *EMBO. J.* **5,** 433–440.
89. Lu, F. and Churchward, G. (1995) Tn916 target DNA sequences bind the C-terminal domain of integrase protein with different affinities that correlate with transposon insertion frequency. *J. Bacteriol.* **177,** 1938–1946.
90. Rudy, C. K., Scott, J. R., and Churchward, G. (1997) DNA binding by the Xis protein of the conjugative transposon Tn916. *J. Bacteriol.* **179,** 2567–2572.
91. Celli, J. and Trieu-Cuot, P. (1998) Circularization of Tn916 is required for expression of the transposon-encoded transfer functions: characterization of long tetracycline-inducible transcripts reading through the attachment site. *Mol. Microbiol.* **28,** 103–117.
92. Bringel, F., Van Alstine, G. L., and Scott, J. R. (1992) Transfer of Tn916 between Lactococcus lactis subsp. lactis strains is nontranspositional: evidence for a chromosomal fertility function in strain MG1363. *J. Bacteriol.* **174,** 5840–5847.
93. Storrs, M. J., Poyart-Salmeron, C., Trieu-Cuot, P., and Courvalin, P. (1991) Conjugative transposition of Tn916 requires the excisive and integrative activities of the transposon-encoded integrase. *J. Bacteriol.* **173,** 4347–4352.
94. Bertram, J., Stratz, M., and Durre, P. (1991) Natural transfer of conjugative transposon Tn916 between Gram-positive and Gram-negative bacteria. *J. Bacteriol.* **173,** 443–448.
95. Gawron-Burke, C. and Clewell, D. B. (1982) A transposon in *Streptococcus faecalis* with fertility properties. *Nature* **300,** 281–284.

96. Norgren, M. and Scott, J. R. (1991) The presence of conjugative transposon Tn916 in the recipient strain does not impede transfer of a second copy of the element. *J. Bacteriol.* **173,** 319–324.
97. Johnson, R. C., Yin, J. C., and Reznikoff, W. S. (1982). Control of Tn5 transposition in *Escherichia coli* is mediated by protein from the right repeat. *Cell* **30,** 873–882.
98. Jaworski, D. D. and Clewell, D. B. (1994) Evidence that coupling sequences play a frequency-determining role in conjugative transposition of Tn916 in *Enterococcus faecalis*. *J. Bacteriol.* **176,** 3328–3335.
99. Manganelli, R., Romano, L., Ricci, S., Zazzi, M., and Pozzi, G. (1995) Dosage of Tn916 circular intermediates in *Enterococcus faecalis*. *Plasmid* **34,** 48–57.
100. Swartley, J. S., McAllister, C. F., Hajjeh, R. A., Heinrich, D. W., and Stephens, D. S. (1993) Deletions of Tn916-like transposons are implicated in tetM-mediated resistance in pathogenic *Neisseria*. *Mol. Microbiol.* **10,** 299–310.
101. Scott, J. R., Bringel, F., Marra, D., Van Alstine,G., and Rudy,C. K. (1994) Conjugative transposition of Tn916: preferred targets and evidence for conjugative transfer of a single strand and for a double-stranded circular intermediate. *Mol. Microbiol.* **11,** 1099–1108.
102. Trieu-Cuot, P., Poyart-Salmeron, C., Carlier, C., and Courvalin, P. (1993) Sequence requirements for target activity in site-specific recombination mediated by the Int protein of transposon Tn 1545. *Mol. Microbiol.* **8,** 179–185.
103. Trieu-Cuot, P., Carlier, C., Poyart-Salmeron, C., and Courvalin, P. (1991) An integrative vector exploiting the transposition properties of Tn1545 for insertional mutagenesis and cloning of genes from gram-positive bacteria. *Gene* **106,** 21–27.
104. Gaillard, J. L., Berche, P., Frehel, C., Gouin, E., and Cossart, P. (1991) Entry of *L. monocytogenes* into cells is mediated by internalin, a repeat protein reminiscent of surface antigens from Gram-positive cocci. *Cell* **65,** 1127–1141.
105. High, N. J., Deadman, M. E., Hood, D. W., and Moxon, E. R. (1996) The identification a novel gene required for lipopolysaccharide biosynthesis by *Haemophilus influenzae* RM7004, using transposon Tn916 mutagenesis. *FEMS Microbiol. Lett.* **145,** 325–331.
106. Nelson, K. E., Richardson, D. L., and Dougherty, B. A. (1997) Tn916 transposition in *Haemophilus influenzae* Rd: preferential insertion into noncoding DNA. *Microb. Comp. Genomics* **2,** 313–321.
107. Shaw, J. H. and Clewell, D. B. (1985) Complete nucleotide sequence of macrolide-lincosamide-streptogramin B- resistance transposon Tn917 in Streptococcus faecalis. *J. Bacteriol.* **164,** 782–796.
108. Perkins, J. B. and Youngman, P. J. (1984) A physical and functional analysis of Tn917, a Streptococcus transposon in the Tn3 family that functions in Bacillus. *Plasmid* **12,** 119–138.
109. Kuramitsu, H. K. and Casadaban, M. J. (1986) Transposition of the gram-positive transposon Tn917 in *Escherichia coli*. *J. Bacteriol.* **167,** 711,712.
110. Mei, J. M., Nourbakhsh, F., Ford, C. W., and Holden, D. W. (1997) Identification of *Staphylococcus aureus* virulence genes in a murine model of bacteraemia using signature-tagged mutagenesis. *Mol. Microbiol.* **26,** 399–407.

111. Schwan, W. R, Coulter, S. N, Ng, E. Y., Langhorne, M. H., Ritchie, H. D., Brody, L. L, et al. (1998) Identification and characterization of the PutP proline permease that contributes to in-vivo survival of *Staphylococcus aureus* in animal models. *Infect. Immun.* **66,** 567–572.
112. Foster, T. J. (1987) The use of mutants for defining the role of virulence factors in-vivo, in *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology*. (Neidhardt, F. C., Ingraham, J. L., Magasanik, B., et al., eds.), American Society for Microbiology, Washington, DC, pp. 173–191.
113. Berg, C. M., Vartak, N. B., Wang, G., Xu, X., Liu, L., MacNeil, D. J., et al. (1992) The μγδ-1 element, a small gd (Tn1000) derivative useful for plasmid mutagenesis, allele replacement and DNA sequencing. *Gene* **113,** 9–16.
114. McLaughlin, R., Spinola, S. M., and Apicella, M. A. (1992) Generation of lipooligosaccharide mutants of *Haemophilus influenzae* type b. *J. Bacteriol.* **174,** 6455–6459.
115. Abu Kwaik, Y., McLaughlin, R. E., Apicella, M. A.,and Spinola, S. M. (1992) Cloning and analysis of lipooligosaccharide synthesis genes of *Haemophilus influenzae* type b that assemble or expose a 2-keto-3- deoxyoctulosonic acid epitope in *Escherichia coli. J. Infect. Dis.* **165,** 195–196.
116. Abu Kwaik, Y., McLaughlin, R. E., Apicella, M. A., and Spinola, S. M. (1991) Analysis of *Haemophilus influenzae* type b lipooligosaccharide-synthesis genes that assemble or expose a 2-keto-3-deoxyoctulosonic acid epitope. *Mol. Microbiol.* **5,** 2475–2480.
117. Lampe, D. J., Churchill, M. E. A., and Robertson, H. M. (1996) A purified mariner transposase is sufficient to mediate transposition in-vitro. *EMBO J.* **15,** 5470–5479.
118. Nassif, X., Puaoi, D., and So ,M. (1991) Transposition of Tn1545-Δ 3 in the pathogenic *Neisseriae*: a genetic tool for mutagenesis. *J. Bacteriol.* **173,** 2147–2154.
119. Korch,C., Hagblom, P., Ohman, H., Goransson, M., and Normark, S. (1985) Cryptic plasmid of *Neisseria gonorrhoeae*: complete nucleotide sequence and genetic organization. *J. Bacteriol.* **163,** 430–438.
120. Sarandopoulos, S. and Davies, J. K. (1993) Genetic organization and evolution of the cryptic plasmid of *Neisseria gonorrhoeae*. *Plasmid* **29,** 206–221.
121. Stein, D. C., Young, F. E., Tenove, F. C., and Clark, V. L. (1983) Characterization of a chimeric beta-lactamase plasmid of *Neisseria gonorrhoeae* which can function in *Escherichia coli*. *Mol. Gen. Genet.* **189,** 79–84.
122. Stein, D. C., Silver, L. E., Clark, V. L., and Tenover, F. C. (1983) Construction and characterization of a new shuttle vector, pLES2, capable of functioning in *Escherichia coli* and *Neisseria gonorrhoeae*. *Gene* **25,** 241–247.
123. Caillaud, F., Trieu-Cuot, P., Carlier, C., and Courvalin, P. (1987) Nucleotide sequence of the kanamycin resistance determinant of the pneumococcal transposon Tn1545: evolutionary relationships and transcriptional analysis of aphA-3 genes. *Mol. Gen. Genet.* **207,** 509–513.
124. Trieu-Cuot, P. and Courvalin, P. (1983) Nucleotide sequence of the *Streptococcus faecalis* plasmid gene encoding the 3'5'-aminoglycoside phosphotransferase type III. *Gene* **23,** 331–341.
125. Kay, R. and McPherson, J. (1987) Hybrid pUC vectors for addition of new restriction enzyme sites to the ends of DNA fragments. *Nucleic Acids Res.* **15,** 2778.

13

Transposon Tn*10*

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1. Introduction

Classical transposons applicable to *Haemophilus influenzae* have had their limitations. Recently, however, advances have been made in the development of transposon systems and their applications, such as signature tagged mutagenesis, to identify in-vivo survival genes, and the GAMBIT strategy, to identify in-vitro essential genes. Over the last two decades, transposon mutagenesis has been developed mainly for *Escherichia coli*. During that time, several transposable gene fusion vector and phage systems were constructed, which targeted different aspects of gene expression, protein subcellular localization, and membrane protein topology ***(1)***. For example, operon and gene fusion systems were established to study transcriptional/translational regulation of target genes. These systems consist of "reporter" genes, such as *lacZ*, *blaM*, *phoA*, *gfp*, *luxA*, or *cat*, lacking either their own transcriptional or translational signals. The reporters are embedded within transposons in such a way that they form transcriptional or translational hybrids with the target gene. For example, systems such as Tn*phoA* ***(2)*** or Tn*bla* ***(3)*** carry the *phoA* or *blaM* reporter genes, encoding alkaline phosphatase or TEM1 β-lactamase, respectively, but lacking signal sequences. Both reporter genes express activity only after an insertion occurs in the reading frame of an expressed gene encoding for an exported or membrane associated protein. A potential advantage of the *blaM* reporter is that gene fusion events can be selected directly after transposition by demanding various levels of resistance to ampicillin or other β-lactam antibiotics.

Two perceived drawbacks of the early available transposable elements have been their large size and their ability independently to transpose more than once. In order to circumvent the problem of "unstable" transposon insertions, "defective minitransposons" were developed. Trans-complementable and defective

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Table 1
Bacterial Strains and Plasmids

Strain or plasmid	Genotype relevant characteristics	Source or reference
Strains		
E. coli XL1-blue	(F'::Tn*10 proA*$^+$*B*$^+$ *lacIq* Δ*(lacZ)M15/ recA1 endA1 gyrA96*(Nalr) *thi hsdR17* (rk$^-$mk$^+$) *supE44 relA1 lac*)	New England Biolabs
H. influenzae	Rd KW20	(A. Wright)
H. influenzae	Type b	Eagan
H. influenzae	*sxy-1*	***(12)***
Plasmids		
pACYC177	Kan^r, Ap^r	***(17)***
pACYC184	Cm^r, Tet^r	***(18)***
pZT344	Tn*10d*-Cam, Amp^r, Cm^r	***(5)***
pJRP4	pACYC184, Cm^r *hel*	***(9)***
pAKbla	pACYC184, Cm^r, Tn*10d-bla*	***(7)***
pAKcat	pACYC177, Kan^r, Cm^r Tn*10d-cat*	***(7)***
pAKlacZcat	pACYC177, Kan^r, Cm^r, Tn*10d-lacZcat*	***(7)***

mini transposable elements based on derivatives of Tn*10* were constructed, such as Tn*10d*-Kan ***(4)*** and Tn*10d*-Cam ***(5)***. However, none of these elements provided transcriptional or translational gene fusions, nor did they target the genes for exported proteins per se. Recently, a mini-Tn*10d* derivative combining the reporter gene and minitransposon concepts, termed Tn*10d-bla*, was developed ***(6)***. This element was used to target membrane or secreted gene products and the insertions remained stably integrated in the target gene due to the lack of a functional transposase.

*1.1. Mini-Tn*10d *Elements and Delivery System for* H. influenzae

*1.1.1. Design and Purpose of the Mini-Tn*10d *Constructs*

A series of mini-Tn*10d* elements was constructed and each tested for transposition activity in *H. influenzae* ***(7)*** (*see* **Note 5**). The mini-Tn*10d* system comprises defective minitransposon elements with different selection markers (*cat* and *amp*) and reporter genes (*lacZ, blaM*) (**Table 1, Fig. 1A**). For example, for the construction of Tn*10d-bla*, plasmid pZT344 served as platform, harboring the Tn*10* transposase under the control of the P_{tac} promoter ***(4,5).*** The P_{tac} promoter activity is inducible by isopropil-β-D-thiogalactopyramoside (IPTG) and

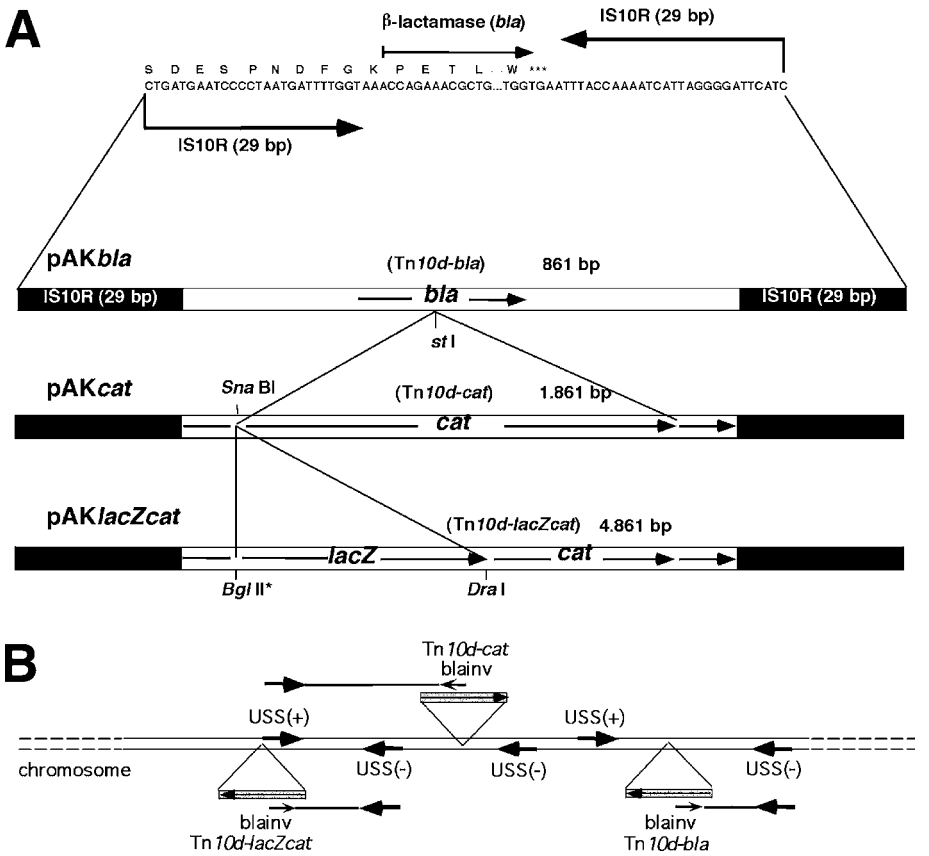

Fig. 1. Tn*10* constructs.(**A**) Tn*10d-bla*, Tn*10d-cat*, Tn*10d-lacZcat*. Based on Tn*10d-bla*, suitable transposons had been constructed and are encoded on pACYC184 or pACYC177 derivatives, respectively. Tn*10d-cat* was obtained by subcloning of a PCR-generated 1.1-kb *cat* gene encoding *PstI* fragment into the *PstI* opened Tn*10d-bla* element. The PCR *cat* fragment also contains the *cat* promoter. Tn*10d-lacZcat* was constructed based on Tn*10d-cat*. Promoterless *lacZ* fragment (blunt ended *Dra*I-*Bgl*II fragment) was obtained from plasmid pMD35 ***(16)***. This fragment was subsequently ligated into an unique *Sna*BI site of Tn*10d-cat*. (**B**) PCR fragment enrichment method. Shown is the generation of PCR fragments consisting of Tn*10d* insertions together with flanking chromosomal DNA due to the specificity of the USS(+), USS(−) and blainv oligonucleotides.

activates the expression of the transposase, which then mobilizes the *in cis* coding minitransposon. This transposition activation acts *in trans*, hence after transposition the transposase encoding gene is no longer associated with the minitransposon. Tn*10*-type transposition acts mainly in a nonreplicative mode

and the donor element normally vanishes ***(8)***. Based on Tn*10d-bla*, the following elements were constructed (**Fig. 1A**): Tn*10d-bla* (pAK*bla*), Tn*10d-cat* (pAK*cat*) and Tn*10d-lacZcat* (pAK*lacZcat*). These elements can be utilized for the following types of mutagenesis: (1) pAK*bla* for protein location, translational regulation, and membrane protein topology; (2) pAK*cat* for knockout phenotypes; and (3) pAK*lacZcat* for determining transcriptional regulation.

1.1.2. Transposon Delivery System

To establish Tn*10d* transposition in *H. influenzae*, it was necessary to deliver the elements on a plasmid able to propagate in *H. influenzae* (p15A origin), a plasmid that can easily be transformed into the organism by virtue of the presence of an uptake signal sequence (USS). The transposon delivery system of all the pAK plasmids is based on plasmid pJRP4 ***(9)***, which is either a pACYC184 or a pACYC177 derivative (**Table 1**). Plasmid pJRP4 contains the *hel* gene and a single USS site. This plasmid was also chosen, because the function of *hel* is essential under laboratory standard growth conditions ***(10)***, hence simple homologous recombination of transposon into the chromosomal *hel* would fail because the resulting mutants would be nonviable. It is desirable to lose the transposon donor immediately after transposition. In our system, we observed that during the initial transposition events, soon after transformation, donor plasmids are usually lost at high frequency. However, we recommend and describe below a procedure to ensure the removal of any persisting donor plasmids (*see* **Subheading 1.2.**).

1.2. Transformation Protocol and Generation of Mutant Pools

1.2.1. Generation of Transposon Mutant Banks in H. influenzae *Rd*

Competent cells have to be produced by standard protocol ***(11)***. Alternatively, use the constitutively competent strain Rd harboring the *sxy-1* mutation, as described by R. Redfield ***(12)***. Always prepare the pAK plasmids from *E. coli* strain XL1, because that strain contains $lacI^q$, which effectively represses the P_{tac} promotor, thereby reducing the likelihood of Tn*10d* transposition in the *E. coli* host. As no *lacI* control is present in *H. influenzae*, the P_{tac} promotor is not repressed, so after transformation, expression of the transposase and subsequent transposition of the elements will occur in *H. influenzae*. In order to get as many different insertions as possible, independent transformations must be performed, and 500–800 independent transformations are feasible utilizing 96-well microtiter plates. Transformation is achieved by simply adding transposon harboring plasmids (pAK) and competent cells to the wells. Such plates are then incubated at 37°C and aliquots taken and plated onto selective growth medium (brain heart infusion [BHI] agar medium) containing the appro-

priate antibiotic (e.g., chloramphenicol). After growth, the colonies are pooled and chromosomal DNA is prepared. This chromosomal DNA represents the pool DNA, which can then be used to back-transform freshly made competent *H. influenzae* or constitutively competent *H. influenzae sxy-1*. Individual screening schemes are designed and transformants screened for the desired phenotype.

1.2.2. Removal of Persisting Transposon Delivery Plasmids

After the transformation of the transposon harboring plasmids into *H. influenzae*, some plasmids will persist (e.g., by mutations of the transposase or IS elements). Removing the remaining plasmids, which may lead to instability of transposon insertions in the chromosomal DNA, can easily be achieved by digestion of the pool DNA with restriction endonuclease *Sma*I. This enzyme is a rare cutter in the *H. influenzae* chromosome with just 16 recognition sites; however, there is a single *Sma*I site in the kanamycin gene of the pAK plasmids. Therefore, *Sma*I digestion effectively destroys pAK plasmids (with the exception of pAK*bla*) and after retransformation of the *Sma*I-digested pool DNA into *H. influenzae,* only negligible amounts of plasmid persists.

*1.3. Determining Mini-Tn***10d** *Sites of Insertion by PCR Fragment Enrichment*

To allow rapid identification of the generated insertion sites, a fragment enrichment method was developed. Polymerase chain reaction (PCR) is used to amplify a junction fragment generated between the mini-Tn*10d* insertions and 5' flanking chromosomal regions. For this method, USS sites were utilized. These are randomly distributed across the chromosome (1465 copies) and contain a 9-bp core consensus sequence AAGTGCGGT ***(13)***. Since the USS sequences exist in two possible orientations (+ or −), it was necessary to synthesize two 16-mer hemirandom oligonucleotides containing the conserved 9 bp core sequence, USS(+): 5'-N_6AAAGTGCGGT-3', and USS(−): 5'-N7ACCGCACTT-3'. Another synthetic oligonucleotide, blainv (5'-CCGTAA GATGCTTTTCT GTGACTGGT-3') was designed, which specifically hybridizes with the complementary 5' oriented Tn*10d-bla*, Tn*10d-cat*, or Tn*10d-lacZcat* encoding DNA (**Fig. 1B**). The production of PCR fragments, consisting of an IS*10*-chromosomal junction fragment, was carried out using the amplification oligonucleotides in a PCR reaction with isolated transposon containing chromosomal DNAs as templates. PCR fragments ranging in sizes from 0.5 to 6 kbp can be obtained from insertions generated by Tn*10d-bla*, Tn*10d-cat*, and Tn*10d-lacZcat*. The PCR fragments are then sequenced to identify the transposon integration sites.

1.4. Identification of Transposon Insertion Sites

The insertion sites of the Tn*10d* transposon elements are determined by the dideoxy nucleotide chain termination method of Sanger et al. ***(14)***. Sequencing is performed by PCR cycling reaction according to Amersham Life Science. Sequence detection is by infrared dye-labeled primer (IRD800), monitoring by the automatic sequencing method of the LiCor-System (MWG). The sequencing primer used is an antiparallel 24-mer oligonucleotide IS*10*seq (5'-CAACT GATCTTCAGCATCTTTTAC-3') of the 5' end of the *blaM* gene, which can be used to detect insertions of Tn*10d-bla*, *-cat*, and *-lacZcat*.

1.5. Applications of Transposon Mutagenesis

1.5.1. Construction of Knockout Mutations

Knockout mutations produced by Tn*10d-cat* insertions can simply be obtained by antibiotic marker selection. Insertions into coding and noncoding regions have been observed ***(7)***. This kind of mutagenesis is suitable for the generation of broad designed phenotype selection, e.g., characterization of physiological properties such as aerobic vs anaerobic mutant selection, substrate uptake, or oxygen stress.

1.5.2. Tagging of Membrane Derived Proteins (Gene Fusion Systems)

The Tn*10d-bla* element can be used to detect genes encoding outer-membrane proteins (OMPs) of *H. influenzae* by insertional mutagenesis. To confer ampicillin resistance to the cell, Tn*10d-bla* has to produce protein fusions in such a way that the mature β-lactamase encoding part of the Tn*10d-bla* is inserted in the translated reading frame of the mature part of the outer-membrane protein. Tn*10d-bla* insertions are produced by transformation of *H. influenzae* with pAK*bla*. Transformants are first selected for chloramphenicol resistance and subsequently for ampicillin resistance. The localization of integration sites can be determined as described in **Subheading 3.2**. The outer-membrane protein (OMP) profile of wild-type and mutant strains can then be compared by SDS-PAGE and silver staining (**Fig. 2**) to identify mutations of major OMPs. Western blot analysis, using β-lactamase-specific antibodies directed against hybrid proteins, allows the detection of minor proteins.

1.5.3. Transcriptional Fusion System

In addition to knockout mutations, mutagenesis with the Tn*10d-lacZcat* element allows one to study the transcriptional regulation of the insertion mutants by applying β-galactosidase assays using *o*-nitrophenyl β-D-galactopyranoside (ONPG). LacZ-expressing cells can also be screened directly on agar medium

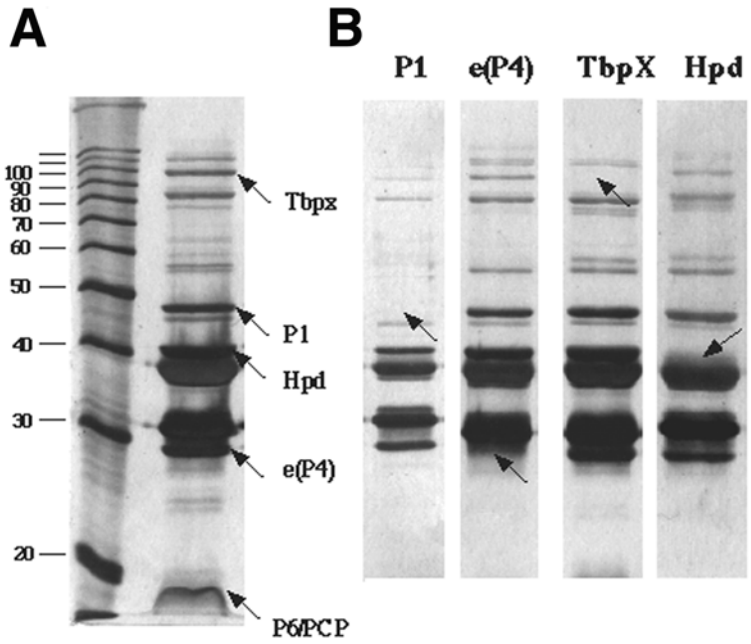

Fig. 2 Outer-membrane protein profiles. A typical outer membrane protein (OMP) profile is shown for *H. influenzae* strain Rd (**A**) and mutant strains (**B**) encoding P1::Tn*10d-cat*, e(P4)::Tn*10d-bla*, HI1217 (TbpX)::Tn*10d-bla*, Hpd::Tn*10d-bla*. A 12% SDS-PAGE was performed and the gels were subsequently stained with a silver staining procedure. The arrows indicate the positions of the identified major OMPs.

containing the chromogenic dye 5-bromo-4-chloro-3-indolyl beta-D-galacto-pyranoside (X-Gal).

1.5.4. *Exchange of Tn*10d-bla *Insertions with Tn*10d-cat *or Tn*10d-lacZcat *Sequences by Transformation and Recombination*

For the purpose of marker exchange or to investigate transcriptional regulation of *-bla* fusions, Tn*10d-bla* insertions can be exchanged with Tn*10d-cat* or Tn*10d-lacZcat* elements. As all transposons described here contain *blaM* sequences (**Fig. 1A**), it is possible to exchange Tn*10d-bla* insertions with Tn*10d-cat* or Tn*10d-lacZcat* via homologous recombination with linear transposon-encoding DNA fragments. PCR generated 1.7- or 4.8-kb DNA fragments of Tn*10d-cat* or Tn*10d-lacZcat* can be used to transform competent *H. influenzae* cells encoding a defined Tn*10d-bla* insertion. Chloramphenicol-resistant and ampicillin-sensitive transformants are then isolated and confirmed by PCR analysis to show that the Tn*10d-bla* insertions have been exchanged with Tn*10d-cat* or Tn*10d-lacZcat*.

2. Materials

2.1. Transformation of Competent H. influenzae with Mini-Tn10d

2.1.1. Preparation of Competent H. influenzae

1. Supplemented brain heart infusion broth (SBHI) i.e., 3.8% BHI (Difco) supplemented with NAD (10 µg/mL, Sigma) and hemin chloride (20 µg/mL, Sigma); M-IV medium (*see* standard protocol ***[11]***).

2.1.2. Transformation Scheme for Generation of a Mutant Bank

1. 150–200 µg miniTn*10d* carrying plasmids (pAK*bla*, pAK*cat*, or pAK*lacZcat*) *(see* **Note 1**) is sufficient for 500 independent transformations (about 200–300 ng per transformation).
2. 96-well microtiter plates.
3. 8-channel pipet, 5–50 µL.
4. 25 mL of M-IV-competent cells of *H. influenzae* strain Rd/Eagan.
5. Prepare sufficient BHI agar plates with chloramphenicol (Cm, 2 µg/mL), usually 24 agar plates per microtiter plate; use polystyrol Petri dishes of 145 mm diameter.
6. BHI medium (about 10 mL per plate).
7. Sterile 100% glycerol.
8. Pooled cell suspension for large-scale preparation of chromosomal DNA.
9. TE buffer (pH 8.0): 10 m*M* Tris-HCl, pH 8.0, and 1 m*M* EDTA, pH 8.0; autoclave and store at room temperature.
10. 1–30 µg chromosomal pool DNA.
11. Restriction endonuclease *Sma*I, 10X restriction buffer (usually supplied with the enzyme) and dH_20.
12. Sterile glass tubes (10 mL) with caps.
13. M-IV-competent *H. influenzae* Rd cells (250 µL per transformation).
14. BHI broth.
15. BHI agar plates (Petri dishes, 80 mm diameter) containing 6 µg/mL ampicillin (Amp) or 2 µg/mL Cm.
16. BHI agar plates with supplements for individually designed screening procedure.
17. BHI agar plates with Cm (2 µg/mL) for pAK*bla*, and with kanamycin (Kan, 10 µg/mL) for pAK*cat* and pAK*lacZcat*

2.2. Localization of Mini-Tn10d Integration Sites

2.2.1. Preparation of Chromosomal DNA

1. 2 mL overnight culture of *H. influenzae* mutant clone(s) in BHI.
2. TNE buffer: 10 m*M* Tris-HCl, pH 8.0, 10 m*M* NaCl, 10 m*M* EDTA, pH 8.0, autoclave and store at room temperature.
3. TNEX buffer: TNE buffer, plus 1% (v/v) Triton X-100 (Sigma); autoclave and store at room temperature.
4. Lysozyme (Sigma): 5 mg/mL in H_2O, freshly prepared.
5. Proteinase K (Sigma) 20 mg/mL in H_2O, store aliquots at −20°C.
6. 5 *M* NaCl (autoclave); absolute ethanol (100%), store at −20°C.

7. 70% ethanol; store at 4°C.
8. TE buffer, pH 8.0 (*see* **Subheading 2.1.2.**).

2.2.2. PCR Fragment Enrichment Method

1. Hemirandom primers (16-mer) containing uptake signal sequences USS(+): 5-N6AAAGTGCGGT-3 (100 pmol), USS(−): 5-N7ACCGCACTT-3 (100 pmol); primer blainv (26-mer): 5-CCGTAAGATGCTTTTCTGTGACTGGT-3 (100 pmol); dNTP-mix: 10 m*M* dATP, dCTP, dGTP, and dTTP each; 25 m*M* $MgCl_2$ (usually supplied with PCR polymerase); PCR polymerase (Taq- or proofreading polymerase); PCR polymerase buffer (also supplied with the enzyme); dH_2O.
2. PCR machine.
3. 0.7% agarose in 1X TAE buffer, running buffer: 1X TAE, staining solution 0.4-g/mL ethidium bromide.
4. UV transilluminator.

2.2.3. Purification of PCR Products

1. 0.7% agarose gel; 1X TAE buffer; staining solution: 0.4-g/mL ethidium bromide.
2. Scalpel.
3. DNA purification kit for recovering DNA from agarose (e.g., QIAquick gel extraction kit, Qiagen; Gene Clean kit, Bio 101, La Jolla, CA).

2.2.4. Sequencing of PCR Derived Products

1. IS*10*seq primer (24-mer): 5-CAACTGATCTTCAGCATCTTTTAC-3 (5-labeled with infrared dye IRD 800 for LiCor-System of MWG, Germany).
2. Sequencing kit (e.g., from Amersham Life Science).

2.3. Exchange of *Tn*10d-bla *Insertion with Tn*10d-cat *or Tn*10d-lacZcat *Sequences*

2.3.1. PCR Amplification of *Tn*10d-cat *or Tn*10d-lacZcat

1. Plasmid pAK*cat* or pAK*lacZca*; primer IS*10*: 5-CTGATGAATCCCCTAA TGATTTTGGTA-3; 10 m*M* dNTP mix; 25 m*M* $MgCl_2$; PCR polymerase buffer (supplied with polymerase); proofreading PCR polymerase; dH_2O.
2. PCR cycler.

2.3.2. Purification of PCR Products

Materials as described in **Subheading 2.2.3.**

2.3.3. Transformation of Competent *Tn*10d-bla *Mutant Strain*

1. M-IV-competent cells of *H. influenzae* Tn*10d-bla* mutant strain.
2. BHI agar plates (Cm, 2 µg/mL).

2.3.4. Verification of Transposon Exchange

1. *See* **Subheading 2.2.1.** for materials required for preparation of chromosomal DNA.
2. *See* **Subheading 2.3.1.**

3. 0.7% agarose gel; 1X TAE buffer; staining solution: 0.4 g/mL ethidium bromide.
4. UV transilluminator.

3. Methods

3.1. Transformation of Competent H. influenzae Cells with Mini-Tn10d Transposons

3.1.1. Preparation of Competent H. influenzae Rd/Eagan

Prepare M-IV-competent cells of *H. influenzae* Rd (or wild-type Eagan) according to standard protocol ***(11)***.

3.1.2. Transformation Scheme for Generating a Mutant Bank

1. Pipette 200–300 ng of pAK*bla*, pAK*cat,* or pAK*lacZcat* (in 10 µL dH_20) into each well of a 96-well microtiter plate.
2. Mix 40 µL of competent *H. influenzae* Rd or Eagan with the plasmid DNA in each well. Incubate for 30–40 min without agitation at 37°C. Then add 150 µL BHI per well and incubate for 180 min at 37°C, first 90 min without agitation and then 90 min with shaking.
3. Use an 8-channel pipet to plate 75 µL per well (8 wells per row × 75 µL = 600 µL) onto 145-mm BHI agar plates supplemented with 2 µg/mL of Cm (*see* **Note 2**). Repeat this step for each row of microtiter wells; 2 agar plates per row; 24 BHI plates. Incubate at 37°C for at least 2 d.
4. After growth, colonies are washed off the plates with a suitable volume of BHI (e.g., 10 mL) and combined to form a mutagenized cell pool. At this point cells can be (1) frozen in 15% glycerol at −80°C, (2) appropriately diluted and plated on selective BHI agar, or (3) used to isolate chromosomal DNA (recommended procedure, and proceed with **step 5**).
5. Centrifuge cells and prepare chromosomal DNA (maxiprep according to standard protocol **[11]**) from the cell pellet. Store chromosomal DNA in TE buffer (pH 8.0) at 4°C.
6. To minimize transformation of transposon carrying plasmid, cleave the chromosomal DNA with *Sma*I. Approximately 10–30 µg (usually 10–50 µL) of chromosomal DNA are cleaved in a total volume of 100 µL with 3 U *Sma*I/µg DNA (overnight at 30°C). It is not necessary to inactivate the restriction enzyme or to reextract the DNA before retransformation.
7. M-IV-competent *H. influenzae* Rd or Eagan is then retransformed with the chromosomal DNA of the mutagenized cell pool. For each transformation, mix all the DNA from **step 6** with 250 µL competent cells in M-IV medium and incubate for 30–40 min at 37°C without shaking. Then add 1 mL of BHI and incubate at 37°C for 90 min without shaking and for additional 90 min with shaking.
8. Plate suitable aliquots of the transformation mixture on selective BHI agar plates (use Amp for Tn*10d-bla*, Cm for Tn*10d-ca*t and Tn*10d-lacZcat*), usually 50–200 µL per plate. When selecting for Amp-resistant mutants, don't plate more than 50–100 µL of the cell suspension, to avoid "background" formation due to dead cells.

