J. L. Dangl (Ed.)

Bacterial Pathogenesis of Plants and Animals

Molecular and Cellular Mechanisms

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Molecular and Cellular Mechanisms

Edited by J. L. Dangl

With 41 Figures and 8 Tables

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Cover Illustration: The left and upper portions of the cover illustrate directional assembly of host actin by Listeria monocytogenes in Vero cells, which contributes to intra- and intercellular spread of this pathogen. The bacteria are labelled in red and host cell actin is labelled in green (see chapter by Sheehan et al. for details). At the lower right is a pepper fruit suffering from spot disease caused by Xanthomonas campestris pv. vesicatoria (see chapter by Bonas for details). By Udo Ringeisen, Cologne.

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Renoir a m' a dit: Quand j' ai arrangé un bouquet pour le peintre je m' arrète sur la côté que je n' avais pas prévu.

(Renoir told me: When I have arranged a bouquet for the purpose of painting it, I always turn it to the side I did not plan)

Henri Matisse, *Jazz*

Once in awhile you get shone the light in the strangest of places if you look at it right.

The Greatful Dead, *Scarlet Begonias*

Preface

The last decade has seen an explosion in our understanding of how bacterial pathogens trick, cajole, usurp and parasitize their various hosts. This renaissance is due to the convergence of molecular and cellular techniques with the power of microbial genetics. The purpose of this volume is to introduce recent advances in understanding selected systems chosen from both plant and animal hosts of bacterial pathogens. This somewhat nonobvious choice of topics was spurred by the recent findings, detailed by several conributors to this volume, of common systems used to secrete virulence factors from pathogens of both plants and animals. These serendipitous findings underscored the importance of basic research approaches to parallel problems in biology. More importantly, they brought together investigators who may not have otherwise become conversant with each other's experimental systems. I, for one, find the kinds of synergism reflected in a volume of this sort to be one of the most pleasant aspects of science and hope that the reader, whether a newcomer to the field or an expert, can find a new slant to old problems in the reviews contained here. It was, however, necessary to limit volume length, and this has forced the exclusion of a number of fascinating bacterial pathosystems. The ones chosen for inclusion are meant to reflect a range of pathogen life strategies, and authors were encouraged to speculate where appropriate on how we might discover more common strategies of bacterial pathogenesis involving plants and animals. The hope, of course, is that by understanding both the commonalities and idiosyncracies of various pathogenesis modes, we may come to better understand host responses and resistance mechanisms.

J. Dangl

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Epiphytic Fitness of Phytopathogenic Bacteria: Physiological Adaptations for Growth and Survival

G.A. Beattie and S.E. Lindow

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1 Introduction

Large numbers of microorganisms flourish on leaves, despite the fact that leaf surfaces are subject to rapid and drastic fluctuations in temperature, radiation and humidity. The bacteria that are found on aerial leaf surfaces are distinct from bacteria in other habitats (Jensen 1971; Stout 1960a,b), including the nearby soil, suggesting that they have particular adaptations allowing them to exploit epiphytic environments. Few studies have tested whether bacteria that are not

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typically found on leaf surfaces can exploit the leaf surface habitat as well as those that are commonly found there. In one such study, O'Brien and Lindow (1989) found that while strains of *Salmonella*, *Escherichia*, and *Aeromonas* species could achieve large population sizes on leaves kept continuously wet under controlled conditions, their populations decreased dramatically upon drying of the leaf surface. In contrast, common epiphytic bacterial species such as *Pseudomonas syringae* maintained large population sizes on dry leaf surfaces. Although such differences in epiphytic behavior can be demonstrated, the traits that contribute to the unique success of epiphytes in the phyllosphere are poorly understood.

In natural environments, epiphytic bacteria can grow very rapidly and individual species can establish large population sizes, often greater than 10^7 cells per gram of leaf. Although many epiphytes can affect plant health under suitable conditions, such as by inciting disease or frost injury, many have no known influence under any conditions. It has long been recognized that phytopathogenic bacteria can develop large populations in the absence of disease (reviewed in Henis and Bashan 1986; Hirano and Upper 1983). The fact that large epiphytic populations contribute to an increased probability of disease incidence has only recently been demonstrated (reviewed in Hirano and Upper 1983, 1990). The existence of such a relationship indicates that knowledge of the factors contributing to the successful establishment and maintainance of large epiphytic populations may be critical to both epidemiological predictions and strategies for disease and frost injury control.

For the purposes of this review, epiphytic fitness is defined as the ability of bacteria to grow and/or survive on leaves exposed to a given environmental regime. Fitness is therefore context-dependent, since it will depend on the environment in which it is being assessed. Thus, we will primarily discuss traits which may individually influence the behavior of bacteria on leaves in a particular environmental context, and only briefly consider fitness in a broader context, in which changes in the environment may require changes in the expression of distinct traits. We therefore will be focusing on traits that affect relatively short-term behavior rather than persistence in an evolutionary time frame. Although the epiphytic fitness traits discussed are related to the unique features of the leaf surface habitat, these traits may not confer a specific ability to survive only on leaf surfaces. For example, while motility may be advantageous to epiphytic bacteria in their acquisition of protected sites and resources, this trait could also be advantageous when the bacteria are in water or soil.

The most common approach to link bacterial traits with epiphytic fitness has been guesswork. In this approach, specific traits are predicted to affect epiphytic fitness, then correlations are drawn between the presence of this trait and specific epiphytic behavior. Often, such correlations are made after examining unrelated strains. Rigorous testing, however, requires comparisons of isogenic strains, which are identical in all traits except the one being tested. Such isogenic strains could be constructed either by gene inactivation or by transferring the genes conferring the phenotype of interest into a recipient strain, then comparing the behavior of the constructed strain to its parental strain. Unfortunately, such

rigorous tests have not yet been performed on many phenotypes hypothesized to be involved in epiphytic fitness. It should be remembered that any one gene or phenotype is unlikely to be sufficient to confer fitness to an epiphytic bacterium in a given situation, but rather it may be one of several necessary contributors to fitness. In such a case, inactivation of the trait in a strain may reduce fitness significantly, but its transfer to another strain not normally possessing this trait would not necessarily provide a significant increase in fitness.

2 Colonization Ability

2.1 Motility and Chemotaxis

While several studies have demonstrated an important role of motility in the invasion of plants by phytopathogenic bacteria (BAYOT and RIES 1986; HATTERMANN and RIES 1989; PANOPOULOS and SCHROTH 1974; RAYMUNDO and RIES 1981), its role as an epiphytic fitness determinant has been evaluated only recently. If cells multiply or survive at particular sites on the surface of the leaf, then the ability to move to such sites would be an important factor in bacterial growth and survival. To test this concept, HAEFELE and LINDOW (1987) evaluated the behavior of nonmotile (Mot$^-$) mutants of *P. syringae* on leaves incubated under various moisture regimes. More Mot$^+$ cells than Mot$^-$ cells moved to distal parts of the leaf or to different leaves of plants exposed to moist conditions. Consequently, at the population level, motility should increase the potential for colonization by increasing access to potentially colonizable sites. This was confirmed when the strains were inoculated singly onto leaves; the population sizes of the Mot$^+$ strain became significantly larger than those of the Mot$^-$ strain after an extended incubation under moist conditions (> 5 days). After coinoculation of the two strains, the Mot$^+$ strain grew at the expense of the Mot$^-$ strain, achieving a higher population size after 2 days of incubation. Motility may therefore confer an advantage in the competition for sites.

HAEFELE and LINDOW (1987) also demonstrated that motility allows bacteria to attain sites that may be protected from environmental stresses. After large populations of the Mot$^+$ and the Mot$^-$ strains had developed under moist conditions, the Mot$^-$ strain experienced a larger and faster decrease in its population size than the parental strain when the plants were transferred to dry conditions. Furthermore, after growth of Mot$^+$ and Mot$^-$ strains on leaf surfaces under moist conditions, a higher fraction of Mot$^-$ than Mot$^+$ cells were killed by subsequent UV irradiation of leaves, although they exhibited identical sensitivities to UV irradiation in vitro. KENNEDY and ERCOLANI (1978) demonstrated a similar phenomenon with a Mot$^-$ mutant of *P. syringae* pv. *glycinea*. Active acquisition of "protected" sites is the most likely reason that Mot$^+$ cells survived better than Mot$^-$ cells on plant surfaces. At these sites, the cells are able to avoid exposure to environmental stresses such as desiccation and UV radiation.

The fact that motile and nonmotile bacteria exhibit substantially different behavior indicates that motility can occur on leaf surfaces. However, the role of chemotaxis rather than motility per se remains unclear. Chemotaxis toward plant extracts has been demonstrated in vitro (CHET et al. 1973; CUPPELS 1988; KLOPMEYER and RIES 1987), but few studies have examined it in planta (MULREAN and SCHROTH 1979). Bacteria appear to occupy distinct sites on leaf surfaces (DE CLEENE 1989; LEBEN 1965; LEBEN et al. 1970; MEW and VERA CRUZ 1986; MEW et al. 1984; ROOS and HATTINGH 1983; SCHNEIDER and GROGAN 1977). It would seem advantageous to a bacterium to move toward these sites in a directed rather than a random manner. However, the existence of nutrient source sites forming gradients, or of chemical signals that facilitate bacterial movement to sites favoring bacterial survival, is unknown. Alternatively, the ability to move in a nondirected fashion may enable bacteria to explore a larger fraction of the leaf surface, thereby increasing their chance of encountering sites where survival might be facilitated.

2.2 Adhesion

Once a bacterium reaches a site favorable for growth or survival, the ability to resist removal may be a selective advantage. The strength of the selective pressure depends on the strength of the removal pressure. It is clear that rain, wind, and temperature-driven convection currents can remove bacteria from leaf surfaces in a plant canopy (BUTTERWORTH and MCCARTNEY 1991; LINDEMANN and UPPER 1985), indicating that some selective pressure exists. However, due to the large variability in the environmental conditions within a plant canopy, and even on a single leaf (BURRAGE 1971, 1976), it is not clear what proportion of epiphytic bacterial cells in a plant canopy is subject to such pressures. Bacteria have two general mechanisms to maintain close proximity to a surface: association and adhesion. Association involves localization, via motility or chemotaxis, and possibly weak, reversible, nonspecific attachment, mainly via electrical charges. Adhesion is a stable, irreversible attachment mediated by specific attachment structures, such as fimbriae, cellulose fibrils or extracellular polysaccharides (EPS). It has been hypothesized that bacteria employ specific adhesins in environments where there are strong physical shear forces, such as on mucosal surfaces in the small intestine, but rely simply on association with surfaces where there are few shear forces, such as on the skin (ARP 1988). Due to the improbability of continually strong shear forces on most leaf surfaces, bacterial adherence probably does not offer a strong selective advantage, especially if the bacteria can multiply at a rate fast enough to compensate for the removal that does occur, or can occupy sites which offer some protection from the existing removal forces.