9. Perform the designed screening procedures (e.g., patching colonies on plates for conditions A, B, etc.).
10. Purify the colonies of interest about 3–4 times by streaking them onto the same selective plates. In parallel, test isolated transformants for loss of plasmid on selective agar plates (for pAK*bla*, test for Cm sensitivity; for pAK*cat* and pAK*lacZcat*, test for Kan sensitivity) (*see also* **Note 3**).

3.2. Localization of Mini-Tn10d Integration Sites

3.2.1. Preparation of Chromosomal DNA

1. Briefly, according to Grimberg et al. ***(15)***, 1 µL from an overnight culture of miniTn*10d* containing *H. influenzae* mutant(s) in SBHI is centrifuged (4000*g*; 4°C).
2. Discard the supernatant and suspend the pellet in 1 mL of TNE buffer.
3. After centrifugation (as **Subheading 3.2.1.1.**), the cell pellet is resuspended in 270 µL of TNEX buffer.
4. Add 30 µL lysozyme and incubate for 20 min at 37°C.
5. After cell lysis, add 15 µL of proteinase K and incubate at 65°C until the solution becomes clear (usually 1–2 h).
6. Then add 15 µL of 5 M NaCl and 2.5 vol of ice-cold ethanol (100%) to precipitate the DNA. Invert tube to mix.
7. Following centrifugation (6000*g*; 10 min), the DNA pellet is washed with cold 70% ethanol and air-dried briefly.
8. Resuspend the DNA pellet in 150–200 µL of TE buffer with shaking.

3.2.2. PCR Fragment Enrichment Method

1. Set up two PCR reactions per clone (for each USS- direction ± as follows: 1 µL template DNA (1:20) (*see* **Subheading 2.2.1.**); 1 µL USS(+) *or* USS(−) primer (1:10); 1 µL blainv primer (1:10); 1 µL dNTP mix; $MgCl_2$ (final conc. 2–4 m*M*); PCR buffer 1X; 1–2 U DNA polymerase; dH_20 to a final volume of 50 µL.
2. Run a touch down PCR program with the following parameters:

a. Initial denaturation:	94°C, 2 min	
b. Denaturation:	94°C, 1 min	
c. Annealing temperature:	5 cycles each at 56/54/52/50°C; 10 cycles at 48°C, 1 min	30 cycles
d. Elongation:	68°C, 6 min (usually 1 min/kb)	
e. Final elongation step:	68°C, 10 min	

3. Run 5–10 µL of PCR products through a 0.7% agarose gel (in 1X TAE buffer), stain gel in ethidium bromide for 5–10 min (wear suitable protection gloves for handling ethidium bromide-stained gel!), and visualize the DNA fragments on a UV transilluminator.

3.2.3. Purification of PCR Products

1. Separate all of the PCR product on a 0.7% agarose gel, stain gel in ethidium bromide.

2. Excise the DNA band corresponding to the junction fragment between mini-Tn*10d* insertion and USS site.
3. Recover DNA from the gel slice using a gel extraction kit system.

3.2.4. Sequencing of PCR Derived Products

1. To determine the insertion sites of the mini-Tn*10d* transposon, the PCR derived fragments are sequenced directly by the dideoxy nucleotide chain termination method according to Sanger et al. ***(14)***. Any manual or automatic method can be used that allows sequencing of PCR products.
2. Cycle sequencing is performed according to the supplier's manual (Amersham Life Science).

3.3. Exchange of *Tn*10d-bla *Insertion with Tn*10d-cat *or Tn*10d-lacZcat *Sequences*

3.3.1. PCR Amplification of *Tn*10d-cat *or Tn*10dlacZcat

To achieve high fidelity of the PCR-generated DNA fragment, it is recommended that one use a DNA polymerase with proofreading activity rather than a Taq polymerase.

1. PCR setup:
   2 µL 1:50 plasmid DNA (pAK*cat* or pAK*lacZcat*); 4 µL IS10 primer (1:10) (*see* **Notes 4** and **5**); 2 µL dNTP-mix (10 m*M*); $MgCl_2$ (final conc. 2–4 m*M*); PCR buffer 1X; 2 U DNA polymerase (with proofreading activity); dH_20 to a final volume of 100 µL.
2. PCR conditions:

a. Initial denaturation:	94°C, 2 min	
b. Denaturation	94°C, 1 min	30 cycles
c. Annealing temperature:	55°C, 1 min	30 cycles
d. Elongation:	68°C, 3 or 6 min (usually 1 min/kb)	30 cycles
e. Final elongation step:	5–10 min	

3.3.2. Purification of PCR Products

1. Run the PCR product on a 0.7% agarose gel (*see* **Subheading 3.2.3.**) (*see* **Note 6**).
2. Excise the 1.7- or 4.8-kb DNA band corresponding to the Tn*10d-cat* or Tn*10d-lacZcat* fragments.
3. Recover DNA by gel extraction.

3.3.3. Transformation of Competent *Tn*10d-bla *Mutant Strain*

1. Transform purified transposon fragment (5–10 µg DNA) with competent cells of *H. influenzae* Tn*10d-bla* mutant strain (M-IV- derived competence ***[11]***) according to the procedure described in **Subheading 3.1.2.**, **step 7**.
2. Plate bacteria on BHI plates containing Cm (2 µg/mL) and incubate at 37°C for 1–2 d (*see* **Note 7**).

3.3.4. Verification of Transposon Exchange

1. Prepare chromosomal DNA (small scale, *see* **Subheading 3.2.1.**) of Cm-resistant transformants.
2. Set up PCR reactions with primer IS*10* using chromosomal DNA derived from the "exchanged" mutant and the original Tn*10d-bla* mutant as a control (*see* **Subheading 3.2.1.**).
3. Finally, separate PCR products on a 0.7% agarose gel, stain in ethidium bromide, and compare the resulting fragment sizes with each other.

4. Notes

1. To generate a mutant bank, the pAK plasmids can be transformed into constitutively competent cells of *H. influenzae* strain *sxy-1* ***(12)***: First inoculate cells in 25 mL of BHI from a fresh overnight grown culture of *H. influenzae* strain *sxy-1* ***(12)*** on chocolate agar to reach an optical density (OD_{490nm}) of cells between 0.05 and 0.1. Incubate culture at 37°C with agitation until it has reached OD_{A490} 0.8–1.3. Proceed with **step 1** in **Subheading 3.1.2**. Then mix 40 µL of competent *H. influenzae (sxy-1)* cells with the plasmid DNA in each well of the microtiter plates. Incubate microtiter plates for 30 min without agitation at 37°C. Adjust plates on a lab shaker and incubate with shaking for an additional 30–40 min at 37°C. Spot 7–10 µL of the transformation mixture 3–4 times per well (in total ~ 40 µL per transformation) onto 145-mm BHI agar plates supplemented with 2 µg/mL of Cm. Air-dry spots on agar plates under a sterile hood and incubate plates at 37°C for at least 2 d. Be sure to get 10–20 colonies per spot. Then continue the procedure with **step 4** in **Subheading 3.1.2**.
2. For transformation of plasmid pAK*bla* spot bacteria on BHI agar plates containing 2 µg/mL Cm. Select for ampicillin resistant transformants only after retransformation of the chromosomal pool DNA, otherwise the frequency of detectable ampicillin resistant bacteria may be low.
3. LacZ-expressing cells of *H. influenzae* can be screened directly on agar plates containing X-Gal. However, X-Gal should not be used when purifying mutants carrying Tn*10d-lacZcat* insertions because X-Gal is toxic to *H. influenzae* at 40 µg/mL.
4. Although we (and others for their Tn*10* minitransposons) have not observed dominant hot spots for transposition of mini-Tn*10d* elements so far, it must be stated that we cannot exclude their existence. When using Tn*10d-lacZcat*, hotspotting may theoretically arise as a consequence of the transposons' relatively large size (4.8 kb). We have noted that the transposition frequency drops significantly for Tn*10d-lacZcat* compared to Tn*10d-bla* (0.8 kb).
5. As the mini-Tn*10d* elements are flanked by inverted IS*10* sequences (29 bps; **Fig. 1A**), the IS*10*primer hybridizes to both ends of the transposons. Therefore fragments of Tn*10d-cat* (1.7 kb) or Tn*10d-lacZcat* (4.8 kb) can be generated by PCR from pAK*cat* or pAK*lacZcat* with only one oligonucleotide of IS*10* sequence.
6. For an exchange of an original Tn*10d-bla* mutant with Tn*10d-cat* or Tn*10d-lacZcat* (*see* **Subheading 3.3.**), the PCR-generated transposon should be purified to remove template plasmid DNA, otherwise additional mutations may arise from transposition form the plasmid.

7. After exchange of a Tn*10d-bla* insertion with Tn*10d-cat* or Tn*10d-lacZcat* (*see* **Subheading 3.3.**), the transformants have to be purified on Cm-containing BHI agar plates for 2–3 times in order to get rid of cells with the original Tn*10d-bla* insertion, which are not killed by the bacteriostatic action of chloramphenicol. Then the marker exchange can be verified by PCR.

References

1. Slauch, J. M. and Silhavy, T. J. (1991) Genetic fusion as experimental tools. *Meth. Enzymol.* **204,** 13–48.
2. Manoil, C. and Beckwith, J. (1985) Tn*phoA:* a transposon probe for protein export signals. *Proc. Natl. Acad. Sci. USA* **82,** 8129–8133.
3. Tadayyon, M. and Broome-Smith, J. K. (1993) Tn*blaM:* a transposon for directly tagging bacterial genes encoding cell envelope and secreted proteins. *Gene* **111,** 21–26.
4. Way, J. C., Davis, M. A., Morisato, D., Roberts, D. E., and Kleckner, N. (1984) New Tn*10* derivates for transposon mutagenesis and for construction of *lacZ* operon fusions by transposition. *Gene* **32,** 369–379.
5. Elliott, T. and Roth, J. R. (1988) Characterization of Tn*10d-Cam:* a transposition-defective Tn*10* specifying chloramphenicol resistance. *Mol. Gen. Genet.* **213,** 332–338.
6. Reidl, J. and Mekalanos, J. J. (1995) Characterization of *Vibrio cholerae* bacteriophage K139 and use of a novel mini transposon to identify a phage-encoded virulence factor. *Mol. Microbiol.* **18,** 685–701.
7. Kraiß, A., Schlör, S., and Reidl, J. (1998) In vivo transposon mutagenesis in *Haemophilus influenzae*. *Appl. Environ. Microbiol.* **64,** 4697–4702.
8. Kleckner, N., Bender, J., and Gottesman, S. (1991) Uses of transposons with emphasis on *Meth. Enzymol.* **204,** 139–180.
9. Reidl, J. and Mekalanos, J. J. (1996) Lipoprotein *e*(P4) is essential for hemin uptake by *Haemophilus influenzae*. *J. Exp. Med.* **183,** 621–629.
10. Reidl, J., Schlör, S., Kraiß, A., et al. (2000) NADP and NAD utilization in *Haemophilus influenzae*. *Mol. Microbiol,* **35,** 1573–1587.
11. Barcak, G. J., Chandler, M. S., Redfield, R. J., and Tomb, J. F. (1991) Genetic systems in *Haemophilus influenzae*. *Meth. Enzymol.* **204,** 321–342.
12. Redfield, R. J. (1991) *sxy-1,* a *Haemophilus influenzae* mutation causing greatly enhanced spontaneous competence. *J. Bacteriol.* **173,** 5612–5618.
13. Smith, H. O., Tomb, J. F., Dougherty, B. A., Fleischmann, R. D., and Venter, J. G. (1995) Frequency and distribution of DNA uptake signal sequences in the *Haemophilus influenzae* Rd genome. *Science* **269,** 538–540.
14. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
15. Grimberg, J., Maguire, S., and Belluscio, L. (1989) A simple method for the preparation of plasmid and chromosomal *E. coli* DNA. *Nucleic Acids Res.* **21,** 88–93.
16. Dziejman, M. and Mekalanos, J. J. (1994) Analysis of membrane protein interaction: ToxR can dimerize the amino terminus of phage lambda repressor. *Mol. Microbiol.* **13,** 485–494.
17. Rose, R. E. (1988) The nucleotide sequence of pACYC177. *Nucleic Acids Res.* **16,** 356.
18. Rose, R. E. (1988) The nucleotide sequence of pACYC184. *Nucleic Acids Res.* **16,** 355.

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In Vivo Expression of Bacterial Genes During Human Infections

Martin Handfield, Trevor Seifert, and Jeffrey D. Hillman

1. Introduction

1.1. Historical Perspectives

The pathogenesis of a microbial infection is a complex, dynamic process, constantly evolving within the host. In many instances, the production of virulence determinants is tightly regulated, and their production is modulated in response to the ever-changing environment encountered at the site of infection. It is unlikely that all regulated virulence determinants of a pathogen can be identified in vitro, because it is technically impossible to determine and mimic all of the different environmental stimuli that occur at the site of an infection. This shortcoming hampers our complete understanding of the virulence mechanisms employed by *Haemophilus influenzae* as well as other human pathogens in general. To overcome this problem, a number of investigators have emphasized the need to study bacterial virulence using organisms engaged in an actual infectious process. Several different methods, such as in vivo expression technology (IVET), signature tagged mutagenesis (STM), differential fluorescence induction (DFI), and microarray analysis, have been created to accomplish this end. Application of these methods to infectious processes caused by various bacterial pathogens has succeeded in identifying collections of genes that define the in vivo lifestyle of a pathogen. These genes can be analyzed and interpreted to gain a deeper understanding of the functions that dictate host specificity, tissue tropism, and disease manifestation ***(1–6)***.

While the methods developed to date for identifying in vivo induced genes have contributed significantly to our understanding of bacterial pathogenesis, they all suffer from several important drawbacks. Notably, they depend on the

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use of animal models to obtain cells of the pathogen growing in an actual site of infection. In most instances, the animal model used does not closely resemble the conditions found within the natural human host. A number of examples exist in the literature of erroneous conclusions being drawn by extrapolation of results from animal models to humans *(5)*. In many other instances, there simply is no suitable animal model for a particular pathogen. Furthermore, many of these schemes are not readily applicable to microorganisms that are not genetically maleable, that is, microorganisms for which there is no well-established or reliable means of genetic manipulation.

We recently reported the design of a novel method called in vivo induced antigen technology (IVIAT) for studying microbial pathogenesis that accomplishes the same goals as IVET, STM, and DFI in identifying in vivo expressed genes *(7,8)*. IVIAT overcomes all of the problems described above and, in particular, does not require the use of potentially misleading animal models. IVIAT is presently being used to study approx 30 different prokaryotic and eukaryotic pathogens.

In this chapter we present a conceptual overview of IVIAT as it might be applied to *H. influenzae*, and we compare it to IVET to provide a basis for focusing on the relative advantages of the IVIAT system. There is no experience currently with application of IVIAT or IVET to *H. influenzae*, but such studies would be valuable in understanding the pathogenesis of this organism.The methods and materials required to apply IVIAT to the study of *H. influenzae* infections are presented. A summary of other methods currently available to identify in vivo induced genes is also provided.

1.2. The IVIAT Strategy

A general overview of the IVIAT scheme is presented in **Fig. 1**. Serum from patients who have experienced an infection caused by the pathogen under study is pooled and repeatedly adsorbed with in vitro grown cells of the pathogen leaving antibodies against antigens that are expressed only in vivo. An expression library of the pathogen's DNA is generated in a suitable host and clones are probed with the adsorbed serum. Reactive clones, which are producing antigens expressed during a natural infection but which are not expressed during in vitro cultivation, are purified and their cloned DNA sequenced. Genes are identified in this fashion as encoding in vivo induced (IVI) antigens. These antigens are purified and used to verify that the IVI antigen is expressed by the pathogen during an infectious process. This can be done in various ways, for example by probing biological samples taken from infected patients using immunofluorescence. A noteworthy feature of IVIAT is the potential it has for defining a timeline for antigen production by the pathogen throughout the course of a natural infection process. Once one or more in vivo induced antigens have been identified and purified according to the scheme in **Fig.1**, sera

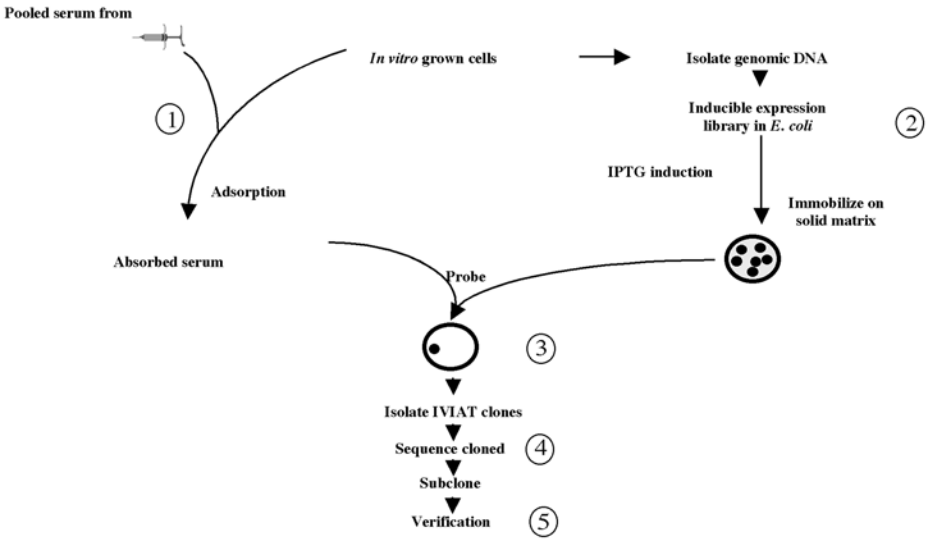

Fig. 1. The IVIAT system.

of individual patients can be probed for reactive antibodies. The pattern of reactivity for each in vivo induced antigen can then be correlated to the stage of infection (early, middle, late) for each individual patient. The staging of individual sera may provide relevant information on the pathogenesis of the microorganism and on the host response to the infection. This may in turn provide valuable insights for rationale vaccine design, diagnostics, and antibiotherapy (Hillman and Handfield, unpublished).

To this point, we have made substantial progress in testing IVIAT with Aa, Pa, *Burkoldheria pseudomallei*, *Candida albicans*, and *Candida glabrata*. Homologs that have been discovered to date are an excellent representation of the sorts of genes we expected to isolate using IVIAT. Several are homologous to previously reported virulence factors. There are also examples of genes for proteins of unknown function, which may well represent entirely new and novel virulence factors. We continue to develop and appreciate the potential repercussions that IVIAT may provide, not only in our understanding of pathogenesis, but as a new instrument to study the virulence of any pathogen directly at the site of a natural infection in the human host.

1.3. Important General Considerations for the Application of IVIAT

For any experimental system intended to identify in vivo induced genes of a pathogen, the choices of strains and serum are important and should receive

careful consideration. Before beginning IVIAT, the following questions should be answered as thoroughly as possible.

1. *Does the pathogen demonstrate clonality?* Clonality refers to genetically stable lineages of a species, some of which may be more pathogenic than others or may relate to the propensity to cause different types or use different routes of infections. If more than one clonotype (as determined by ribotyping, serotyping, RFLP, MLEE, or any other typing scheme) has been correlated with disease, you may wish to use representatives of each in construction of the genomic libraries. In instances where the main thrust of the work is directed toward identifying in vivo expressed proteins that are likely candidates for vaccine or diagnostic work, an antigen common to all pathogenic clonotypes may be desired. In this case, it is reasonable to construct your genomic library usings DNA from a single, pathogenic strain. In instances where the main thrust of the work is directed toward obtaining the broadest possible understanding of the pathogenic mechanisms used by the microorganism, we recommend using one or more representatives of each clonotype, to maximize the chances of identifying the broadest array of virulence genes. The more strains you use in library construction, the more clones you will have to screen to be sure that you have seen everything once. But the screening is the easy part, and IVIAT is very sensitive. *Haemophilus influenzae* possesses substantial genetic diversity ***(9)***, and exhibits a clonal population that varies geographically ***(10)***. The main clonotype associated with disease is the *H. influenzae* type b serotype, followed by the other serotypes, and finally NTHi causes localized disease. NTHi can occasionally cause invasive disease.
2. *What are the criteria for selecting the best sera for use in the IVIAT screening?* A good clinical history for every serum sample is essential. Knowledge of the site of infection, length of infection prior to sample collection, age of patient, antibiotic therapy, etc., is essential information that will ultimately help in the interpretation of your data. The more serum samples that you can obtain from patients representing different routes of infection and/or different stages of infection, the better the chances will be of recovering the broadest array of in vivo induced genes, including transiently expressed genes. The dilution factor caused by pooling sera obviously can have a negative effect on sensitivity. Preliminary titering of each individual serum may give you some feel for the overall reactivity, but says nothing about reactivity for particular in vivo induced antigens. Using 5 pools of 4 sera is more sensitive than using 1 pool of 20, but also requires more time and expense to perform a comprehensive screening. Counting heavily on the sensitivity of the chemiluminescence screening method employed in IVIAT, we have pooled as many as 20 serum samples for *Pseudomonas aeruginosa* (Pa) work.
3. *Does the pathogen have a surface localized Ig-binding protein?* This was the case with the prototype pathogen, *Actinobacillus actinomycetemcomitans* (Aa), notably from the same family as *H. influenzae*. In this instance we chose simply to ignore the presence of the Ig-binding protein. There was vastly more non-Aa-specific Ig present in serum than there was Aa-specific Ig, so the binding proteins would most certainly be saturated with "uninteresting" antibodies. If you so

desired, you could treat your cells with control sera or Ig before using them to adsorb your experimental sera. Potential problems in obtaining complete adsorption of the serum using whole cells because of steric hindrance problems created by nonspecific binding of Ig are no doubt eliminated by adsorbing the serum with cell extracts bound to membranes. With regard to the screening step, we were concerned that we might get false positives from clones expressing the Ig-binding proteins. This has not happened either, probably in part because the size of the cloned fragments does not result in a sufficiently large expressed product to be biologically active. Finally, you do need to bear in mind that the Ig-binding proteins are there if and when you do Western blots on the extracts of your microorganism of interest. You can readily identify and discount these proteins by their reactivity with control sera.

4. *Does your pathogen have a surface localized fibronectin binding protein?* Some species produce fibronectin-binding proteins. As with the Ig-binding proteins, probably the biggest concern is the possible steric hindrance that might interfere with complete serum adsorption using whole cells. But, as in the case of Ig-binding proteins, this is unlikely to be a problem since you will also do adsorptions with lysates bound to membranes. They should not interfere with the screening and should not yield false positives.

1.4. IVIAT Compared with Other Methods for Determining Virulence Genes in Bacterial Pathogens

1.4.1. IVET

The prototype in vivo expression technology was essentially a fusion of promoterless genes whose products confer a phenotype that can be positively selected for in vivo. Both antibiotic [*cat, tetA(Q)*2] and auxotrophic (*purA, purEK, thyA, asd*) selections as well as recombinase-mediated excision were used as a mean for in vivo selection. A variety of reporter systems (*lacZ, gfp, galK*) were also used to monitor gene expression in vivo and in vitro. IVETs essentially work as promoter traps, where genomic DNA of the microorganism of interest is cloned into a suicide plasmid, upstream of the selectable promoterless genes. Reintroduction of the random libraries in a mutant strain of the microorganism of interest, auxotroph for the corresponding selection gene, or sensitive to the selectable antibiotic-resistance marker creates a merodiploid by homologous recombination. In this genetic construct, a copy of the cloned insert drives the expression of the promoterless selection genes, while the other copy drives the gene(s) under the natural control of this promoter. The pool of random promoter fusions is then subjected to selection in various environmental conditions, in cell cultures or simply in animal models of infection. These specific physicochemical conditions will positively select for fusions turned "on" as the auxotroph and the fusions without an active promoter are not viable under these specific conditions.

Although extremely powerful as an approach, IVETs still require a great number of elements to allow convenient use in a given microorganims. First and foremost, there is as absolute need for a convenient genetic selection, which is still problematic for a number of genetically "undomesticated" microorganisms. A complementable auxotroph or a convenient antimicrobial resistance gene may not be readily available for all systems and may require some engineering. Nevertheless, a great number of teams have successfully used IVETs to study a broad array of pathogens, including Gram-positives, Gram-negatives, fungi, and some fastidious bacteria including Mycobacterium.

1.4.2. Differential Fluorescence Induction (DFI)

DFI is essentially a simpler method to accomplish the same goals as IVET. It is also based on the construction of gene fusions to promoterless reporter systems. In this specific case, the green fluorescence protein (gfp) is used as a reporter system, as the protein naturally fluoresces in a great number of biological systems studied thus far ***(6)***. Although DFI suffers from the same pitfalls as IVETs, it nevertheless has a number of advantages. Notably, the gfp activity can be detected by an automated fluorescence-activated cell sorter (FACS). For this reason, this system allows the detection of downregulated as well as upregulated gene products. DFI requires less genetic manipulations than IVET and therefore may be more quickly and simply applicable to a greater number of systems. Unfortunately, some of the characteristics of the gfp may impair its general use in certain systems. The protein is very stable and requires oxygen for activation. Therefore, there may be a certain bias introduced to study transient gene expression and its use for the study of anaerobic microorganisms may be impaired. Although its use in animal models of infection has been demonstrated, it may be better suited for in vitro studies or in cell culture systems because of the technical difficulties associated with FACS analysis in tissue homogenates.

1.4.3. Signature Tagged Mutagenesis (STM)

STM combines insertional mutagenesis and in vivo selection to identify attenuated mutants in virulence-associated loci. The signature tags are short DNA sequences that contain invariable arms, which allow specific polymerase chain reaction (PCR) amplification, flanking a variable region that allows the distinction between two mutants. These tags were designed in each individual mutant to allow the identification of mutants from a complex pool. DNA isolated from tagged mutants is immobilized in arrays. These arrays are probed by colony or dot-blots with pools of mutants recovered before and after a specific natural selection such as defined culture conditions or animal models of infection. This comparative hybridization allows the identification of mutants that

cannot survive the selective pressure. One conceptual limitation of this technique is that essential genes are missed because of the insertional inactivation of transposons. Obviously, this technique is still restricted to a few organisms for which transposons and delivery systems are available. Nevertheless, STM has been useful in a number of systems to identify in vivo induced genes including Gram-positive, Gram-negative and a few fastidious microorganisms including Mycobacterium.

1.4.4. DNA Microarrays, Proteomics, and Functional Genomics

As complete genome sequences become available, more techniques have been designed to study gene expression at the genomic level. Most current array-based research focuses on expression-level monitoring. Microarrays are typically constructed by fixing on a solid matrix (a glass slide or silicone-based "chip") DNA from reversed-transcribed mRNA isolated from a specific tissue or a collection of oligonucleotides designed from the known sequences obtained in genomic databases. mRNA is isolated from bacterium grown in different environmental conditions or isolated from infected tissues and probed against these arrays to screen for those genes transcriptionally induced or repressed in different conditions. Computerized algorithms provide a means to relate sequence data to putative function based on a compilation of all known sequences in databases. However, posttranscriptionaly regulated products may be missed. This is one reason why many are actually more interested in proteomic approaches rather than genomics. Proteomics refers to the collection of proteins expressed at a given point in time. A common technical problem to arrays and proteomics is that it is fairly difficult to isolate enough mRNA or proteins from bacteria isolated from an actual site of infection. It is therefore still technically impossible to date to study gene regulation in vivo with these systems.

1.4.5. Genomic Analysis and Mapping by In Vitro Transposition (GAMBIT)

GAMBIT was developed to identify essential genes in organisms whose genomes have been sequenced. In short, selected regions of the chromosome are amplified by PCR and subjected to transposon mutagenesis. Bacteria are subsequently transformed with mutagenized PCR products and grown under selective conditions. PCR is performed on the postselection pool of transformants using a transposon-specific primer and primer to a known location on the chromosome. The resulting PCR products are analyzed to determine which genes in the region are necessary for survival in the conditions set for the selection. A drawback of GAMBIT is the requirement of designing primers for each region of chromosome to be studied (approx130 primers per megabase).

1.4.6. Recombinase-Based In Vivo Expression Technology (RIVET)

RIVET is an IVET-like promoter trap in which in vivo induced promoters drive expression of a resolvase gene. Expression of the site-specific resolvase leads to the excision of an antibiotic-resistance cassette flanked by recognition sequences that can then be screened for. Initially the system was overly sensitive, as basal levels of expression would result in the resistance marker being immediately resolved. However, recent modifications have reduced the sensitivity, making it possible to identify temporal patterns of expression.

1.4.7. Selective Capture of Transcribed Sequences (SCOTS)

SCOTS uses a combination of selective and differential hybridization. Biotinylated microbial genomic DNA, preblocked with microbial rDNA, is used to selectively capture cDNA generated from microbial RNA extracted from infected host cells or tissue. The captured cDNA is then hybridized to genomic DNA fragments that have been prehybridized with rDNA and cDNA generated from in vitro grown microbial RNA. Captured cDNA is then eluted, PCR amplified, and, after several rounds of SCOTS, cloned. Clones that hybridize to host tissue grown, but not from in vitro grown bacterial cDNA are then sequenced. SCOTS can be applied to any microbial pathogen from which total RNA can be obtained. A notable advantage of SCOTS is that it can be used to detect in vivo expressed genes from a small number of bacteria obtained from any infected tissue sample.

1.4.8. Linear-Expression Elements (LEE)

LEE were designed as PCR amplicons from a pathogen of interest linked to two additional PCR fragments containing a host-specific promoter and a terminator system. When injected into animals, the coding regions are highly expressed and lead to an immune response by the host. Using an entire genomic library of LEE for a given pathogen, it is possible to systematically identify antigens that produce a protective immune response. The bigger drawback of LEE is the need to construct primers to amplify each coding region, which, of course, requires a sequenced genome.

1.4.9. Differential Display-Reverse Transcriptase Polymerase Chain Reaction (DD-RTPCR)

DD-RTPCR of mRNA species is the basis of this technique. It is typically carried out by reverse transcription of mRNA species isolated from microorganisms subjected to diverse environmental conditions. Subsequent PCR amplification of these DNA fragments, using arbitrarily primed random PCR

primers, results in an electrophoretic pattern that reveals induction and repression of the various mRNA species present in the sample. Again, the technical difficulty of isolating a large amount of good-quality RNA is limitating when applied to animal models of infection.

1.4.10. Differential/Subtractive Hybridization Techniques

Hybridization techniques have been used to recover differentially expressed genes. The technical challenge associated with these techniques has, however, limited their general applicability. Differential hybridization essentially relates to the screening of genomic libraries with different probes. These probes can be cDNA reverse-transcribed from RNA isolated from bacteria grown in different conditions or tissues. Alternatively, a differential library can be constructed from the cDNA resulting after absorption with immobilized cDNA from a reference growth condition.

2. Materials

1. Bacterial strain of interest, for instance, *H. influenzae* type b, cultured in supplemented brain heart infusion (SBHI).
2. Acute and convalescent serum obtained from patients who have recovered from disease, for instance, invasive type b disease, epiglotitis, or meningitis.
3. 100 µL phosphate-buffered saline (PBS) containing 0.02% sodium azide.
4. 8 mm Nitrocellulose membrane (Gibco-BRL).

3. Methods

We have constructed the following guidelines for efficient screening of virtually any microbial genome. Some representative data are presented here for illustration purposes and stem from our projects on *Actinobacillus actinomycetemcomitans* (Aa) and *Pseudomonas aeruginosa* (Pa). The steps refer to **Fig. 1**.

3.1. Adsorbing Convalescent Patient Sera with In vitro-Grown H. influenzae *(Step 1)*

1. Serum for probing the genomic library is obtained from appropriate patients and pooled using the guidelines provided above. It is advisable to check the titer of each serum using an ELISA procedure vs whole cells or French press extracts to assess the quality of the starting material. You should note that some whole cells do not make good targets for ELISA, as was the case with Pa, which we believe did not bind to plates in a uniform manner. So far, we have found that cell-free extracts made using a French press have performed uniformly well in this capacity. Equal amounts of each reactive serum are then pooled to create the starting material shown in **step 1** of **Fig. 1**.
2. The pooled serum is then adsorbed to remove antibodies that are reactive with proteins made by the pathogen during in vitro cultivation. Absorptions are performed using both whole cells and cell extracts immobilized on nitrocellulose

membranes. Briefly, 500 µL of pooled serum is typically subjected to five successive direct absorptions with cells grown in BHI broth at 37°C. Each absorption consists of an overnight incubation with mild agitation at 4°C of the pooled serum with approx 10^{11} bacteria in 100 µL PBS/0.02% sodium azide. The cells are removed by centrifuging in a microfuge for 2 min at 4°C and recovering the serum. For this and the remaining steps, we have typically used cells grown in rich broth. Logically, the use of minimal medium for growth of the cells used in the adsorption steps should decrease the number of housekeeping genes ultimately recovered in the IVIAT screening. To this point, however, very few of the genes that we have recovered are clearly of the housekeeping variety, perhaps because these proteins tend to be relatively well conserved throughout evolution and perhaps as a consequence tend not to be highly immunogenic.

3. The serum is further adsorbed by exposing it to a nitrocellulose membrane saturated with extracts of the pathogen prepared by French press treatment. Bacteria (10^{11}) are suspended in 1 mL of PBS/0.02% sodium azide and treated at 14,000 psi. The entire volume of the extract (do not spin out the cell debris) is incubated for 1 h with a nitrocellulose membrane at room temperature with gentle agitation. The membrane is then repeatedly washed with PBS/0.5% Tween-20 prior to use. These coated membranes can be kept for 1 wk in PBS/0.5% Tween-20 at 4°C until used. The adsorbtion is performed by spreading 1 mL of the serum directly onto the membrane in a Petri dish and incubating overnight at 4°C with gentle agitation on a rocking platform. The serum is recovered by draining the membrane and washing it with 500 µL of PBS. The aspirated samples are pooled.
4. A final absorption step is then carried out using the same French pressure cell extract prepared in the previous step except that it is heat-denatured in a boiling-water bath for 10 min before immobilization on the nitrocellulose membrane in order to expose additional linear immunoreactive epitopes.
5. To test the efficiency of the absorption steps, French pressure cell extracts are immobilized in microtiter wells and, following an ELISA procedure, are reacted with serial dilutions of serum samples taken at different points in the adsorption process. Peroxidase-conjugated goat anti-human affinity-purified immunoglobulin (Cappel, ICN), reactive with all classes of human immunoglobulins, is used to determine the reactive antibody titers. As shown in **Fig. 2**, the successive adsorptions can decrease the reactive antibody titer by a factor greater than 10^4, essentially removing all of the antibodies specific for in vitro grown bacterial constituents. We have typically included an untreated serum sample from a healthy, normal subject as a control in this ELISA. In the case of Pa, the fully adsorbed sera from cystic fibrosis patients had a lower titer than this control, so do not assume that serum from a healthy subject reflects the background level of reactivity. Depending on the results obtained, additional adsorption steps using whole cells or extracts can be added. Also, adsorption of the serum with the *Eschericia coli* host strain, BL21(DE3), may decrease the background observed in the screening step that follows. This in large part depends on the reactivity of the serum to *E. coli*, which may vary from subject to subject. To be safe, we now

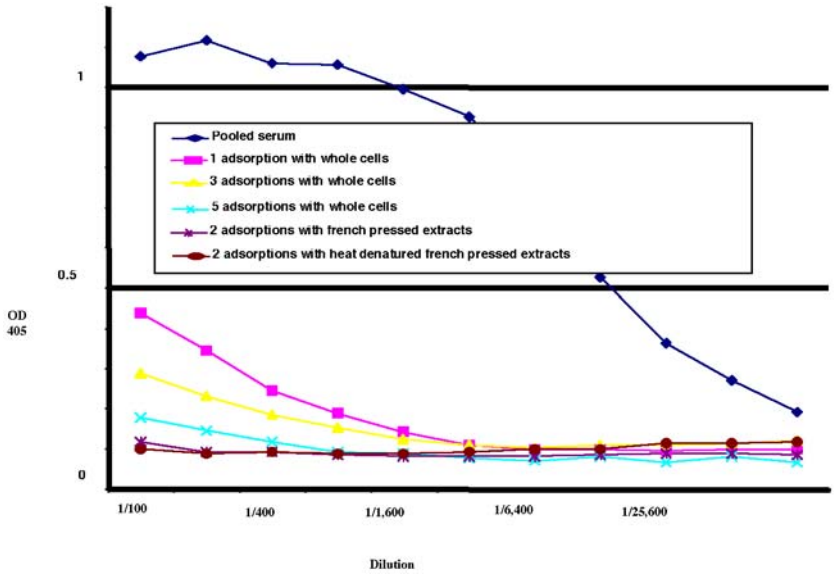

Fig. 2. Sequential adsorption of pooled serum to eliminate antibodies reactive with in-vitro expressed antigens. The pooled sera from periodontitis patients were subjected to 5 successive adsorptions with whole cells. The serum was further adsorbed with Aa strain HK1651 extracts prepared by French press. A final adsorption step was carried out using the same extract which was heat-denatured in order to expose additional immunoreactive epitopes. An ELISA was used to determine the reactive antibody titers.

routinely include this step, but use a single adsorption to a heat-denatured French pressure extract of BL21(DE3) immobilized to a nitrocellulose membrane. The resulting adsorbed serum is aliquoted and stored at −80°C.

3.2. Constructing a Genomic Expression Library in E. coli *(Step 2)*

1. Genomic DNA is purified using a Qiagen kit or other suitable protocol and trial partial digestions are performed with *Sau3A* to optimize production of 0.5–1.5-kb fragments. This step takes a bit of playing around with, but most people have had this experience in previous work so we won't belabor the point. On a 1% agarose gel, your digested DNA should appear as an intense smear with no obvious banding. Once the optimal conditions for digestion are found, we typically scale the digestion up to use approx 20 µg of starting DNA. This is best done by replicating the optimized conditions in 20 individual tubes, rather than multiplying everything by 20-in. one tube.
2. The fragments are then pooled and gel-purified on low-melting NuSieve GTG agarose (FMC BioProducts) in Tris-acetate EDTA (TAE) buffer, trying to fit as much DNA as possible in the fewest number of tracks to limit the amount of agarose subsequently requiring digestion. Avoid short-wavelength UV light to

illuminate your DNA, since this is likely to introduce mutations. Use long-wavelength UV to identify the gel fragment of interest as indicated by appropriate MW markers, and cut it out using a clean scalpel blade. To recover the DNA, many alternatives are available. We prefer to use β-agarase digestion (New England Biolabs) following the manufacturer's instructions, and recover and concentrate the DNA using a Centricon-100 size-exclusion filter (Amicon). After initial recovery, we have found that an additional phenol–chloroform extraction step, followed by a chloroform extraction step, greatly increases the efficiency of ligation. After the extractions, recover and wash the DNA again using Microcon-30-size exclusion filters (Amicon). Purity and yield are then evaluated by electrophoresis of 5% of the final volume through a 1% agarose gel (typically 2.5 µL of 50 µL).

3. 3–5 µL of the pET30 expression vectors (Novagen), a, b, and c, are individually digested with *BamHI* to completion and then treated with calf intestinal phosphatase (CIP) according to the manufacturer's instructions. After phenol–chloroform and chloroform extractions, the DNA is washed and concentrated using a Microcon-30 as described above. Purity and yield are again tested by electrophoresis of 1–5% of the final volume through a 1% agarose gel (typically 2.5 µL of 50 µL).
4. The insert DNA is then ligated into the *Bam*HI-digested, CIP-treated pET30 expression vectors to create three separate genomic libraries, one each for pET30 a, b and c. Ligation conditions are empirical. Reaction conditions should be varied in order to achieve a minimum of 5000 independent clones per reaction. After ligation, the mixture is typically washed and concentrated on a Microcon-30 to 2–5 µL final volume, and electroporated into electrocompetent *E. coli* BL21(DE3), the expression host for pET30. The entire volume of the electroporation reaction is spread onto brain heart infusion (BHI) plates containing 50-µg/mL kanamycin. After overnight incubation at 37°C, the plates are scraped to collect the cells, which are aliquoted and stored in BHI broth containing 50% glycerol at −80°C.
5. For screening, the library is thawed and dilutions spread on BHI agar containing 50-µg/mL kanamycin to obtain plates containing approx 500 colonies. The plates are incubated for 12–14 h at 37°C.

3.3. Library Screening (Step 3)

1. The genomic expression library created in **step 2** is serially diluted and plated on BHI medium, so that each plate contains approx 500 colonies. These plates are then replicated using sterile velvet onto duplicate BHI plates containing kanamycin and IPTG (1 m*M*) and incubated for 5 h at 37°C to induce expression of the cloned genes.
2. To partially lyse the bacteria and expose induced proteins, the colonies are exposed to chloroform vapors for 15 min in a hermetic container (we use a dessicator with chloroform-saturated paper towels) and then overlaid with nitrocellulose membranes (Gibco BRL) for 15 min at room temperature. The membranes are then carefully removed and saturated with 5% nonfat skim milk in PBS-Tween. The duplicate membranes are then reacted with either unadsorbed (this is optional—it is gratifying to see that most of the clones react with

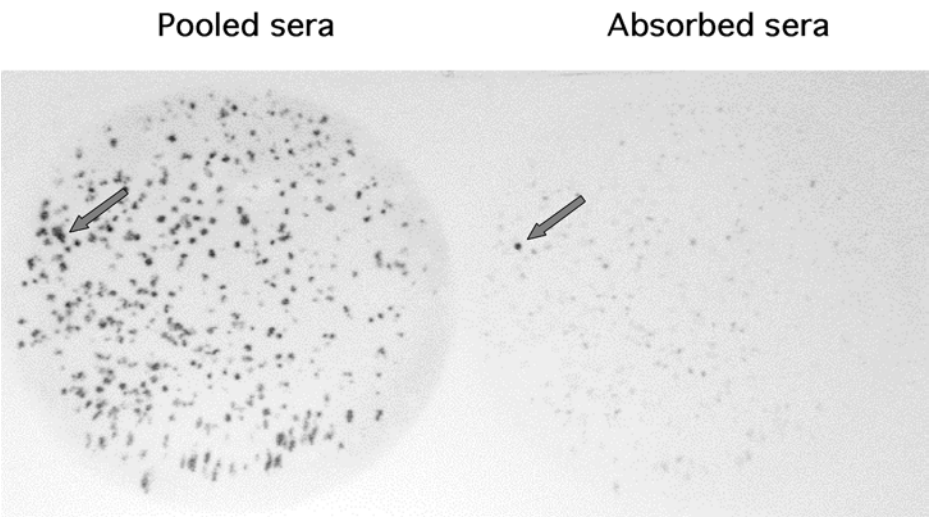

Fig. 3. Immunological reactivity against duplicate blots of a partial Aa library in pET30abc/BL21(DE3). Colonies were reacted with either the pooled unadsorbed or pooled adsorbed sera and visualized by chemiluminescence.

unadsorbed serum, indicating that the absorption process actually eliminated antibodies directed against in vitro induced genes) or adsorbed sera created in **step 2** using a 1/5000–1/10,000 dilution (obviously, this depends on the titer of the starting material) in PBS-Tween. The membranes are incubated with the sera at room temperature for 1 h with mild agitation. Reactive clones are detected using peroxidase-conjugated goat antihuman immunoglobulin (reactive with all classes of human immunoglobulins) (Cappel/ICN) at a 1/5000 dilution. Our conditions for probing with the primary and secondary antibodies are optimized for use with the ECL chemiluminescence kit and Hyperfilm ECL (Amersham), so some adjustment may be required if other labeling methods are used. The chemiluminescent method provides greater sensitivity and a lower background than colorimetric peroxidase assays. High sensivity was desirable because, among other reasons, the availability of the human serum is potentially limiting.

3. **Figure 3** demonstrates the reactivity observed when unadsorbed and adsorbed sera were used to screen a representative set of duplicate membranes containing a partial Aa library. Virtually all of the clones reacted with the unadsorbed serum, indicating that patients produced antibodies or had cross-reactive antibodies against a broad array of Aa proteins. Only a small subset of colonies (i.e., 20 out of 10,000 clones, representing 25% of the Aa genome) tested to date reacted with the adsorbed antiserum. These IVIAT clones were isolated and purified as candidates likely to be expressing Aa proteins that are made during a natural infectious process in human hosts but not during routine cultivation in vitro.

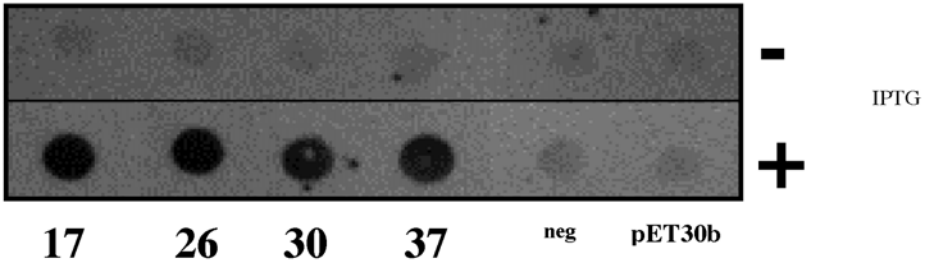

Fig. 4 IPTG induction of some Aa IVIAT clones. Several IVIAT clones and controls were grown with (+) or without (−) IPTG and probed by colony blot with the adsorbed serum.

3.4. Step 4

1. Reactive clones are identified by their position on the master plates and purified on kanamycin medium. A second screening to verify reactivity is recommended, particularly if there are other nearby colonies. Vector DNA from the reactive clones is purified using a plasmid isolation kit (Maxi-Prep, Qiagen Tip-500). The cloned DNA inserts are sequenced in both directions using pET30 primers (Novagen). The sequences are analyzed to determine unique inserts. MacVector v.6.0.1 was used to identify open reading frames (ORFs). In the case of Aa, potential open reading frames were present on the cloned inserts oriented in the same and opposite direction relative to the pET promoter. In order to determine which of these was most likely to be the one expressing the antigen reactive with the adsorbed serum, we first determined if the level of expression of the antigen was regulated by IPTG. The IVIAT clones were grown overnight at 37°C in BHI broth containing kanamycin (50 µg/mL), and then 1 µL of each culture was spotted on BHI agar plates containing kanamycin with or without IPTG (1 m*M*). Following incubation of the plates at 37°C for 5 h, proteins were immobilized on nitrocellulose membranes by colony blot after chloroform treatment of the plates as described above and probed with adsorbed serum as described in **step 3**. Two negative controls were included in this IPTG induction assay, namely, pET30b/BL21(DE3) with no cloned insert, and −, which has an Aa DNA insert but which was nonreactive with adsorbed antiserum. As illustrated in **Fig. 4**, this assay demonstrated that all of the IVIAT clones expressed genes that were induced by IPTG and therefore are likely to be cloned in the same orientation as the pET30 promoter. Their translation may be initiated either at the pET30 ribosome binding site or at their own ribosome-binding site.
2. Subcloning of the open reading frames in the same orientation as the pET promoter was then performed to positively identify the one that was expressing the protein reactive with the adsorbed serum. Subcloning was accomplished by PCR

amplification of each ORF, which was then cloned into pET30 using the one-step (ligationless) EkLIC system (Novagen). The resultant subclones were retested as above for their reactivity with the adsorbed serum. Positive clones were shown to be IPTG regulatable. In most every instance, the open reading frame that was immediately downstream of the pET promoter and that was in-frame with the pET translation start codon proved to be the one expressing the protein reactive with the adsorbed serum.

3.5. Step 5

1. Our scheme for isolation of an in vivo induced antigen using absorbed serum does not eliminate the possibility that the reactive antibodies were originally raised against a cross reacting protein. The following step is optional, but it would discount this possibility before investing significant time and resources in the analysis of a particular IVIAT protein.
2. Several possible approaches can be used to independently confirm that a protein discovered by IVIAT is actually expressed by the pathogen during a human infection. The following method provides the most direct proof. IVIAT subclones are grown for 1–3 h in the presence of IPTG to determine the time of maximum protein production. Based on the results obtained, inclusion body preparations ***(11)*** are made from 10-mL batch cultures of the clone incubated with IPTG. SDS-PAGE and Western blot analysis are performed to confirm that the overexpressed protein is reactive with the adsorbed serum used in the original screening. The reactive band is excised from the Coomasie-stained gel and used to raise polyclonal antibodies in mice. Murine polyclonal IgG from ascites fluid is purified using a protein A affinity column. The purified IgG is shown to react in Western blots with a protein of the appropriate size in extracts and inclusion body preparations made from the subclone. The antibodies are labeled directly with fluorescein isothiocyanate (FITC) (Molecular Probes, Eugene, OR, USA). A murine monoclonal antibody specific for the pathogen under study is labeled with Texas red (TR) ***(11)***.
3. Biological tissues or fluids from infected patients containing the microorganism of interest are mixed with 1 mL of BHI broth containing 50% (v/v) glycerol and, after removing 0.05 mL for microbiological analysis, are quick-frozen in a Dry Ice/ethanol bath. This ensures preservation of the antigens present on the bacteria at the time they were isolated from the infection site. Expression of IVIAT proteins in biological specimens isolated from infected patients are confirmed using the frozen biological samples described above. They are thawed and homogenized by brief vortexing at 4°C. Samples are probed using a 1/1000 dilution of the FITC-labeled polyclonal antibody directed against each in vivo induced antigen and are simultaneously probed with a 1/1000 dilution of a pathogen-specific monoclonal antibody labeled with TR. After probing for 1 h at 4°C with mild agitation, the cells will be washed by centrifugation in the cold and resuspended in cold PBS.
4. Samples are examined by fluorescence microscopy (excitation wavelength 488 nm). Double color analysis, using matched differential emission filters, is performed to

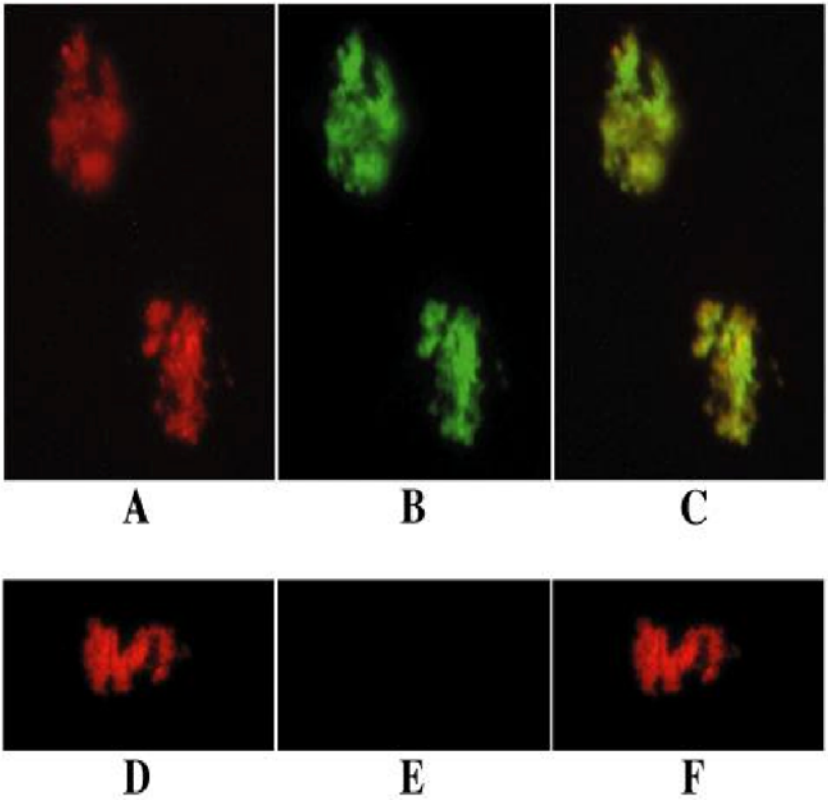

Fig. 5. Immunofluorescence assay. Demonstration of specific in-vivo induction of Aa2008. Plaque samples and matched in-vitro grown *A. actinomycetemcomitans* isolates were probed in parallel with FITC-labeled monospecific antibody directed against antigen Aa2008 and the TR-labeled *A. actinomycetemcomitans*-specific monoclonal antibody. Samples were examined by fluorescence microscopy. Bacterial cells in plaque that reacted with TR-labeled anti-*A. actinomycetemcomitans* monoclonal antibody (**A**) also reacted with FITC-labeled anti-Aa2008 antibody (**B**) and these signals were found to colocalize (**C**). In contrast, matched in vitro grown *A. actinomycetemcomitans* reacted with the TR-labeled anti-*A. actinomycetemcomitans* monoclonal antibody (**D**) but not with FITC-labeled anti-Aa2008 antibody (**E**) and therefore no colocalization was observed (**F**).

identify individual cells that are simultaneously labeled with FITC and TR. In the event that these are observed, they would serve as direct and definitive proof that the cloned IVIAT genes identified in specific aim 1 are expressed by the microorganism of interest during an actual infectious process in the human host. For this

set of experiments, negative controls for the anti-IVI antigen and anti-pathogen antibodies will include, respectively, in vitro grown pathogen (each clinical and reference strain) and *E. coli* BL21(DE3). **Figure 5** shows that IVIAT protein Aa2008 is present on the surface of Aa present in dental plaque of a patient with localized juvenile periodontitis, but is not present on in vitro grown cells ***(8)***.

3.6. Conclusions

1. Unlike IVET and related strategies, IVIAT avoids the use of animal models by using pooled serum from patients who have experienced disease caused by the pathogen under study.
2. IVIAT is also technically very simple. Aside from the requirement for sera from infected patients, IVIAT requires only the construction of an expression library of the pathogen's DNA in *E. coli* or other suitable host. IVIAT permits the construction of genomic libraries using multiple strains of a pathogen, and the libraries can be screened using pooled sera from patients infected by different routes. By contrast, IVET requires construction of promoter probe libraries that may be more or less difficult to accomplish depending on the pathogen under study. For this reason, IVET and related strategies are usually restricted to examination of a single clonotype engaged in a single route of infection.
3. By using serum samples from patients in different stages of infection, IVIAT should enable us to identify transiently expressed in vivo induced genes. Most IVET and related strategies identify do not have this advantage.
4. Logically, IVIAT can be conveniently applied to any prokaryotic or eukaryotic pathogen that elicits an antibody response. Because of their technical difficulties, other strategies are not well suited for studies of every prokaryotic or eukaryotic pathogen.

References

1. Mekalanos, J. J. (1992) Environmental signals controlling expression of virulence determinants in bacteria. *J Bacteriol.* **174,** 1–7.
2. Mahan, M. J., Slauch, J. M., and Mekalanos, J. J. (1993) Selection of bacterial virulence genes that are specifically induced in host tissues. *Science* **259,** 686–688.
3. Finlay, B. B. and Falkow, S. (1997) Common themes in microbial pathogenicity revisited. *Microbiol. Mol. Biol. Rev.* **61,** 136–69.
4. Heithoff, D. M., Conner, C. P., and Mahan, M. J. (1997) Dissecting the biology of a pathogen during infection. *Trends Microbiol.* **5,** 509–513.
5. Smith, H. (1998) What happens to bacterial pathogens in vivo? *Trends Microbiol.* **6,** 239–243.
6. Handfield, M. and Levesque, R. C. (1999) Strategies for isolation of in-vivo expressed genes from bacteria. *FEMS Microbiol. Rev.* **23,** 69–91.
7. Handfield, M., Brady, L. J., Progulske-Fox, A., and Hillman, J. D. (2000) IVIAT: a novel method to identify microbial genes expressed specifically during human infections. *Trends Microbiol.* **8,** 336–339.

8. Handfield, M., Brady, L J., Progulske-Fox, A., and Hillman, J. D. Probing for bacterial antigens induced specifically in human infections. *Infect. Immun.* Submitted.
9. Porras, O., Caugant, D. A., Lagergard, T., and Svanborg-Eden, C. (1986) Application of multilocus enzyme gel electrophoresis to *Haemophilus influenzae*. *Infect. Immun.* **53,** 71–78.
10. Musser, J. M., Kroll, J. S., Granoff, D. M., Moxon, E. R., Brodeur, B. R., Campos, J., et al. (1990) Global genetic structure and molecular epidemiology of encapsulated *Haemophilus influenzae*. *Rev. Infect. Dis.* **12,** 75–111.
11. Coligan, J. E., Dunn, B. M., Ploegh, H. L., Speicher, D. W., and Wingfield, P. T. eds. (1997) Chemical modification of proteins: basic protocol #2. Addition of isothiocyanati, in *Current Protocols in Protein Science*. John Wiley, New York, p. 15.2.7.

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ELISA

Joyce S. Plested, Philip A. Coull, and Margaret Anne J. Gidney

1. Introduction

Enzyme linked immunosorbent assay (ELISAs) has been used as a convenient routine immunological assay for many specific antigens to monitor antibody responses to vaccination, and has replaced other methods, e.g., radioimmunoassay (FARR assay) ***(1,2)***. For example, ELISA using tetanus toxoid, diphtheria toxoid, *Haemophilus influenzae* Type b (Hib) polysaccharide and *Neisseria meningitidis* A/C polysaccharide are used to determine serum antibodies following vaccination. In these assays, the antigen, e.g., toxoid (tetanus, diphtheria) or polysaccharide (Hib PRP, Men C) alone or conjugated to poly-L-lysine or methylated human serum albumin (mHSA), is coated onto plastic of 96-well microtiter plates ***(3–5)***. By direct or indirect methods the antibodies in serum samples can be detected using enzyme-linked secondary antibody conjugates, e.g., horseradish peroxidase or alkaline phosphatase, and appropriate substrates using color reaction at specific absorbance e.g., OD_{A405}. The principle of the indirect ELISA is shown in **Fig. 1** ***(6)***. Using dilutions of a standard serum or monoclonal antibody with known concentration of total/specific antibody or given arbitrary units, it is possible to compare dilutions of unknown samples against a standard curve. In this chapter we demonstrate how to establish an ELISA for antibody to *H. influenzae* (*Hi*) inner-core lipopolysaccharide (LPS) epitopes.

Our experience with *Neisseria meningitidis* (*Nm*) inner-core LPS epitopes, e.g., mAb NmL3B5 raised to *NmL3 galE* LPS, demonstrated the use of a monoclonal antibody (mAb) and purified LPS to develop a specific, reproducible ELISA to detect inner-core LPS antibodies in human sera. This assay detected significant *L3galE* LPS antibodies in adult and infant sera and increased concentrations in antibodies following invasive *Nm* disease ***(1)***. This assay was

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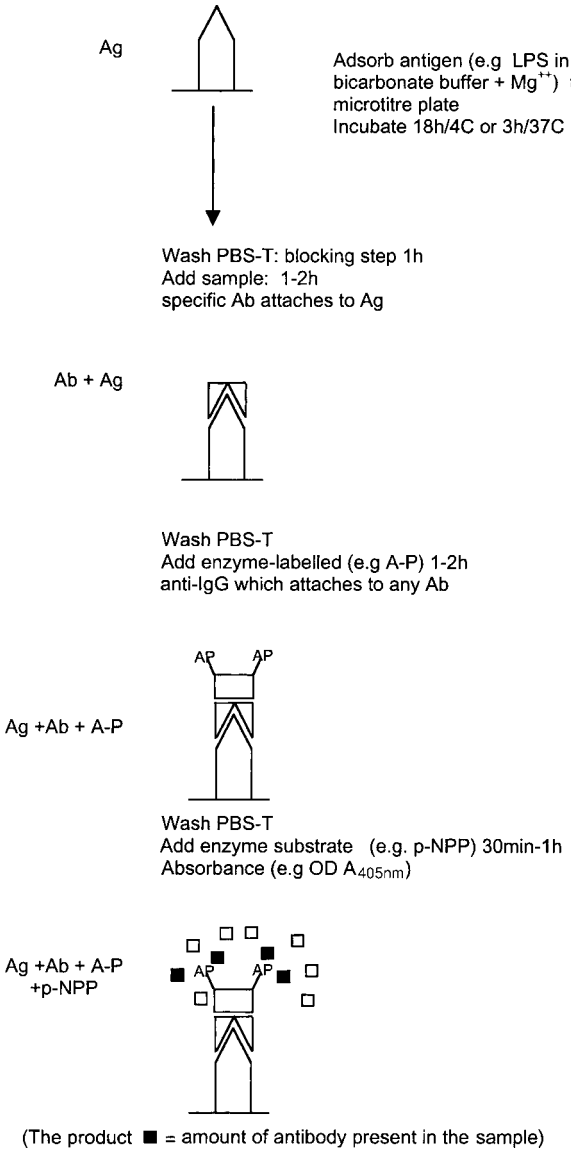

Fig. 1. Principle of non-competitive indirect ELISA (for antibody detection) applied to inner-core *H.influenzae* lipopolysaccharide (LPS) antibodies (adapted from **ref. *6***).

also used to determine antibody response to vaccination with inner-core LPS epitopes using monoclonal and polyclonal mouse antibodies. These principles could then be directly applied to *Haemophilus influenzae* (*Hi*) inner-core LPS with the availability of appropriate mAbs.

Other methods are available to detect antibodies in monoclonal and polyclonal sera or can be used to detect the presence of specific epitopes using appropriate monoclonal/polyclonal antibodies e.g., immuno-dot blots with whole-cell lysates ***(8,9)***. Unless using a mAb to a defined epitope, these assays are not specific (they detect antibodies to the whole cell), and they can be difficult to interpret and to determine reactivity reproducibly. However, they are useful indicators of an appropriate antibody response and allow greater numbers of strains to be screened, for example, to give an idea of conservation of an epitope. Immunofluorescence microscopy with specific mabs can provide valuable information on accessibility of epitopes in whole-cell bacteria, e.g., *Nm galE* LPS epitope in strain MC58 ***(10)***. This information can be used in conjunction with whole-cell assays to determine the candidacy of potential vaccine antigens prior to further immunogenicity studies.

1.1. Optimizing Parameters Affecting ELISA

Each stage of the ELISA will influence the final result and therefore great care must be taken to optimize and then standardize the method. The most important influencing factors are antigen coating, choice of plates, choice of blocking agent, and choice of secondary antibodies and detection system. Each of these factors will vary with each antigen being used.

1.1.1. Antigen Coating Concentration and Conditions

Optimal conditions with regard to antigen coating concentration and conditions were determined using mAbs raised to inner-core LPS of *Hi*, for example mAb L6A9 ***(11)***. The optimal concentration of LPS used to coat the plates was determined with each batch of LPS, for example, a range from 100 to 1 µg/mL was tested with mAb L6A9. The minimum LPS concentration that could differentiate concentrations of mAb L6A9 ranged from 25 to 5 µg/mL and 10 µg/mL was chosen as the optimal concentration (*see* **Fig. 2**). The optimal coating buffer for preparing the LPS solution for *Nm* was 0.05 *M* carbonate buffer with 0.02 *M* magnesium chloride (pH 9.8) ***(7)*** and this was also suitable for *Hi* LPS. A combination of different antigen coating conditions, including buffers and antigen concentration, needs to be compared to determine the optimal conditions for the particular antigen, for example, for LPS or polysaccharide (**Table 1**).

1.1.2. Choice of Microtiter Plates

Earlier studies with *Neisseria meningitidis* (*Nm*) inner-core (*galE*) LPS compared a number of plates from different sources with the same antigen coating method. A range of 96-well microtiter plates was compared, for example Nunc, Dynatech, Costar, Falcon with the standard ELISA protocol ***(7)***. These plates could be divided into two groups based on specific and non-

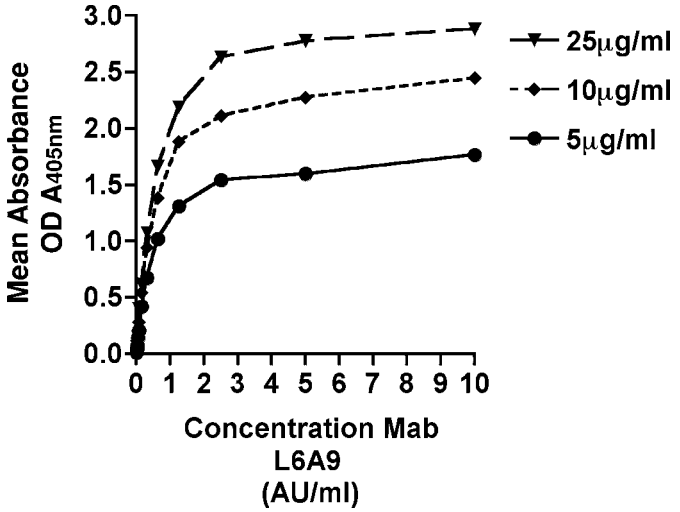

Fig. 2. The effect of three different coating concentrations of *H. influenzae* (*Hi*) inner-core lipopolysaccharide (LPS) (25,10,5 μg/mL indicated by solid triangle, diamond, and circle respectively) with a range of concentrations of *Hi* mAb L6A9 ascites fluid (Arbitrary units AU/mL). Mean absorbance (A_{405nm}) as determined by the standard LPS ELISA (as outlined in **Subheading 3.5.**).

specific antibody binding. The plate type chosen for optimal binding with *Nm galE* LPS ELISA was from group 1 (Nunc Maxisorp) (**Table 2**). For a new antigen it is best to compare a range of plates and many manufacturers will provide samples to try out. The decision on the best plate type is usually a compromise between high specificity for the antigen of interest and low–medium nonspecific binding (NSB) to the plate (**Table 2**). For example, Nunc Maxisorp plates give low-NSB but the highest specific binding (group 1 type plate).

1.1.3. Choice of Blocking Agents

The choice of blocking agent depends on the combination of antigen, plate, coating buffer, and species of test sera, and ideally should be compared for each new antigen used in ELISA. For example, previous studies using inner-core LPS from *Nm galE* using the standard ELISA compared human and murine antibodies on the chosen plate type with a range of different blocking agents (unpublished data, J. Plested). These included dilutions of bovine serum albumin (BSA) of different purity, normal horse serum, fetal calf serum, skimmed milk, gelatin, and Tween-20. For example, a comparison of the different blocking agents is shown in **Fig. 3**. for the *Hi* inner-core mAb L6A9. In most cases, the

Table 1
Coating Buffers and Conditions Used for Bacterial Lipopolysaccharide ELISAs

Antigen species	Coating buffer	Coating conditions	Plate type	Ref.
E. coli J5; *N. gonorrhoea*; LPS	0.01 *M* $MgCl_2$, pH 6.6, NaCl (0.45%)	200 µL per well (200 µg/mL) 1 h at 37°C	Falcon	***24***
E.coli J5; Core glycolipid	0.05 *M* bicarbonate buffer, pH 9.6	50 µg/mL 5.5 h at 37°C, overnight at 4°C	Dynatech F lexivinyl	***25***
E.coli Lipid A, LPS	0.01 *M* PBS pH 7.3 or chloroform/ ethanol 1:9 v/v	100 µg/mL Evaporate 18 h at room temp in hood	Falcon Probind	***26,27***
N. meningitidis: LPS,	0.1 *M* poly-L-lysine in PBS, liposomes	10 µg/mL 18 h at 8°C pH 7.2	Nunc Polysorb	***28***
Strep. pneumioniae (6A,9,14, 23, 3, 19)	Poly-L-lysine-PRP using cyanuric chloride as coupling agent	1 µg/mL 18 h at 4°C	Plastic tubes	***3*** (applied to Hib PRP ELISA Dynatech Immulon-1)

(continued)

Table 1 *(continued)*

Antigen species	Coating buffer	Coating conditions	Plate type	Ref.
E. coli J5; *S. minnesota*; LPS inner-core, lipid A	BSA-LPS 0.5% Triethylamine, 0.05 *M* carbonate pH 9.6	10 µg/mL (1 mg/mL LPS: 10 mg/mL BSA), 18 h at room temp	Dynatech Immulon-2	***24***
E. coli J5; *S. minnesota*; LPS	BSA-LPS coupling	10 µg/mL 18 L/h at 3°C	Costar 3596	***30***
E. coli; LPS; lipid A	0.5% Triethylamine	40 µg/mL, evaporate for 12 h at room temp in fume hood	Nunc Maxisorp	***31***
E. coli; LPS	0.4 *N* HCl	1–5 µg/mL 15–30 min at 4°C	Not known	***32***
Synthetic peptides (HIV-1 vpu) protein	Alcian Blue (1% w/v) in 3% acetic acid, 0.05 *M* bicarbonate buffer with 0.2 *M* $MgCl_2$ pH 9.8	10 µg/mL 30 min at room	Immulon-1 Nunc Maxisorp	***33***
N. meningitidis; LPS	0.2% Trichloroactetic acid in pBS pH 7.4	3–10 µg/mL 18 h at 37°C	Costar	***34***

Table 2
Comparison of Specific and Nonspecific Binding of Adult Pooled Sera (IgG) on Different Types of ELISA Plate

Group[b]	Plate type	Temp °C	Coating buffer [c] A	B	C	D	E	F	G	H
1	Dynatech Im-1	4	++ (+)[a]	+(−)	++(+)	+(−)	+(−)	nd[d]	nd	nd
		20	++ (+)	++(+)	+(+)	+(+)	+(+)	++(+)	++(+)	−(−)
2	Dynatech Im-2	4	++(+)	+(−)	++(++)	+(+)	++(++)	nd	nd	nd
		20	+++(++)	++(++)	+++(++)	−(−)	++(++)	++(+)		−(−)
1	Dynatech Im-3	4	++(+)	+(−)	++(+)	+(−)	+(−)	nd	nd	−(−)
		20	++(+)	+(−)	++(+)	−(−)	+(−)	+(−)		
2	Dynatech Im-4	4	+++(++)	++(+)	++(++)	++(++)	+++(+)	nd	nd	nd
		20	+++(++)	++(−)	++(++)	++(+)	+++(+)	++(+)		+(+)
2	Falcon F3912	4	++(++)	+(+)	++(+)	++(+)	+++(+)	nd	nd	nd
		20	++(++)	++(−)	++(++)	+(+)	++(++)	++(+)		+(+)
1	Falcon F3915	4	++(+)	+(−)	++(+)	+(−)	+(+)	nd	nd	nd
		20	+(−)	++(−)	++(+)	−(−)	+(+)	++(−)		−(−)
1	Nunc Maxisorp	4	+++(+)	++(+)	++(++)	++(+)	++(++)	nd	nd	nd
		20	+++(+)	++(−)	+++(++)	+(+)	++(++)	++(+)	++(+)	−(−)

[a]Binding was compared between seven plate types with eight different coating buffers (A–H) for *Nm* inner-core (*galE*) LPS with overnight incubation at 4°C or 20°C at a coating concentration of 10 µg/mL of LPS in appropriate buffer. Binding was scored from: +++, $OD_{A405nm} > 1.0$; ++, $OD_{A405nm} > 0.5$; +, $OD_{A405nm} = > 0.2$; −< $OD_{A405nm} < 0.1$. Specific binding is shown without brackets and nonspecific binding is indicated in the brackets.

[b]Group: type of plate 1, high-medium specific binding and low nonspecific binding (< OD 0.1); 2, high-medium specific binding and high nonspecific binding (> OD 0.5)

[c]Buffers: A, magnesium chloride 0.02 *M*; B, bicarbonate buffer 0.5 *M*; C, poly-L-lysine in PBS; D, trichloroacetic acid; E, hydrochloric acid; F, chloroform/ethanol; G, alcian blue dye; H, triethylamine.