Bacteria have been demonstrated to attach to specific surface sites. Microscopic examinations showed that *P. syringae* pv. *phaseolicola* cells adhered preferentially to the stomata of bean leaves whereas *P. syringae* pv. *syringae* cells were found uniformly distributed over the bean leaf surface (NURMIAHO-

LASSILA et al. 1991; ROMANTSCHUK 1992). Also, *Xanthomonas campestris* pv. *hyacinthi* cells, as well as fimbriae from those cells, attached preferentially to the stomata of hyacinths (VAN DOORN et al. 1991). Thus, adhesion may play a role in localizing epiphytic bacteria to specific sites on the leaf surface, which may be important for meeting specific growth or survival requirements or for invasion by particular phytopathogens.

Many epiphytic bacteria are able to adhere to plant surfaces in in vitro assays and in fact elaborate structures that permit attachment. Pili and/or fimbriae have been identified on at least some strains of both *P. syringae* and *X. campestris* (ROMANTSCHUK 1992; STEMMER and SEQUEIRA 1987; and VAN DOORN et al. 1991). Nonpiliated mutants of various *P. syringae* pathovars were greatly reduced in their adherence to leaf surfaces, and super-piliated strains attached more efficiently in in vitro assays (MILLS et al. 1991; NURMIAHO-LASSILA et al. 1991; ROMANTSCHUK and BAMFORD 1986). While these results demonstrate that pili can mediate adhesion to leaf surfaces, the role of pili in epiphytic fitness has not been tested. In addition to pili and/or fimbriae, most epiphytic bacteria can produce EPS, which can function in attaching bacterial cells to surfaces. However, evaluation of EPS-mediated adhesion in epiphytic fitness is extremely difficult due to the multiple functions EPS may have in epiphytic growth and survival.

3 Extracellular Polysaccharide Production

There is considerable evidence that many epiphytic bacteria are surrounded by a layer of EPS while on leaf surfaces. Scanning electron micrographs reveal strands of amorphous material that emanate from and between bacterial cells on leaves (DICKINSON 1986; HATTINGH et al. 1986; TIMMER et al. 1987). These strands probably represent the dehydrated remnants of a more complete matrix which originally surrounded the cell. This matrix is presumably composed of one or more types of EPS molecules. Plant pathogenic bacteria produce a range of EPS types in culture, most commonly levan and various alginates (FETT et al. 1986; GROSS and RUDOLPH 1987; GROSS et al. 1992), but appear to produce a distinct range in planta (OSMAN et al. 1986). EPS may not only anchor cells to the leaf surface (TAKAHASHI and DOKE 1984), prevent cells from desiccation (WILSON et al. 1965), and protect cells from damage by UV radiation (LEACH et al. 1957), but may also modify the physical and chemical environment around the cell to one more favorable for bacterial growth or survival. The matrix hypothesized to exist on leaf surfaces may have many analogies to that of biofilms in which many aquatic micro-organisms are found. Biofilms have ion-exchange capabilities which can concentrate nutrients from dilute sources in the vicinity of the cell (COSTERTON et al. 1987). The biofilm can also provide protection from predators and shield cells from the action of lytic enzymes, antibiotics, and other inhibitory compounds (ANWAR et al. 1989; CALDWELL and LAWRENCE 1986). The production of such a matrix by epiphytic bacteria could be highly advantageous for the cell.

Studies on the involvement of EPS production in epiphytic fitness have provided equivocal results. In one study, Lindow et al. (1993) identified *P. syringae* transposon mutants that were altered in EPS production in culture and were reduced in epiphytic fitness. In a second study, Haefele and Lindow (unpublished) identified *P. syringae* chemical mutants that were EPS-deficient in culture but were identical to the parental strain in their epiphytic growth and survival. Interpreting the results of these experiments can be complicated for several reasons. First, pleiotropic alterations commonly occur with alterations in EPS production (Brumbley and Denny 1990; de Crécy-Lagard et al. 1990; Tang et al. 1991). For example, of nine EPS-altered epiphytic fitness mutants identified by Lindow et al. (1993), five expressed pleiotropic alterations. Additional alterations were not identified in the remaining four, but the possibility exists that they were altered in unexamined phenotypes. Second, strains that are EPS-deficient in culture may produce EPS on plants. Previous studies have identified mutants that were EPS-deficient on rich media but produced EPS in planta (Coplin and Cook 1990). Third, bacterial mutants can produce not only altered amounts of EPS, but also altered types. It is plausible that various types of EPS molecules play distinct roles in the phyllosphere. Such qualitative changes have rarely been evaluated in culture, much less in planta. Due to the complex nature of EPS and the regulation of its production in bacteria, we have much to learn before we can determine the role of EPS production in the growth or survival of epiphytes.

4 Stress Tolerance

4.1 Osmotolerance

If solutes are abundant in the free water on a leaf surface, they could become sufficiently concentrated upon drying to stress the resident bacteria. An extensive list of organic and inorganic substances have been identified in leaf leachates (Tukey 1970); however, useful quantitative data of their concentrations on leaf surfaces are not available. Skowlund and Lindow (unpublished) produced transposon mutants of a *P. syringae* strain that were reduced in their osmotolerance in culture. The mutants grew similarly to the parental strain both in media with low osmolarities and on moist leaves. When colonized plants were transferred to dry conditions, in about half of the experiments the mutants experienced similar population decreases to the parental strain, and in the other half they experienced larger population decreases. These results suggest that osmotic conditions on leaf surfaces may be highly variable. In another study, Lindow et al. (1993) identified 14 transposon mutants of *P. syringae* that were reduced in their osmotolerance in culture and in their ability to survive on dry leaves. Although seven of these mutants carried additional phenotypic alterations, the behavior of the remaining seven suggests that osmotolerance can contribute to

bacterial survival on dry leaves. Lastly, a locus was identified in *P. syringae* that can function in the production of periplasmic glucans (LOUBENS et al. 1992; MUKHOPADHYAY et al. 1988), which are compounds that have been shown to confer osmotolerance. Inactivation of this locus resulted in a reduced ability to grow in planta (MILLS et al. 1985). Thus, several studies indicate that osmotolerance can contribute to epiphytic growth and survival. However, the fact that preexposure of cells to a high osmolarity medium did not increase epiphytic fitness (WILSON and LINDOW 1993) suggests that induction of osmotolerance on leaves may require more complex signals than simply high osmoticum.

4.2 Matric Stress Tolerance

Osmotic stress is only one component of the total water stress that a bacterium may encounter on a leaf surface. The total leaf water potential is a quantitative term reflecting water availability. It is a sum of the osmotic potential, which is due to the interaction of water with the available solutes (molecules that can penetrate a membrane), and the matric potential, which is due to the interaction of water with the leaf surface (or molecules that cannot penetrate a membrane) (SOROKER 1990). Matric potential has been studied primarily in soils, where it is strongly influenced by the soil texture and structure (GRIFFIN 1981). Similarly, the leaf matric potential is probably strongly related to the texture and structure of the leaf surface. Some studies have demonstrated that the stress imposed by a low matric potential has a stronger influence on a bacterial cell than an equivalent osmotic potential (MCANENEY et al. 1982; RATTRAY et al. 1992). For example, in cultures grown at various water potentials, *Escherichia coli* cells survived to almost –40 bars under salt stress, but were nonrecoverable at –8 bars under matric stress (MCANENEY et al. 1982). The mechanisms of water stress tolerance may explain this phenomenon. Bacteria tend to survive osmotic stress by a combination of accumulating solutes, via an influx through the membrane, and synthesizing compatible solutes, such as glutamate, trehalose, or glucan (reviewed in CSONKA and HANSON 1991). Bacteria can survive matric stress only by synthesizing solutes or EPS (ROBERSON and FIRESTONE 1992; SOROKER 1990); therefore, survival requires a greater input of energy. Functionally, matric stress can be imposed in culture by adding a compound that cannot be transported through the bacterial membrane, such as a large polymer like polyethylene glycol (PEG) (MCANENENY et al. 1982). Beattie and Lindow (unpublished) identified a transposon mutant of *P. syringae* that was reduced in its tolerance of matric stress, as determined by its reduced growth in the presence of PEG, and was reduced in its ability to survive on leaves under low relative humidity. These results demonstrate that differences in matric stress tolerance can be identified in epiphytic bacteria, and that matric stress tolerance may be epiphytically advantageous. However, this mutant was also reduced in its osmotolerance, as determined by its reduced growth in the presence of high salt concentrations.

This pleiotropy suggests that the mechanistic basis of matric stress tolerance may be explicitly tied to that of osmotolerance, thus complicating the identification of the quantitative contribution of each to epiphytic fitness.

The ability to survive on dry leaves probably is one of the defining characteristics of epiphytic versus saprophytic bacteria, since strains of *P. syringae*, *Aeromonas*, *Escherichia*, and *Salmonella* all grew equally well on wet leaves, but *P. syringae* survived significantly better on dry leaves (O'Brien and Lindow 1989). Although the production of highly hygroscopic polysaccharides may help prevent desiccation, the primary survival strategy of epiphytes is probably localization in crevices that retain water when the leaf surface dries. There is some evidence supporting this hypothesis. First, surface populations of *P. syringae*, i.e., those in leaf sonicates, comprised a much larger proportion of the total bacterial population in dry leaves than in wet leaves, indicating a poorer survival rate in sites allowing bacterial release by sonication than in sites preventing it (O'Brien and Lindow 1989). Secondly, populations of two pathovars of *X. campestris* in leaf washings were found to decrease over time on field-grown tomato plants, while populations in leaf homogenates remained constant or increased (Timmer et al. 1987).