[d]nd, not determined.

blocking buffer can be used as the diluent buffer for test sera/mabs or PBS without Tween can be used. The washing buffer used is phosphate-buffered saline (PBS) with Tween (0.05% or 0.1%), determined empirically. There are many grades of BSA; for most ELISAs the lower grade is suitable, but, for the *Nm galE* LPS ELISA a higher grade (gamma globulin free) 1% BSA-PBS (Sigma A-4161) was the best for mouse mabs and human antibodies. The species of antibodies you are interested in measuring also affects the choice of blocking agent, e.g., if you are assaying bovine antibodies, then BSA and skimmed milk are not suitable and Normal Human Serum may be used. The optimal incubation time and temperature is determined empirically, usually 1–2 h at room temperature or overnight at 4°C or 30 min at 37°C. This is usually a compromise in terms of optimal binding and convenience for the researcher.

1.1.4. Choice of Secondary Antibodies and Substrate Detection Systems

Comparison of enzyme-labeled antibody conjugates and color detection systems should be determined empirically for each antigen ELISA. The dilution of conjugate should be determined empirically for each new batch (IgG/IgM) using an appropriate standard curve and control sera using a checkerboard of dilutions. For inner-core LPS ELISAs, horseradish peroxidase (HRP) conjugate with tetra methylene blue substrate (TMB) and alkaline phosphatase (AP) labeled with *p*-nitrophenylphosphate (*p*-NPP) in diethanolamine buffer have been directly compared. The latter system appeared to be more sensitive, gave lower nonspecific bindary, and gave reproducible results with control sera.

1.2. Specificity

1.2.1. Inhibition Studies Using ELISA

Specificity of the assay can be determined by pre-incubation of mAb/human sera with purified LPS of known concentration for 1 h at room temperature or overnight 4°C and then assaying the supernatant for reactivity. This was shown previously with *Nm* L3 *galE* LPS ELISA using mAb *Nm* L3B5 and *Nm* L3 *galE* LPS *(7)*. Using such inhibition studies, a dose-dependent decrease in reactivity from 500 µg-1 ng/mL could be observed with mAb L6A9 with *Hi* Eagan inner-core LPS in the *Hi* inner-core ELISA (**Fig. 4**). Results can be expressed as OD_{A405nm}, or percent inhibition calculated from:

$$OD_{A405\,nm} \text{ of nonadsorbed} - OD_{A405mn}\text{absorbed}/OD_{A405mn}\text{nonadsorbed} \times 100$$

For example, for mAb L6A9, 50% inhibition of reactivity was achieved approx 10–100 ng/mL with inner-core LPS by ELISA (**Fig. 4**). Alternatively, competition ELISAs can be performed using an unknown sera and mAb of known specificity to epitope on ELISA plate, detected with commercially avail-

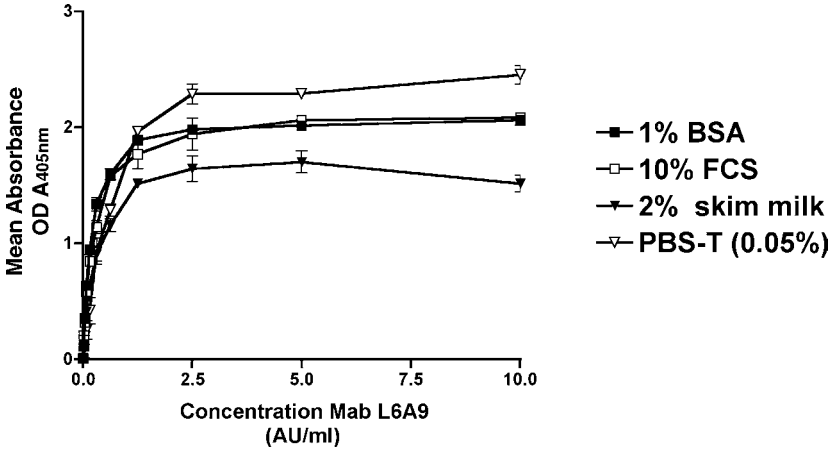

Fig. 3. Comparison of four different blocking agents (bovine serum albumin [BSA] 1%, fetal calf serum [FCS] 10%, skim milk 2%, or Tween-20 [PBS-T] 0.05%) diluted in PBS with serial dilutions of *H. influenzae* (*Hi*) mAb L6A9 (starting at 1/1000 dilution equivalent to 10 AU/mL). These are indicated by solid square, open square, solid triangle, and open triangle, respectively. Mean absorbance (A_{450nm}) was determined by standard LPS ELISA using coating concentration of 10 μg/mL of *Hi* inner-core LPS.

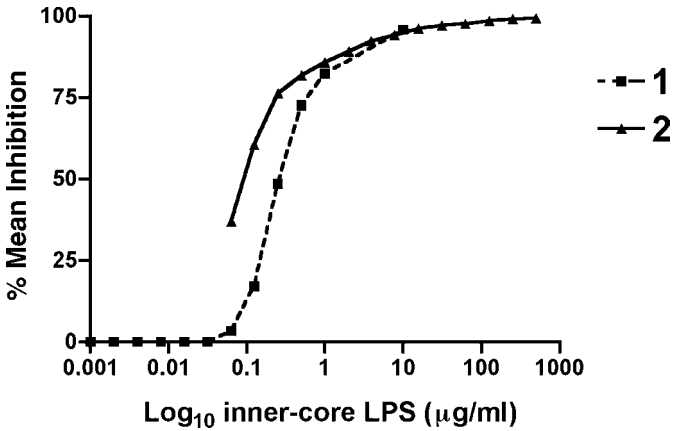

Fig. 4. Competitive inhibition experiments with inner-core lipopolysaccharide (LPS): mean inhibition (%) of *H. influenzae* (*Hi*) mAb L6A9 to inner-core *Hi* LPS by serial dilutions of mAb pre-incubated with different concentrations of purified *Hi* inner-core LPS (500 μg/mL to 10 ng/mL) determined by standard inner-core LPS ELISA. Percent inhibition for each dilution was calculated from absorbance of non-absorbed mAb L6A9. Results of two independent experiments are shown as 1 and 2.

able enzyme-labeled secondary antibody or mAb conjugated directly to enzyme ***(7,12)***.

1.2.2. Avidity Studies Using ELISA

The standard ELISA protocol can be adapted to determine relative avidity of the antibody by the addition of chaotropic agent. This can be achieved by either: (1) an additional 15-min incubation step using ammonium thiocyanate (NH_4SCN) after the primary antibody incubation, before conjugate/detection step ***(13,14)*** or (2) inclusion of ammonium thiocyanate in the diluent buffer ***(15)***. The relative difference in antibody binding to inner-core LPS with the addition of NH_4SCN (0.5 *M*) compared to without NH_4SCN can give an indication of relative avidity of the antibody or mAb. The relative avidity can be expressed as an avidity index ***(16)*** (*see also* **Note 8**).

1.3. Interpretation of Results

In order to quantitate the antibody response (IgG, IgM) to a specific vaccine antigen there is first a need to develop an immunogenicity assay to detect specific antibodies that bind to the antigen, e.g., inner-core LPS. In the absence of a vaccine, as is the case with non-typeable *Hi*, and where there are no known standards, the assay must be developed using arbitrary units (AU). Monoclonal antibodies raised to specific antigens provide invaluable tools to assign AU and therefore develop and test the specificity of a new ELISA.

Arbitrary units can be assigned to a known dilution of pooled sera or mAb and, using a standard curve and AU/mL can be assigned to an unknown sample. These units can then be quantified in terms of µg/mL. For example, from a known concentration of IgG (µg/mL) in a particular standard, determined following antibody purification and protein assay, the equivalent concentration of antibody can be assigned to the AU. Heterologous ELISAs can also be used to compare standards of known antibody concentration with unknown sera/mAbs in a semi-quantitative way. This is determined by comparing OD results of ELISAs performed at the same time on the same plate, for example, Hib standard (FDA standard) for IgG or IgM on a Hib ELISA can be compared to an *Hi* ELISA to semi-quantitative unknown serum samples.

1.4. Applications

In this chapter we describe the use of an LPS ELISA to determine quantity, quality, and specificity of inner-core LPS antibodies in healthy and diseased individuals (adults and children) with *H. influenzae* infections. For example, the prevalence of inner-core LPS *Hi* antibodies in healthy adult (HC) ($n = 3$), and infant sera ($n = 7$) were compared using *Hi* inner-core LPS ELISA. Adult sera have a range of *Hi* inner-core LPS antibodies, reflecting exposure or car-

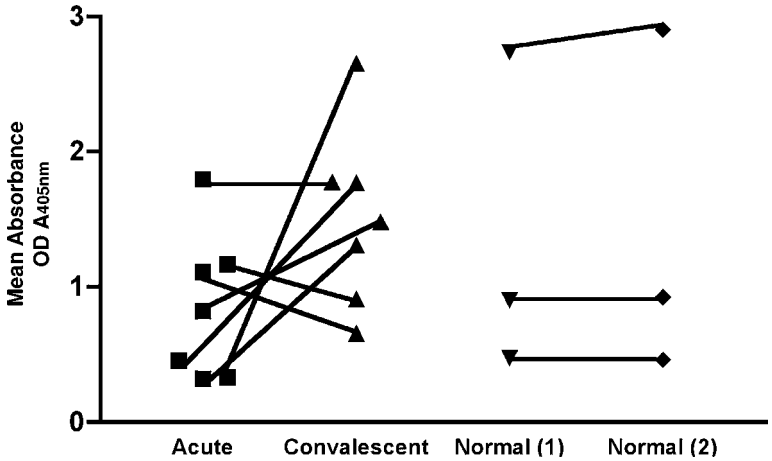

Fig. 5. Inner-core *H. influenzae* (*Hi*) lipopolysaccharide (LPS) antibodies in paired infant sera serum samples taken early (acute) and late (convalescent) during invasive *Hi* type B disease indicated by solid squares and solid up triangles respectively ($N = 7$). Mean absorbance (A_{450nm}) was determined using the standard *Hi* LPS ELISA. Inner-core *Hi* LPS antibodies in sera from three healthy adults were determined in two independent experiments (normal 1, normal 2) indicated by solid down triangle and solid diamond.

riage of the organism at some time (**Fig. 5**). There was a significant difference in the mean OD between acute and convalescent sera in these infants ($P < 0.05$, suggesting the acquisition of inner-core LPS antibodies with exposure to *Hi* (**Fig. 5**). These results suggest that the inner-core LPS epitope is accessible to *Hi* and may have a role in protection. The function of these antibodies can be determined using opsonophagocytosis (OP) and serum bactericidal assays (SBA) as correlates of protection.

The ELISA can be used to screen an age-stratified serum bank to determine sero-epidemiology of potential vaccine candidates, especially for *Hi*. The use of fine structural analysis of LPS by mass spectrometry (MS) and nuclear magnetic resonance (NMR) (*see* Chapter 11) combined with genetics (*see* Chapter 10) can help to identify and map potential epitope(s) in detail to identify vaccine candidates.

To determine the specificity and cross-reactivity of the ELISA, we have been able to use mAbs raised to defined LPS structures and defined LPS of known structure derived from genetically defined mutants (*see,* for example, **ref. *16***). Fine structural analysis of LPS by electrospray-mass spectrometry (ES)-MS and NMR can define the structural epitope recognized by the mAb. For example, cross-reactivity can be seen with *Hi* mAbs and a range of truncated inner-core LPS of known structure. A series of 4 mAbs raised to the inner-core

LPS demonstrated three different patterns of cross-reactivity with different truncated inner-core LPS. mAb 2 and mAb 4 recognize structure "a" only, whereas mAb1 cross-reacts with structures "a," "b," and "c" and mAb 3 recognized only "a" and "b" (**Table 3** and **Fig. 6**). An understanding of the detailed structures of these LPS can be used to determine the epitope(s) for each of the mAbs (**Fig. 6**) ***(16–18).*** This approach has also been successfully used with inner-core LPS of *Nm* to determine the epitope for *Nm* mAb L3B5 ***(10)***.

2. Materials

2.1. Bacterial Strains and Culture

LPS samples for ELISA and inhibition studies were prepared from *H. influenzae* wild-type Eagan (disease isolate from USA) ***(19)***; and *Hi* inner-core LPS mutant) derived from Eagan RM153 *Hi* wild-type strain (*see* **Subheading 3.1.**).

2.2. Purified Lipopolysaccharide

LPS samples for ELISA and inhibition studies were obtained as previously described using standard hot phenol/water extraction ***(20)***. (*See* **Subheading 3.2.**). Stock solutions were prepared from 1 mg/mL purified LPS dissolved in PBS or coating buffer.

2.3. Source of Human Sera

Venous blood obtained from healthy control sera from adults and infants, and from infants with Hib disease, were separated and serum frozen at −20°C in aliquots.

2.4. Murine Monoclonal Antibodies to Inner-Core LPS

Monoclonal antibody, designated mAb L6A9, was selected from a set of 13 murine monoclonal antibodies raised to formalin-killed whole cells of *Hi* inner-core LPS mutant strains. *See* **Subheading 3.4.** for standard preparation of mAb and screening.

2.5. Microtiter Plates for ELISA

Nunc Maxisorp plates were used for this assay. Optimal antigen coating concentration was 10 µg/mL LPS in bicarbonate coating buffer.

2.6. Preparation of Buffers for ELISA

1. Coating buffer: 0.05 *M* carbonate buffer pH 9.8 with 0.02 *M* $MgCl_2$. Check pH 9.8 (very important for solubilizing LPS).
2. Blocking buffer: 1% bovine serum albumin (Sigma, A-4161) prepared in phosphate-buffered saline (prepared from 10 tablets per 1 L distilled water, Oxoid). Washing buffer: 0.05% Tween-20 in phosphate buffered saline (PBS-T).
3. For affinity step: 0.5 *M* ammonium thiocyanate, prepared fresh each time.

Table 3
Cross Reactivity Studies with Monoclonal Antibodies to a d*Hi* Inner-Core Lipopolysaccharide

	OD_{A405nm} LPS Structure on ELISA plate[a]		
MAbs (isotype)	a	b	c
MAb 1 (IgG_{2b})	+++[b]	++	+++
MAb 1(IgG_3)	++	−	−
MAb 3 (IgG_3)	+	++	−
MAb 4 (IgG_3)	++	−	−

[a]*See* **Fig. 6** for structures of inner-core LPS a, b, c.
[b]+++, OD_{A405nm} 1.8; ++, OD_{A405nm}; +, > OD_{A405nm} 0.5; −, Negative > OD_{A405nm} 0.1.

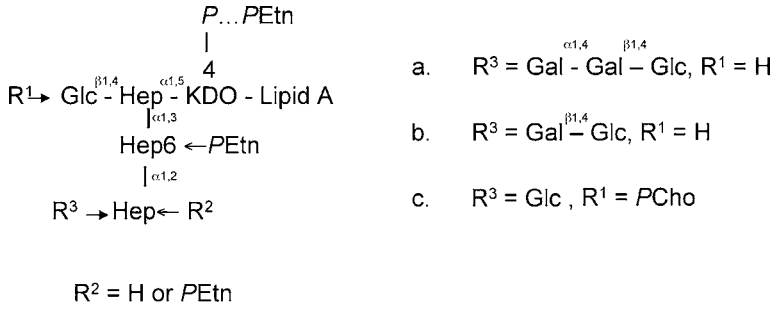

Fig. 6. Structural model of *H. influenzae* LPS showing the conserved L-*glycero*-D-*manno*-heptosyl trisaccharide inner-core region (*Hep*I–*Hep*III) observed in every strain investigated to date (*see* ***17***, and references therein). *Hep*I is linked to the lipid A portion of the molecule via a 2-keto-3-deoxyoctulosonic acid residue (KDO). KDO carries a phosphate (*P*) or pyrophosphoethanolamine (*P*-*P*Etn) substituents at the O-4 position. The triheptosyl inner-core unit is substituted at a β-D-glucose residue (Glc) at the O-4 position of *Hep*I and by a phosphoethanolamine residue (*P*Etn) at the O-6 position of *Hep*II. In some *Hi* strains, this Glc residue is substituted at the O-6 position by phosphocholine (R1 = *P*Cho) (*see* ***18***). In the LPS from the strain used for generating the MAbs used for cross reactivity studies in **Table 3**, *Hep*III was partially substituted by *P*Etn (R2 = *P*Etn or H.) Structural analysis indicated that in the major LPS glycoform population *Hep*III is substituted at the O-2 position by globotriose side chains (**a**). Strains elaborating LPS contained lactose (**b**), and glucose (**c**) side chains were generated by specific mutation of genes involved in oligosaccharide chain biosynthesis (*see* **ref. 16***).* These LPS were used for cross reactivity studies (**Table 3**).

2.7. Preparation of Secondary Antibodies

Alkaline phosphatase conjugates antihuman or anti-mouse IgG and IgM (Sigma) were prepared in PBS-T diluted 1/1000–1/2000, determined empirically.

2.8. Preparation of Substrate Reagent

P-nitrophenylphosphate tablets (Sigma 1 mg/mL) were solubilized in diethanolamine buffer, pH 9.8 (Don Whitley) (2 tablets per 10 mL) and protected from light with foil. Prepared fresh and thoroughly mixed prior to use.

2.9. Stopping Buffer

3 *M* NaOH was prepared from 9 *N* NaOH in distilled water.

2.10. Equipment

Plates were washed using a plate washer (Thermo LifeSciences) programmed for dispensing 3 × 400 µL per well of PBS-T. Plates were read at OD_{A405} using Dynatech ELISA plate reader (Thermo LifeSciences) and data collected using Revelations software.

3. Methods

3.1 Bacterial Culture

Hi were grown overnight under standard conditions at 37°C in brain heart infusion (BHI) broth supplemented with hemin (10 mg/mL) and NAD (2 mg/mL) (5 L batches) (*see* **Note 1**).

3.2. LPS Extraction

Briefly, cells from 5-L batch cultures were harvested from an overnight growth and LPS was extracted by the hot-phenol/water method as described ***(20)*** and purified from the aqueous phase by repeated ultracentrifugation (105,000*g*, 4°C, 2 × 5 h) ***(21)***. Stock solutions of LPS were prepared in PBS or coating buffer (1 mg/mL) (*see* **Note 2**). MS analysis of purified *Hi* LPS was carried out on O-deacylated LPS ***(17)*** using ES-MS and NMR analysis ***(16)***.

3.3. Human Serum Samples

Adult control sera were obtained from healthy adult volunteers, pooled, and stored at −20°C for the standard curve. Normal adult sera were collected from healthy volunteers and used as normal controls (high, medium, low titers) (*see* **Note 3**). Paired acute and convalescent sera were collected from children with Hib disease from the British Paediatric Surveillance Unit/Hib vaccine failure study.

3.4. Protocol for Preparation of Murine Monoclonal Antibodies

Briefly, 6–8-wk-old BALB/c mice were immunized three times intraperitoneally followed by once intravenously, with formalin-killed whole cells of

inner-core LPS mutant of Eagan (10^8 cells per injection). Hybridomas were prepared by fusion of spleen cells with SP2/O-Ag 14 ***(22)*** as described by Carlin et al. ***(23)***. mAbL6A9 was screened with inner-core LPS ***(11)***. mAb L6A9 was used to show the specificity of inner-core LPS ELISA by direct inhibition using purified inner-core LPS. A second series of *Hi* mAbs was generated in a similar way using whole cells of a *Hi* inner-core LPS mutant as described in **Subheading 2.4.** Three fusions were screened using a number of truncated LPS from inner-core LPS mutants.

3.5. Standard Protocol for Inner-Core LPS ELISA

This method was adapted from ELISA using inner-core LPS from *Nm galE* mutant ***(7)***. Briefly, flat-bottom 96-well microtiter plates (Nunc Maxisorp) were coated (100 µL/well) with inner-core LPS, extracted from LPS inner-core mutant, diluted in 0.05 *M* carbonate buffer (pH 9.8) with 0.02 *M* $MgCl_2$ (10 µg/mL) (*see* **Note 4**). Plates were sealed with clear tape and incubated for 3 h at 37°C and stored at 4°C for 1–2 wk (*see* **Note 5**). The antigen was discarded, nonspecific binding sites were blocked for 1 h at room temperature with 1% BSA-PBS (Sigma A4161) (*see* **Note 6**) and then the plate was washed three times with PBS-T (0.05% v/v Tween-20). Dilutions of test or control sera (1/50) or murine MAb, e.g., mAb *Hi* L6A9 in 1% BSA-PBS were added to the plate (100 µL/well), and incubated for 1 h at room temperature. The plate was washed three times with PBS-T. Human antibodies were detected using alkaline phosphatase conjugated to goat antihuman IgG (Fc) or IgM (Sigma) for 1 h at room temperature followed by washing (*see* **Note 7**), and 100 µL *p*-nitrophenylphosphate (Sigma) in diethanolamine buffer, pH 9.8 (1 mg/mL) for 30 min at room temperature (Don Whitley Scientific). The reaction was stopped with 3 *M* NaOH (50 µL) and absorbance was read at a wavelength of 405 nm (Titertek Multiscan MCC/240). Mouse antibodies were detected using alkaline phosphatase conjugated to goat anti-mouse IgG (Fc) (Sigma). A log-linear standard curve was plotted using arbitrary units, and antibody concentration of test samples was calculated using Revelations software (Thermo LifeSciences). Arbitrary units were assigned to neat MAb L6A9 culture supernatant of 10,000 AU.

3.6. Adaption for Avidity ELISA

As standard protocol, except after primary antibody and washing with PBS-T, there is an additional 15 min step before secondary antibody conjugate/detection, adding 100 µL to each well of 0.5 *M* ammonium thiocyanate. After exactly 15 min, wells are washed with PBS-T and secondary antibody added as usual. It is best to use half plate with/without SCN to calculate relative avidity index on the same plate (*see* **Note 8**).

3.7. Statistical Analysis of Results

Student's *t* test was used to assess the statistical significance of differences in IgG and IgM measured between the groups. The independent samples test was used when the comparison was between two separate groups of individuals (healthy and diseased), whereas the paired *t* test was used when the comparisons were between two time points for the same children (acute and convalescing). Each group is summarized using mean OD. Two-tailed *p* values are quoted throughout. Analysis was performed using Stata software (Stat Corp 97, Stat Statistical Software: Release 5.0, Stata, College Station, TX).

4. Notes

1. Bacterial growth conditions are important to standardize and growth in BHI broth was preferred to solid agar for extracting LPS from *H .influenzae*.
2. LPS batches can vary in purity. Each new batch of antigen should be tested for purity on SDS-PAGE and tested with the new plates to determine best coating concentration.
3. Each new plate batch should be tested prior to extensive use with appropriate control sera (high, medium, low titer) to determine inter- and intracoefficient of variations and to detect any variations in assay reproducibility.
4. The pH of the coating buffer is important for solubilizing LPS (pH 9.8 optimal). You may need to mix LPS coating solution well by pipet, by vortex, or by sonication to disperse evenly in solution, avoiding overheating and aerosol formation. Sometimes LPS solution goes cloudy, but this does not affect LPS coating of plate.
5. Shelf life for coated plates at 4°C is 1–2 wk.
6. The blocking step can be carried out overnight at 4°C or for 1–2 h at room temperature or for 30 min at 37°C. The purity of the BSA can affect the assay (fraction V). For *Nm* and *Hi,* higher-grade BSA gave lower nonspecific binding than lower ELISA grade.
7. With each new lot, the optimal concentration of secondary antibody should be empirically tested using a checkerboard of dilutions, e.g., a range of dilutions 1/500–1/10,000 with the standard curve.
8. Avidity index (AI) can be calculated as (OD_{A405nm} without NH_4SCN — OD_{A405nm} with NH_4SCN/OD_{A405nm} without $NH_4SCN \times 100$) (%), or 50% AI can be determined using a range of ammonium thiocyanate from 0.5 *M* to 5 *M*.

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References

1. Farr, R. S. (1958) A quantitative immunochemical measure of the primary interaction between I*BSA and antibody. *J. Infect. Dis.* **103,** 239–262.
2. Phipps, D. C., West, J., Eby, R., Koster, M., Madore, D. V., and Quataert, S. A. (1990) An ELISA employing a *Haemophilus influenzae* type b oligosaccharide-human serum albumin conjugate correlates with radio-antigen binding assay. *J. Immunol. Meth.* **135,** 121–128.
3. Gray, B. M. (1979) ELISA methodology for polysaccharide antigens: protein coupling of polysaccharides for adsorption to plastic tubes. *J. Immunol. Meth.* **28,** 187–192
4. Carlone, G. M., Frasch, C. E., Siber, G. R., et al. Multicenter comparison of levels of antibody to the *Neisseria meningitidi*s Group A capsular polysaccharide measured by using an enzyme-linked immunosorbent assay. *J. Clin. Microbiol.* **30,** 154–159.
5. Gheesling, L. L., Carlone, G. M., Pais, L. B., et al. (1994) Multicenter comparison of *Neisseria meningitidis* serogroup C anti-capsular polysaccharide antibody levels measured by a standardised enzyme-linked immunosorbent assay. *J.Clin. Microbiol.* **32,** 1475–1482.
6. Wood, H. C. and Wreghitt, T. G. (1990) Techniques, in ELISA in the Clinical Microbiology Laboratory (Wreghitt, T. G., and Morgan-Capner, P., eds.), Laverham Press, Whaddon, Salisbury (Public Health Laboratory Service series).
7. Plested, J. S., Gidney, M. A. J., Coull, P. A., et al. (2000) Enzyme linked immunosorbent assay (ELISA) for the detection of serum antibodies to the inner-core lipopolysaccharide of *Neisseria meningitidis* Group B. *J. Immunol. Methods* **237,** 73–84.
8. Abdillahi, H. and J. T. Poolman. (1988) Typing of group-B *Neisseria meningitidis* with monoclonal antibodies in the whole-cell ELISA. *J. Med. Microbiol.* **26,** 177–180.
9. Pollard, A. J., Galassini, R., Rouppe van der Voort, E. M., et al. (1999) Humoral immune responses to *Neisseria meningitidis* in children. *Infect. Immumol.* **67,** 2441–2451.
10. Plested, J. S., Makepeace, K., Jennings, M. P., et al. (1999) Conservation and accessibility of an inner-core lipopolysaccharide epitope of *Neisseria meningitidis*. *Infect. Immunol.* **67,** 5417–5426.
11. Gidney, M. A. J., Lacelle, S., Cox, A. D., Martin, A., Moxon, E. R., and Richards, J. J. Production and characterisation of a monoclonal antibody (L6A9) against inner-core epitope in *Haemophilus influenzae* lipopolysaccharide. (in preparation).
12. Estabrook, M. M, Baker, C. J., and Griffis, J. M. (1993) The immune response of children to meningococcal lipooligosaccharides during disseminated disease is directed primarily against two monoclonal antibody-defined epitopes. *J. Infect. Dis.* **167,** 966–970.
13. Goldblatt, D., Vaz, A. R., and Miller, E. (1998) Antibody avidity as a surrogate marker of successful priming by *Haemophilus influenzae* type b conjugate vaccines following infant immunization. *J. Infect. Dis.* **117,** 1112–1115.
14. Anttila, M., Voutilainen, M., Jantti, V., et al. (1999) Contribution of serotype-specific IgG concentration, IgG subclasses and relative antibody avidity to

opsonophagocytic activity against *Streptococcus pneumoniae*. *Clin. Exp. Immunol.* **118,** 402–407.

15. Granoff, D. M., Maslanka, S. E., Carlone, G. M., et al. (1998) A modified enzyme-linked immunosorbent assay for measurement of antibody responses to meningococcal C polysaccharide that correlate with bactericidal responses. *Clin. Diagn. Lab. Immunol.* **5,** 479–485
16. Hood, D. W., Cox, A. D., Wakaarhuk, W. W., et al. (2001) Genetic basis for expression of the major globotetraose-containing lipopolysaccharide from *H. influenzae* strain Rd (RM118). *Glycobiol.* **11,** 957–967.
17. Mansson, M., Bauer, S. H., Hood, D. W., et al. (2001) A new structural type for *Haemophilus influenzae* lipopolysaccharide from nontypeable *Haemophilus influenzae* strain 486. *Eur. J. Biochem.* **268,** 2148–2159.
18. Risberg, A., Masoud, H., Martin, A., et al., (1999) Structural analysis of the lipopolysaccharide oligosaccharide epitopes expressed by a capsule-deficient strain of *Haemophilus influenzae* Rd. *Eur. J. Biochem.* **261,** 171–180.
19. Anderson, P., Johnston, R., and Smith, D. (1972) Human serum activities against *Haemophilus influenzae* type b. *J. Clin. Invest.* **51,** 31–38.
20. Westphal, O., and Jann., J. K. (1965) Bacterial lipopolysaccharides extraction with water-phenol and further applications of the procedure. *Methods Carbohydr. Chem.* **5,** 83–91.
21. Masoud, H., Moxon, E. R., Martin, A., et al. (1997) Structure of the variable and conserved lipopolysaccharide oligosaccharide epitopes expressed by *Haemophilus influenzae* serotype b Eagan. *Biochemistry* **36,** 2091–2103.
22. Shulman, M., Wilde, C. D., and Kohler, G. (1978) A better cell line for making hybridomas secreting specific antibodies. *Nature* **276,** 269–270.
23. Carlin, N. I., Gidney, M. A., Lindberg, A. A., and Bundle, D. R. (1986) Characterisation of Shigella flexneri-specific murine monoclonal antibodies by chemically defined glycoconjugates. J. *Immunol.* **137,** 2361–2366.
24. Ito, J. I., Wunderlich, A. C., Lyons, J., et al. (1980) Role for magnesium in the enzyme-linked immunosorbent assay for lipopolysaccharides of rough *Escherichia coli* strain J15 and *Neisseria gonorrhoeae*. *J. Infect. Dis.* **142,** 532–537.
25. Law, B. J. and Marks, M. I. (1985) Age-related prevelance of human serum IgG and IgM antibody to the core glycolipid of *Escherichia coli* strain J5, as measured by ELISA. *J. Infect. Dis.* **151,** 988–994.
26. Freudenberg, M. A., Fomsgaard, A., Mitov, I., and Galanos, C. (1989) ELISA for antibodies to lipid A, lipopolysaccharides and other hydrophilic antigens. *Infection* **17,** 322–328.
27. Kuhn, H. M., Brade, L., Appelmelk, B. J., et al. (1992) Characterisation of the epitope specificity of murine monoclonal antibodies directed against lipid A. *Infect. Immunol.* **60,** 2201–2210.
28. Petrov, A. B., Semenov, B. F., Vrtanyan, Y. P., et al. (1992) Toxicity and immunogenicity of *Neisseria meningitidis* lipopolysaccharide incorporated into liposomes. *Infect. Immunol.* **60,** 3897–3903.

29. Appelmelk, B. J., Verweij-Van Vaught, A. M., MacLaren, D. M., and Thijs, L. G. (1985) An enzyme-linked immunosorbent assay (ELISA) for the measurement of antibodies to different parts of the gram-negative lipopolysaccharide core region. *J. Immunol. Meth.* **82,** 199–207.
30. Kirkland, T. M., Colwell, D. E., Michalek, S. M., et al. (1986) Analysis of the fine specificity and cross-reactivity of monoclonal anti-lipid A antibodies. *J. Immunol.* **137,** 3614–3619.
31. Stoll, C., Schedel, I., and Peest, D. (1985) Serum antibodies against common antigens of bacterial lipopolysaccharides in healthy adults and in patients with multiple myeloma. *Infection* **13**, 115–119.
32. Erich, T., Schellekends, J., Bouter, A., et al. (1989) Binding characteristics and cross-reactivity of three different antilipid A monoclonal antibodies. *J. Immunol.* **143,** 4053–4060.
33. Lagace, J., Arsenault, S., and Cohen, E. H. (1994) Alcian blue-treated polystyrene microtitre plates for use in an ELISA to measure antibodies agains synthetic peptides. *J. Immunol. Meth.* **175,** 131–135.
34. Hardy, E., Ohlin, M., and Llano, M. (1994) Enhanced ELISA sensitivity using TCA for efficient coating of biologically active lipopolysaccharides for lipid A to the solid phase. *J. Immunol. Meth.* **176,** 111–116.

16

Opsonophagocytosis Assay Using Flow-Cytometry

Joyce S. Plested and Philip A. Coull

1. Introduction

We describe the development of a flow-cytometric-based opsonophagocytosis (OP) assay that is capable of looking at antigen-specific antibodies, that is, complement-dependent, not complement-mediated. Using the OP assay we are able to look at uptake of bacteria by neutrophils simultaneously with oxidative burst within the cell. This assay using human donor peripheral blood polymorphonuclear cells (PMNs) is rapid, reproducible, and specific. Other OP assays described use HL-60 cell lines that are technically more difficult to use and require tissue culture facilities ***(1)***.

The assay was originally developed from methods described by Lehmann et al. ***(2)*** using ethanol-fixed *Neisseria meningitidis* (*Nm*) and lipopolysaccharide-coated beads ***(3)*** and has been applied to *Haemophilus influenzae* (type b and nontypeable). The principle of OP assay is outlined in **Fig. 1** (bacteria stained with rhodamine green X or rhodamine red X). Adaptation of the OP assay to look at oxidative burst with addition of dihydrorhodamine (DHR) with Rhodamine red beads/bacteria is outlined in **Fig. 2** ***(2,3)***.

Other functional assays are available to study serum antibodies, e.g., serum bactericidal assays but these are dependent on target strain and source of complement used ***(4,5)***. Other assays that measure function of antibodies in the presence of PMNs include the OP killing assay ***(1)***, and the whole blood assay ***(6)***.

1.1. Optimizing Variables of OP Assay

1.1.1. Preparation of the Bacteria

For practical reasons it is safer to use fixed rather than live bacteria in the OP assay. Previous workers have compared methods of fixation, including heat, ethanol, or formaldehyde, and concluded that similar results were seen with

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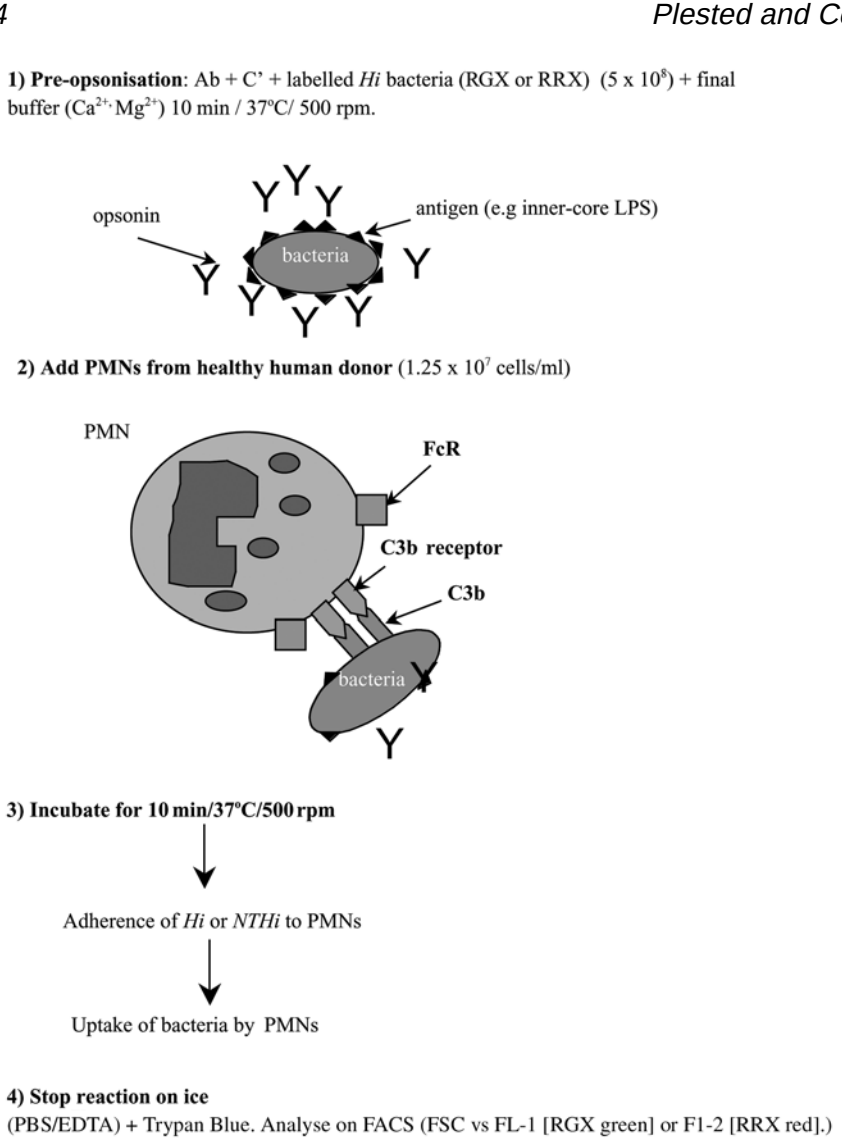

Fig. 1. Opsonophagocytosis assay protocol using a flow-cytometric based assay.

ethanol-fixed bacteria ***(2)***. In our experience with *Nm* comparing live and ethanol-fixed bacteria in surface-labeling experiments with mAb B5, similar trends were observed, with highest binding with live vs fixed bacteria ***(3)***. Batch-to-batch variations in the staining of bacteria can occur, and each new batch should be tested. Optimal staining was with 10^9–10^{10} *Nm* or *Hi* organisms/mL. Growth phase may also have an effect on the efficiency of labeling. For reproducibility of staining, stationary-phase organisms were used for these studies.