4.3 Tolerance to UV and Visible Radiation

The phylloplane is exposed to substantially higher amounts of electromagnetic radiation than most other microbial habitats. Solar rays reaching the earth's surface include UV, visible, and infrared radiation. Wavelengths in the far-UV range (< 300 nm) are known to be among the most lethal to bacteria, primarily due to their damaging effects on DNA; however, very little far-UV radiation actually reaches the earth's surface. Thus, tolerance to radiation in the near-UV range (300–400 nm) is likely to be of greater importance to epiphytic bacteria. The mechanisms by which near-UV wavelengths kill cells are not known, but are known not to be via a direct effect on cellular DNA. Laboratory studies examining factors that may contribute to UV tolerance in epiphytic bacteria almost universally assay using far-UV radiation (usually 254 nm). At this assay wavelength, the *recA* gene has been found to contribute to UV tolerance. The *recA* gene, which is involved in the repair of DNA damage, has been cloned from a number of epiphytic bacteria including *P. syringae* (Hickman et al. 1987). The elimination of the *recA* function in such strains decreased their tolerance to UV radiation in culture by several orders of magnitude (Willis et al. 1988). Similarly, elimination of the production of a siderophore, a compound known to function as a UV chromophore (Torres et al. 1986), decreased the tolerance of a *P. syringae* strain to UV radiation in culture (Loper and Lindow 1987). On bean leaves, both the siderophore-deficient mutant and its parental were equally sensitive to UV radiation; however, the role of the siderophore in UV tolerance remains unclear since no evidence was found for siderophore production by the parental strain on leaves. Lastly, UV absorption by crude exudate from *X. campestris* pv. *phaseoli*

cultures indicates that it could provide protection against UV radiation (LEACH et al. 1957), but direct evidence for a role for EPS in UV tolerance has not been demonstrated.

Visible light can also be lethal to bacteria, usually by reacting with a photosensitizing compound (any organic molecule able to absorb light of wavelengths of 320–900 nm) to generate highly reactive oxygen derivatives. An effective mechanism of tolerance in bacteria is the production of protective compounds, the most significant of which are carotenoids and other pigments. These compounds function by neutralizing the reactive oxygen derivatives. Several studies have found that a majority of the bacteria isolated from leaf surfaces produce pigments in culture (AUSTIN and GOODFELLOW 1978; STOUT 1960a,b). When genes encoding pigment production were transferred from the epiphyte *Erwinia herbicola* into *E. coli*, the constructed strain showed an increased tolerance to near-UV radiation in culture (TUVESON et al. 1988). Melanin has been proposed to offer protection from UV and visible light in fungi (DICKINSON 1986). The production of melanoid-type pigments in some epiphytic bacteria (BASU 1974) leaves open the possibility that it serves a similar role in bacteria.

If bacteria are commonly located in crevices on the leaf surface, as is observed by scanning electron microscopy (BLAKEMAN 1985; DE CLEENE 1989; ROOS and HATTINGH 1983), then they may survive UV and visible radiation by simply avoiding it. BARNES (1965) demonstrated that exposure of a colonized soybean leaf surface to UV radiation resulted in an initial decrease in the recoverable bacteria in leaf homogenates, as expected, since the bacteria were sensitive to UV irradiation in culture. However, after 15 min, continued exposure to UV radiation had no further effect on the recoverable population. It was hypothesized that the surviving cells (approximately 10^4 bacteria per 12 mm leaf disk) were those in the intercellular spaces and other sites not exposed to the incident UV radiation. SZTEJNBERG and BLAKEMAN (1973) observed a similar phenomenon on beetroot plants.

4.4 Production of Protective Enzymes

The probable abundance of toxic oxygen derivatives and plant-derived antimicrobial substances (BLAKEMAN and ATKINSON 1981) must make the phylloplane a relatively hazardous environment for bacteria. Consequently, epiphytic bacteria must have many methods of self-protection. For example, almost all species of bacteria that are good epiphytes, including all *Xanthomonas* and *Erwinia* spp. and all *P. syringae* pathovars, are oxidase-negative. Cytochrome oxidase has been hypothesized to be highly detrimental to epiphytic bacteria due to its involvement in the conversion of plant-produced phenols to bactericidal or bacteriostatic quinones (MOUSTAFA and WHITTENBURY 1970). The absence of oxidase therefore may be a strong requirement for epiphytic survival. Catalase may also be important in the phyllosphere, primarily for detoxifying the high amounts of hydrogen peroxide on plant surfaces. All aerobic bacteria produce catalase;

however, they differ in their induction, activity, and number of catalase isozymes (Katsuwon and Anderson 1992; Moustafa and Whittenbury 1970). While catalase activity has not been examined in the phyllosphere, Anderson et al. (1992) demonstrated that catalase activity increased and catalase isozymes were differentially expressed in *Pseudomonas putida* upon contact with root surfaces. The presence of a high number of antimicrobial substances, especially phenolic compounds, in leaf exudates (Blakeman and Atkinson 1981) could make the possession of detoxification enzymes epiphytically advantageous; however, such enzymes have not been identified.

5 Competition for Resources

Bacteria of different species often occur together in mixtures on leaves. If these species occupy the same microsites, then the survival of any one depends on its ability to successfully compete for shared resources, and/or its ability to coexist, by utilizing resources distinct from the others. A wide array of compounds have been found in leaf exudates, including a large number of amino acids, polysaccharides and organic acids (Morgan and Tukey 1964; Weibull et al. 1990). Thus, a broad nutrient utilization profile, which is common in epiphytic bacteria (Morris and Rouse 1985), should increase the potential for an organism to survive in the presence of others. Furthermore, the sole ability to utilize an abundant resource should be highly advantageous to an organism, as long as other resources are not limiting. For example, similar to studies that have been done in the rhizosphere (Savka and Farrand 1993), Wilson and Lindow (unpublished) have found that the ability of a strain to utilize mannopine allowed this strain to establish much larger populations than a non-mannopine catabolizing strain on mannopine-producing plants. Also, a *P. putida* strain that was able to catabolize salicylate achieved larger populations than a non-salicylate catabolizing strain on leaves in the presence of exogenously applied salicylate (Wilson and Lindow, unpublished). These results demonstrate that a unique ability to utilize the nutrients that are available can be epiphytically advantageous.

Competition for a shared resource also occurs among epiphytes. Studies using bacterial agents to control plant disease or frost injury have demonstrated that colonization by one bacterial strain can exclude subsequent colonization by another (e.g., Lindow et al. 1983; Thomson et al. 1976). The strength of the competition between two strains may depend on the degree of overlap in the resource needs of the strains (Lindow 1985a, 1987; Wilson and Lindow 1991, unpublished) and on the availability of those resources. Thus, a strain may have a competitive advantage if it is able to grow at a faster rate or at lower nutrient concentrations than its competitors, or if it has a superior ability to acquire nutrients, such as an ability to actively acquire iron by producing siderophores. Unfortunately, the contribution of these various traits to the ability of an organism to compete for resources in the phyllosphere has not been directly examined.

5.1 Siderophores

Microorganisms require Fe^{3+} for growth (NEILANDS and LEONG 1986). While iron is abundant in nature, only low amounts of ionic Fe^{3+} are thought to be available to microorganisms, due to the propensity of iron to form insoluble oxidation products under normal environmental conditions (LINDSAY and SCHWAB 1982; RAYMOND and CARRANO 1979). Siderophores are low molecular weight compounds with high affinity for iron that are excreted by microorganisms. When their production is coupled with a specific uptake system, siderophores allow microbial acquisition of iron in environments where the free element is in low concentrations (NEILANDS 1981). The concentration of iron in the microhabitats exploited by bacteria on leaves is unknown; therefore, it is unclear whether siderophores are a necessary fitness factor for acquisition of iron, and if so, whether their production confers a competitive advantage over non-siderophore producing strains.

LOPER and LINDOW (1987) conducted a study to determine whether siderophores were required for the colonization of leaf surfaces. Chemical mutants of a *P. syringae* strain were identified that lacked the ability to produce a siderophore under iron-limiting conditions in culture. The siderophore-deficient mutants grew to similar population sizes and produced as many bacterial brown spot lesions as the parental strain on bean plants under greenhouse conditions. Furthermore, four siderophore-deficient mutants survived as well or better than their respective parental strains when inoculated onto bean plants under field conditions. Although these results suggest that siderophores were not a necessary fitness factor under these growth conditions, it was not known whether the parental strain actually produced the siderophore under these conditions. In more recent studies, LOPER and LINDOW (1994) observed that a gene required for siderophore production was transcribed at a low level on leaves, suggesting that at least under some conditions iron may be sufficiently limited in leaf surface microsites to induce siderophore production. Whether siderophore production contributes to fitness under such conditions is still unknown.

5.2 Antibiotic Production

If microbes must compete for limiting resources on leaf surfaces, production of a biocide such as an antibiotic could improve their competitive ability. Many bacteria, including the common epiphytes *Erwinia* and *Pseudomonas* spp., produce antibiotics or bacteriocins in culture (JENSEN 1971; VIDAVER et al. 1972). However, little is known about the production of such compounds in nature. BLAKEMAN (1991) reasoned that antibiotic production is probably not important in the interactions among microorganisms on leaves due to insufficient nutrient levels to support significant antibiotic production. Lack of evidence for a role of antibiosis in bacterial antagonism on leaf surfaces supports Blakeman's statement. LINDOW (1988) isolated mutants deficient in antibiotic production of 25

epiphytic strains known to be highly antagonistic toward a particular *P. syringae* strain on leaves. When they were inoculated prior to the *P. syringae* strain, the antibiotic-deficient mutants and their parental strains were similar in their ability to inhibit *P. syringae* growth on plants in the greenhouse. The mutants also reached population sizes similar to their parental strains in the presence of other competing bacteria. Thus, while antibiosis has been demonstrated to be a contributing factor to the competitiveness of several bacterial biocontrol agents on roots (reviewed in FRAVEL 1988), its contribution to the competitive advantage of a producing strain over a nonproducing strain on leaves has not yet been demonstrated.

6 Ice Nucleation Activity

Some pathogenic and nonpathogenic bacteria can cause ice formation (LINDOW 1983, 1986; LINDOW et al. 1978; MAKI and WILLOUGHBY 1978; MAKI et al. 1974). Ice formation in sensitive plants can cause injury (LINDOW 1983; LINDOW et al. 1982) and may facilitate the entry of phytopathogenic bacteria into plants. SÜLE and SEEMÜLLER (1987) have therefore suggested that ice nucleation is a temperature-conditional virulence factor. Whereas maintaining the ability to produce ice nuclei must have a metabolic and genetic cost, the production of ice nuclei has not been found to confer any measurable benefit to the cells. LINDEMANN and SUSLOW (1987) showed that Ice^- mutants of *P. syringae* and *Pseudomonas fluorescens* were equally competitive with their Ice^+ parental strains on plant surfaces. Similarly, Ice^- mutants of *P. syringae* grew and survived like the parental strain on all plant species tested under moist and dry conditions in the greenhouse (LINDOW 1985a). Lastly, Ice^- mutants were similar to their parental strains in their ability to survive repeated freezing and thawing cycles both in an aqueous environment and on leaves (LINDOW 1985b). Therefore, even on plants subjected to freezing conditions, ice nucleation activity has not been demonstrated to influence epiphytic behavior.