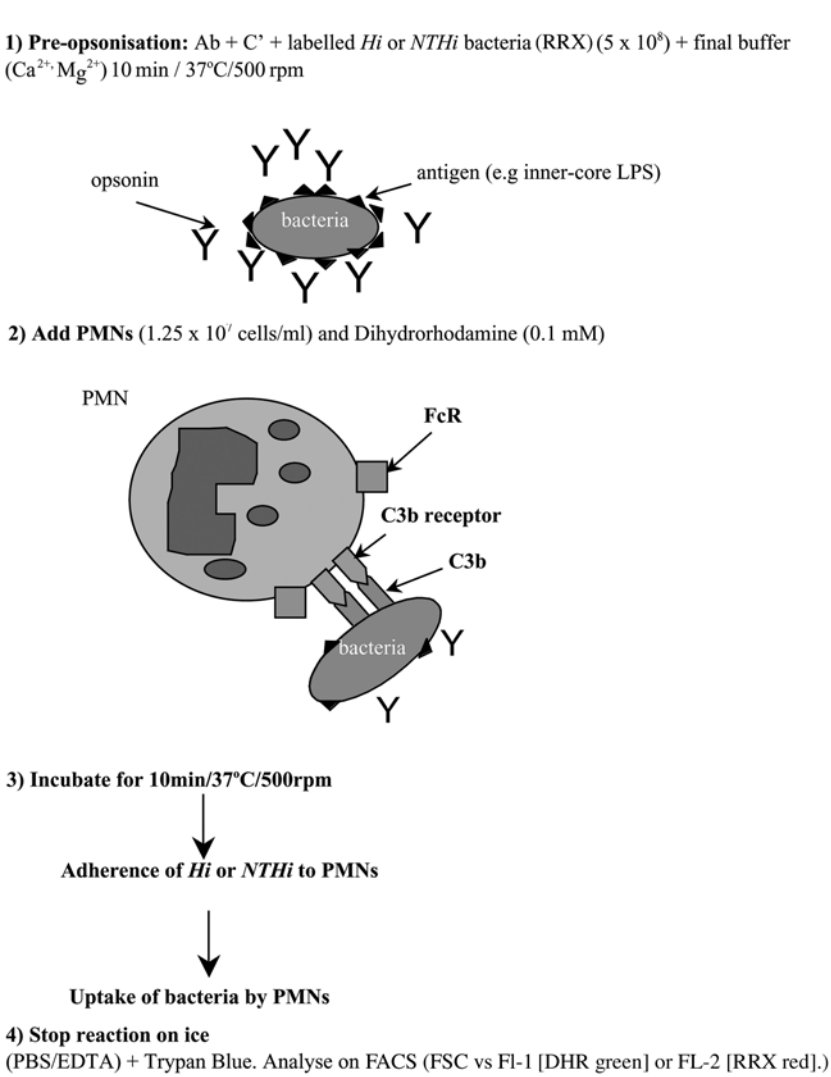

Fig. 2. Opsonophagocytosis assay with oxidative burst using dihydrorhodamine.

1.1.2. Source of Donor Neutrophils.

For these studies, human donor PMNs were used as the neutrophil source and found to be highly reproducible ***(2,3)***. Alternatives to human donors are various cell culture lines, such as HL-60 cells ***(1)***. These cell lines are technically far more difficult to maintain, have problems of contamination and obtaining differentiated stage cells, and need tissue culture facilities; however, these are being used in a standardized *Streptococcus pneumoniae* OP assay that is

flow-cytometric-based *(7)*. Previous workers have shown that the ratio of bacteria to neutrophils is crucial for the OP assay *(2)*, with a ratio of 40:1 optimal in our hands *(3)*. The method of preparation of the PMNs is key to the success of the assay; unlike other assays that use lymphopreps to separate lymphocytes, both lymphocytes and nonlymphocytes (granulocytes and monocytes) are used in the assay, and gates are set on the flow cytometer to look at the cell populations of interest. These gates can be checked using suitable surface markers to identify neutrophil (granulocyte, monocyte) population (e.g., CD15). The PMNs must be prepared from freshly lysed heparinized blood and gently treated with suitable buffers to avoid clumping effects (*see* **Note 5**).

1.1.3. Temperature of Incubation of the OP Assay

The phagocytosis rate has been shown to increase exponentially with temperature up to 39–40°C *(2)*; however, for reproducibility, the best temperature is 37°C. Slight fluctuations in temperature during the incubation time can greatly affect the uptake of bacteria by the PMNs and should be carefully controlled. It is important that the cells and opsonized bacteria are agitated gently during the incubation time, as this interaction is important for uptake.

1.1.4. Complement Source

The role of complement is key to the OP assay, but unlike the serum bactericidal assay (SBA), in which the assay is totally complement-dependent, in the OP assay it is the combination of antigen-specific antibodies and complement that is required. Complement controls alone show very low activity, demonstrating that it is the synergistic effect of specific antibodies in the presence of complement that is being measured with this assay. The source of complement and storage are important for a reproducible assay (*see* **Note 2**).

1.2. Specificity of the OP Assay

The ability to measure the function of specific antibodies is one of the advantages of the OP assay over other functional assays, e.g., the SBA. Correlations can be shown between the presence of specific antibodies and function in the OP assay (*see* **Note 3**). Inhibition studies with purified LPS can be used to show specificity of antibodies in the OP assay (*see* **Subheading 1.2.1.**). Other specific surface antigens of bacteria may have effects on the OP assay, e.g., *Nm* opacity protein binding to CD66 receptor *(8)*. There are many advantages to using genetically defined bacteria of known phenotype to eliminate these other possible factors, for example, *Nm* Opa- Opc- Pil- Cap- deficient mutant can compared to wild-type *Nm* to demonstrate that antibodies to these components are not contributing to OP activity *(3)*.

1.2.1. Inhibition Studies Using Purified Lipopolysaccharide

Preincubation of serum with purified antigen, for example, lipopolysaccharide (LPS) derived from LPS mutant, compared to nonadsorbed serum, can give an indication of the specific OP activity attributed to these specific LPS antibodies (*see* **Note 3**). It is important to use appropriate controls for inhibition studies to ensure that reduction in OP activity is not due to toxic effects of LPS on the PMNs. For example, for inner-core *Hi* antibodies *Eschericia coli* LPS was used as a control LPS to determine effect of lipid A toxicity on PMNs in an OP assay. Monoclonal antibodies (mAbs) that are are opsonic against target bacteria are useful tools to show specificity of the OP assay, for example, mAb B5 and *galE* LPS for *Nm* wild-type and mutant ***(3)***.

1.3. Applications

The flow-cytometric based OP assay can be applied to any Gram-negative or Gram-positive bacteria that can be labeled, to look for opsonins or identify potential vaccine candidates. The OP assay can be used to quantify OP activity and oxidative burst (intracellular killing) within the neutrophil simultaneously. Adaptation of the assay to use specific antigen-coated beads enables quantitation of exact numbers of beads taken up by a neutrophil ***(2)***. Single antigen coating of different fluorescently stained latex beads can enable more than one antigen to be studied simultaneously, e.g., for OP antibodies to type specific *S. pneumoniae* polysaccharides ***(7)***. Studies with Men C-conjugate specific antibodies demonstrated good correlation between OP and serum bactericidal activity for capsular polysaccharide antibodies postvaccination (unpublished data). The OP assay may prove to be a suitable replacement for the SBA as a correlate of protection in some cases, for example, where specific antibodies are opsonic not bactericidal, but are able to protect against disease. OP activity can be determined in sera with complement intact (stored immediately at −80°C) or in sera with complement inactivated (by heat or storage conditions [4°C]) with addition of heterologous complement source. *Hi* LPS antibodies in healthy control sera with addition of heterologous human complement source have opsonic activity against target bacteria, for example, *Hi* inner-core LPS mutant, and Eagan wild-type and NTHi using the OP assay (*see* **Fig. 3**). The use of mAbs to show specificity can be demonstrated using *Hi* mAb L6A9 raised to inner-core LPS mutant, which has opsonic activity against target bacteria *Hi* inner-core LPS mutant but not *Hi* wild-type (**Fig. 4**).

2. Materials

2.1. Bacterial Strains

H. influenzae wild-type Eagan and inner-core LPS mutant derived from Eagan; *Hi* wild-type strain 375, and inner-core LPS mutant derived from strain

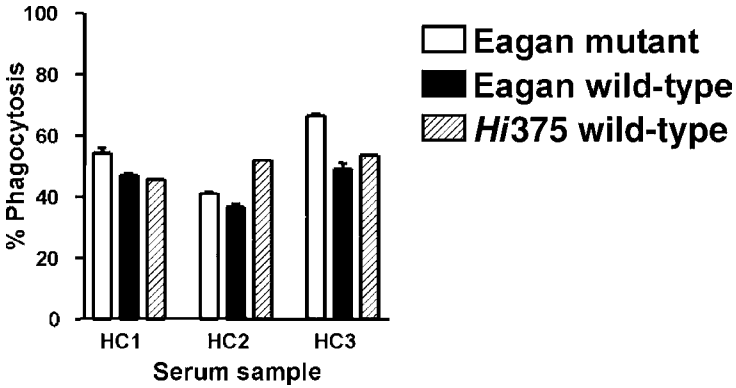

Fig. 3. Opsonophagocytosis assay with healthy control sera and *Hi* Eagan wild-type, inner-core LPS mutant, or *Hi*375 wild-type. Mean percentage phagocytosis of ethanol-fixed, labeled *H. influenzae Hi* inner-core mutant, Eagan wild-type, and *Hi* 375 wild-type with three healthy adult control sera (HCl-3) and human donor neutrophils in the presence of human complement source. Complement alone was 2.43%, 2.8%, 3.04%, and final buffer alone was 2.1%, 1.86%, 1.13% for Eagan wild-type, Eagan mutant, and *Hi*375 wild-type, respectively.

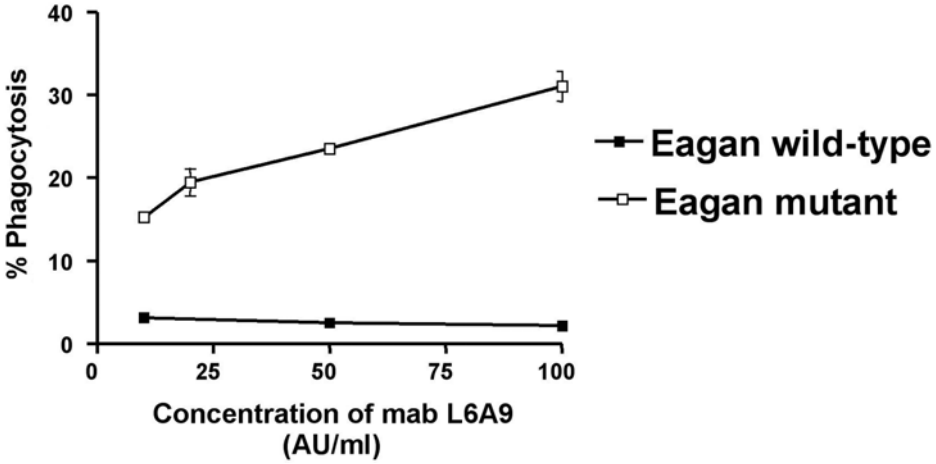

Fig. 4. Opsonophagocytosis assay with mAb L6A9 with *Hi* Eagan wild-type and inner-core LPS mutant. Mean percentage phagocytosis (% uptake) of ethanol-fixed, labeled *H. influenze* Eagan wild-type and Eagan inner-core (LPS) mutant. Uptake is measured against dilutions of murine mAb L6A9, specific for Eagan inner-core LPS mutant, in the presence of human complement. Complement alone 2.8%, 2.43%, and final buffer 0.86%, 2.1%, for Eagan mutant and wild-type, respectively.

NTHi were grown in brain heart infusion (BHI) broth as described previously (*see* Chapter 15). Bacteria were washed once in phosphate-buffered-saline (PBS) and then ethanol-fixed (70% v/v) for 30 min prior to labeling (*see* **Subheading 3.1.**).

2.1.1. Preparing Stock Solutions for Labeling of Bacterial Strains Using Rhodamine Green-X (RGX) and Rhodamine Red-X (RRX)

1. 0.9% NaCl to wash cells (70% ethanol-fixed 30 min at room temperature).
2. 0.2 *M* sodium bicarbonate buffer, pH 8.2, freshly prepared.
3. Stock solutions: rhodamine green X (Molecular Probes, R6163); rhodamine red X (Molecular Probes, succinimidyl ester, mixed isomers, R6160) (5 mg/3 mL dimethyl sulfoxide [DMSO] stored at −80°C).
4. Stopping buffer: hydroxylamine-HCl (pH 8.5).
5. Nytal mesh (11 µm).
6. Microfuge (14,000*g*).
7. Eppendorf tubes (1.5 mL).
8. Plastic Pasteur pipets (wide-end), for suspending cells.

2.1.2. FACS Surface Labeling

1. 1% w/v bovine serum albumin (BSA) in PBS.
2. FITC-conjugated $F(ab)_2$ goat anti-mouse (Sigma F2772).
3. 1% v/v formaldehyde.

2.2. LPS Preparation

LPS samples for OP assay were obtained as previously described using standard phenol/water extraction ***(9,10)*** (*see* **Subheading 3.2.**). Stock solutions were prepared from 1 mg purified LPS dissolved in 1 mL PBS or coating buffer (as ELISA).

2.2.1. Passive Coating Beads

1. Polystyrene microspheres with a diameter of 1 µm contained a red (polychromatic red fluorescent, PC red) fluorescent dye within the polymer (Polysciences, Fluoresbrite PC red, cat. no. 18660) (0.25 mL), stored at 4°C.
2. Blocking solution (10 mg/mL BSA in borate buffer).
3. Borate buffer: 0.1 *M* borate buffer, pH 8.5.
4. Storage buffer: 0.1 *M* PBS pH 7.4, containing 10 mg/mL BSA, 0.1% sodium azide, 5% glycerol.

2.3. Opsonins

2.3.1. Source of Human Sera from Disease/Carrier Cases

Healthy adult sera and sera from infants with Hib disease were separated and stored in aliquots at −20°C.

2.3.2. Source of Complement

For the OPA, complement sources were: baby rabbit serum (Sigma S7764, rabbit HLA-ABC or Pel-freeze) or a human source (patient with hypogammaglobulemia [IgG: 0.6 g/L, IgM 0.2 g/L, IgA <0.1 g/L; normal range IgG 6–13 g/L, IgM 0.8–2.5 g/L, IgA 0.8–3g/L]). Aliquoted on ice and stored at −80°C. Each aliquot, once thawed, is not reused in the assay.

2.3.3. Murine Monoclonal Antibodies to Inner-Core LPS in H. influenzae

mAb L6A9 was prepared as described previously (*see* Chapter 15, **Subheading 3.4.**) using formalin-killed whole cells of Eagan inner core LPS ***(11)***.

2.3.4. Preparation of Human Peripheral Blood Neutrophils: Buffers

1. RPMI media (1640 HEPES modification, Gibco) instead of PBS.
2. Washing solution: Dulbecco's buffer: 8 g/L NaCl, 1.44 g/L $Na_2HPO_4 \cdot 2H_2O$, 0.2 g/L KCl, 0.2 g/L KH_2PO_4 or PBS tablets (Oxoid), 10 tablets per 1 L distilled water.
3. Final buffer: Dulbecco's buffer (PBS) supplemented with 0.5% BSA, 0.1 g glucose 12.1 mg $MgSO_4 \cdot 7H_2O$, 13.2mg $CaCl_2 \cdot 2H_2O$ per 100 mL.
4. Lysis buffer (0.15 *M* ammonium chloride, 0.1 *M* sodium bicarbonate, 0.002 *M* EDTA·$2H_2O$, pH 6.8; 8 g/L NH_4Cl, 0.8 g/L $NaHCO_3$, 0.88 g/L EDTA·$2H_2O$, pH 6.8; use 1/10 dilution of buffer with freshly drawn heparinized venous blood.

2.4. Opsonophagocytosis Assay (Flow Cytometric Based): Buffers, Equipment

1. Microtiter plates (Costar): sterile 96-well (U-bottom).
2. Vortex/platform stirrer (MS1 Minishaker, IKA, Esslab).
3. Repeat 8 channel repeat pipet (25 L).
4. Centrifuge (1500*g*).
5. Incubator set to 37°C
6. Access to Coulter counter or flow cytometer for differential cell count.
7. Final buffer: per 100 mL Dulbecco's buffer add 0.5 g BSA, 0.1 g glucose, 12.1 mg $MgSO_4 \cdot 7H_2O$, 13.2mg $CaCl_2 \cdot 2H_2O$, pH 7.4.
8. PBS/EDTA.
9. Trypan blue dye (4 mg/mL).
10. FACScan (Becton-Dickinson).
11. Cellquest software.

2.5. Dihydrorhodamine (DHR): Reagents

1. Dihydrorhodamine 123 (DHR) (Molecular Probes, Eugene, OR) prepare stock 10 m*M*, aliquots stored at −80°C.

3. Method

3.1. Bacterial Strains

LPS samples for OP assay were prepared from *H. influenzae* wild-type Eagan and inner-core LPS mutant derived from Eagan; *Hi* wild-type strain 375, and mutant inner-core LPS mutant from strain 375 (*see* **Subheading 2.1.**).

3.1.1. Preparation of Stock Solutions for Labeling of Bacterial Strains Using Rhodamine Green-X (RGX) and Rhodamine Red-X (RRX)

This preparation was adapted from the method of Lehmann et al., ***(2)***, except that rhodamine red X-labeled bacteria were used for the oxidative burst assay. Briefly, a suspension of ethanol-fixed bacteria (total 10^{10} organisms in 4 mL) was washed with 0.9% NaCl and allowed to settle on the bench. The saline was removed and the bacteria were suspended in 3 mL of 0.2 *M* sodium bicarbonate buffer, pH 8.2. 100 µL of stock solution of rhodamine green X (Molecular Probes, R6163) or rhodamine Red X (Molecular Probes, succinimidyl ester, mixed isomers, R6160) (5 mg/3 mL DMSO stored at −80°C) was added dropwise to the suspension and mixed gently at room temperature for 1–1.5 h covered in foil. The reaction was terminated with 400 µL hydroxylamine-HCl (pH 8.5, 5 *M*) centrifuged at 6K rpm for 10 min in the microfuge (14,000*g*) and washed 3× in ice-cold 0.9% NaCl. The mixture was finally suspended in 5 mL final buffer used for the OP assay to give approx 5×10^8 cells/250 µL aliquots, filtered using Nytal mesh (11 µm) to remove clumps of bacteria, and stored at −80°C.

3.1.2. FACS Surface Labeling of Bacteria

The method was adapted from Moe et al., ***(11)***, except no sodium azide was included in the blocking buffer step ***(3)***. To prepare labeled bacteria, organisms were grown overnight by standard conditions at 37°C on BHI agar plates and gently suspended in PBS. OD_{A260} was adjusted to give the required concentration, e.g., 5×10^9 organisms/mL. 100 µL bacterial cells were added to each tube (5×10^8 organisms) and an equal volume of diluted sera (1/100 mAb in 1% BSA/PBS) was added. Tubes were incubated for 2 h at 4°C and cells centrifuged for 5 min at 14,000*g*. The supernatant was discarded and cells were washed with 200 µL of 1% BSA/PBS. 100 µL of FITC-conjugated $F(ab)_2$ goat anti-mouse (Sigma F2772) was added, diluted 1/100 in 1% BSA/PBS, and tubes were incubated for 1 h at 4°C. Cells were centrifuged at 14,000*g* for 5 min and washed by addition of 200 µL of 1% BSA/PBS. The supernatant was discarded and the cells were suspended in 1% v/v formaldehyde. Samples were transferred to tubes and read. For ethanol-treated bacteria, bacteria were grown as before, gently suspended in PBS, OD_{A620} was adjusted to give 5×10^9 organisms/mL and fixed in ethanol (70% v/v). The same labeling procedure and flow cytometric analysis was used as described previously.

3.2. LPS Preparation

For inhibition studies in the OPA, LPS was obtained as described previously ***(9).*** Briefly, *Hi* bacteria were grown overnight on BHI broth and prepared from 2 × 5 L bacterial culture as described in Chapter 15 (**Subheading 2.2.**). Crude LPS was extracted from the bacterial pellet using the standard hot

phenol/water method ***(10)*** and purified from the aqueous phase by ultracentrifugation (105,000*g*, 4°C, 2 × 5 h) ***(13)***.

3.2.1. Coating Beads

The method was adapted from the method of Lehmann et al ***(2)***, except that beads were coated with purified LPS in bicarbonate buffer ***(3)***. Polystyrene microspheres with a diameter of 1 µm contained a red (polychromatic red fluorescent, PC red) fluorescent dye within the polymer (Polysciences, Fluoresbrite PC red, cat. no. 18660) (0.25 mL). These were washed 3 × in 1 mL 0.1 *M* borate buffer, pH 8.5, centrifuged for 6 min at 15,000*g*, and suspended in 1 mL coating buffer (pH 9.68). 250 µg LPS prepared from stock solution (2.5 mg/mL) was added to washed beads to give a final volume of 1.25 mL and mixed overnight at room temperature. The bead mixture was centrifuged for 10 min at 14,000*g* and the supernatant retained for LPS determination. The pellet was resuspended in 1 mL blocking solution (10 mg/mL BSA in borate buffer) and incubated for 30 min at room temperature with gentle mixing. The mixture was then centrifuged for 6 min at 14,000*g* and the blocking step repeated. The pellet was resuspended in 1 mL storage buffer (0.1 *M* PBS, pH 7.4, containing 10 mg/mL BSA, 0.1% sodium azide, 5% glycerol) at 4°C. 50 µL of stock solution of beads in storage buffer were added to 0.95 mL final assay buffer and centrifuged for 6 min at 14,000*g*. Beads were washed 3× in PBS alone.

3.2.2. Inhibition Studies Using Purified LPS

Dilutions of sera or mab were preincubated overnight at 4°C with a range of concentrations of purified LPS (0.5–1.0 mg/mL), centrifuged at 14,000*g* for 10 min, and the supernatent used for the OP assay ***(3)***.

3.3. Opsonins

3.3.1. Source of Human Sera from Disease/Carrier Cases

Healthy control adult sera (HC 1–3) from laboratory staff and sera from infants with known Hib disease (British Paediatric Surveillance Unit).

3.3.2. Source of Complement

Sources for the OPA complement were: baby rabbit serum (Sigma S7764, rabbit HLA-ABC) or a human source (patient with hypogammaglobulemia (0.6 g/L IgG, 0.2 g/L IgM, <0.1 g/L IgA, normal range: 6–13 g/L IgG, 0.8–2.5 g/L IgM, 0.8–3 g/L IgA).

3.3.3. Murine Monoclonal Antibodies to Inner-Core LPS in H. influenzae

Dilutions of mAb L6A9 raised to Eagan inner-core LPS mutant (as described for ELISA) were used in the OP assay with wild-type and mutant Eagan strains.

3.4. Preparation of Human Peripheral Blood Neutrophils

Peripheral leucocytes were prepared from 20 mL heparinized venous blood from healthy adult donors. The method used was adapted from Lehmann et al. *(2)*, except that cells were washed in RPMI media instead of PBS *(3)*. Briefly, heparinized blood was diluted 1/10 in lysis buffer (0.15 *M* ammonium chloride, 0.1 *M* sodium bicarbonate, 0.002 *M* EDTA·$2H_2O$, pH 6.8) and left at room temperature for exactly 10 min, then centrifuged at 350*g* for 5 min. The supernatant was discarded and the pellet of cells was washed with RPMI medium (1640 HEPES modification) and centrifuged as before. The lysis step was repeated for 2–5 min if a layer of intact erythrocytes was still present. Cells were washed again in RPMI medium, centrifuged, and suspended in 10 mL PBS. A differential cell count for total numbers of nonlymphocytes and lymphocytes was determined using the Coulter counter, and the percentage of granulocytes was calculated using the flow cytometer. The dilution factor was calculated to give a concentration of 1.25×10^7 granulocytes (hereafter referred to as PMNs) per milliliter. The cells were suspended in final buffer immediately before addition to the assay.

3.5. Opsonophagocytosis Assay (Flow Cytometric Based): Buffers, Equipment

The method was adapted from Lehmann et al. *(2)* in 96-well Costar plates except that RGX/RRX-stained Eagan wild-type, Eagan mutant, or *Hi*375 wild-type were used. Final buffer, diluted sera, bacteria, and PMNs were placed in a final volume of 25 µL for each assay, instead of 20 L. Stained bacteria were pre-opsonized with serum and/or complement in final buffer for 10 min at 37°C in wells in a microtiter plate and mixed on a shaking platform at 500 rpm. PMNs were added to the mixture in wells and incubated for a further 10 min at 37°C. The reaction was stopped on ice with 150 µL ice-cold PBS/EDTA per well and transferred to tubes containing 50 µL trypan blue solution (4 mg/mL) immediately prior to analysis by FACS (*see* **Note 5**). 10,000 nonlymphocytes were collected using FACScan and Cellquest software. PMNs were analyzed by FSC vs FL-1 or FL-2 to determine percentage uptake (% phagocytosis). Reproducibility of the assay was assessed with high- and low-titer control sera (*see* **Note 6**).

3.6. Dihydrorhodamine (DHR): Reagents

This method was adapted from the method of Lehmann et al. *(14)*. Briefly, the same method as for the OPA was followed, except that RRX was used instead of RGX to label the bacteria and the oxidative burst substrate dihydrorhodamine 123 (DHR) (Molecular Probes, Eugene, OR) was added immediately before addition of PMNs to a final dilution of 0.1 m*M* (1/100

dilution from 10 m*M* stock). DHR 123 is converted intracellularly to green fluorescent rhodamine 123 (R-123) by reactive oxygen intermediates ***(15)***. FACS analysis was performed on measurements of forward-angle light scatter (FSC) vs green R-123 fluorescence (FL-1 channel between 505 and 545 nm) for oxidative burst and FSC vs red fluorescence (FL-2 channel between 560 and 590 nm) for uptake of bacteria (% phagocytosis).

4. Notes

1. Preparation of PMNs is optimal with washes using RPMI medium and cells should not be suspended in final buffer until immediately prior to assay, as this affects the function of the neutrophils in the assay and can cause clumping effects. PMNs can be prepared and left in 1 mL RPMI at 37°C for 1–2 h prior to use, until dilution in final buffer immediately prior to adding to the assay. Cells are best added to the microtiter plates in the incubator using a multichannel pipet to avoid fluctuations in temperature affecting the assay.
2. Complement source is crucial for the OP assay. This can be selected by screening human sera for low titers of inner-core LPS antibodies or obtaining sera from a patient with hypogammaglobulemia. Baby rabbit complement can be used as a more practical and available alternative. Aliquots must be stored at −80°C and not thawed and refrozen.
3. Opsonic activity of antibodies to inner core LPS can be correlated to inner-core *Hi* LPS antibody titers determined by ELISA (*see* Chapter 15).
4. Specificity of OP assay can be shown using *Hi* mAbs and sera by inhibition of OP activity following preincubation of antibody sample with concentrations of purified inner-core LPS.
5. Addition of trypan blue (TB) immediately before analysis by flow cytometer is useful for exclusion of surface-associated bacteria and reduces background (nonspecific uptake) for 10-min incubation time. However, if incubation times of 30 min or longer are chosen, nearly all bacteria are taken up, and addition of TB is not required.
6. To ensure that OP assay is reproducible, a number of healthy control sera (usually high, medium, and low titer) are always run in every assay, and negative controls of final buffer alone or complement alone. The performance of the assay can be expressed as intra- and intercoefficient of variation for control sera.

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References

1. Romero-Steiner, S., Libutti, D., Pais, L. B., et al. (1997).Standardization of an opsonophagocytic assay for the measurement of functional antibody activity against *Streptococcus pneumoniae* using differentiated HL-60 cells. *Clin. Diagn. Lab. Immunol.* **4,** 415–422.
2. Lehmann, A. K., Halstenen, A., Holst, J., and Bassoe, C.-F. (1997) Functional assays for evaluation of serogroup B meningococcal structures as mediators of human opsonophagocytosis. *J. Immunol. Methods.* **200,** 55–68.
3. Plested, J. S., Ferry, B. L., Coull ,P. A., et al. (2001) Functional opsonic activity of human serum antibodies to inner core lipopolysaccharide (*galE*) of serogroup B meningococci measured by flow cytometry. *Infect. Immun.* **69,** 3203–3213.
4. Goldschneider, I., Gotschlich, E. C., and Artenstein, M. S. (1969) Human immunity to meningococcus I. The role of humoral antibodies. *J. Exp. Med.* **129,** 1307–1326.
5. Zollinger, W. D., and Mandrell, R. E. (1983) Importance of complement source in bactericidal activity of human antibody and murine monoclonal antibody to meningococcal group B polysaccharide. *Infect. Immunol.* **40,** 257–264.
6. Ison, C. A., Anwar, N., Cole, M. J., et al. (1999) Assessment of immune response to meningococcal disease: comparison of a whole-blood assay and the serum bactericidal assay. *Microb. Path.* **27,** 207–214.
7. Martinez, J. E., Romero-Steiner, S., Pilishvili, T., et al. (1999) A flow cytometric opsonophagocytic assay for measurement of functional antibodies elicited after vaccination with 23-valent pneumococcal polysaccharide vaccine. *Clin. Diagn. Lab. Immunol.* **6,** 581–586.
8. Virji, M., Watt, S. M., Barker, S., Makepeace , K., and Doyonnas. R. (1996) The N-domain of the human CD66a adhesion molecule is a target for Opa proteins of *Neisseria meningitidis* and *Neisseria gonorrhoeae*. *Mol. Micro.* **22,** 929–939.
9. Plested, J. S., Makepeace, K., Jennings, M. P., et al. (1999) Conservation and accessibility of an inner core lipopolysaccharide epitope of *Neisseria meningitidis*. *Infect. Immun.* **67,** 5417–5426.
10. Westphal, O. and Jann, J. K. (1965) Bacterial lipopolysaccharides extraction with water-phenol and further applications of the procedure. *Methods Carbohydr. Chem.* **5,** 83–91.
11. Gidney, M. A. J., Lacelle, S., Cox, A. D., Martin, A., Moxon, E. R., and Richards, J. J. Production and characterisation of a monoclonal antibody (L6A9) against inner-core epitope in *Haemophilus influenzae* lipopolysaccharide. In preparation.
12. Moe, G. R., Tan, S., and Granoff, D. M. (1999) Differences in surface expression of NspA among *Neisseria meningitidis* group B strains. *Infect. Immun.* **67,** 5664–5675.
13. Masoud, H., Moxon, E. R., Martin, A., Krajcarski, D., and Richards, J. C. (1997) Structure of the variable and conserved lipopolysaccharide oligosaccharide epitopes expressed by *Haemophilus influenzae* serotype b Eagan. *Biochemistry.* **36,** 2091–2103.
14. Lehmann, A. K., Halstenen, A., and Bassoe, C.-F. (1998) Flow cytometric quantitation of human opsonin-dependent phagocytosis and oxidative burst responses to meningococcal antigens. *Cytometry.* **33,** 406–413.
15. Rothe, G., Oser, A., and Valet, G. (1988) Dihydrorhodamine 123: a new flow cytometric indicator for respiratory burst activity in neutrophil granulocytes. *Naturwissenschaften* **75,** 354–355.

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In Vitro Models of Infection I–Human Respiratory Tissue Organ Culture

Andrew M. Middleton, Paula Keig, and Robert Wilson

1. Introduction

The interaction of bacteria with mucosal surfaces of the respiratory tract is thought to be critical in their pathogenesis. Bacteria must overcome local defenses that include physical barriers such as mucus, beating cilia, and an intact epithelial surface; antibacterial substances such as defensins, secretory immunoglobulin A, and lysozyme; and resident phagocytes. Organ cultures can be used to study the initial interaction of bacteria with the mucosal surface and subsequent events, in particular the effects of bacteria on the structure and function of the respiratory epithelium. Organ cultures have a near-normal ratio of differentiated cell types that maintain their three-dimensional relationships with the extracellular matrix and submucosal tissues, and by incorporating an air interface they closely simulate in vitro the physiological conditions of the respiratory mucosa found in vivo.

1.1. Organ Culture vs Harvested Cells or Cell Culture Monolayers

The complexity of host–bacterial interactions that occur during mucosal infections precludes the use of a single model as a universal tool with which to study them. Therefore, care must be taken to choose an appropriate model to answer the questions being posed during experimental investigations. Options include the use of dissociated cells (e.g., buccal epithelial cells or nasal epithelium), cell lines, primary cell cultures, organ cultures, and whole animal models. Each of these approaches has advantages and disadvantages. For example, when studying bacterial adherence, cell lines are readily available and provide a homogeneous cell population that is well characterized, but local defenses are absent and the surface receptors may be very different from those present

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in vivo. It has been shown that bacterial interactions with cell monolayers can be compromised by the cell system's simplicity, and the absence of mucus is important because some bacterial species show tropism for it ***(1–6).***

Normal epithelial function depends on the retention of cell junctions, the polarity of the epithelium, and the orientation of the epithelium on the connective tissue substratum and extracellular matrix ***(7)***. The production of mediators by epithelial cells differs significantly between tracheal organ cultures and primary tracheal cell cultures ***(8),*** and loss of the three-dimensional organization can affect secretion and other physiological responses ***(9,10)***. Organ cultures maintain the arrangement of folds, grooves, and glands present on the epithelial surfaces in vivo and possess a population of leukocytes, although the lifespan of the leukocytes may be limited ***(11,12)***. Boat et al. (1977) demonstrated secretion of mucus and glycoconjugates from epithelial explants for longer than 10 d, and Jackson et al. (1996) showed that epithelial integrity remained intact for at least 20 d ***(13,14)***. Therefore, organ cultures provide the best opportunity to study interactions between bacteria and the intact mucosa under in vitro conditions. In addition, because of their longevity, they can be manipulated to study the effect of a prior treatment or insult on subsequent infection ***(15)***.

1.2. Air Interface Organ Cultures vs Submerged Organ Cultures

Broadly speaking, organ cultures that have been used to study bacterial infection are constructed in three ways: pieces of tissue suspended in culture medium, in which dissected tissue surfaces will be exposed to bacteria ***(11)***; pieces of tissue immersed in medium, but in some way oriented so that only the mucosal surface is exposed ***(2)***; or an air interface ***(4)***. Since bacteria adhere to nonluminal cell surfaces and extracellular matrix after disruption of the epithelial barrier of organ cultures ***(4)***, interpretation of results obtained from organ cultures with exposed nonluminal surfaces must take into account the possibility that bacteria may invade the tissue without penetrating the epithelial barrier, and their products may affect the epithelium from both apical and basolateral aspects.

Immersion in medium has several disadvantages. It may reasonably simulate conditions in the intestinal tract, but replacement of the air-mucosal interface of the respiratory tract with fluid represents a significant departure from the normal physiology of the airways ***(16,17)***. Microbial adherence to cell surfaces is influenced by the pH and ionic concentrations in the culture medium ***(18,19)***. Bacterial pathogens replicate in the medium of immersed organ cultures, and continual exposure to such replicating bacterial populations, together with their products, may damage epithelium independent of bacterial adherence. Competition for available nutrients occurs because bacteria and the host

tissue derive their nutrition from the same source and bacterial replication is likely to place the tissue under conditions of nutritional stress, which may increase its susceptibility to damage or alter its morphological features. It is not surprising, therefore, that bacterial interactions with the respiratory mucosa are significantly altered by immersion of tissue in culture medium. Jackson et al. (1996) compared the adherence of *Haemophilus influenzae* type b to immersed organ cultures and adherence to organ cultures maintaining an air interface ***(20)***. It was shown that *H. influenzae* adherence to mucus, damaged epithelium, and unciliated cells was significantly higher in the air- interface organ cultures, and total bacterial adherence was over 50 times greater than in the immersed organ cultures.

1.3. Human vs Animal Tissue

Human tissue is the most appropriate choice for organ culture studies of human pathogens, but it is often difficult to obtain, it is derived from a heterogeneous population and it is usually acquired following surgery for medical indications so it cannot be considered normal. Animals offer an alternative source of tissue. Healthy tissue from animals is more readily available than human tissue, and because it can be derived from inbred populations it may give less variable results. However, bacterial adherence can vary depending on the source of tissue, particularly for pathogens such as *H. influenzae*,for which humans alone are the natural hosts ***(21)***. The response of animal tissue to an experimental challenge may also vary compared to human tissue. For example, serum from cystic fibrosis patients slows rabbit cilia, which for a time led to an erroneous hypothesis that a serum factor was the cause of delayed mucociliary clearance in the condition, until human cilia were shown not to be affected ***(22)***. Results obtained with animal tissue must therefore be interpreted with caution, and care taken when extrapolating to human conditions.

1.4. Applications of the Organ Culture

Variation in the tissues used to assemble organ cultures can create problems when comparing results obtained with different bacterial strains. This can be reduced by examining tissue by phase-contrast light microscopy prior to the assembly of the organ culture. For example, by screening tissue pieces for active ciliary beating unciliated, organ cultures can be avoided. Morphometric counting techniques should be used when analyzing results obtained after bacterial infection of the mucosa. This will provide data that is objective rather than descriptive. A sufficient number of experiments should be performed to ensure that the observed results are reproducible. Tissue availability may be a major limiting factor, because a series of organ cultures used in a single experiment should be derived from the same donor to ensure that any differences that

are observed are not due to variation in the donor tissue. Experiments are usually time-consuming and require the use of good aseptic technique. We have used the air-interface organ culture described in this chapter to study *H. influenzae* ***(20,23)***, *Streptococcus pneumoniae* ***(5)***, *Pseudomonas aeruginosa* ***(4,24)***, *Niesseria meningitidis* ***(25)***, *Mycobacterium avium-intracellulare* ***(6)***, and *Bordetella pertussis* ***(26)***.

1.4.1. Bacterial Interactions with Respiratory Mucosa

Tissue can be removed from the model and examined by light microscopy, scanning electron microscopy (SEM), and transmission electron microscopy (TEM). The organ culture model, as described below, can remain structurally and functionally intact for 4 d without a change of the culture medium, and for up to 3 wk with medium replaced daily ***(13)***. The organ culture can therefore be used to investigate a variety of factors influencing the actions of bacteria: viruses, oxygen metabolites, pharmacological agents, and cigarette smoke. Organ cultures are sufficiently sensitive to discriminate between the interactions of closely related bacterial strains and so provide a powerful tool by which to investigate the importance of a bacterial toxin or a surface structure involved in adherence during infection of the mucosa ***(5,23)***.

1.4.1.1. Light Microscopy

Light microscopy is used to assess bacterial adherence and the structural integrity of the epithelium at a relatively low magnification. Paraffin wax-embedded sections can also be used to differentiate cell types and to perform immunohistochemical staining, e.g., cytokine responses and *in situ* hybridization ***(27–29)***.

1.4.1.2. Scanning Electron Microscopy (SEM)

Morphometric analysis by SEM allows epithelial features to be quantified and results obtained with uninfected control organ cultures compared with infected tissue. The number of adherent bacteria are counted and the features to which bacteria adhere can be examined (**Fig. 1**). Primary antibodies to epithelial components, e.g., fibronectin, or to microorganisms can be incubated with tissue, followed by secondary antibodies conjugated to microscopic gold particles. Using an SEM with a backscatter detector and video scanning unit, the immunogold pattern can be combined with the standard SEM image.

1.4.1.3. Transmission Electron Microscopy (TEM)

Analysis by TEM allows examination of the ultrastructure of epithelial cells. For example, nuclear chromatin, dilation of the nuclear envelope, cytoplasmic blebbing, description of tight cell junctions, mitochondrial abnormalities, vacu-

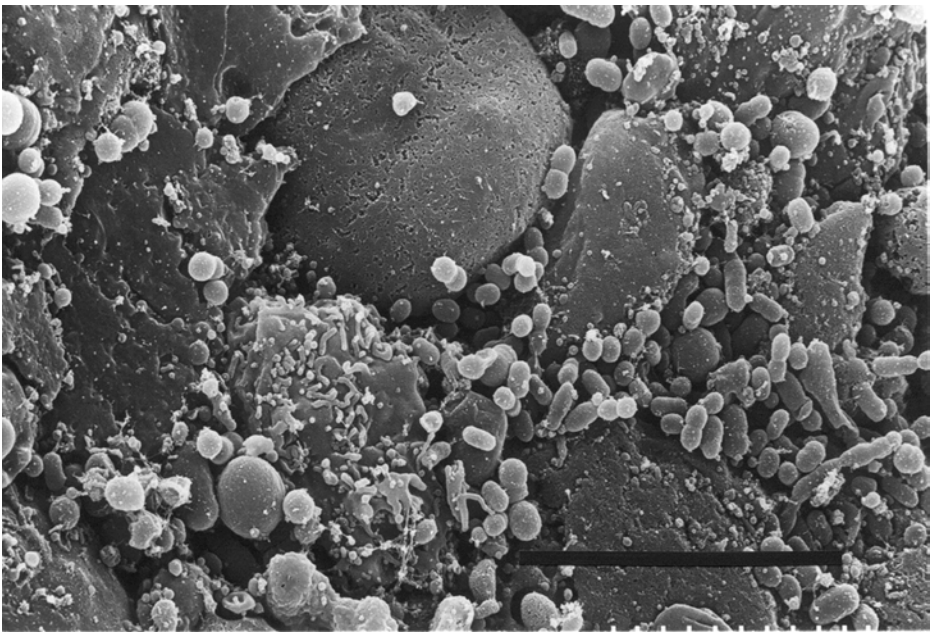

Fig. 1. Electron microscopy of *Haemophilus influenzae* adhering to tissue. Nontypeable *Haemophilus influenzae* are seen as cocco-bacilli adhering to damaged human respiratory epithelium. Scale bar 5 μm.

olation of the cytoplasm and reduction in the density of cilia on ciliated cells all occurred during *H. influenzae* infection ***(23)***. TEM sections can also be used to study bacterial invasion of the epithelium by endocytosis or between cells ***(5,25)***.

1.4.1.4. Cell Responses

The effects of microorganisms and their products on the ciliary beat frequency can be measured in the organ culture. In submerged organ cultures the surrounding maintenance medium can be assayed for inflammatory mediators, whereas staining techniques can be used in the air-interface model. Mucus produced by the model to various stimuli, including bacterial infection, can be collected and assayed.

2. Materials

2.1. Preparation of Bacteria for Inoculation

1. Bacteria.
2. Sterile phosphate-buffered saline (PBS).
3. Culture medium.

2.2. Tissue Collection

2.2.1. Transport of Tissue

1. Human adenoid, bronchial, or nasal turbinate tissue.
2. Eagle's minimum essential maintenance medium (MEM).
3. Antibiotics: 50-μg/mL streptomycin, 50-IU/mL penicillin, and 50-μg/mL gentamicin. (An antifungal can be added if contamination proves to be a problem after longer incubation periods, for example, 50-μg/mL amphotericin B).

2.2.2. Dissection of Tissue

1. Tissue.
2. MEM with and without antibiotics.
3. Class II laminar-flow cabinet.
4. Sterile plastic loops.
5. Sterile scalpels.
6. Sterile Petri dishes.

2.3. Assembly of Organ Culture

1. Dissected tissue pieces.
2. MEM.
3. Sterile Petri dishes: 3.5 and 6 cm.
4. Whatman No. 1 filter paper strips: 5 × 70 mm, sterile.
5. 1% sterile purified agar.

2.4. Sterility or Purity of Bacterial Growth at End of Experiment

1. Sterile PBS.
2. Sterile plastic loops.
3. Columbia agar base supplemented with 5% defibrinated horse blood, "blood agar."
4. Appropriate solid medium for bacteria studied (if not blood agar).

2.5. Preparation of Tissue for Further Analysis

2.5.1. Culture

1. Sterile PBS.
2. Sterile tissue grinder.
3. Bacterial culture medium.
4. Graduated glass centrifuge tube.

2.5.2. Light Microscopy

1. 10% formaldehyde.
2. 70, 90, and 100% methanol.
3. Molten paraffin wax.
4. Solvent (e.g., chloroform).

2.5.3. Scanning Electron Microscopy

1. 2.5% glutaraldehyde.
2. 70, 90, and 100% methanol.
3. Acetone.
4. Distilled water.
5. 1% osmium tetroxide.
6. 0.05 *M* sodium cacodylate buffer.

2.5.4. Immunogold Labelling

1. 0.05% glutaraldehyde.
2. 2.5% glutaraldehyde.
3. 2% *para*-formaldehyde.
4. PBS.
5. Wash buffer (0.8% bovine serum albumin [BSA], 0. 1% fish gelatin).
6. Blocking buffer (wash buffer plus 5% fetal calf serum).
7. Primary antibody for selected target, i.e., epithelia) or bacterial feature.
8. Secondary antibody, conjugated to gold particles, which is specific against primary antibody.
9. Silver enhancement (if required).
10. High-resistance (or BPLC-grade) water (if required).

2.5.5. Transmission Electron Microscopy

1. 2.5% glutaraidehyde.
2. 0.05 *M* sodium cacodylate buffer.
3. 1% osmium tetroxide.
4. Distilled water.
5. 70, 90, and 100% methanol.
6. Propylene oxide.
7. Araldite.
8. 1% alkaline toluidine blue.
9. Uranyl acetate.
10. Lead citrate.

3. Methods

3.1. Preparation of Bacteria

The method of preparation of bacteria for inoculation of the organ culture will vary depending on the species studied. We have used a number of techniques, including overnight broth cultures, aliquoted batch cultures that are frozen for storage and thawed prior to inoculation, and scraping bacteria directly from agar plates. The results obtained with stored bacteria should be compared to fresh cultures to ensure that they are the same. The bacteria should be centrifuged and washed in sterile PBS to produce a homogenous suspen-

sion. The viable count of the inoculum should be known and, when comparing strains/mutants of the same species, the inocula need to be adjusted so they are all approximately the same number. This can be achieved by measuring light absorbance, e.g., at 600 nm using a spectrophotometer, or comparison of turbidity, e.g., using McFarland's standards.

3.2. Tissue Collection and Transport

The organ culture is constructed using tissue obtained from routine surgical operations. In most cases human nasal turbinate is obtained from patients undergoing surgery for nasal obstruction, human adenoid tissue from children with adenoid hypertrophy, and human bronchial tissue from patients undergoing tumor resection (*see* **Note 1**). Hospital ethical committee approval must be sought and patients must give signed consent to their tissue (which is removed for clinical purposes and would otherwise be discarded) being used for research.

1. Tissue is transported from the surgical theatre to the laboratory in MEM containing antibiotics (*see* **Subheading 2.2.1.** and **Note 2**).
2. Dissection of the tissue yields small squares of mucosa approx 3 mm^2 in area and 2–3mm thick (*see* **Note 3**).
3. Immerse tissue in MEM without antibiotics for at least 1 h in order to remove the antibiotics (*see* **Note 4**).
4. Examine the dissected pieces of tissue using phase-contrast light microscopy. Normal ciliary beating along at least one entire edge should be seen. Discard tissue that does not fulfil this criterion (*see* **Notes 5** and **6**).

3.3. Assembly of an Organ Culture with an Air–Mucosal Interface

Assembly of the organ culture should be performed under aseptic conditions in a class II laminar-flow cabinet (*see* **Figs. 2** and **3**).

1. Place the base of a sterile Petri dish 3.5 cm in diameter aseptically within a sterile 6-cm Petri dish.
2. Place a strip of sterile Whatman No. 1 filter paper (5 × 70 mm) soaked in MEM, without antibiotics, across the diameter of the inner Petri dish (*see* **Note 7**).
3. Place a single piece of dissected tissue (from **Subheading 3.2.**), epithelial surface facing upward, on the center of the filter paper strip in the inner dish.
4. Pipet 30 µL of molten 1% purified agar, cooled to 40ºC, carefully around the edge of the organ culture (*see* **Note 8**).
5. Pipet 4 mL of antibiotic-free MEM into the outer Petri dish and replace the outer lid (*see* **Note 9**).

3.4. Organ Culture Inoculation and Incubation

When studying the effects of bacterial infection:

1. Pipet 2-µL of washed bacterial suspension onto the organ culture surface (*see* **Notes 10** and **11**).

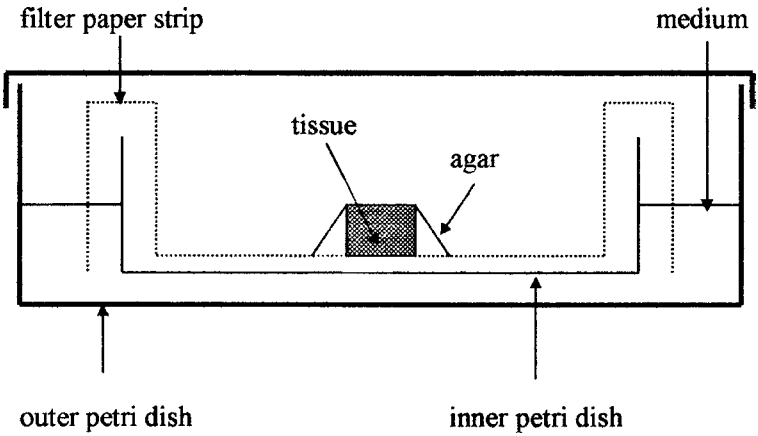

Fig. 2. Diagrammatic representation of an organ culture with an air–mucosal interface.

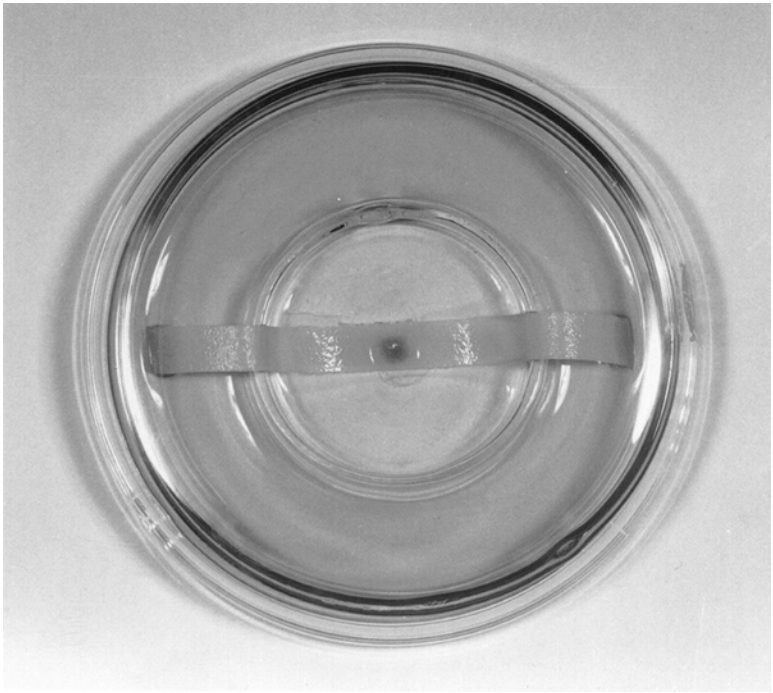

Fig. 3. An organ culture system that incorporates human respiratory tissue and an air interface (*see* text for details).

2. Incubate organ cultures at 37ºC in a humidified atmosphere of 5% CO_2 (*see* **Note 12**).
3. Wash unattached bacteria from the surface of the tissue by pipetting 100-mL sterile PBS onto the tissue (×3).
4. Check for contamination by touching each of the four edges of the tissue with a sterile plastic loop and plating onto solid medium appropriate to the studied bacteria. Also include a blood agar plate to detect contamination by other species if this is not the chosen appropriate medium.
5. Plate three 20 µL samples of the MEM reservoir onto blood agar.
6. Remove the tissue from the organ culture by cutting the filter paper around the tissue with a sterile scalpel (*see* **Note 13**).

3.5. Analysis of Tissue

3.5.1. Culture

1. Tissue is washed 3× by gently pipetting 100 µL of sterile PBS.
2. The volume of the tissue is measured by liquid displacement of PBS in a graduated glass centrifuge tube.
3. Tissue is placed in 1 mL of sterile PBS and macerated in a tissue grinder.
4. Viable counts are performed on solid medium appropriate to the bacteria being studied. Viable counts are calculated taking into account PBS dilutions and the volume of the tissue.

3.5.2. Light Microscopy

1. Tissue is fixed in 10% formaldehyde.
2. Tissue is processed using standard histological techniques for paraffin embedding, e.g.,
   a. 70, 90, and 100% methanol.
   b. Solvent (e.g., chloroform).
   c. Molten wax.

3.5.3. Scanning Electron Microscopy

Dehydration and critical-point drying is carried out according to standard protocols ***(2)***. Dehydration is usually through a gradient of increasing methanol concentrations incorporating a second fixation in osmium tetroxide and ending with acetone. For example,

1. Cacodylate buffer wash (×4; 5, 10, 15, and 20 min).
2. 1% Osmium tetroxide (60 min).
3. Distilled water wash (×3; 5, 10, and 20 min).
4. 70% methanol (×3; 5, 10, and 20 min).
5. 90% methanol (×3; 5, 10, and 20 min).
6. 100% methanol (×3; 5, 10, and 20 min).
7. 100% acetone (×3; 20, 30, and 60 min).

The method of SEM morphometric analysis described below is the standard method used in our laboratory for the study of respiratory pathogens such as

H. influenzae. The magnification used and the representative area covered can be adjusted depending on a number of factors, for example, the size of the microorganism studied and the frequency with which bacterial adherence occurs; i.e,. if bacterial adherence is a rare event then a larger area of the organ culture surface should be examined.

1. Place a transparent A4 acetate, with a grid of 100 squares printed on it, over one VDU of the SEM (*see* **Fig. 4**).
2. At ×50 magnification, select a recognizable feature on the tissue surface with which to orientate the grid. Place this feature in the first of the 40 squares numbered as shown in **Fig. 4**.
3. Increase magnification to ×3000.
4. Record the percentage coverage (1% represented by each square of the acetate grid) of the mucosal features, for example, mucus, ciliated cells, unciliated cells, and damaged epithelium (*see* **Note 14**). The number of bacteria associated with each mucosal feature is also recorded (*see* **Note 15**). If a square contains more than one feature, we use the convention that the feature occupying the greater proportion of the square is scored.
5. Decrease the magnification to ×50. Move the selected surface feature to the second of the 40 squares in the predetermined pattern (**Fig. 4**). Repeat **steps 1–5**. Continue this process for the remaining squares.

The proportion of the epithelium covered by the individual features is calculated as a mean of the counts for each of the 40 squares. The total number of bacteria adhering to each mucosal feature can be recorded, and the density of bacteria adhering to each mucosal feature can be calculated (*see* **Note 15**).

3.5.4. Immunogold Labeling

Preliminary experiments will be necessary to establish the optimum dilutions of primary and secondary antibody, and to establish the extent of nonspecific binding (*see* **Note l6**). Tissue preparation and bacterial inoculation are as described in **Subheadings 3.1.–3.4**.

1. After organ culture incubation, fix tissue in cacodylate-buffered 0.05% glutaraldehyde and 2% *para*-formaldehyde for 60 min (*see* **Note 17**).
2. Wash tissue in wash buffer (×3, 5 min); *see* **Subheading 2.5.4.**
3. Incubate tissue in blocking buffer (*see* **Subheading 2.5.4.**) for 60 min at room temperature.
4. Wash tissue in wash buffer (×3, 5 min).
5. Incubate tissue with primary antibody at the predetermined dilution, for 60 min (*see* **Note 18**).
6. Wash tissue in wash buffer (×3, 5 min).
7. Incubate tissue with secondary antibody, conjugated to gold particles, at the predetermined dilution, for 60 min (*see* **Note 18**).
8. Wash tissue in wash buffer (×3, 5 min).

1				21					11
	2				22			12	
	38	3		23			13		
			4		24	14		37	
32	31	30	29	5	15				
				16	6	33	34	35	36
	39		17	25		7			
		18			26		8		
	19			27			40	9	
20					28				10

Fig. 4. Grid used for morphometric analysis. An acetate sheet with 100 squares is used for assessment of tissue by scanning electron microscopy. The numbers represent the predetermined pattern of 40 squares used for morphometric anlaysis.

9. Fix tissue in 2.5% glutaraldehyde for a minimum of 60 min.
10. Perform silver enhancement, if required, according to the manufacturer's instructions (*see* **Note 18**).
11. If silver enhancement is used then washing with high-resistance (or HPLC-grade) water (×3, 5 min) is necessary to deactivate the enhancer.
12. Go to **Subheading 3.5.3**.

3.5.5. Transmission Electron Microscopy

The embedding of tissue and production of ultrathin sections (70–90 nm) is carried out using the protocol outlined below.

1. Cacodylate buffer wash (2.1% osmium tetroxide [60 min]. 3; 5, 10, and 20 min).
2. 2.1% osmium tetroxide (60 min).
3. Distilled water (×3; 3, 5, and 10 min).
4. 70% methanol (×2; 5 and 20 min).
5. 90% methanol (×2; 10 and 20 min).
6. 100% methanol (×3; 10, 20, and 40 min).
7. 100% propylene oxide (×2; 30 and 30 min).

8. Propylene oxide and araldite in a ratio 3:1 (20 min).
9. Propylene oxide and araldite in a ratio 1:1 (30 min).
10. Propylene oxide and araldite in a ratio 1:3 (30 min).
11. 100% araldite (×3; 3 h each).
12. Place tissue in molds, cover with araldite, and polymerize at 60°C for 48 h.
13. Cut 1-µm thick sections and float them on a drop of distilled water on a glass microscope slide.
14. Allow the water to evaporate and stain sections with 1% alkaline toluidine blue.
15. Using light microscopy, select an appropriate area for ultrathin (70–90 nm) sections, i.e., areas of intact epithelium.
16. Cut ultrathin sections and place them on copper grids (200 squares per grid).
17. Stain ultrathin sections with uranyl acetate and lead citrate.

TEM sections can be used to perform morphometric analysis of the epithelial integrity. The study of the state of nuclear chromatin, dilation of the nuclear envelope, cytoplasmic blebbing, integrity of tight cell junctions, mitochondrial abnormalities, vacuolation of the cytoplasm, and density of cilia on ciliated cells can be recorded using a morphometric scoring system of each individual cell.

3.6. Troubleshooting

The most important aspect of organ culture assembly is to maintain an aseptic environment. Any contamination will result in the need to discard the organ cultures and repeat the experiment. The possibility of contamination needs to be carefully monitored, because subsequent analysis of the tissue may show morphological changes or increased numbers of adherent bacteria not associated with the intended bacteria. The use of biohazard category 3 pathogens prohibits the use of class II laminar-flow cabinets. Instead, class I safety cabinets (situated in a containment level 3 laboratory) must be used. These protect the operator from the cabinet contents rather than the contents from the environment. As a result, the amount of contamination of the organ cultures in these experiments is in our experience higher. To limit the possibility of contamination in these experiments, the organ culture can be assembled using glass Petri dishes (*see* **Note 19**).

Pipetting of the molten agar seal must be carried out with care. The use of too much agar will result in a rim being formed around the tissue, preventing ciliary clearance of mucus, bacteria, and debris from the tissue surface. Too little agar and the cut edges of the tissue will be left exposed as a likely target for bacterial adherence and perhaps resulting in tissue damage and invasion. Improperly scaled edges may also result in dehydration of the organ culture. Purified agar should be used, as any form of nutrient agar may promote the multiplication of the microorganisms.

There are a number of opportunities for the epithelium to become damaged other than due to the study treatment. Damage occurring during surgi-

cal removal from the patient is beyond the researcher's control, although screening of the tissue by light microscopy can enhance selection of healthy ciliated organ cultures, and careful handling of the tissue after receipt in the laboratory is obligatory. However, experiments must incorporate adequate controls to differentiate between preexisting damage and any deleterious effects of the studied microorganism.

As tissue becomes available for experiments, even if the microorganisms to be studied are not ready for inoculation, it is better to assemble and maintain the tissue within the organ culture rather than delaying the dissection or prolonging the incubation of dissected tissue in antibiotics.

The bacterial inoculurn can be difficult to standardize. Optical densities measure dead and live bacteria, and may not be accurate. Some bacterial species tend to clump. If bacteria are taken from agar plates, they should be fresh. Time spent optimizing this step will be well spent, and a range of viable counts that are acceptable may be defined.

When analyzing the interaction of bacteria with the organ culture, the experiment needs to be repeated with tissue from different donors. In our experience, six experiments are usually sufficient for statistical analysis of the data. A complete series of experiments should be finished and the tissue samples coded and randomized before commencing the analysis. This avoids observer bias.

The incubation times of the organ culture, to study the effect of bacterial infection will vary depending on the bacteria studied. In all cases it is recommended that one organ culture be studied immediately after inoculation to investigate bacterial adherence, and then after an interval to study the effect of infection. We have incubated *Pseudomonas aeruginosa* in the organ culture for 8 h, *H. influenzae* for 24 h, *Bordetella pertussis* for 72 h and *Mycobacterium tuberculosis* for up to 21 d. The incubation time can be judged from the speed of growth in vitro.

Some bacteria, for example, *Streptococcus pneumoniae*, can be confused with epithelial features such as mucus droplets and cytoplasmic blebbing, particularly when working with SEM. There are a number of solutions to this problem. The size of the bacteria can be measured against sizes quoted in standard texts, or pure culture of the bacteria can be observed under the electron microscope in order for the observer to get a "feel" for the size, or the bacteria can be immunogold-labeled on the organ culture to distinguish them from the epithelial features.

4. Notes

1. Adenoid tissue is the most readily available tissue type, but only a small proportion of the tissue received in the laboratory is useful for the organ culture, as adenoid is also the most damaged, either by disease or surgical removal. Turbi-

nate tissue is easier to handle but is less readily available, due to the increasing surgical practice of diathermy rather than cutting as the method of removal. Bronchial tissue may be the most physiological by relevant tissue for respiratory pathogens, but specimens are less frequently available because the surgery required for its removal is major (lung resections), and careful liaison with the pathologist/surgical team is recommended.

2. The presence of antibiotics is required to eliminate the host's commensal bacterial flora, and the tissue should be kept in MEM with antibiotics for a minimum of 4 h which we have found is long enough to eliminate commensal flora in most cases. Tissue arriving at the laboratory late in the day can be kept in antibiotic medium overnight and used the following morning.
3. The tissue dimensions are important, since internal necrosis can accompany the culture of large pieces of tissue ***(30)***. This may affect epithelial cell function. The size of the dissected tissue should be kept to a minimum but needs to be adequate to allow easy mechanical manipulation.
4. We have demonstrated that antibiotics are effectively removed by this protocol by a simple agar plate assay. Tissue was placed on a blood agar plate (Columbia agar base containing 5% defibrinated horse) covered with a lawn of Oxford *Staphylococcus aureus* NCTC6571. There was no zone of inhibition of the *S. aureus* after overnight incubation at 37ºC.
5. Upon receipt of tissue, a visual inspection can indicate whether it is likely to be healthy enough to be used in organ culture. Adenoid tissue is often too damaged to be able to discern what represents the epithelium. In our experience, between 70% and 90% of adenoids received are too damaged to be used in organ culture. Turbinate tissue with a smooth, light-colored surface is usually healthy, but dark, "bruised"- looking tissue often shows a high percentage of damage when examined by SEM. Bronchial tissue is the easiest of tissues to dissect. It arrives as a tube. Associated connective tissue is carefully removed and the tube is simply cut down its length, laid flat, and dissected into squares. However, once dissected, the bronchial tissue has the most delicate epithelium and needs to be manipulated with extreme care.
6. Under some experimental circumstances, use of tissue that does not fulfil the criterion of "healthy" may be valid—for example, when studying the effect of prior epithelial damage on bacterial adherence.
7. The filter paper strip is manipulated with sterile forceps so that its middle portion adheres to the base of the inner dish and each of its moistened ends adheres to the base of the outer dish.
8. As the agar cools it forms a seal around the edges of the tissue, protecting the tissue from dehydration and preventing bacterial adherence/invasion to nonepithelial structures. Care should be taken to avoid formation of a rim of agar above the level of the tissue surface, which would then form a reservoir for material pipetted onto the organ culture.
9. The filter paper strip acts as a wick to draw medium from the outer Petri dish to the underside of the tissue, maintaining an air interface with the epithelial surface.

10. Each experimental series should include at each time point a negative control organ culture of uninfected/untreated tissue, in addition to a positive control of untreated tissue (i.e., no pharmacological agent) infected with parent microorganism (i.e., bacterium without genetic manipulation).
11. 2 μL is chosen as the drop size because larger drops are seen to "run off" the organ culture.
12. The time of incubation will depend on the bacteria/pharmacological agent studied and the purpose of the experiment.
13. Care should be taken not to dislodge the surrounding agar, as this may result in mucus being torn from the surface of the tissue, biasing any subsequent morphological analysis of the epithelial features and enumeration of adherent bacteria.
14. Cells can be categorized according to the presence or absence of cilia on their apical surfaces. Extruded cells, extracellular matrix, dead cells and cell debris, collagen, basement membrane, and open cell junctions can be scored together as damage or scored separately.
15. An approximation of the number of bacteria may be needed when they are present in sheets. In these instances it may be difficult to determine which mucosal component(s) the bacteria are associated with. Observation of the surrounding tissue may identify the underlying feature(s). The density of bacteria associated with each mucosal component can be calculated, in order to remove bias produced by different proportions of the organ culture surface being occupied by a particular mucosal feature. For example, a bacterium may adhere to mucus, but the amount of mucus on an organ culture may vary from 5% to 50%. Bacterial density is calculated by dividing the total number of bacteria adhering to a given mucosal feature by the number of squares on the acetate sheet that mucosal feature occupied at 2.1% osmium tetroxide (60 min). 3000 magnification. The number of squares occupied by a mucosal feature is calculated from the percentage of the organ culture occupied by that mucosal feature, giving the number of bacteria adhering per one square or unit area calculated as 3.55×10^2 μm2 (*see* example).

    Example of bacterial density calculation:

    100 bacteria adherent to mucus. Percentage mucus coverage of epithelium was 50%.
    100% of the organ culture analyzed = 40 grid squares at ×3000.
    +=> 50% mucus coverage occupies 20 grid squares at ×3000.
    100 bacteria/20 squares = no. of bacteria adhering per one grid square density of bacteria adherent to mucus = 5/U area (1 grid square).
    At ×3000 magnification 1 grid square = 3.55×10^2 μm^2.
    Density of bacteria adhering to mucus = 5 per 3.55×10^2 μm^2.
16. The optimum antibody dilutions are achieved when the epithelial feature or bacteria are labeled with the minimum of background nonspecific labeling. We have not established an exact definition for the optimum dilutions, but through using a combination of ranges of primary and secondary gold- conjugated antibody from 1:50 to 1:1000, a judgment can be made of the optimum.
17. This fixation replaces the 2.5% glutaraldehyde fixation immediately after tissue is removed from the organ culture.

18. For example, primary antibody of rabbit anti-bacterial antibody diluted 1:100 in PBS. Secondary antibody of mouse anti-rabbit antibody conjugated to gold particles, diluted 1:100 in PBS. The combination of antibodies will be dictated by either those commercially available or made in-house. Antibodies are available conjugated to different-sized gold particles. If the gold particles remain difficult to detect by SEM, they can be enhanced using silver enhancement. The longer the enhancement time, the larger the gold particles become, as the amount of silver builds up. For most requirements, 10 min at room temperature is the optimum.
19. The organ culture is constructed as described in **Subheading 3.3**., minus the tissue and the 4 mL MEM, sealed in an autoclave bag, and sterilized at 121°C for 15 min. This reduces the number of manipulations required at the point most likely to introduce contamination. There are a number of other possible explanations for repeated experimental contamination: the stock solutions or incubator could be contaminated; or the antibiotics may have lost activity. If repeated contamination occurs, it may be worthwhile changing/disinfecting everything and starting from scratch. If fungal contamination is a problem, the introduction of an antifungal agent into the MEM with antibiotics could be considered. For example, we have used 50-µg/mL amphotericin B.

References

1. Birkness, K. A., Swisher, B. L., White, E. H., Long, E. G., Ewing, E. P. Jr., and Quinn, F. D. (1995) A tissue culture bilayer model to study the passage of *Neisseria meningitides. Infect. Immun.* **63,** 502–409.
2. Read, R. C., Wilson, R., Rutman, A., et al. (1991) Interaction of nontypable *Haemophilus influenzae* with human respiratory mucosa in vitro. *J. Infect. Dis.* **163,** 549–558.
3. Yamamoto, T., Albert, M. J., and Sack, R. B. (1994) Adherence to human small intestine of capsulated *Vibrio cholerae* 0139. *FEMS Microbiol.Lett.* **119,** 229–235.
4. Tsang, K. W. T., Rutman, A., Tanaka, E., et al. (1994) Interaction of *Pseudomonas aeruginosa* with human respiratory mucosa in vitro. *Eur. Respir. J.* **7,** 1746–1753.
5. Rayner, C. F. J., Jackson, A. D., Rutman, A., et al. (1995) The interaction of pneumolysin sufficient and deficient isogenic variants of *Streptococcus pneumoniae* with human respiratory mucosa. *Infect. Immun.* **63,** 442–447.
6. Middleton, A. M., Chadwick, M.V., Nicholson, A.G., et al. (2000) The role of *Mycobacterium avium*. complex fibronectin attachment protein in adherence to the human respiratory mucosa. *Mol. Microbiol.* **38,** 381–391.
7. Chambard, M. Verrier, B., Gabrion, J., and Mauchamp, J. (1984) Polarity reversal of inside-out thyroid follicles cultured within collagen gel; re-expression specific functions. *Biol. Cell* **51,** 315–326.
8. Kelsen, S. G., Johnson, R. A., Mest, S., et al. (1993) Explant culture of rabbit tracheobronchial epithelium: structure and prostaglandin metabolism. *Am. J. Respir. Cell. Mol. Biol.* **8,** 472–479.
9. Rodriguez-Boulan, E. and Nelson, W. J. (1989) Morphogenesis of the polarized epithelial cell phenotype. *Science* **245,** 718–725.