7 Plant Hormone Production

Many plant-associated bacteria can produce plant growth hormones (e.g., ERNSTSEN et al. 1987; FETT et al. 1987; LOPER and SCHROTH 1986). Gall-forming plant pathogens, including *P. syringae* pv. *savastanoi*, *E. herbicola* pv. *gypsophilae*, and *Agrobacterium tumefaciens*, can produce both auxins and cytokinins and thus cause hyperplasia (MANULIS et al. 1991; ROBERTO and KOSUGE 1987; THOMASHOW et al. 1984; WEILER and SCHRODER 1987). While such galls might provide refuges for the survival of these pathogens, it is unclear whether non-gall-forming bacteria actually produce plant growth hormones in nature, and if so, what ecological benefit they derive from their production. Exogenous auxins can induce plant-cell-

wall loosening and membrane leakiness (LEOPOLD and KRIEDEMANN 1975). Thus, perhaps auxin production benefits bacteria by improving their habitat via an increase in the rate of plant leaching or induction of local anatomical changes conducive for growth or survival.

Efforts focused on elucidating the role of plant growth hormone production in epiphytic behavior have focused on 3-indoleacetic acid (IAA). Several studies indicate that IAA production may contribute to the epiphytic survival of *P. syringae* pv. *savastanoi* (SILVERSTONE et al. 1993; VARVARO and SURICO 1984). Recently, *E. herbicola* strains have been identified that produce higher quantities of IAA in culture than do gall-forming pathogens (CLARK and LINDOW 1989) and achieve large population sizes when inoculated onto pear trees (Lindow, unpublished). Chemical mutants of one such *E. herbicola* strain were found that produced reduced amounts of IAA in culture. These mutants achieved smaller population sizes than the parental strain on pear trees under field conditions (Lindow, unpublished), suggesting that IAA production may help condition epiphytic fitness in this strain.

8 Pathogenicity

The contribution of intercellular growth to epiphytic fitness is unclear, especially since the dynamics between internal (e.g., substomatal cavities and intercellular spaces) and external bacterial populations are so poorly understood. To complicate the issue, epiphytic bacteria are frequently functionally defined as those that can be removed from above ground plant parts by washing (HIRANO and UPPER 1983), and the representation of populations from internal sites in these washings is not known. Numerous studies suggest that surface application of pathogens results in internal colonization (e.g., CAFATI and SAETTLER 1980; ROOS and HATTINGH 1983; STADT and SAETTLER 1981), proving that there is active exchange, at least in the inward direction, between the two populations. If there is outward exchange to any extent, then phenotypes that contribute to high intercellular populations would directly contribute to higher surface populations, and thus to the epiphytic fitness. For this reason, several bacterial characteristics whose primary influence is on internal population sizes will be discussed here.

Although many of the bacteria that are isolated from leaf surfaces are phytopathogenic, pathogenicity itself is not a requirement for epiphytic growth. Epiphytes have been identified that were not pathogenic on any plant examined. For example, *P. syringae* strains Cit7 and TLP2, which were isolated from healthy citrus and potato leaves, respectively, were capable of establishing and maintaining large epiphytic populations but did not produce symptoms on any of 75 plant species tested (LINDOW 1985b; LINDOW and PANOPOULOS 1988). Also, numerous studies have demonstrated that epiphytic plant pathogens are capable of growth on nonhost plant species (e.g., ERCOLANI et al. 1974; O'BRIEN and LINDOW

1989). And lastly, since 1959 when CROSSE (1959) reported finding high populations of *P. syringae* pv. *morsprunorum* on healthy cherry leaves, it has been demonstrated and accepted that most pathogens are able to grow well on hosts without producing diseases symptoms (e.g., LEBEN et al. 1968b; TIMMER et al. 1987).

Pathogenicity may not be required for, but it may contribute to, epiphytic fitness, since the existence of a pathogenic host relationship correlates well with the ability of a pathogen to attain large epiphytic population sizes. Almost without exception (RIDÉ et al. 1978), pathogens have been found to grow to larger populations on susceptible than on resistant varieties of the host plant species (CAFATI and SAETTLER 1980; MCGUIRE et al. 1991; MEW and KENNEDY 1971; STADT and SAETTLER 1981). Unfortunately, this correlation does not distinguish between the possibility that bacterial expression of pathogenicity is causal to abundant epiphytic growth, or that a susceptible host provides an environment that is more conducive to bacterial multiplication than that of a resistant host. Symptom expression, per se, probably does not contribute significantly to an increase in population size, at least in the laboratory, since the largest population increases after inoculation usually occur before symptoms are visible (KLEMENT et al. 1964; LEBEN et al. 1968a; OLIVEIRA et al. 1991; STADT and SAETTLER 1981; WYMAN and VANETTEN 1982).

An evaluation of the epiphytic behavior of pathogenic-deficient mutants ($Path^-$) mutants should indicate whether or not bacterial pathogenicity contributes to epiphytic fitness. Pathogenicity is a complex process and probably requires, and is influenced by, a large number of bacterial traits. Thus, it should not be surprising that most $Path^-$ mutants of epiphytic bacterial pathogens are pleiotropic, making the identification of causal relationships difficult. For example, *lemA* mutants of *P. syringae* pv. *syringae*, agent of bacterial brown spot in beans, were deficient not only in the ability to form lesions, but also in their production of a phytotoxin and a protease in vitro (WILLIS et al. 1990). While *lemA* mutants grew like the parental strain on bean plants under moist conditions in the greenhouse (WILLIS et al. 1990), they grew to 10- to 100-fold smaller population sizes on bean plants under field conditions (HIRANO et al. 1992). Unfortunately, the pleiotropic effects of *lemA* inactivation make it difficult to identify the specific contribution of pathogenicity to epiphytic fitness under field conditions in these mutants. Other $Path^-$ *P. syringae* pv. *syringae* mutants have been identified which are similarly able to grow like the parental strain on wet leaves but exhibit reduced population sizes on dry leaves (YESSAD et al. 1992). Although it is possible that $Path^-$ mutants are compromised specifically in their ability to tolerate environmental stresses, there is a more likely explanation for their apparent inability to maintain large populations on leaves subjected to environmental stresses. If the requirements for bacterial growth are different in internal versus external sites, the $Path^-$ mutants may have a reduced ability to grow in the internal sites, which may offer protection from the environmental stresses imposed under field conditions, but may be unaltered in their ability to grow in exposed sites, where the majority of growth may occur on wet leaves.

The interactions between bacterial pathogens and their plant hosts fall into two categories: compatible, in which disease develops, and incompatible, in which no disease develops. The incompatible interaction is often correlated with the induction of a hypersensitive reaction (HR), characterized by a rapid local necrosis after introduction of high numbers of bacteria (> 10^6 cells/ml) into leaf tissue. The majority of characterized $Path^-$ mutants of epiphytic bacteria are also unable to induce an HR when infiltrated into incompatible plants. These *hrp* mutants (for hypersensitive reaction and pathogenicity; see chapters by Bonas and Collmer and Bauer, this volume) are almost uniformly reduced in their ability to grow in compatible plants (Bertoni and Mills 1987; Huang et al. 1991; Kamoun and Kado 1990b; Lindgren et al. 1986; Rahme et al. 1991; Somlyai et al. 1986). Studies by Atkinson and Baker (1987) provide an elegant mechanistic explanation for this reduced growth. They showed that the presence of a pathogen in a compatible plant is associated with a K^+ efflux/H^+ influx exchange across the plant plasma membrane. Mutants unable to induce this exchange were reduced in their ability to multiply in host tissue, and the strength of the exchange response induced by various mutants correlated well with their rate of growth. Further work provided evidence for a model in which bacteria trigger an imbalance in the plant plasma membrane H^+ gradient, disrupting the normal active uptake of nutrients from the intercellular fluid. The resulting nutrient accumulation promotes bacterial multiplication, causing higher K^+/H^+ exchange rates, further destruction of the H^+ gradient, and further nutrient accumulation. Thus, pathogenicity may confer an enhanced ability to multiply in the intercellular spaces of host tissues by influencing the pH and nutritional status of the intercellular fluid. Although a K^+/H^+ exchange also occurs in incompatible interactions, differences in the rate and degree of exchange may account for quantitative differences in bacterial growth in incompatible versus compatible hosts (Atkinson and Baker 1987). The role of *hrp* genes in epiphytic fitness has not been examined.

8.1 Avirulence Genes

Avirulence (*avr*) genes confer an incompatible interaction between plant pathogens and plants having corresponding resistance genes (see chapter by J. L. Dangl, this volume). Since bacterial growth within the intercellular spaces of incompatible leaf tissue is extremely limited (generally less than 100-fold population increase in a 48 h period), the effect of introducing an *avr* gene into a pathogen should be to reduce its growth in the intercellular spaces of a plant carrying the corresponding resistance gene. This has been observed with *avr* genes from a variety of phytopathogenic bacteria (Debener et al. 1991; Dong et al. 1991; Parker et al. 1993; Ronald et al. 1992; Wanner et al. 1993; Whalen et al. 1991). Similarly, inactivation of an *avr* gene has been found to result in increased growth of the pathogen (Carney and Denny 1990). Thus in general, *avr* genes are detrimental to intercellular multiplication in hosts carrying the corresponding resistance gene. At least one avirulence gene, however, contributes to growth in

susceptible hosts. KEARNEY and STASKAWICZ (1990) found that loss of *avrBs2* from *X. campestris* pv. *vesicatoria* resulted in reduced intercellular growth in susceptible plants. These results clearly illustrate the dependence of fitness on environmental context: while the *avrBs2* gene was detrimental to bacterial growth in one plant cultivar, it was a major contributor to growth in another. The effect of *avr* genes specifically on epiphytic fitness has not been addressed.