10. Simons, K. and Wandinger-Ness, A. (1990) Polarized sorting in epithelia. *Cell* **62,** 207–210.
11. Farley, M. M., Stephens, D. S., Mulks, M. H., et al. (1986) Pathogenesis of IgA protease-producing and non-producing *Haemophilus influenzae* on human naopharnygeal organ cultures. *J. Infect. Dis.* **154,** 752–759.
12. Forsgre, J., Samuelson, A., Ahlin, A., Jonnason, J., Rynnel-Dagoo, B., and Lindbergh, A. (1994) *Haemophilus influenzae* resides and multiplies intracellularly in human adenoid tissue as demonstrated by in situ hybridization and bacterial viability assay. *Infect. Immun.* **62,** 673–679.
13. Boat, T. F., Cheng, P., and Wood, R. E. (1997) Tracheobronchial mucus secretion in vivo and in vitro by epithelial tissues from cystic fibrosis and control subjects. *Mod. Probl. Paediatr.* **19,** 141–152.
14. Jackson, A. D., Rayner, C. F. J., Dewar, A., Cole, P. J., and Wilson R. (1996) A human respiratory-tissue organ culture incorporating an air interface. *Am. J. Respir. Crit. Care. Med.* **153,** 1130–1135.
15. Plotleowski, M. C., Beck, G., Tournier, L. M., Bernardo-Fillio, M., Marques, E. A., and Puchelle, E. (1989) Adherence of *Pseudomonas aeruginosa* to respiratory epithelium and the effect of leucocyte elastase. *J. Med. Microbiol.* **30,** 285–293.
16. Johnson, A. P., Clark, L. B., and Osborn, M. F. (1983) Scanning electron microscopy of the interaction between *Haemophilus injluenzae* and organ cultures of rat trachea. *J. Med. Microbiol.* **16,** 477–482.
17. Tamaoki, J. (1991) Endothelin stimulates ciliary beat frequency and chloride secretion in canine cultured tracheal epithelium. *Cell. Mol. Biol.* **4,** 426–431.
18. Marcus, H., Austria, A., and Baker, N. R. (1989). Adherence of *Pseudomonas aeruginosa* to tracheal epithelium. *Infect. Immun.*. **57,** 1050–1053.
19. Grant, M. M., Niederman, M. S., Peklman, M. A., and Fein, A. M. (1991) Characterization of *Pseudomonas aeruginosa* adherence to cultured hamster tracheal epithelial cells. *Am. J. Respir. Cell. Mol. Biol.* **5,** 563–570.
20. Jackson, A. D., Cole, P. J., and Wilson, R. (1996) Comparison of *Haemophilus influenzae* type b interaction with respiratory mucosa organ cultures maintained with an air interface or immersed in medium. *Infect. Immun.* **64,** 2353–2355.
21. Wilson, R. and Moxon, E. R. (1988) Molecular basis of *Haemophilus influenzae* pathogenicity in the respiratory tract, in *Bacterial Infections of Respiratory and Gastrointestinal Mucosae*, (Griffiths, E., Donachie, W., and Stephens, J., eds.), Society for General Microbiology, IRL Press, pp. 29−40.
22. Rutland, J., Penketh, A., Griffin, W. M., Hodson, M. E., Batten, J. C., and Cole, P. J. (1983) Cystic fibrosis serum does not inhibit human ciliary beat frequency. *Am. Rev. Respir. Dis.* **128,** 1030–1034.
23. Rutman, A., Dowling, R., Wills, P., Feldman, C., Cole, P. J., and Wilson, R. (1998) Effect of dirithromycin on *Haemophilus injluenzae* infection of the respiratory mucosa. *Antimicrob. Agents. Chemother.* **42,** 772–778.
24. Dowling, R. B., Rayner, C. R. L., Rutman, A., et al. (1997) Effect of salmeterol on *Pseudomonas aeruginosa* infection of respiratory mucosa. *Am. J. Respir. Crit. Care Med.* **155,** 327–336.

25. Rayner, C. F. J., Dewar, A., Moxon, E. R., Virji, M., and Wilson, R. (1995) The effect of variations in the expression of pili on the interaction of *Neisseria meningitidi*s with human nasopharyngeal epithelium. *J. Infect. Dis.* **171,** 113–121.
26. Soane, M.C., Jackson, A., Maskell, D., et al. (2000). Interaction of *Bordetella pertussis* with human mucosa in vitro. *Resp. Med.* **94,** 791–799.
27. Buttery, L. D. K., Springall, D. R., and Chester, A. H. (1996) Inducible nitric oxide synthase is present within atherosclerotic lesions and promotes the formation and activity of peroxynitrite. *Lab. Invest.* **75,** 77–85.
28. Robbins, R. A., Sisson, L. K., and Springall, D. R. (1997) Human lung mononuclear cells induce nitric oxide synthase in murine epithelial cells in vitro: role of TNFα and IL1β. *Am. J. Resp. Crit. Care Med.* **155,** 268–273.
29. Marshall, B. G., Wangoo, A, Cook, H. T., and Shaw, R. J. (1996) Increased inflammatory cytokines and new collagen formation in cutaneous tuberculosis and sarcoidosis. *Thorax* **51,** 1253–1261.
30. Johnson, R. A., Stauber, L., Hilfer, S. K., and Kelsen, S. G. (1994) Organization of trachea epithelium in the cartilaginous portion of adult-rabbit and its persistence in organ-culture. *Anatom. Rec.* **238,** 463–472.

18

In Vitro Models of Infection II—Human Umbilical Vein Endothelial Cells (HUVECs) System

Mumtaz Virji and Darryl J. Hill

1. Introduction

Haemophilus influenzae type b, a colonizer of the human respiratory tract, is capable of breaching the epithelial cellular barrier of this niche, as well as endothelial barriers of the vasculature to enter the blood system, prior to dissemination to other tissues including the brain ***(1)***. Besides typeable *H. influenzae, acapsulate* (nontypeable; NTHi) stains, especially those belonging to the clonal biogroup aegyptius, are also capable of causing disseminated infections ***(2)***. The specificity of the organism for human respiratory mucosa and the mechanisms that allow it to transmigrate across both epithelial and endothelial barriers are not fully understood. Bacteria may interact directly with host cells, or they may first interact with phagocytic cells and cross the barrier as passengers within phagocytes. In the former case, interactions with target cell receptors may lead to adhesion, followed by cell signaling events that allow either entry into the cells and transcytosis or opening of cell–cell junctions and paracytosis. Investigations at the cellular level that define the precise components involved in the interactions have been facilitated by the use of immortalized cell lines as in the case of epithelial cells or limited culture of primary human cells as in the case of endothelial cells. Although increasingly more endothelial cell lines are becoming available, human umbilical vein endothelial cells (HUVECs) remain a popular choice of primary endothelial cells and will be described here in detail.

1.1. The HUVECs: Historical Considerations

In early attempts at isolation of endothelial cells, two main problems had been encountered: (1) maintenance of cells as pure cultures for reasonable

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periods, and (2) lack of suitable markers for their identification. In the 1960s, Maruyama ***(3)*** and later Fryer et al. ***(4)*** isolated endothelial cells using trypsin but the primary cultures failed to grow and exhibited progressive degeneration. The main progress in this field followed the introduction of collagenase in place of trypsin to dislodge endothelial cells from cord veins. Collagenase acts on the basement membrane with minimal trauma to cells. Jaffe et al. ***(5–7)*** described a method of isolation based on that of Maruyama but using collagenase. They also used a number of criteria to distinguish umbilical vein endothelial from muscle cells and fibroblasts. The behavior and cytology of HUVECs has been well described both by Jaffe et al. ***(5–7)*** and by Gimborne et al. ***(8)***, and our own procedures are based on these reports.

Observations of these and other authors, summarized below, are worth noting before undertaking the isolation and use of endothelial cells.

1. *To achieve successful establishment of cultures:* Reasonable lengths of cords (20 cm or over) are required to obtain optimal seeding density. Primary inocula of less than 3×10^5 viable cells per 25-cm^2 flask usually fail to become established. Cells released from a 20-cm cord will usually produce a confluent monolayer in a 25-cm^2 flask within 5 d.
2. *Ease of cultivation in vitro:* This is apparently also related to the presence of multicellular aggregates ***(8)***, therefore prolonged collagenase/trypsin treatment should be avoided. 1 mg/mL of collagenase in phosphate-buffered saline (PBS) for 10–20 min is sufficient to yield a uniform population of cells in small clumps, with high viability (ca. 90%); 20–50% of these attach to the substratum with 12 h in culture.
3. *To avoid contaminating cells:* Cord sections should be carefully selected to avoid damaged areas, since these will release contaminating cells that will outgrow endothelial cells. Note that isolation of muscle cells from cords involves deliberate damage of cords with repeated clamping to release muscle cells ***(7)***.
   Occasional giant contaminating cells are seen (in $<10\%$ cultures), which are usually lost during subsequent subcultures, that use trypsin digestion to lift cells. However, when harvesting endothelial cells for subculture, it is important to minimize the trypsin digestion step (3–4 min) in order to maintain viability.
4. *Endothelial cells in culture:* At confluency, cultured endothelial cells reach quiescence, typical of endothelial cells of the adult vasculature. Trauma induced during accidental damage to monolayers (e.g., during washing procedures) results in increased DNA synthesis ***(8,9)*** and expression of specific cellular markers; this should be borne in mind in both employing endothelial cell models and during their utilization. In addition, in quantitative experiments, errors may be introduced, since damage to confluent monolayers reduces cell numbers available for bacterial attachment and exposes extracellular matrix (to which bacteria may bind avidly).
5. *Endothelial markers:* Factor VIII-related antigen, von Willebrand factor (vWF) ***(6,10)***, has been used as a marker for HUVECs and is routinely used in our laboratory to monitor the state of the culture **(Fig. 1)**. By electron microscopy, Weible-Palade bodies (storage organelles for vWF) can also be observed ***(11)***.

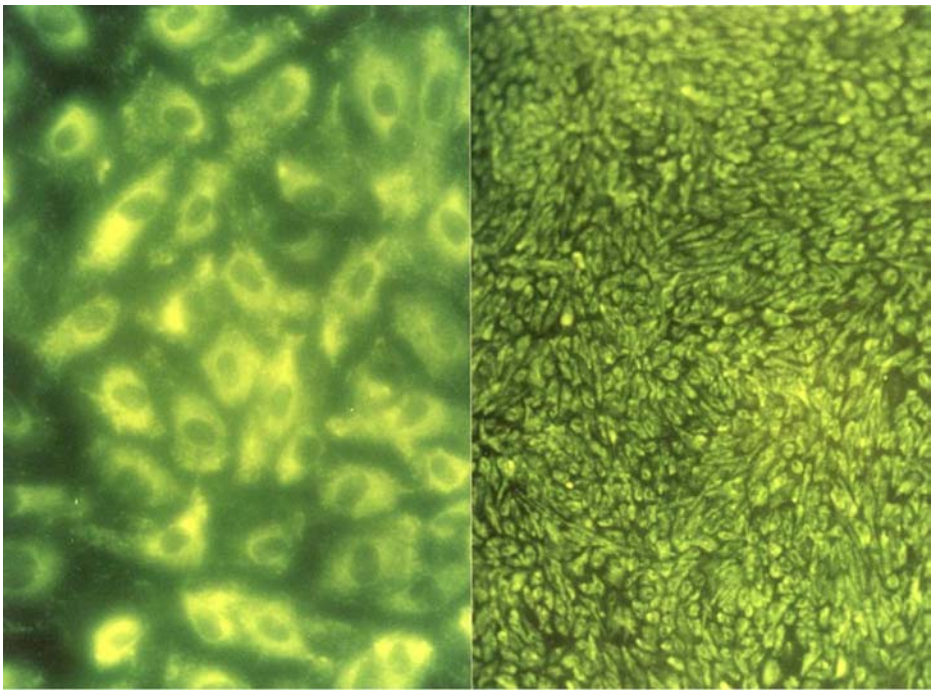

Fig. 1. Low- and high-power magnifications of HUVECs in culture stained for von Willebrand factor expression. Confluent cells at passage 1 were fixed in absolute methanol for 5 min, washed and stained with anti-vWF antibody (Dako), followed by secondary antibody conjugated to FITC.

6. *Length of use of HUVECs in culture:* Primary cells and first-passage cells behave similarly in their growth rates and stain strongly for vWF. Their ability to form cobblestone appearance **(Fig. 2)** and tight monolayers gradually deteriorates, and vWF stain appears progressively patchy with increasing passage number. It is advisable to use cells from early passages (1–3), HUVECs beyond passage 4 should not be used.
7. *Choice of tissue:* As mentioned above, to many scientists, HUVECs still represent the only readily available primary cell line that can be isolated and cultured with ease. Since endothelial cells of distinct tissue origins are heterogeneous with respect to surface receptor expression, tight junction characteristics, and response to various stimuli ***(9,12)***, results obtained with HUVECs may not represent those with endothelial cells at other sites. Routine isolation of endothelial cells from other sites (brain, lung) is clearly not feasible for most investigators if prolonged use is envisaged. Also, in such situations, commercially available cells become prohibitively expensive. In addition, most available cells are transformed and may have altered receptor repertoire. Therefore, many laboratories have devel-

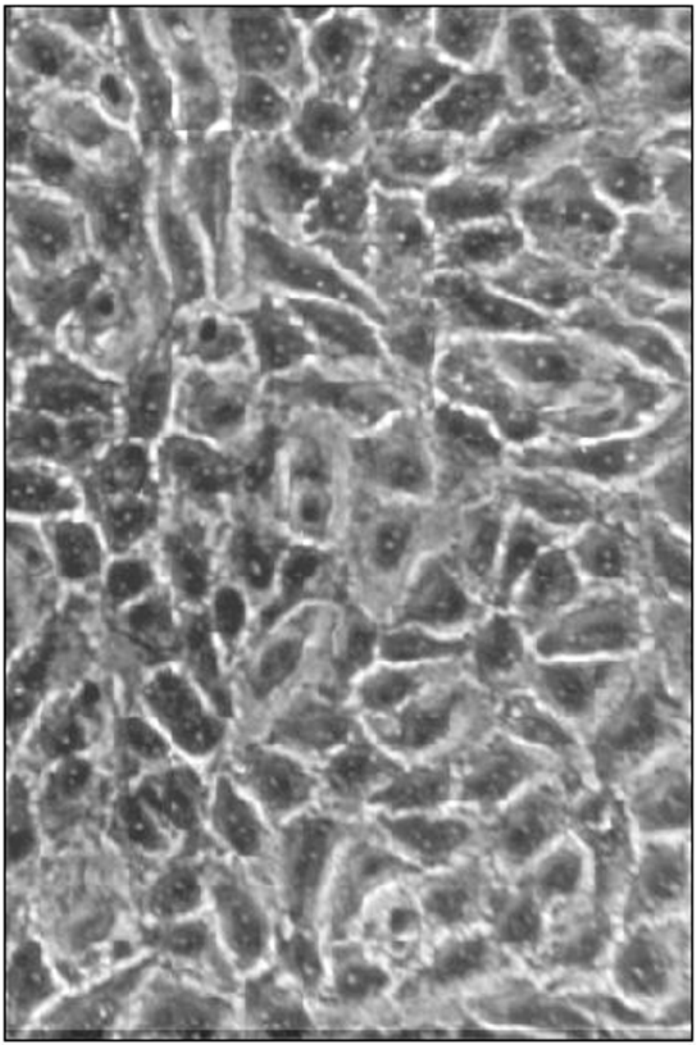

Fig. 2. Typical cobblestone appearance of HUVECs at confluency light microscopy.

oped initial studies using HUVECs and verified important findings using endothelial cells of distinct origins in a limited number of investigations. In our case, the primary role of pili in meningococcal adhesion to human endothelial cells was established using HUVECs, and was confirmed using human capillary endothelial cells.

8. *Choice of medium:* Primary endothelial explants require a rich medium supplemented with high levels of fetal calf serum and brain extracts. Since distinct serum

sources vary in their growth-supporting property, each batch requires careful screening before use. This involves lengthy procedures to test different batches of sera for their ability to support growth (e.g., using 3H thymidine incorporation rates, cell doubling time, etc.). Recently, however, sera have become commercially available. In addition, considerable advances have been made in the development of pretested complete media for endothelial culture, which are available at reasonable cost. All media must be free of endotoxin or mycoplasma contamination.

9. *Choice of extracellular matrix:* Traditionally, gelatin has been used to coat culture dishes, to enhance endothelial attachment to plastic surfaces. However, we found no need for this when seeding HUVECs in EGM as described below (*see* **Subheading 2.2.**).

1.2. Overview of Methods Used to Study Cellular Interactions

1.2.1. Cell Association Studied by the Use of Endothelial Monolayers Cultured in Polystyrene Tissue Culture Dishes

Flat-well 96-well tissue culture plates can be used to investigate bacteria–host cell association, which can be quantified using viable count assays and visualized by immunofluorescence microscopy.

Using specific configuration of plates and assay layout, several phenotypes of bacteria can be analyzed simultaneously on a single plate, and triple or quadruple estimations of each test can also be easily incorporated.

To assess cellular invasion, gentamicin protection assays can also be conducted. These use the aminoglycoside antibiotic gentamicin to eliminate extracellular bacteria since it does not enter eukaryotic cells to a significant extent. After removal of the antibiotic, internalized protected bacteria can be released (using a detergent such as saponin, which acts on eukaryotic but not prokaryotic cells) and colony-forming units (cfu) counted following overnight culture. Microscopic methods can also be used that employ an ordinary fluorescence microscope and require bacteria prelabeled with fluorescein. In this case, after incubation, washing, and fixation, the fluorescence of extracellular bacteria can be quenched using ethidium bromide or trypan blue. Intacellular bacteria appear green and extracellular bacteria orange-red ***(13,14)***. The use of confocal microscopy is described later.

1.2.2. The use of Endothelial Cultures Established on Microporous Filters to Investigate Mechanisms of Transmigration

Several types of filters mounted on suspended wells are available for such investigations (*see* **Note 1**). Using appropriately sized filters, bacterial migration across endothelial monolayers can be studied. This requires that endothelial cells be confluent and the cell barrier be complete. Electrical resistance measurements as well as dyes or small particles have been used to establish the integrity of the

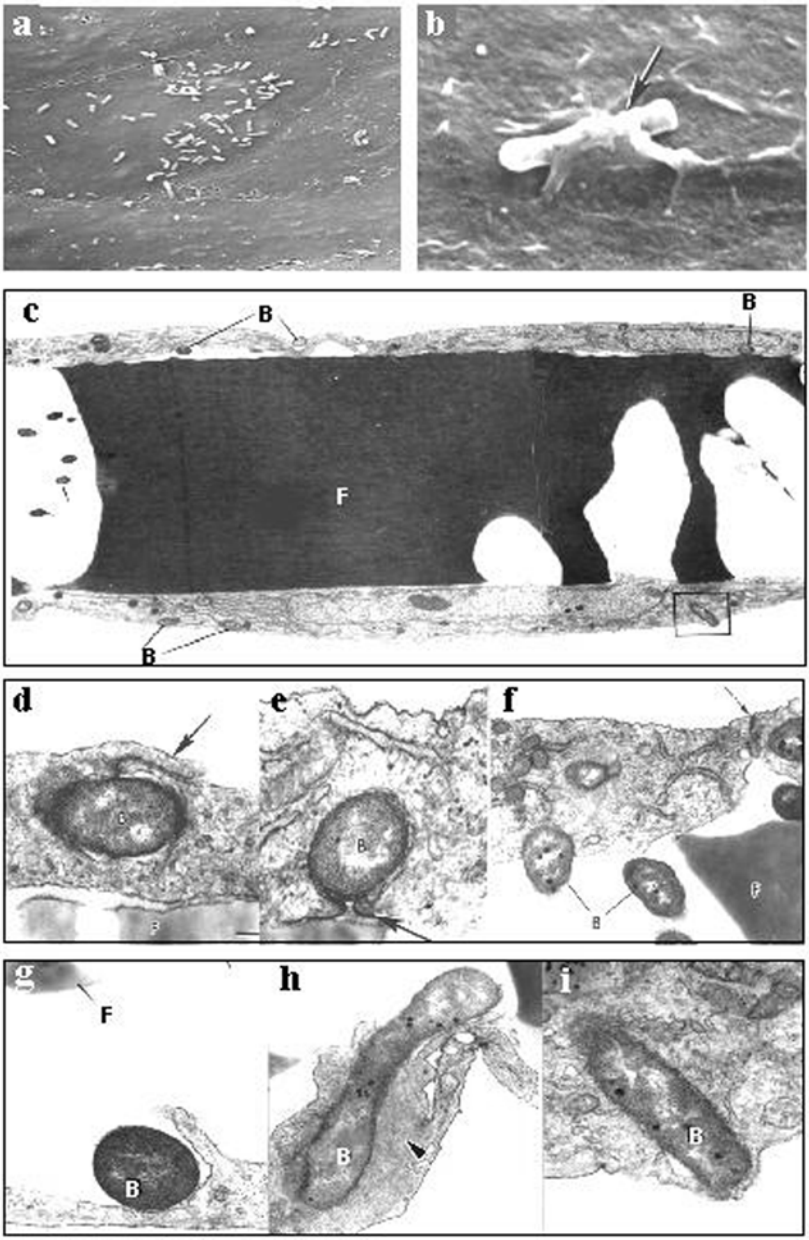

Fig. 3. Electron microscopic study of the interactions of *H. influenzae* with cultured HUVECs. (**A**) A scanning electron micrograph (SEM) showing a large number of bacteria adherent to some but not all cells in the monolayer. (**B**) A higher-magnification SEM showing a bacillary *H. influenzae* in the process of being taken up by an endothelial cell. Cellular processes can be seen overlapping the bacterium (arrow).

monolayers. Confluent monolayers are infected from above (apical infection), and samples are withdrawn from below to estimate bacterial content. The filters can also be sliced for electron microscopy observations (**Fig. 3**).

One interesting observation made with HUVECs was that, if they were cultured on filters with a 3-µm pore size, the cells migrated through the pores and established confluent monolayers on both sides, thus complicating the assay (**Fig. 3**). Intermediate filters (0.4-and 1-µm pore size) are available to reduce this possibility. We used bilayers of HUVECs established on both sides of 3-µm pore filters in conjunction with electron microscopy to follow the passage of *H. influenzae* across HUVECs ***(15)*** (**Fig. 3**).

1.2.3. Confocal Scanning Laser Microscopy

Over the last few years, the amount of information that can be obtained from bench-top microscopy has greatly advanced with the advent of confocal laser imaging. In confocal microscopy, a scanning laser is focused onto a small section of a specimen labeled with fluorescence-coupled antibodies or probes. Collected fluorescent light is passed through a pinhole aperture, eliminating reflected light outside the focal plane. This technique greatly improves the resolution obtainable (down to 0.1-µm focal plane) compared to light microscopy.

The improved resolution obtainable with confocal microscopy allows the study of co-localization of bacterial and host cell molecules and manipulation of host cells to be observed, provided suitable antibodies/probes exist for this purpose. It also allows a three-dimensional image of a cell monolayer to be compiled. Thus the adhesion to and traversal of HUVEC monolayers by *H. influenzae* can be compared qualitatively (possibly semi-quantitatively) by this technique in concert with the quantitative techniques described in this chapter.

Fig. 3. *(continued)* (**C**) Transmission electron micrograph (TEM) showing HUVEC monolayers established on both sides of a 3-µm filter support (F). The top monolayer was infected from above and the filter incubated for several hours before processing for electron microscopy ***(15)***. Bacteria (B) can be seen located at the basolateral side of the top monolayer and both the basal and apical sides of the lower monolayer. The bacterium located in the box is shown at a higher magnification in **i**. (**D–F**) Top monolayer: close-up of bacteria being taken up at the apical surface (**D**), in the process of exit at the basolateral surface (**E**), and located beneath the monolayer in which tight junctions (arrow) remain intact (**F**). (**G–I**) Lower monolayer: close-up of *H. influenzae* interactions at the basolateral surface of the lower monolayer (**G**), a bacillary form entering a HUVEC (**H**), and in the process of transcytosis at the apical surface. The electron micrographs were produced in collaborations with Dr. D. J. P. Ferguson.

2. Materials

2.1. Equipment and General Materials

1. Appropriate facilities for tissue culture and handling of microorganisms and their safe disposal (*see* **Note 2**).
2. Many of the tissue culture-grade materials can be obtained from Sigma. All media use tissue culture-grade water.
3. General disposable plasticware such as sterile pipets, 50-mL tubes, 20-mL universal bottles, and 7-mL bijoux bottles; microbiological culture loops will be required.
4. Eight-pronged pipets (e.g., Finnpipette, Labsystems).

2.2. Materials for Isolation of HUVECs

1. Tissue culture cabinet.
2. Humidified 5% CO_2 incubator set at 37°C.
3. Inverted microscope suitable for examination of tissue culture flasks and plates (e.g., Olympus CK40).
4. Centrifuge (e.g., IEC Centra CL2).
5. Sterile clamps, scalpels, and scissors.
6. Dissection board (sterilized by presoaking in 70% ethanol).
7. Large sterile Petri dishes (Sterilin, cat. No. SC-261).
8. Sterile gauze (10-cm^2 pieces, Vernon Carus, cat. no. 41200) and suture thread (Ethicon, cat. No. W211).
9. 25 cm^2 tissue culture flasks (Corning, cat. no. 25100).
10. 50-mL polypropylene conical tubes (Becton Dickinson, cat. No.352070).
11. 2 L pot with 1 L 1% Virkon (Appleton Woods, cat. no. gc380) for solid waste, 1 L pot with 300 mL 2% Virkon for liquid waste and wash bottle with 70% ethanol.
12. Umbilical cord collection medium: 500 mL Hanks' balanced salt solution (HBSS) (Sigma, cat. no. H-9269); 6 mL gentamicin (10-mg/mL; Sigma, cat. no. G-1272); 5 mL penicillin-streptomycin solution (1000 U/mL; Sigma, cat. no. P-0906); 5 mL sodium pyruvate (11-mg/mL in PBS; Sigma, cat. no. S-8636); 5 mL glutamine (200-mM; Sigma, cat. no. G-7513).
13. Fresh umbilical cords of a good length are collected in cord medium in 250-mL screw-top pots (Sterilin, cat. no. 190C) (see **Note 2**).
14. PBS: sterile Dulbecco's phosphate-buffered saline, 250 mL per cord (Sigma, cat. no. D-8537), for the collagenase incubation step.
15. 30 mL of HBSS containing 2% fetal bovine serum (FBS; GibcoBRL, cat. no. 10106-169) in appropriate syringes: 3 syringes required per cord.
16. Collagenase (Sigma, cat. no. C-5138) freshly diluted in HBSS to a final concentration of 0.5 mg/mL, 10 mL required per cord in a 10-mL syringe.
17. Endothelial cell growth medium (EGM) (Clonetics, cat. no. CC-3124)–containing endothelial cell basal medium (EBM) (CC-3121), 3-mg/mL bovine brain extract (BBE) (CC-4092), 10 mg/mL hEGF, 1-mg/mL hydrocortisone (CC-4035), FBS (CC4101), 50-mg/mL gentamicin, and 50-µg/mL amphotericin-B (CC-4081).

2.3. Additional Materials for Maintainance and Subculture of HUVECs

1. Trypsin-EDTA solution (10X; Sigma, cat. no. T-4174).
2. Trypan blue (Sigma, cat. no. T-6146), 0.4% solution in PBS, filter sterilize and store at 4°C.
3. Improved Neubauer Haemocytometer (Weber Scientific, cat. no. hc001).
4. 96-well tissue culture plates (Costar, cat. no. 3595).
5. 24-well tissue culture plates for cell culture insert (Falcon, cat. no. 3504). Cell culture inserts with 1 μM pore filter (Falcon, cat. no. 3104) or as preferred.
6. Cryovials (Simport Plastics, cat. no. T311-2).
7. Freezing medium: medium 199 containing 20% FBS and 10% dimethyl sulfoxide (DMSO; Sigma, cat. no. D-2650).

2.4. Bacteriological and Other Reagents and Equipment

1. Brain heart infusion (BHI) agar (Difco, cat. no. 0037-17) supplemented with 10% (v/v) heated horse blood (TCS, cat. no. HB035; heated at 100°C for 45 min and then centrifuged at 3000*g* for 30 min) and NAD (nicotimamide adenine dinucleotide; Sigma, cat. no. N-7004) supplement 0.75 g/L.
2. Freezing medium for bacteria: 1% (w/v) proteose peptone or nutrient broth containing 10% (v/v) glycerol. Autoclaved stocks stored at 4°C.
3. PBSB: Sterile Dulbecco's phosphate-buffered saline containing 0.9 m*M* $CaCl_2$ and 0.5 m*M* $MgCl_2$ (Sigma, cat. no. D-8662).
4. Saponin (BDH, cat. no.43650-2L) 10% stocks made in PBS, sterilize by filtration and store at 4°C.
5. Infection medium: Medium 199 (Sigma, cat. no. M-2154) supplemented with 2% FBS.
6. Paraformaldehyde: 2% (w/v) in PBSB, pH 7.4.
7. Absolute methanol.
8. BSA block: 3% (w/v) bovine serum albumin (BSA) (Sigma, cat. no. A-2058) in PBS containing 0.05% (v/v) Tween-20 (Sigma, cat. no. P-1379) and 0.05% sodium azide (Sigma, cat. no. S-2002). Prepared in bulk and stored at 4°C.
9. 1% BSA-PBST (diluent for antibodies): 1% (w/v) bovine serum albumin (BSA) (Sigma, cat. no. A-2058) in PBS containing 0.05% (v/v) Tween-20 (Sigma, cat. no. P-1379) and 0.05% sodium azide (Sigma, cat. no. S-2002). Prepared in bulk and stored at 4°C.
10. Tris-buffered saline (TBS): 0.05 *M* Tris, 0.15 *M* NaCl (pH 8.5), autoclaved and stored at room temperature.
11. NaCl-Tween: 0.9% (w/v) NaCl and 0.05% (v/v) Tween-20. 10× stock (9% NaCl, 0.5% Tween-20) stored at room temperature and diluted in distilled water as required.
12. Antibodies
    a. Monoclonal antibodies and polyclonal antisera against *H. influenzae*
    b. Secondary antibodies:
        i. Goat anti-mouse IgG conjugated to tetramethylrhodamine isothiocyanate (TRITC) (Sigma, cat. no. T-5393).

ii. Goat anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC) (Sigma, cat. no. F-2012).
iii. Rabbit anti-rat IgG conjugated to fluorescein isothiocyanate (FITC) (Sigma, cat. no. F-1763).
iv. Goat anti-rabbit confugated to tetramethylrhodamine isothiocyanate (TRITC) (Sigma, cat. no. T-6778).

13. Fluorescein isothiocyanate (FITC; Sigma, cat no. F4272).
14. Trypan blue (Sigma, cat. no. T0776).
15. Ethidium bromide (Sigma, cat. no. E8751).
16. Microscope for viewing immunofluorescence: Inverted phase contrast (e.g., Olympus IX70) with fluorescence attachment and a camera attached to a special port or other cell imaging accessories.
17. Spectrophotometer.
18. Colony counter.
19. Tissue volt/ohmeter (World Precision Instruments, cat. no. EVOM).

3. Methods

3.1. Preparation of HUVECs

For safety aspects and discard procedures, *see* **Note 2**.

1. Prewarm PBS (250 mL per cord), medium 199 (10 mL per cord), and EGM (5 mL per cord) in a 5% CO_2 incubator at 37°C.
2. Dispense 30-mL volumes of HBSS containing 2% FBS (3 required per cord) and 10 mL of freshly diluted collagenase (*see* **Subheading 2.2.**) in appropriate syringes.
3. Place all other required materials in a tissue culture hood.
4. Remove an umbilical cord from the collection medium, clean the outside with 70% ethanol, wipe with a gauze, and place on the dissection board. Examine the cord carefully for clamp marks or punctures and avoid these areas (*see* **Subheading 1.1.**)
5. Holding the cord firmly with gauze, cut off the ends and any clamp marks with a scalpel. Pace the cord in a large Petri dish.
6. Locate the vein and wash through with 60 mL HBSS containing 2% FBS into the Petri dish to remove all traces of blood. Discard the wash into liquid disposal container.
7. To one end of the cord vein; secure the syringe with collagenase solution. Gently fill the vein; when the solution appears at the other end, clamp with a metal clamp and continue to fill the vein.
8. Tie the second end of the cord with suture thread.
9. Lower the cord into warmed PBS and incubate for 10 min at 37°C, keeping the ends outside the bath.
10. Place the cord on the dissecting board and gently massage to release endothelial cells.
11. Lower the thread end into a 50-mL tube, remove the clamp, and hold this end firmly with a piece of gauze. Cut off the thread end to release the contents. Wash the vein through with 30 mL of HBSS containing 2% FBS.

12. Pellet endothelial cells by centrifugation at 250*g* for 5 min at room temperature.
13. Remove the supernatant and resuspend the pellet in 5 mL of EGF growth medium, transfer to 25-cm^2 tissue culture flask.
14. Incubate at 37°C in a 5% CO2 incubator (*see* **Note 3**).
15. Observe the cells after overnight incubation, wash and feed with fresh medium the next day.

3.2. Maintainance of HUVECs

1. Feed the cells every 2–3 d by replacing half of the medium.
2. Examine daily, and when cells have reached confluency (usually within 7 d), passage at a split ratio of 1:3. Discard the flask if the cells are not confluent by d 10 (*see* **Note 4**).
3. To passage cells, remove EGM and wash the monolayer with PBS.
4. Add 1 mL of 1× trypsin-EDTA solution diluted in sterile PBS and incubate for 3 min at 37°C.
5. Dislodge adherent cells by gently tapping the sides of the T-25 flask and add 5 mL HBSS containing 2% FBS.
6. Remove cells by pipet to a 25-mL universal tube and centrifuge at 250*g* for 5 min at room temperature.
7. Decant supernatant and resuspend endothelial cells in 15 mL EGM. Transfer 5 mL to new 25-cm^2 culture flasks and maintain by feeding as above until required for adhesion/invasion experiments or freezing stocks.
8. To seed cells in multiwell plates for experiments, remove cells from culture flask and collect by centrifugation as above.
9. Resuspend cells in an appropriate volume of EGM (usually 1 mL) and dilute a 2–µL aliquot by the addition of 8 µL trypan blue (0.4% in PBS). Count viable cells on a Neubauer haemocytometer.
10. Adjust the cell density to ca. 10^5/mL and dispense 100 µL per well in a 96-well tissue culture plate for adhesion experiments or 24-well transwell plates on appropriate-pore size filters for transcytosis experiments (*see* **Note 5**).
11. Unused cells may be frozen at this time. Pellet cells by centrifugation at 250*g* for 5 min and resuspend in freezing medium at an appropriate density (usually 10^6/vial), then aliquot into cryovials. Transfer to a polystyrene container and place in a –70°C freezer overnight. For longer storage, transfer to liquid nitrogen until required (*see* **Note 6**).

3.3. Adhesion Assay

3.3.1. Isolation and Characterization of Distinct Bacterial Phenotypes

1. Phase and antigenic variations occur at high frequencies in a large number of surface molecules of *Haemophilus,* including the surface polysaccharides (capsule, LPS-linked glycans) as well as outer membrane adhesins ***(16).*** Therefore, it becomes imperative that bacterial phenotype is carefully examined when studying molecular mechanisms of adhesion—at least with respect to the major surface components. The high frequency of antigenic/phase variation in some

surface ligands also makes it necessary to apply strict handling procedures to any isolated strains, variants, or mutants. One advantage of phase variation is that isolates with distinct surface characteristics can be obtained with relative ease. As with *Neisseriae,* expression of some of the surface structures affects bacterial colony morphology ***(17)***, and this knowledge can help in isolation of adhesion variants. Other methods have used adhesive cell surfaces or phagocytic cells for derivation of variants with distinct characteristics. For example, to select an invasive phenotype, endothelial cells may be infected with the bacteria and incubated for several hours. Then the extracellular bacteria can be eliminated by selective methods and intracellular bacteria isolated.