8.2 Phytotoxin Production

While phytotoxins produced by plant pathogenic bacteria have been investigated primarily for their role in pathogenicity, there is increasing ecological interest in their contribution to the pathogenic life-style of the bacteria (MITCHELL 1991). The majority of characterized toxins produced by epiphytic bacteria are produced by pathovars of *P. syringae*, although toxins from *Erwinia amylovora* and *X. campestris* pathovars have been reported (MITCHELL 1991). Non-toxigenic mutants of several *P. syringae* pathovars have provided good evidence that toxin production can affect in planta populations. For example, both coronatine- and tabtoxin-deficient mutants initially grew like the wild-type strains in leaves, but after 3 or 4 days their populations decreased while the wild-type populations remained constant or continued increasing (BENDER et al. 1987; TURNER and TAHA 1984). These results suggest that production of particular toxins is not important for establishing large populations, but may be important for maintaining them. Although syringomycin has been proposed to benefit intercellular bacterial growth by inducing a K^+/H^+ exchange similar to that involved in HR (GROSS 1991), most syringomycin-deficient mutants, as well as phaseolotoxin-deficient mutants, grew like their parental strains in planta for 3–5 days after inoculation (PATIL et al. 1974; XU and GROSS 1988); however, the population dynamics in the subsequent week were not examined.

The fact that most pathovars of *P. syringae* have genes to produce one or more phytotoxins suggests that the epiphytic environment may exert a selective pressure for toxigenic strains. Several toxins, including phaseolotoxin, syringotoxin and syringomycin, have antibacterial and/or antifungal activities in vitro (reviewed in MITCHELL 1991). Therefore, they may contribute to the competitiveness against other phylloplane micro-organisms. Unfortunately, these antimicrobial activities have not been demonstrated on plant surfaces.

9 Model of Coordinate Regulation

Bacteria may respond to changes in their environment by coordinately altering a range of phenotypes. For example, if a cell arrives on a leaf, via airborne deposition or migration from another plant part, and encounters a site hospitable for growth, it probably requires a different set of attributes for colonization of that site than it required for survival during transit. The behavior of a bacterium during

the various phases of its life probably depends on differential expression of distinct, but overlapping, sets of phenotypes during each phase. LEBEN (1981) described several phases in the life of pathogenic bactera. In the *resident phase*, cells multiply on the surfaces of apparently healthy plants. In the *pathogenic phase*, they induce disease symptoms in their host, with or without multiplication. Cells can survive for years in the *survival phase*, exhibiting a very low metabolic rate and an increased resistance to inhibitors relative to actively dividing cells. They probably enter this state slowly. For example, the plant material surrounding a cell in an aging lesion may dry slowly and slowly embed the cell; the cell may then remain in this matrix until the next season. The concept that differential expression of a range of phenotypes governs the transition between phases has been explored primarily for the transition between the resident and pathogenic phases. Phenotypes that are required specifically for the pathogenic phase have been termed "pathogenicity factors." It seems quite possible that similar blocks of traits could dictate bacterial behavior during the survival and resident phases as well.

Much to the chagrin of those who prefer straightforward causal relationships, numerous studies on mutants of phytopathogenic bacteria have discovered pleiotropic mutations. So many, in fact, that pleiotropy may be more the norm than the exception. These findings may be the key to identifying coordinately regulated blocks of bacterial traits. Based on these studies, we have composed a model describing both the range of phenotypes that may be expressed during two distinct phases of the life of an epiphyte and the molecular mechanisms that may direct the phase transitions (Fig. 1). Although we define the *survival phase* as described above, we define the *multiplication phase* as that phase in which the cells are actively dividing. The multiplication phase thus includes the resident phase, but may also include the pathogenic phase.

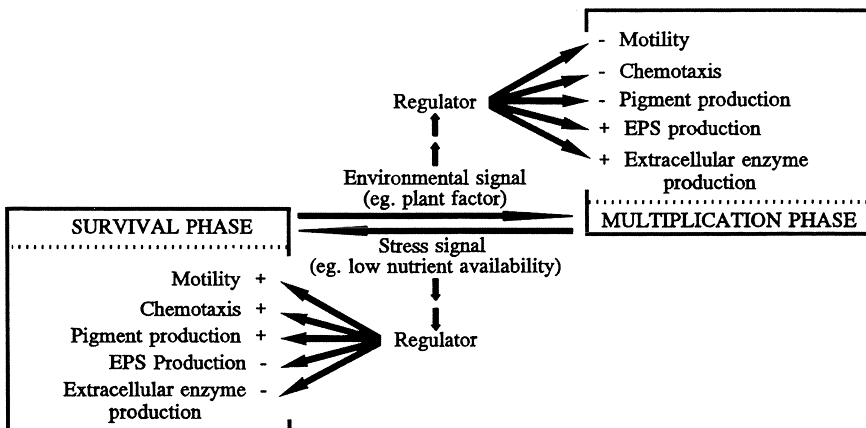

Fig. 1. Model of coordinately regulated phenotypes involved in two distinct phases of the life cycle of epiphytic bacteria. EPS, extracellular polysaccharides

The association of particular phenotypes with each phase is based on observations of simultaneously altered phenotypes in several pleiotropic mutants of phytopathogenic bacteria. For example, KAMOUN and KADO (1990a) identified spontaneous variants of strains of five *X. campestris* pathovars that were EPS-deficient and chemotactic on laboratory media as compared to the parental strains that produced EPS and were nonchemotactic. Assuming that actively growing cells on laboratory media are in the multiplication phase, then their results suggest that expression of these two phenotypes was coordinately regulated, and that chemotaxis was not expressed and EPS production did occur in cells that were in the multiplication phase. To derive the model shown in Fig. 1, similar logic was applied to pleiotropic mutants, both spontaneous and constructed, of both epiphytic and nonepiphytic plant pathogens (BRUMBLEY and DENNY 1990; DE CRÉCY-LAGARD et al. 1990; LIAO et al. 1993; MORALES et al. 1985; TANG et al. 1990, 1991; THORPE and SALMOND 1993), bearing in mind that all of the mentioned phenotypes were not examined for each mutant. In almost every case, reduced EPS production was associated with reduced secretion of extracellular enzymes, such as amylase, endoglucanase, polygacturonate lyase and protease in *X. campestris* pv. *campestris* (TANG et al. 1991), and pectate lyase and protease in *Pseudomonas viridiflava* (LIAO et al. 1993). Also, reduced EPS and exoenzyme production was associated with increased pigment (DE CRÉCY-LAGARD et al. 1990) or IAA (MORALES et al. 1985) production and increased motility (BRUMBLEY and DENNY 1990). The model that emerges from these mutants is that, in the survival phase, bacteria express phenotypes that may increase their resistance to harsh environmental conditions, such as pigment production for protection from light damage, and phenotypes for locating a more favorable environment, such as motility and chemotaxis. Although EPS has been proposed to increase desiccation tolerance and thus would seem beneficial in the survival phase, de novo production is likely prohibitively carbon- and energy-intensive for a cell operating at its minimum metabolic rate, but this does not exclude a role for preexisting capsular EPS. In the multiplication phase, de novo production of EPS may be critical for attachment of cells to their newfound site and/or concentrating the available nutrients to promote multiplication. The extracellular enzymes may function in degrading available plant debris, or perhaps in degrading the plant cell wall at locations where they can avoid or penetrate the cutinous layer.

The transition from survival to multiplication phase probably occurs in response to a different signal than transition in the opposite direction, and that signal is probably transduced through a distinct pathway. For example, a signal indicating that a cell has arrived at a favorable environment for growth, such as a diffusible plant factor, may trigger a switch to the multiplication phase. Two possible components of such a signal transduction pathway in phytopathogenic bacteria have been identified. The *rpf* (regulation of pathogenicity factors) region in *X. campestris* pv. *campestris*, which has homology in other *X. campestris* pathovars, positively regulates the production of EPS and four extracellular enzymes, with some indication that the regulation occurs at the transcriptional level (TANG et al. 1991; see chapter by Dow and Daniels, this volume). Second, the

phcA (phenotype conversion) region in *Pseudomonas solanacearum*, which has homology in diverse *P. solanacearum* strains, not only positively regulates the production of EPS and endoglucanase, but also negatively regulates motility (BRUMBLEY and DENNY 1990). The transition from multiplication to survival phase is likely triggered by conditions that are no longer favorable for growth, such as low nutrient availability sensed via starvation. Evidence for such a signal rests in the fact that when actively growing laboratory cultures, which are likely in the multiplication phase, are incubated for extensive periods without any nutrient addition, spontaneous variants arise. These variants display many of the phenotypes of cells in the survival phase (KAMOUN and KADO 1990a; MORALES et al. 1985). Furthermore, reversion was detected only in planta (KAMOUN and KADO 1990a), supporting the hypothesis that a plant signal may be involved in the transition from survival to multiplication phase. Possible components of a signal transduction pathway involved in the multiplication to survival phase transition have also been identified. *X. campestris* pv. *campestris* contains a region that negatively regulates the production of EPS and extracellular enzymes (TANG et al. 1990), and two *P. solanacearum* strains each contain a region that negatively regulates EPS production and either positively (HUANG and SEQUEIRA 1990) or negatively regulates polygalacturonase production (NEGISHI et al. 1993). The survival and growth of an epiphytic bacterium probably depends not only on its ability to express distinct phenotypes during various phases of its life cycle, but also on its ability to make transitions at appropriate times to the various phases.

10 Identification of Novel Fitness Traits

The previous sections identified methods by which traits hypothesized to be involved in epiphytic fitness could be evaluated. However, novel or unanticipated traits may condition survival or growth of bacteria on leaves. Rather than testing genes that confer known phenotypes for their contribution to epiphytic fitness, it is possible to identify genes directly contributing to epiphytic fitness and use them to identify potentially novel epiphytic fitness traits. A similar approach was used to identify previously undetected genes that confer virulence in phytopathogenic bacteria (LINDGREN et al. 1986). In contrast to evaluating the virulence of individual mutants based on the qualitative presence or absence of disease, evaluating the epiphytic fitness of individual mutants requires estimating the population size of each mutant on plant surfaces. For example, it would be necessary to measure the population size of about 5000 mutants of a typical epiphyte such as *P. syringae* to ensure that there is an 80% chance that a given gene (out of the 3000 genes contained in such a strain) is inactivated in such a collection. The logistical constraints of ascertaining the population size of such a large number of mutants on plants have prevented this approach from being used until recently.

A novel approach has been developed which permits the rapid estimation of the population size of ice nucleation active bacteria on leaf surfaces. HIRANO et al. (1985) used an analysis of the distribution of freezing temperatures of a collection of leaves to estimate the frequency of leaves that had large populations of Ice+ bacteria. Since ice nucleation activity at warm freezing temperatures (> –3° C) occurs very rarely in a population of cells of an Ice+ bacterial strain, only a leaf with a large population size of Ice+ bacteria has a high probability of freezing at such a warm temperature. LINDOW (1986) showed that there was a direct relationship between the logarithm of the epiphytic population size of Ice+ bacteria and the average nucleation temperature of a collection of leaves. After 5300 mutants of an Ice+ *P. syringae* strain were individually inoculated onto bean plants and the plants were subjected to alternating wet and dry conditions, the epiphytic population of each mutant was rapidly estimated based on the freezing temperature of the colonized leaves (LINDOW 1993; LINDOW et al. 1993). In this manner, 82 epiphytic fitness mutants were identified. While 50% of the mutants were altered in phenotypes that could be measured in culture, 50% of the mutants were not altered in any in vitro phenotype examined. This study indicates that many interesting traits which have large individual effects on the epiphytic fitness of bacteria remain to be discovered. It also indicates that it may be very difficult to guess which phenotypes are important in epiphytic fitness.

11 Conclusions and Future Directions

We are only beginning to identify bacterial characteristics that influence the growth or survival of individual cells on leaf surfaces. Unfortunately, identification itself is only a beginning to understanding exactly how these characteristics influence epiphytic behavior under various environmental conditions. This knowledge, coupled with the extensive studies on the dynamics of epiphytic populations (reviewed in HIRANO and UPPER 1983, 1990), may be critical for predicting foliar population responses to changing environmental conditions and designing rational disease control strategies.

In this review, we have discussed many traits that could be involved in epiphytic fitness, and have attempted both to evaluate the evidence for a role of each trait in bacterial growth or survival on leaves and to explore the potential mechanistic basis for its involvement. Unfortunately, many studies of these traits have been largely descriptive. The recent advance of molecular genetic techniques has made more rigorous testing possible. For example, comparisons of mutants with their isogenic parental strains have demonstrated a role for motility and osmotolerance, as well as a lack of a role for ice nucleation activity, in survival under particular environmental conditions. Even with these techniques, however, identification of individual epiphytic fitness determinants may be hampered by several obstacles. First, alterations in individual traits may

result in changes too small to be detected with current methodology; thus, better methods for accurately measuring bacterial growth and survival in planta are needed. Second, since epiphytic fitness is dependent on environmental context, subtle variations in the epiphytic environment may increase the variability in the observed behavior and obscure detection of true changes in epiphytic fitness. Last, the fact that inactivation of a single gene frequently results in alterations in more than one phenotype greatly complicates the establishment of clear, causal relationships. Pleiotropy may occur at the transcriptional level, e.g., a regulatory gene influencing the transcription of other genes, or at the phenotypic level, e.g., a cell surface alteration influencing motility and extrusion of exocellular enzymes and EPS. If a regulatory gene is involved, then the contribution of each regulated gene should be evaluated individually.

One of the biggest challenges ahead is evaluating exactly how particular traits contribute to epiphytic fitness. Molecular genetic techniques are likely to be of invaluable use for such studies. For example, the expression of genes in bacterial cells on a leaf surface can be evaluated using reporter genes. In planta induction of general epiphytic fitness traits was illustrated by the fact that bacterial cells that were harvested from leaf surfaces survived better than cells harvested directly from laboratory culture after inoculation onto leaf surfaces (WILSON and LINDOW 1993). Understanding the exact conditions that influence the expression of a gene should indicate when its encoded trait is important for cell growth or survival and thus provide insight into the mechanistic contribution of that trait to fitness. Due to the rapid and extreme changes that frequently occur on the leaf surface, it is important to consider the possibility that bacteria coordinately regulate a range of phenotypes in response to these changes.

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Pathogenicity Determinants and Global Regulation of Pathogenicity of *Xanthomonas campestris* pv. *campestris*

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1 Introduction

Xanthomonas campestris pathovar *campestris* (hereafter *X.c. campestris*), the causal agent of black rot of crucifers, is a phytopathogenic bacterium of great economic importance (Williams 1980; Onsando 1992; Schaad and Alvarez 1993). Molecular genetic methods have now allowed a number of genes encoding pathogenicity determinants of *X.c. campestris* and related pathovars to be identified. In this chapter we will briefly review some of these and discuss our current understanding of the regulation of pathogenicity in this bacterium.

Two major strategies have been widely employed to isolate pathogenicity genes. In "black box" methods, populations of mutagenised bacteria are screened for individuals showing loss of symptoms, usually in simplified plant assays. Identification of DNA fragments complementing these nonpathogenic mutants or of flanking regions (if the mutants are transposon-tagged) allows isolation of the gene(s). The second approach is to test the role of suspected pathogenicity determinants by cloning and specific mutation of the cognate genes, followed by plant tests. A third strategy has been to isolate plant-inducible genes through use of a promoter probe plasmid (Osbourn et al. 1987). The rationale here is that a proportion of such genes are likely to be involved in

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pathogenesis. This can be tested by specific mutagenesis of the genes and subsequent plant assays.

The choice of plant material for inoculation, the method of inoculation and levels of bacteria used undoubtedly influence the outcome of these pathogenicity tests in that different aspects of the disease process are probably being tested (SHAW and KADO 1988; DANIELS and LEACH 1993). In natural infections, *X.c. campestris* enters the plant principally through the hydathodes at the leaf margin, although secondary entry sites can develop in wounds or roots (COOK et al. 1952). This allows the bacteria to gain access to the vascular system of the plant. Here the bacteria are usually confined to the xylem (BRETSCHNEIDER et al. 1989), except in the later phases of the disease in which severe tissue degradation takes place. The characteristic symptoms of the disease in the field consist of V-shaped chlorotic lesions spreading from the leaf margins within which are blackened leaf veins. Diseased tissue eventually becomes brown and parched. Pathogenicity assays in which bacteria are introduced into the mature leaf through cut vein endings or by inoculation into the leaf petiole mimic the natural infection pathways and disease symptoms more closely than assays employing seedlings or inoculations into the leaf lamina (SHAW and KADO 1988; DOW et al. 1990). However, *X.c. campestris* is capable of substantial growth in the mesophyll tissue of crucifers and this is an experimentally easier system in which to study bacterial growth and concomitant plant defence responses (COLLINGE et al. 1987; CONRADS-STRAUCH et al. 1990). Assays in which seeds are soaked in bacterial suspensions before sowing may mimic another aspect of the disease cycle as contaminated seed is believed to be a major source of infection (SCHAAD and ALVAREZ 1993). In our laboratory, for mass screening we have mainly used assays in which seedlings are stab-inoculated with mutant bacteria. A major class of pathogenicity mutants we have identified using this assay are pleiotropically defective in the production of extracellular enzymes (see below). This may suggest that seedling tests indicate tissue degradation capacity, corresponding to a late phase of the natural disease; it is likely that, using only this assay, genes involved in more subtle effects would be missed.

In addition to symptom production and bacterial growth, more detailed studies of host responses, in particular defence-related gene expression, is certainly warranted. Recent work in our laboratory on the induction of β-1,3-glucanase gene transcription in response to a number of defined pathogenicity mutants of *X.c. campestris* has reflected the undoubted complexity of host-bacterial interactions. Certainly no simple relationship between bacterial growth, symptom production and triggering of defence-related gene expression exists.

Below we review the pathogenicity determinants so far revealed with the exception of *hrp* genes and protein export genes which are covered in other chapters of this volume (by BONAS and COLLMER and BAUER). In the second part of the review we discuss regulatory genes involved in pathogenicity and the complex of interconnected regulatory circuits in *X.c. campestris*.

2 Production of Extracellular Enzymes

In common with many plant pathogens *X.c. campestris* produces a range of enzyme activities capable of degrading plant tissue. These include endoglucanase (cellulase), protease, polygalacturonate lyase, amylase and lipase (reviewed by DANIELS et al. 1988). These enzymes are good candidates for pathogenicity determinants in black rot pathogenesis in which degradation of the plant tissue is evident in the later phases of the disease. As with other plant pathogens, multiple enzyme activities have been observed for protease and polygalacturonate lyase (DOW et al. 1987,1990). All of these enzymes are exported from *X.c. campestris* by a signal sequence-dependent mechanism encoded by a cluster of at least 12 genes, some of which have been characterised (DUMS et al. 1991; HU et al. 1992). Closely related protein export systems are found in a wide range of gram-negative organisms (reviewed by PUGSLEY et al. 1990; SALMOND and REEVES 1993). Mutations within the *X.c. campestris* gene cluster cause retention of the enzymes within the bacteria and give a nonpathogenic phenotype on plants associated with reduced symptom expression (DOW et al. 1987; HU et al. 1992). This provides strong circumstantial evidence for a role for extracellular enzymes in disease. However, export-defective mutants do grow at similar rates to the wild-type when low numbers of bacteria are introduced into the leaf lamina, which is consonant with a role for the enzymes only in the later phases of the disease process.

The genes for several individual enzymes (endoglucanase, one isoform of polygalacturonate lyase, a serine protease and lipase) have been cloned and the effects of specific mutations in these genes on pathogenicity has been assessed (GOUGH et al. 1988; DOW et al. 1989; TANG et al. 1987 and unpublished results). None of these mutations alone causes a change in bacterial growth or symptom expression in a wide range of pathogenicity tests. Although a protease-deficient strain was shown to have severely attenuated disease symptoms in some plant tests (DOW et al. 1990), subsequent examination of other protease-deficient mutants failed to confirm a role for protease in the early phases of the disease process. It is likely that the original observations were due to secondary mutations involving extracellular polysaccharide production (see below) or to a pleiotropic effect of the mutation. The role of other enzymes, particularly the major isoform of polygalacturonate lyase which may contribute greatly to tissue disintegration, remains to be tested. However it is possible that several enzymes contribute synergistically to pathogenicity, or that other unidentified exported proteins (e.g. proteins induced only in planta) are responsible. Work on other pathovars of *X. campestris* has suggested no role for polygalacturonate lyase or protease in pathovars *vesicatoria* and *glycines,* respectively (BEAULIEU et al. 1991; HWANG et al. 1992). The diseases caused by these bacteria (leaf spot of pepper and tomato and pustule formation on soybean) do not however involve generalised destruction of the plant tissue.