2. In addition to selection of naturally occurring variants, specific mutations can be created in genes encoding the ligands of interest. Deletion insertion constructs containing kanamycin or other antibiotic-resistance genes have been used widely. These methods are similar to those used for meningococci and have been described in detail recently ***(18)***.

3.3.2. Bacterial Culture

1. *H. influenzae* are grown routinely on BHI agar containing 10% heated horse blood supplemented with NAD. Frozen stocks are generally established after a minimum number of subcultures.
2. Bacteria are frozen in 10% glycerol broth and stored in liquid nitrogen. For experiments, aliquots are scraped out (using sterile flat metal spatulas) from frozen culture stocks, inoculated on agar plates, and cultures used after 16–18 h of growth.

3.3.3. Preparation of Bacterial Suspensions

The following stages should be performed in a microbiological safety cabinet.

1. Grow the strain of interest overnight (16–18 h) on agar plates at 37°C. in 5% CO_2.
2. Using a 10 mL culture loop, make a suspension of the bacteria in 2 mL PBSB. Mix by gently shaking; do not vortex.
3. Remove large bacterial aggregates by centrifugation at 150*g*, for 1 min, at room temperature.
4. Without disturbing the pellet, remove the homogeneous suspension (top 1 mL) into a sterile tube.
5. Solubilize an aliquot of the suspension (10–50 µL) in 1 mL of 1% SDS 0.1 *M* NaOH and measure the nucleic acid content by determining the absorbance of the solution at 260 nm (A_{260}).
6. For *H. influenzae* strains, A_{260} of 0.8 corresponds to a bacterial density of ca. 1×10^9 colony-forming units (cfu)/mL in our laboratory.
7. Dilute the bacterial suspension to an A_{260} of 0.12 in 0.5 mL medium 199 containing 2% FCS (equivalent to 1.5×10^8 bacteria/mL) for use in experiments described in **Subheading 3.3.4.**

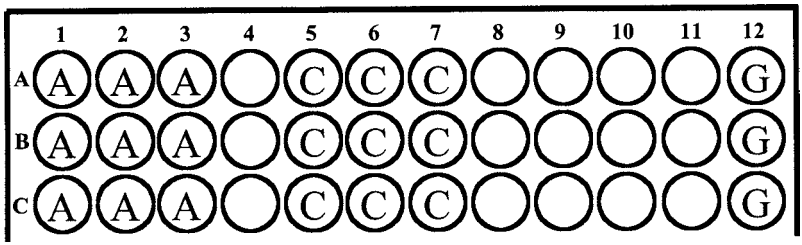

Fig. 4. Diagram showing a possible arrangement of 96-well tissue culture plate for adhesion experiment. Wells marked with A and G contain target cells and bacteria; those marked C contain bacteria alone.

3.3.4. Adhesion Assay

For each strain/variant used, the following wells in a 96-well tissue culture plate are required (enough wells for 3 phenotypes are shown in **Fig. 4.**).

Wells contain: A, confluent cells in growth medium 5 Association wells; C, growth medium with no cells 5 Control association wells; G, confluent cells in growth medium 5 "Growth wells" (set up to monitor numbers of bacteria at the end of the incubation).

1. Prepare bacterial suspensions as described in **Subheading 3.3.3.**
2. Wash all the wells 3× with HBSS to remove growth medium containing antibiotics. Use a multichannel pipet to remove medium, taking care not to disturb the monolayers.
3. Add 50 µL of medium 199 containing 2% FBS to each adhesion and control adhesion well and 40 µL to the growth well.
4. Add 50 µL of the bacterial suspension to each well (A, C, and G), giving 100 µL/well total volume for A and C and 90 µL in well G (*see* **Note 7**).
5. Gently tap the plate to distribute the bugs evenly over the monolayer/well surface and incubate in the 5% CO_2 incubator at 37ºC for 3 h or as required.
6. Immediately following this, estimate cfu in the inoculum. Pipet 90 µL PBSB into 5 wells of a new 96-well plate. Use these wells to serially dilute 10 µL of each bacterial suspension 5 times; a multichannel pipet can be used for simultaneous manipulation of all suspensions, taking care not to cross-contaminate. Pipet 10 µL of dilution 4 (10^{-4}) and 5 (10^{-5}) in duplicate on agar plates. Plates can be sectored to apply 2–4 samples per plate. Transfer to a CO_2 incubator.
7. During the course of the incubation, prepare further dilution plates for cfu estimation of adherent bacteria. Add 90 µL of PBSB to the appropriate number of wells for each strain used. Normally, 4 dilution wells for each A well, 2 for each C well, and 5 for each G well are sufficient.
8. Label appropriate numbers of agar plates.
9. At the end of the 3-h incubation, add 10 µL of 10 % saponin to each of the growth wells (G) (bringing the volume to 100 µL and saponin to the required concentration of 1%).

10. Remove the medium from each of the A and C wells using a multichannel pipet. Wash each well 4× with 150 µL of HBSS carefully, to ensure removal of nonadherent bacteria while not damaging the monolayers.
11. Add 40 µL 1% saponin to each A and C well. Incubate at 37°C for 10 min and thoroughly resuspend the contents of each well.
12. Prepare serial dilutions and plate out 10 µL volumes as above.
13. Incubate the agar plates at 37°C overnight to obtain cfu.
14. Estimation of cell adherence should take into account the volume of saponin, dilution factors, and adherence to blank vessel walls.

3.3.5. Quantification of Internalized Bacteria

Extra wells (U, uptake) set up as for association (A above).

1. After 3 h incubation of bacteria with HUVEC monolayers (**Subheading 3.3.4., steps 1–12**), wash the U wells 4× with HBSS and add 250 µL of 200-µg/mL gentamicin in medium 199 to each well (*see* **Note 8**).
2. Incubate the plate for 1.5 h at 37°C, wash 4× with HBSS, and treat the monolayers with 1% saponin as in **step 11** in **Subheading 3.3.4.**
3. Prepare 10-fold serial dilutions and plate 10 µL of undiluted sample as well as dilutions 1 and 2. (*see* **Note 9**).
4. Validation of gentamicin uptake experiment (*see* **Note 10**).

3.3.6. Calculation of Adherent cfu

1. Determine cfu in the inoculum and G wells.
2. Determine cfu/well in A and C wells, adjusting for dilution and volume of saponin.
3. Since approximately 60% of the total well area (containing 100 µL vol) (i.e., well walls in A wells are exposed to bacterial suspension), subtract 60% of C value from A. This is specific adhesion to cells (*see* **Note 11**).
4. Cell association may be expressed as percent of inoculum associated or number of bacteria/cell (*see* **Note 12**).

3.3. Immunofluorescence Detection of Cell-Associated Bacteria

1. Carry out **steps 1–10** as described in **Subheading 3.3.4.**
2. After washing, fix the monolayers with 2% paraformaldehyde solution for 30 min or absolute methanol for 10 min prior to detection by immunolabeling (*see* **Note 13**).
3. Wash the fixed monolayers and block nonspecific sites for 1 h or more with BSA block.
4. Incubate the fixed monolayers with the antibodies against bacteria (e.g., mouse monoclonal antibody against LPS or anticapsular antibody), diluted in 1% BSA-PBST for 1 h. Wash 3× in NaCl-Tween.
5. Add a secondary antibody conjugated to TRITC or FITC to detect specific binding of the primary mabs to bacteria.
6. Incubate the plates at room temperature in the dark for 1 h, then wash 4× with NaCl-Tween. Add 50 µL/well of 50 m*M* TBS, pH 8.5, containing 0.05% sodium azide. Plates can be stored at 4°C in the dark for considerable periods.

7. Examine the labeled preparations in the plates using an appropriate inverted microscope with fluorescence and camera attachments. Images can be recorded using an ordinary camera attachment and Kodak Gold (400ASA) or a similar film.

3.4.1. Detection of Internalized Bacteria by Direct Labeling

1. Suspend *H. Influenzae* in PBSB as described in **Subheading 3.3.3.** and wash twice by centrifugation for 15 min at 2772*g*.
2. Label bacteria with FITC by incubation of 10^9 bacteria in 1 mg/mL FITC isomer in 50 m*M* $NaHCO_3$, pH 9.2, with 0.1% (v/v) glycerol at 25°C. for 15 min.
3. Bacteria were washed 3× in PBSB and overlaid on HUVEC monolayers as described in **Subheading 3.3.4.**
4. Following removal of unbound bacteria, internal bacteria can be detected by the addition of 50-mg/mL ethidium bromide, or 200-µg/mL trypan blue to quench external fluorescence.
5. Cell can now be examined as for immunofluorescence, with external bacteria appearing a red-orange and internal bacteria fluorescing bright green.

3.5. Measurements of Bacterial Traversal Across Filter-Grown Monolayers

1. Seed HUVECs at or near confluency (ca. 3×10^4) on 1-µm-pore filters in a 24-well tissue culture plate. Place 1 mL of EGM into the well and 0.5 mL onto the cells on suspended filters.
2. Feed the cells as above (*see* **Subheading 3.2.**) (*see* **Note 14**).
3. Measure electrical resistance across the monolayer at the same time to assess the confluency of the HUVEC monolayer (*see* **Note 15**).
4. Once the electrical resistance has reached a plateau, the monolayers can be used for transcytosis experiments.
5. Resuspend *H. influenzae* strains as in **Subheading 3.3.3.** and dilute to 2×10^8 bacteria in 1 mL in medium 199 containing 2% FBS.
6. Wash the monolayer gently 3× with HBSS to remove antibiotics and rinse the bottom of the insert in sterile HBSS. Then place inserts in a new 24-well plate containing 1 mL medium (199 containing 2% FBS).
7. Overlay HUVEC monolayers with 0.5 mL of bacterial suspension and incubate for at 37°C
8. At 30-min to 1-h intervals, wash the monolayer gently 3× to remove nonadherent bacteria. Transfer the filter to a new well with fresh medium. Plate out samples from the previous lower chamber to estimate the numbers of bacteria in the lower chamber.

3.6. Confocal Imaging

Filter-grown cells can be used for confocal microscopy, alternatively, cells can be grown on glass cover slips inside tissue culture wells.

1. Label filters after washing by the method described in **Subheading 3.5.**

2. Cut off filters after washing with a sharp scalpel and transfer to a glass slide, cell side down into mountant. Glass cover slips can be treated in an identical manner.
3. View using a confocal microscope. To assess bacterial localisation within the cell, the *X–Z* plane can be viewed, although the flattened nature of endothelial cells makes this assessment difficult.

3.7. Conclusion/Summary

The method for the culture of HUVECs described here has been made easier over the last decade with the improvement of culture medium commercially available and the more reliable consistency of media supplements such as FBS. Although the studies of *H. influenzae* adhesion to HUVECs give us an insight into interactions and traversal of endothelial barriers by these bacteria, it is important to remember that variations in such interactions and the subsequent molecular events may occur in other endothelial types, such as microvascular endothelial cells. This point aside, these studies enable general bacterial-stimulated endothelial events to be studied with relative ease, yielding reproducible quantitative data for adhesion and invasion of *H. influenzae* and the effects of various reagents on such events. It is also an advantage to perform appropriate immunocytochemistry or immunofluorescence studies, which can be examined qualitatively (or semiquantitatively) by light microscopy or confocal laser scanning microscopy, thus providing photographic evidence in support of the quantitative studies. The techniques for adhesion/invasion studies described in this chapter are fairly versatile and can be applied to many cell types/ bacterial species as required by the user with the appropriate adjustments.

4. Notes

1. Transwell filters of 3- or 0.4-μm pore size are available from Costar, and 1-μm pore size are available from Beckton Dickinson.
2. Due to the risk of infection from human tissues, all material discarded during HUVEC preparation should be soaked in 1% Virkon solution prior to autoclaving and disposal. All surfaces and equipment used during HUVEC preparation should be washed with 1% Virkon solution prior to normal washing/sterilization procedures.
3. Up to 4 HUVEC preps can be comfortably managed by one person at a time.
4. If after 10 d the cells have not reached confluency, the chances are that they are traumatized and will not produce lasting cultures.
5. A confluent 25-cm^2 culture flask will contain ca. 2.5×10^6 HUVECs. At confluency, each well of a 96-well culture plate will contain 3×10^4 HUVECs. The size of the filter insert is similar to that of a microwell from the above suppliers.
6. It is advisable to use HUVECs from early passages. Passage 1 cells are best for transcytosis experiments. Cells that have undergone more than 4 passages should, as a rule, not be used. Cells are normally only frozen at passage 1 and seeded at high density for use in experiments. Careful monitoring of their viability and growth rate is important.

7. Overlaying 50 µL of 1.5×10^8 bacteria/mL gives a multiplicity of infection of ca. 250 bacteria/cell. Obviously, this number can be adjusted by diluting the bacteria suspension appropriately.
8. Each strain/variant to be used should be tested for gentamicin sensitivity (using a range of 50–200 µg/mL for 30 min to 2 h).
9. Dilutions required will obviously vary with the phenotype and will require repeat experiments with appropriate dilutions if a higher-invasive phenotype is used.
10. When gentamicin survival assay is used to obtain "internalized" phenotype, it is necessary to carry out a control experiment to establish that the bacteria isolated were truly internalized. This is necessary because in some situations, e.g., when bacteria tend to form large clumps on the target cell surface, many may escape the action of gentamicin. Since cytoskeletal activity is required in most cases for bacterial uptake, the following control experiment will establish if survivors have escaped the antibiotic action by cell entry or via other means.
    Host cells are preincubated with 2-µg/mL cytochalasin D (CD) in infection medium for 30 min prior to infection with bacteria. CD is present throughout the infection period at 1-µg/mL. Pretreatment with CD prevents cytoskeletal activity, and bacteria usually remain externally adherent to the target cells. The subsequent gentamicin treatment should effectively eliminate all bacteria. If a significant number survives, then the assay cannot be used to study internalization.
11. It may not be necessary to undertake this in every experiment if nonspecific adhesion to plastic is too low to be of significance. However, the cfu estimation will be greatly affected depending on the relative cell adherence vs nonspecific interaction with the well.
12. Usually there is limited extent of growth under these infection conditions, but it is important to establish if the inoculum is viable or has grown extensively during the course of the experiment.
13. Use of a fixative depends on the antibody specificity. Paraformaldehyde preserves structures and may be better in some cases, whereas methanol may expose buried epitopes.
14. Replace the medium in the lower chamber, as well as the upper chamber. When changing medium in the upper chamber take care not to touch the monolayer or disrupt it when fresh medium is added.
15. Transendothelial resistance measurements carried out according to the manufacturer's instructions. The electrode should be cleaned frequently in 70% ethanol and washed in fresh sterile medium. Unlike some epithelial cells, HUVECs do not produce a high level of electrical resistance.

References

1. Moxon, E. R. and Ostrow, P. T. (1977) *Haemophilus influenzae* meningitis in infant rats: role of bacteremia in pathogenesis of age-dependent inflammatory responses in cerebrospinal fluid. *J. Infect. Dis.* **135,** 303–307.
2. Quinn, F. D., Weyant, R. S., Worley, M. J., White, E. H., Utt, E. A., and Ades E. A. (1995) Human microvascular endothelial tissue culture cell model for studying pathogenesis of Brazilian purpuric fever. *Infect. Immun.* **63,** 2317–2322.

3. Maruyama, Y. (1963) The human endothelial cell in tissue culture. *Z. Zellforsch. Mikrosk. Anat.* **60,** 69.
4. Fryer, D. G., Birnbaum, G., and Luttrell, C. N. (1966) Human endothelium in cell culture. *J. Atheroscler. Res.* **6,** 151.
5. Jaffe, E. A., Nachman, R. L., Becker, and Minick, R. C. (1972) Culture of human endo thelial cells derived from human umbilical cord veins. *Circulation* **46 II,** 253.
6. Jaffe E. A., Hoyer, L. W., and Nachman, R. L. (1973) Synthesis of antihemophilic factor antigen by cultured human endothelial cells. *J. Clin. Invest.* **52,** 2757–2764.
7. Jaffe, E. A, Nachman, R. L., Becker, C. G., and Minick C. R. (1973) Culture of human endothelial cells derived from umbilical veins. *J. Clin. Invest.* **52,** 2745–2756.
8. Gimborne, M. A., Cotran, R. S., and Folkman, J. (1974) Human vascular endothelial cells in culture. *J. Cell Biol.* **60,** 673–684.
9. Bicknell, R. (1996) *Endothelial Cell Culture: Handbooks in Practical Animal Cell Biology*, Cambridge University Press, pp. 1–5.
10. Booyse, F. M., Sedlak, B. J., and Rafelson, M. E.(1975) Culture of arterial endothelial cells. Characterisation and growth of bovine aortic cells. *Thromb. Diathes. Ahemorrh.* **34,** 825–839.
11. Weibel, E. R. and Palade, G. E. (1964) New cytoplasmic components in arterial endothelia. *J. Cell Biol.* **23,** 101–112.
12. Scott, P. A. E. and Bicknell, R. (1993) The isolation and culture of microvascular endothelium. *J. Cell Sci.* **105,** 269–273.
13. Drevets, D. A. and Campbell, P. A. (1991) Macrophage phagocytosis: use of fluorescence microscopy to distinguish between extracellular and intracellular bacteria. *J. Immunol. Meth.* **142,** 31–38.
14. McNeil, G., Virji, M., and Moxon, E. R. (1994) Interactions of *Neisseria meningitides* with human monocytes. *Microbial pathogen.* **16,** 153–163.
15. Virji, M., Kayhay, H., Ferguson, D. J. P., Alexandresc, C., and Moxon E. R. (1991) Interactions of *Haemophilus influenzae* with cultured human endothelial cells. *Microbial Pathogen.* **10,** 231–245.
16. Moxon, E. R., et al. (1994) Adaptive evolution of highly mutable loci in pathogenic bacteria. *Curr. Biol.* **4,** 24–33.
17. Prasadarao, N. V., Lysenko, E., Wass, C. A., Kim, K. S., and Weiser, J. N. (1999) Opacity-associated protein A contributes to the binding of *Haemophilus influenzae* to change epithelial cells. *Infect. Immun.* **67,** 4153–4160.
18. Virji, M. and Serino, L. (2001) Molecular recognition mechanisms of meningococci: identifying receptor-ligand pairs involved in microbe-host interactions, in *Meningococcal Disease, Methods in Molecular Medicine, vol. 66* (Pollard, A. J. and Maiden, M. C. J., eds.), Humana Press, Totowa, NJ.

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Animal Models

1. Introduction

The two animal models mainly used for the study of *Haemophilus influenzae* infections are the 5-d-old infant rat, for invasive disease, and the chinchilla, for local infection. We describe the 5-d-old infant rat model here and give references for the reader for the chinchilla model.

1.1. The Infant Rat Model

The outbred, specific pathogen-free, Sprague-Dawley 5-d-old infant rat is believed to be a biologically relevant model of invasive human *H. influenzae* infection. Established in the 1970s, the model has valuable characteristics: the route of entry (where intranasal inoculation is employed) and mode of dissemination appear to be the same as in humans; the course and duration of infection are predictable and reproducible within the limits of a natural infection; and the technique of inducing infection is simple ***(1,2)***. It has been well described, validated, and is the standard model for testing the virulence of *H. influenzae* mutants ***(3–5)***. Nasopharyngeal or peritoneal (ip) inoculation leads to bloodborne dissemination and meningitis, although the usual inoculum differs for each route: 10^6 –10^8 organism nasopharyngeal compared with 10^2–10^3 organisms ip. After nasopharyngeal inoculation with 10^7 Eagan, approx 75% of infant rats became bacteremic whether the rats were 5, 10, or 20 d old; however, the magnitude of the bacteremia was inversely proportional to animal age at time of inoculation ***(2)***. The minimal lethal ip dose for newborn rats is less than 10 organisms, compared with 10^3–10^4 at 5 d old and 10^7–10^8 at 10 d old ***(6)***. Five-d-old infant rats given an ip dose of 10^8 Eagan die within 2 h, and 10^3 bacteria is the LD_{50}, with death occurring at 48–72 h. Bacteremia can be detected within minutes of a 10^3 ip injection, and it reaches a magnitude of 2×10^4 organisms by 12 h and 7×10^5 by 24 h. Meningitis is always preceded by several hours of bacteremia, consistent with the notion that CSF entry is from the blood, involving crossing of the blood–brain barrier ***(2)***. Normally, only encapsulated

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H. influenzae are able to cause disease in the model ***(4,7)***, but occasional highly virulent nontypeable *H. influenzae* may also do so ***(8,9)***. For most studies an ip dose of around 10^2 organisms is given and the rats are bled at 48 h ***(10–12)***. This dose means that the rats do not usually die before 48 h. A 10–20 µL tail bleed at 48 h is the standard method of recovering *H. influenzae*, and colony counts with this method compare within 1 log of counts from jugular and cardiac puncture ***(13)***.

1.2. In Vivo Survival Biology

There appear to be some consistent features to *H. influenzae* invasive disease: the chance of infection correlates with the size of the inoculating dose; there is a delay between the inoculation and the infection becoming established (lag phase); inoculating a small number of organisms tends to result in infection by a single organism, whereas inoculating a large number produces a mixed infection; and disease is unlikely to arise from the inoculation of a single organism ***(2,14)***.

In 1935, Halvorson proposed two different models to explain how identical hosts could have different outcomes when challenged with varying doses of a pathogenic organism ***(15)***. The two models were later termed the cooperative action hypothesis and the hypothesis of independent action ***(16)***. In the cooperative action hypothesis, inoculated organisms cooperate to saturate host defences, so that infection is unlikely if the inoculum is small, yet occurs once an individual effective dose (IED) has been delivered. In the hypothesis of independent action, inoculated organisms act independently, the events leading to infection being stochastic. Some evidence in support of the hypothesis of independent action arose when Meynell challenged mice with *Salmonella typhimurium* and determined that inoculation of an IED in divided doses carried the same risk of infection as when the whole dose was given ***(17,18)***. The birth-death model built on this latter hypothesis and assumed that outcome was determined by successive random events that continually operated as long as even one organism was viable in the host ***(19,20)***.

The cooperative action hypothesis never successfully explained how pathogens such as *H. influenzae* could cause infection when the inoculum was small and clearly insufficient to overwhelm host defences ***(14)***. Thus it is likely that *H. influenzae* acts according to the hypothesis of independent action. There might be a role, though, for the hypothesis of cooperative action mechanism in near-terminal stages of infection. For instance, a partially virulent organism that has remained viable after inoculation but is unable to cause disease may be capable of multiplication when host defenses are diminished. This is the partial synergism hypothesis.

If the independent action hypothesis is a true reflection of the early infection process, at a low inoculum of mixed organisms by chance only one or a few clones will be successful in causing disease, whereas when a larger inoculum

is given, more clones will cause disease. And, when the inoculum is very large, the same number of clones will cause disease as are in the inoculum. When considering a pool of mixed clones, if the total dose of the input inoculum is low, the output clones are likely to be a random subset of the survivors.

The standard *H. influenzae* Eagan inoculum for the infant rat is around 1×10^2 to 1×10^3 colony-forming units (cfu), a dose that ensures all animals develop disease as long as the organism is fully virulent. Animals given an ip inoculum greater than 1×10^3 cfu often die before 48 h. The individual effective dose may be much smaller than 1×10^3 cfu, for instance, an ip inoculum of less than 10 Eagan will often cause disease.

1.3. The Chinchilla Model

Nontypeable *H. influenzae* (NTHi) account for 25–30% of otitis media, inflammation of the middle ear; initial colonization of the mucous membranes of the oropharynx is followed by retrograde ascent up the Eustachian tube and then invasion of the middle ear. The chinchilla model of otitis media involves direct inoculation of NTHi into the middle ear; alternatively, nasopharyngeal instillation of NTHi allows the study of nasopharyngeal colonization ***(21,22)***.

2. Materials

Five-day old Sprague-Dawley infant rats are used in the standard type b *H. influenzae* bacteremia model, but can also be employed for the study of nasopharyngeal colonization.

2.1. Bacterial Culture

1. The following bacterial strains are examples of those employed: RM118 (Rd; capsule-deficient type d strain, nonvirulent in the infant rat model); RM153 (Eagan; type b encapsulated); RM154 (streptomycin-resistant Eagan); RM133 (Rd-b+:01; capsule-deficient type d strain, transformed with whole genomic DNA from Eagan conferring type b capsule on an Rd background); RM135 (Rd-b+:02; as Rd-b+:02, but type b capsule and a background strain more akin to Eagan).
2. Brain heart infusion (BHI) broth is made by reconstituting 37 g/L BHI granules in distilled water and autoclaving at 120°C for 15 min (make in batches of 200–400 mL).
3. Immediately before use, add NAD (2 µg/mL; stock solutions of 1 g/mL stored at −20°C) and hemin (20 µg/mL; stock solutions of 2 g/mL stored at +4°C).

2.2. Bacterial Dilution

1. Autoclave phosphate-buffered saline (PBS; Oxoid) at 120°C for 15 min, and store the stock at 4°C. Open aseptically to maintain sterility with recurrent use.
2. Gelatin: 2% stock solution in distilled water, autoclaved at 120°C for 15 min, and stored at 4°C. Prewarm to 50°C before each use. Add 2 mL to 38 mL PBS to give a 0.1% solution. This can be maintained for a few hours at 4°C until used.

2.3. Animal Inoculation and Bacterial Recovery

1. Syringes (1 mL; Plastipack, Becton Dickinson) and needles (25 gage; Microlance3, Becton Dickinson)
2. Neonatal lancets (Owen Mumford) for tail-vein blood sampling.

2.4. Agar Plates

1. BHI agar is made as BHI broth but with the addition of 6 g/400 mL bacteriological agar (Oxoid).
2. Antibiotics are added according to the resistance profiles of the *H. influenzae* strains and mutants. Typical concentrations are: 10 µg/mL kanamycin, 5 µg/mL nalidixic acid, 4 µg/mL tetracycline, 2 µg/mL chloramphenicol, 6 µg/mL ampicillin, and 100 µg/mL streptomycin.
3. Dry plates upright with the lids off for 20 min in a laminar-flow hood or for slightly longer upside down at 37°C.

3. Methods

Vivisection should only be undertaken when experiments cannot be performed in cell or tissue culture. Experiments involving animals must be performed by investigators who have been appropriately trained and who are holders of animal licenses; in the U.K., a home office license can only be obtained after attending an animal welfare course. All animal experiments should be undertaken humanely and in category III pathogen facilities. All bacterial preparation prior to animal inoculation needs to be undertaken in a category II laboratory.

3.1. Invasive Disease Assay (ip Inoculation)

1. Culture bacteria overnight in BHI broth, subculture the following morning to fresh broth, containing antibiotics for mutant strains, and incubate for 2–3 h until the OD_{A490} reaches ~ 0.2–0.4.
2. Dilute bacteria in PBSG to give an estimated inoculum of 20–200 cfu (*see* **Note 1**).
3. Plate an aliquot of the input bacteria to determine the true inoculum.
4. Draw up the bacterial suspensions into prelabeled 1 mL syringes and place on ice until inoculated (*see* **Note 1**).
5. Weigh and label infant rats with a permanent laboratory marker pen (i.e., Pentel N50). Some investigators prefer to use subcutaneous inoculation of India ink in a pattern of dots that distinguishes each infant; however, for 5-d-old rats this is unnecessary. Pen marking will remain visible for at least 2 d. If follow-up for individual rats is required for longer than 48 h, they should be re-marked every 2 d.
6. Inoculate a bacterial suspensions (100 µL) ip into the right lower quadrant of the abdomen of individual infant rats.
7. Check the animals at least daily for signs of disease.
8. At 48 h, the animals are bled via tail-vein puncture using a stylet. The recovered blood can usually be plated directly on to BHI agar that does not contain antibiotics with minimal risk of contamination by skin flora (*see* **Note 2**); plate 5 µL neat and 5 µL diluted in 995 µL PBSG (*see* **Note 3**).

9. Culture the recovered bacteria overnight at 37°C and count the cfu.
10. For bacteria harboring antibiotic resistance, such as defined mutants, a representative proportion can be subcultured to antibiotic-containing medium to ensure that the mutation has remained stable.
11. Use a nonparametric test, such as the Mann-Whitney *U* test, with two-tailed *P* value, for calculating the significance of the difference between survival of the mutant and wild-type organisms.

3.2. Local Infection Assay (Nasopharyngeal Inoculation)

1. For intranasal inoculation, both wild-type and mutant bacteria are first made streptomycin-resistant by transformation with whole chromosomal DNA from Eagan RM154.
2. Prepare the bacteria as above for ip inoculation, but dilute to approx 10^1–10^2 cfu and plate to gage the exact inoculum given.
3. Inoculate 10 µL of the 10^8/100 µL dilution into the right nostril, giving an inoculum of ~10^7 cfu.
4. At 48 h, 20 µL sterile PBSG is inoculated into the right nostril and as much of the PBSG is recovered as bubbles out of the left nostril. Plate on to streptomycin (100 µg/mL) containing agar (*see* **Note 4**). Mutants are subsequently plated on to agar containing the relevant antibiotic.

4. Notes

1. Assume that OD_{A490} 0.4 equates to 2×10^9 cfu/mL. Pellet bacteria that have reached OD_{A490} 0.2–0.4 at 1800*g* and resuspend in a volume of PBSG that gives 2×10^9cfu/mL. For accurate dilution of bacteria, resuspend the pellet carefully but completely in PBSG at room temperature, vortex for ~1–2 s, then add 100 µL of bacteria to 900 µL fresh PBSG, vortex again briefly, and repeat the steps serially until 2×10^3 cfu/mL (2×10^2 cfu/100 µL) is reached. Store bacterial suspensions on ice until inoculated, but make this time interval as short as possible, preferably 1 h. Some bacterial strains have a slightly different density of bacteria at OD_{A490} 0.4; rather than perform growth curves, it is possible to determine the cfu at this OD and assume that there will be a linear relationship between 0.2 and 0.4. Thus, 0.4 represents 2×10^9 cfu/mL for Eagan, and 0.2 and 0.6 equate to 1×10^9 cfu/mL and 3×10^9 cfu/mL, respectively. However, outside the range 0.2–0.6, the relationship between OD and cfu may not be linear.
2. If agar plates inoculated with blood become contaminated with skin flora from the infant rats, bacitracin (200 U/mL) can be added to the agar, and will inhibit the growth of Gram-positive organisms.
3. Infant rats are placed in a warming chamber held at around 30°C while awaiting inoculation or prior to venepuncture. Sprague-Dawley adult rats are docile and do not attempt to bite, provided they are handled calmly and confidently. Infant rats should be separated from their mothers for the briefest interval as possible, to avoid distress to the mother. After inoculation or venepuncture of each infant rat, it is immediately returned to the mother.

4. Assessment of survival in the nasal colonization model is only semiquantitative. It is useful for defining that a mutant is completely unable to survive. Better methods for defining adherence or colonization defects are the chinchilla model or one of the in vitro-tissue culture models (the explanted nasopharyngeal tissue model or HUVECs).

References

1. Moxon, E. R. (1975) An animal model for experimental meningitis in infant rats, in *New Approaches for Inducing Natural Immunity to Pyogenic Organisms* (Robbins, J. B., ed.), National Institutes of Health, Bethesda, MD, pp. 76–80.
2. Moxon, E. R., Glode, M. P, Sutton, A., and Robbins, J. B. (1977) The infant rat as a model of bacterial meningitis. *J. Infect. Dis.* **136(Suppl.),** S186–S190.
3. Moxon, E. R., Zwahlen, A., Rubin, L. G., Hoiseth, S., and Connelly C. (1984) Pathogenesis of meningitis: experimental studies on the molecular basis of *Haemophilus influenzae* infection. *Infection* **12(Suppl. 1),** S23–S28.
4. Moxon, E. R. and Vaughn, K. A. (1981) The type b capsular polysaccharide as a virulence determinant of *Haemophilus influenzae*: studies using clinical isolates and laboratory transformants. *J. Infect. Dis.* **143(4),** 517–524.
5. Moxon, E. R. (1982) Experimental studies of *Haemophilus influenzae* infection in a rat model, in *Haemophilus influenzae* (Sell, S. H. and Wright, P. F., eds.), Elsevier, New York, pp. 59–71.
6. Smith, A. L., Smith, D. H., Averill, D. M., Marino, J., and Moxon, E. R. (1973) Production of *Haemophilus influenzae* b meningitis in infant rats by intraperitoneal inoculation. *Infect. Immun.* **8,** 278–290.
7. Zwahlen, A., Kroll, J. S., Rubin, L. G., and Moxon, E. R. (1989) The molecular basis of pathogenicity in *Haemophilus influenzae*: comparative virulence of genetically-related capsular transformants and correlation with changes at the capsulation locus cap. *Microb. Patho.* **7(3),** 225–235.
8. Rubin, L. G., Gloster, E. S., and Carlone, G. M. (1989) An infant rat model of bacteremia with Brazilian purpuric fever isolates of *Hemophilus influenzae* biogroup aegyptius. Brazilian Purpuric Fever Study Group. *J. Infect. Dis.* **160(3),** 476–482.
9. Nizet, V., Colina, K. F., Almquist, J. R., Rubens, C. E., and Smith, A. L. (1996) A virulent nonencapsulated *Haemophilus influenzae* (published erratum appears in *J. Infect. Dis.* 1998 Jul;**178** +**,** 296). *J. Infect. Dis.* **173(1),** 180–186.
10. Hood, D. W., Deadman, M. E., Allen, T., et al. (1996) Use of the complete genome sequence information of *Haemophilus influenzae* strain Rd to investigate lipopolysaccharide biosynthesis. *Mol. Microbiol.* **22(5),** 951–965.
11. D'Mello, R. A., Langford, P. R., and Kroll, J. S. (1997) Role of bacterial Mn-cofactored superoxide dismutase in oxidative stress responses, nasopharyngeal colonization, and sustained bacteremia caused by *Haemophilus influenzae* type b. *Infect. Immun.* **65(7),** 2700–2706.
12. Bishai, W. R., Howard, N. S., Winkelstein, J. A., and Smith, H. O. (1994a) Characterization and virulence analysis of catalase mutants of *Haemophilus influenzae*. *Infect. Immun.* **62,** 4855–4860.

13. Moxon, E. R., Smith, A. L., Averill, D. R., and Smith, D. H. (1974) *Haemophilus influenzae* meningitis in infant rats after intranasal inoculation. *J. Infect. Dis.* **129,** 154–162.
14. Moxon, E. R. and Murphy, P. A. (1978) *Haemophilus influenzae* bacteremia and meningitis resulting from survival of a single organism. *Proc. Natl. Acad. Sci. USA* **75(3),** 1534–1536.
15. Halvorson, H. O. (1935) The effect of chance on the mortality of experimentally infected animals. *J. Bacteriol.* **30,** 330–359.
16. Rubin, L. G. (1987) Bacterial colonization and infection resulting from multiplication of a single organism. *Rev. Infect. Dis.* **9,** 488–493.
17. Meynell, G. G. (1957) The applicability of the Hypothesis of Independent Action to fatal infections in mice given *Salmonella typhimurium* by mouth. *J. Gen. Microbiol.* **16,** 396–404.
18. Meynell, G. G. and Stocker, B. A. D. (1957) Some hypotheses on the aetiology of fatal infections in partially resistant hosts and their application to mice challenged with Salmonella paratyphi-B of *Salmonella typhimurium* by intraperitoneal injection. *J. Gen. Microbiol.* **16,** 38–58.
19. Armitage, P., Meynell, G. G., and Williams, T. (1965) Birth–Death and other models for microbial infection. *Nature* **207,** 570–572.
20. Williams, T. and Meynell, G. G. (1967) Time-dependence and count-dependence in microbial infection. *Nature* **214,** 473–475.
21. TF dM. Animal models for nontypeable *H. influenzae* otitis media. *Pediatr. Infect. Dis.* **8,** S40–S42.
22. de Maria, T. F. A. M., Nichols, W. A., and Leake, E. R. Evaluation of the virulence of nontypeable *Haemophilus influenzae* lipooligosaccharide htrB and rfaD mutants in a Chinchilla model of otitis media. *Infect. Immun.* **64,** 4431–4435.