3 Extracellular Polysaccharides

In common with many plant pathogens, *Xanthomonas campestris* produces extracellular polysaccharide (EPS), specifically the acidic polymer xanthan. Interest in xanthan as an industrial product is considerable and clusters of genes involved in xanthan biosynthesis have been isolated and characterised (reviewed by SUTHERLAND 1993). However there has been relatively little work reported on the role of xanthan in pathogenesis. Immunogold electron microscopy with xanthan-specific antibodies have clearly shown that xanthan encapsulates *X.c. vesicatoria* cells in both compatible and incompatible interactions with pepper within a few hours of inoculation (BROWN et al. 1993). Genetic evidence for a role for xanthan has been provided the work of BARRÈRE et al. (1986) and RAMIREZ et al. (1988). Although EPS mutants of *X.c. campestris* were indistinguishable from the wild type in symptom production on turnip seedlings, when the bacteria were introduced into the veins of mature plants through cuts at the leaf margins, considerable reduction in symptoms were seen. The growth of EPS mutants after inoculation into turnip and *Arabidopsis* leaves at low initial densities was also severely reduced compared to the wild type. Overall these results suggest a role for xanthan, particularly in the early phases of the disease process.

4 *hrpX*

Kado, Kamoun and coworkers have identified a plant-inducible gene in *X.c. campestris, hrpXc,* which is required for pathogenesis on crucifers and for a hypersensitive response on non-host plants (KAMOUN and KADO 1990; KAMOUN et al. 1992; KAMDAR et al. 1993). The *hrpXc* gene is not part of the *hrp* cluster of *X.c. campestris*, which is discussed elsewhere in this volume (BONAS). Homologous genes which are functionally equivalent to *hrpXc* are present in *X.c. armoraciae* and *X.o. oryzae* and probably in other pathovars of *X. campestris* but not in other genera of plant pathogenic bacteria (KAMDAR et al. 1993). *hrpX* mutants of *X.c. campestris, X.c. armoraciae* and *X.o. oryzae* can be complemented by heterologous *hrpX* genes. In *X.c. campestris*, mutation of the *hrpX* gene causes the normally compatible bacteria to trigger a necrotic reaction in the vascular tissue which has been termed a vascular hypersensitive response (KAMOUN et al. 1992). Growth in the mesophyll tissue is also impaired. These observations have suggested that the role of the *hrpX* gene product is to circumvent host recognition or to suppress host defence responses in the early phases of disease. Sequence analysis of the gene suggests that the gene product might be a substrate for myristoyl transferase, an enzyme found in plants but not in prokaryotes (KAMDAR et al. 1993). This raises an intriguing possibility that the plant enzyme modifies the bacterial cell surface during pathogenesis, a hypothesis which further work on the biochemistry of the HrpX protein both in planta and in vitro could address.

5 Avirulence Genes

Although avirulence genes have been defined in the context of resistance responses in plants to incompatible pathogens, it is clear that, in a least some cases, these gene products contribute to pathogen fitness in the compatible reaction (see chapter by J.L. Dangl, this volume). Sequences related to *avrBs2*, the avirulence gene complementary to the resistance gene *Bs2* in pepper, have been found in all races of *X.c. vesicatoria* (both tomato and pepper pathogens) and in a number of other pathovars of *Xanthomonas campestris*, including pv. *campestris*, that do not cause disease on peppers (Kearney and Staskawicz 1990). Mutation of this gene in *X.c. vesicatoria* causes a reduction of virulence on susceptible pepper lines as shown by decreased bacterial growth. Complementation with plasmid-borne *avrBs2* restored growth to near wild-type levels. Mutation of the *avrBs2* gene of *X.c. alfalfae* was also shown to reduce the virulence of this pathogen to a susceptible alfalfa line (Kearney and Staskawicz 1990). The contribution to pathogenicity of the homologous gene in *X.c. campestris* has yet to be tested. In the converse fashion, a pathogenicity gene from *X. citri* has been shown to render *X.c. phaseoli* avirulent to bean (its normal host) and to confer cultivar-specific avirulence to cotton on *X.c. malvacearum* (Swarup et al. 1992). However this gene, *pthA*, did not have a homologue in either of the two strains of *X.c. campestris* tested. A number of avirulence genes may indeed encode products which act as pathogenicity factors in particular interactions. However this need not always be the case. A gene from *X.c. raphani* that renders *X.c. campestris* avirulent to most accessions of *Arabidopsis thaliana* has a homologue in *X.c. campestris* (Parker et al. 1993). Mutation of this homologous gene has no effect on growth of *X.c. campestris* or symptom expression in both *Arabidopsis* and *Brassica* (C.E. Barber and M.J. Daniels, unpublished).

6 Additional Pathogenicity Genes

A number of other pathogenicity genes of pathovars of *Xanthomonas campestris* have been identified by mutagenesis and the genes characterised. Osbourn et al. (1990a) have described a gene in *X.c. campestris* in which mutation causes a loss of pathogenicity although extracellular enzymes and EPS remain at wild-type levels. Numbers of recoverable mutant bacteria drop by about tenfold within 24 h of introduction into the plant and then increase to a plateau level which is less than that attained by the wild type. This suggests that the gene, which is expressed in rich nutrient medium as well as in planta, is required in the early phases of establishment of the disease. The mutant bacteria trigger an earlier accumulation of transcript of β-1,3-glucanase, a defence-related gene in *Brassica* (M.-A. Newman and J.M. Dow, unpublished). These results may suggest that the bacterial gene is involved in suppression of the host defence responses or in

avoidance of triggering of those responses, as suggested for *hrpX* (see above). However the mutants still show the wild-type phenotype on incompatible pepper lines, and there is no sequence homology to the *hrpX* gene, to the *hrp* cluster of *X.c. campestris* or to any other gene in the database.

Genes involved in the pathogenicity of *X.c. glycines* on soybean have been characterised by HWANG et al. (1992) by complementation of nonpathogenic mutant that did not grow in susceptible soybean cotyledons followed by subcloning and transposon mutagenesis of the complementing clone. A 10 kb DNA fragment restored pathogenicity to the mutant; sequences in this DNA fragment were conserved among a number of *X. campestris* pathovars including pathovar *campestris.* Three regions were involved in restoring pathogenicity. The sequence of two of these showed the presence of two potential open reading frames. Although the cellular functions of the two encoded proteins are not known, both contain hydrophobic domains and one has amino acid sequence similarity to the γ subunit of oxaloacetate decarboxylase. This enzyme is involved in sodium ion transport in *Klebsiella pneumoniae.* It is tempting to speculate that these products have a role in the adjustment of the bacteria to the changed ionic environment of the plant where levels of H^+, K^+, Na^+, Ca^{2+} and Mg^{2+} may differ drastically from those found in nutrient medium.

X.c. armoraciae is a mesophyllic pathogen of crucifers and causes a necrotic response in the vascular system with no subsequent invasion. A 5.3 kb DNA fragment from *X.c. campestris* when introduced into *X.c. armoraciae* alters the symptom expression in mature plants and seedlings such that symptoms typical of *X.c. campestris* are seen (ROBERTS and GABRIEL 1992). This observation provides preliminary evidence that the DNA fragment carries a potential systemic movement factor required for vascular proliferation. It will be interesting to see the result of mutation of the gene(s) carried on the 5.3 kb fragment on the growth and spread of *X.c. campestris* both in the vascular system and in mesophyllic tissue. Differences in the expression pattern of conserved protease genes between *X.c. armoraciae* and *X.c. campestris* are also found (DOW et al., 1993). However manipulation of the pattern of protease production of *X.c. armoraciae* to that resembling *X.c. campestris* by introduction of cloned protease genes did not allow *X.c. armoraciae* to invade the vascular system, suggesting no relationship of the pattern of protease production to the mode of pathogenesis.

7 Regulatory Genes

We noted above that a large proportion of *X.c. campestris* mutants isolated by screening for altered symptoms following stab inoculation of bacteria into seedlings showed pleiotropic defects in extracellular enzyme production. The mutations lie either in secretion genes or in regulatory genes. One of the first *X.c. campestris* mutants to be studied failed to produce protease, polygalacturonate

lyase, endoglucanase and amylase, and gave low levels of EPS (xanthan). Production of all these factors, and pathogenicity, were restored by a cosmid, pIJ3020, containing cloned wild-type DNA (DANIELS et al. 1984; TANG et al. 1991). Analysis of the cloned DNA by transposon mutagenesis, subcloning and sequencing indicated the presence of at least eight genes, *rpfA-H,* mutation in any of which caused a reduction to less than 10% of wild-type levels of enzymes and xanthan. The organisation of these genes is shown in Fig. 1.

Sequence data are available for *rpfF, C, H, G,* and *D; rpfA, B,* and *E* have not yet been characterised in detail. Between *rpfG* and *rpfD* lie two genes which are equivalent to *lysU* and *prfB* of *Escherichia coli.* These genes encode a lysyl tRNA synthetase and peptide release factor, respectively. Mutations in these genes in *X.c. campestris* have little discernible effect on pathogenicity, and it is not known whether their location within a cluster of pathogenicity-related genes has any significance. *lysU* and *prfB* are linked in *E. coli.*

Comparison of the sequences of *rpf* F, *rpfH* and *rpfD* with databases give no clues as to the function of the gene products. However *rpfC* and *rpfG* encode members of a two-component, histidine protein kinase-response regulator system (TANG et al. 1991, and unpublished data). RpfG is a typical response regulator protein, although no DNA-binding motifs are apparent in the peptide sequence. RpfC belongs to a small subclass of histidine protein kinases (class ITR in the nomenclature of PARKINSON and KOFOID 1992). In addition to transmembrane domains and a conserved sensor domain, the protein also contains a region characteristic of the receiver domain of response regulators (RpfG also has such a feature). The function of the duplicated receiver domain in the RpfC/RpfG pair, as with other similar pairs in class ITR, is not understood. A similar gene encoding a protein of this class, *lemA*, is required for pathogenicity of *Pseudomonas syringae* pv. *syringae,* regulating the production of toxin and protease (RICH et al. 1992).

Two component regulatory systems usually function to modulate gene expression in response to external stimuli. The nature of the signal in the case of RpfC/G is unknown. Attempts to address the question directly by studying the biochemical basis of regulation, protein phosphorylation, have met with little success.

Recent experiments have given some information about the function of the *rpfF* gene. Protease and cellulase production by an *rpfF* mutant were restored by growth on plates in proximity to *rpfF*$^+$ strains, suggesting that phenotypic correction by cross-feeding by a diffusible substance was occurring (Fig. 2). It has been shown that a low molecular weight metabolite can be extracted from *X.c. campestris* culture supernatant fluids which increases by a factor of ten enzyme production by the *rpfF* mutant. It is likely that the diffusible substance belongs to the class of "autoinducers" which are now being found to be produced by numerous bacteria. These compounds are N-acylhomoserine lactone derivatives and were first discovered as regulators of luminescence in marine vibrios. Examples have recently been found in the plant pathogens *Agrobacterium* and *Erwinia* (PIPER et al. 1993; ZHANG et al. 1993; PIRHONEN et al. 1993; JONES et al. 1993).

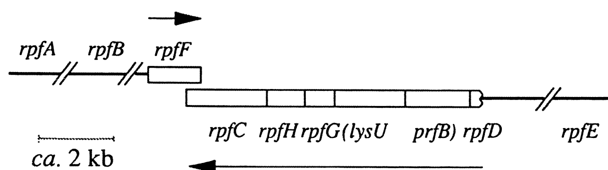

Fig. 1. Organisation of positive regulatory gene cluster of Xanthomonas campestris pathovar campestris. Boxes indicate regions which have been sequenced. Arrows indicate direction of transcription

The regulatory effects of the autoinducers are only exerted at concentrations found in dense bacterial cultures. Hence autoinducers may be viewed as regulators responding to high density, or "quorum sensors" (FUQUA et al. 1994). Much more research will be needed to provide a full understanding of the role of autoinducers in the disease process. For pathogens such as *Erwinia* and *Xanthomonas* one possibility is that when the bacteria are at a low density in plant tissues they can obtain adequate nutrients from the solutes in the extracellular fluids. However high concentrations of bacteria would quickly exhaust these resources and further growth and maintenance would be dependent on nutrients released by enzymatic degradation of plant cell components.

Synthesis of extracellular enzymes and xanthan in *X.c. campestris* is also subject to coordinate negative regulation (TANG et al. 1990). Mutations in a gene designated *rpfN* give overproduction of the enzymes and xanthan, and, conversely overexpression of *rpfN* causes coordinate repression. *rpfN* encodes a protein of 46 KDa and is required for binding of a protein to sequences upstream of the promoter of the protease and endoglucanase structural genes. It is not known whether the RpfN protein itself binds to the DNA (S.D. SOBY, B. HAN and M.J. DANIELS, in preparation).

X.c. campestris contains a gene (*clp*) encoding a DNA-binding protein similar to the catabolite activation protein (CAP) of *E. coli* (DE CRÉCY-LAGARD et al. 1990). CAP is a "broad spectrum" regulator of a large number of genes, and mutants are unable to use certain carbon sources for growth. A *clp* mutant of *X.c. campestris*, in contrast, was unaffected in its ability to utilise all carbon sources tested (unpublished data) and was not responsive to cyclic nucleotides. Xanthan production was reduced in the *clp* mutant, and the residual polysaccharide had different viscosity properties and altered acetyl and pyruvyl content compared with the product of wild-type bacteria. Endoglucanase and polygalacturonate lyase activities were reduced, amylase was unchanged, but protease was overproduced by the *clp* mutant. The ability to cause disease in turnip, measured by several inoculation techniques, was also reduced (DE CRÉCY-LAGARD et al. 1990).

Two-component regulatory proteins such as RpfC and RpfG have conserved amino acid sequence domains. OSBOURN et al. (1990b) designed oligonucleotides which would encode the conserved regions in *X.c. campestris* and used these as hybridisation probes to isolate further regulatory genes of this class. Mutation of one of the new regulator genes reduced xanthan synthesis, but did not affect

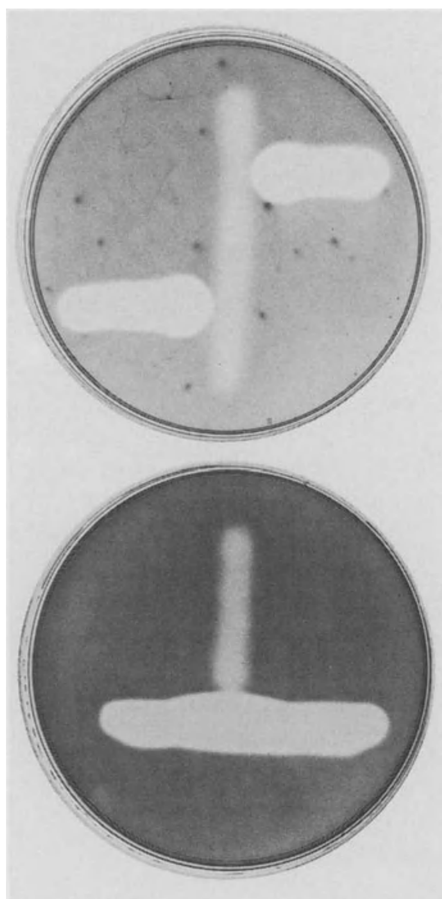

Fig. 2. Cellulase production by an rpfF mutant is enchanced by a diffusible factor from an rpfF$^+$ strain. Nutrient agar plates containing carboxymethyl cellulose were inoculated with a cellulase-deficient rpfF$^+$ strain (horizontal streak) and an rpfF mutant (vertical streak). Cellulase activity, revealed as clear zones after staining with Congo red, was enhanced in that part of the rpfF mutant colony nearest to the rpfF$^+$ colony

extracellular enzyme levels. There was no discernible effect on pathogenicity, presumably because the residual xanthan was adequate for pathogenesis.

There are indications that the above list of regulatory genes effecting enzymes and xanthan is incomplete. Other *X. campestris* and *X. oryzae* pathovars are known to possess DNA sequences which hybridise with the *rpfA-H* region of *X.c. campestris.* SAWCZYC et al. (1989) showed that pathogenicity could be restored to a *X.c. campestris rpf* mutant by either of two cloned DNA fragments from *X.c. translucens* (a cereal pathogen). One of the two fragments hybridised with the *X.c. campestris rpf* cluster, but the other did not. However the latter did hybridise with total DNA of *X.c. campestris*, suggesting that a true homologue exists. This putative additional regulatory gene has not yet been studied.

To summarise, extracellular enzymes and polysaccharide, a subset of pathogenicity factors of *X.c. campestris,* are subject to coordinate regulation of synthesis by several independent sets of regulatory genes. In addition, individual enzymes may be induced or repressed by substrate or product-related effectors.

Other pathogenicity genes such as *hrp* genes do not seem to be regulated by *rpf* and *clp* genes, and, conversely, mutations in *hrp* genes do not affect enzyme and xanthan synthesis (ARLAT et al. 1991). With the exception of *hrp* mutants, all *X.c. campestris* "pathogenicity" mutants provoke a normal hypersensitive response on non-host plants such as tobacco and pepper.

8 Conclusion

Genetic studies with *X. campestris* pathovars have shown that, as with other genera of pathogens, many genes are required for, or contribute to, pathogenicity. Although in many cases the nature of the gene product is known, in no case can we be certain how the gene product interacts with plants to play its part in the overall process of pathogenesis.

A subset of *X.c. campestris* pathogenicity factors consists of extracellular enzymes and polysaccharide. These are particularly useful because their synthesis and regulation are easily studied at the level of genes and proteins. From these studies, as we have indicated above, it has become clear that the production of the factors is regulated in a complex manner, with overlapping coordinate systems as well as individual regulation loops. Since the existence of regulatory systems implies the need to adapt to changes encountered during the life of the bacteria, it is worthwhile considering the black rot disease cycle from this point of view. Outbreaks of disease usually originate from contaminated seed. The bacteria must survive in dry seed coats for long periods of time. Once the seeds germinate the bacteria colonise the surfaces of growing plants, and under favourable conditions enter hydathodes via guttation fluid. Colonisation of the xylem follows and finally the plant tissue becomes necrotic. Little is known about the chemical environment on leaf surfaces. It is certain that it will change in response to changing weather, for example rain will wash leached substances from leaf surfaces, and as dew evaporates, solute concentrations in the surface water film will increase. Some partial analyses of the composition of guttation fluid and xylem sap have been reported, and while it is difficult to generalise from disparate data, it is likely that the bacteria will experience large changes in the composition of their "growth medium" as they move from the leaf surface through the hydathode into the xylem. Once tissue degradation sets in during the later stages of the disease, the bacterial environment will change rapidly. If the regulatory systems operate to enable the bacteria to make the best of the prevailing "growth medium" it will be instructive to determine what environmental triggers are detected by the several systems. Another set of pathogenicity genes in the major *hrp* cluster are also subject to environmental regulation. Early experiments suggested that *hrp* gene expression in *X.c. campestris* is induced by nutrients such as sucrose (ARLAT et al. 1991). However more recent experiments suggest that starvation is the principal factor

stimulating expression (S.A. Liddle and M.J. Daniels, unpublished data). The *hrp* genes do not appear to interact with the *rpf* regulon.

Further studies of gene regulation in *X.c. campestris* will not only increase our understanding of pathogenesis to plants but also contribute to the growing body of information on microbial physiology.

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Erwinia chrysanthemi and *Pseudomonas syringae*: Plant Pathogens Trafficking in Extracellular Virulence Proteins

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1 Introduction

Extracellular proteins are primary weapons in the parasitic attack of bacteria on eukaryotic hosts. Virulence proteins released through accessory secretion pathways enable bacteria to acquire nutrients, invade host tissues, and defeat host defenses. Because of their general importance, these proteins and their secretion pathways provide efficient starting points in the molecular exploration of bacterial pathogenesis; and virulence protein traffic represents a common denominator in

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the study of bacterial pathogens of plants and animals. This is particularly true with gram-negative pathogens, which use broadly conserved components for the specific secretion of virulence proteins, and in some cases use remarkably similar modes of parasitic attack and virulence protein deployment. Consequently, researchers exploring pathogens of one eukaryotic kingdom now are looking with increasing interest at work on pathogens of the other kingdom.

This review will focus on two plant pathogenic, gram-negative bacteria that parasitize plants in strikingly different ways. *Erwinia chrysanthemi* attacks a wide range of plant species, causes extensive host tissue necrosis and maceration, and secretes a battery of tissue-disintegrating pectic enzymes. *Pseudomonas syringae*, in contrast, encompasses a collection of host-specific strains that generally do not macerate host tissues, cause only delayed necrosis in their hosts, and secrete few if any pectic enzymes. *E. chrysanthemi* is typical of necrotrophic parasites that can kill host tissues with molecular brutality and feed off the dead cells (THROWER 1966). *P. syringae* is typical of biotrophic parasites that obtain their nutrients more deviously from living host cells. *E. chrysanthemi* and *P. syringae* thus represent two extremes in the plant pathogenicity of gram-negative bacteria in the genera *Erwinia*, *Pseudomonas*, and *Xanthomonas*. Members of these genera are responsible for the majority of bacterial diseases of plants, and determinants shared between *E. chrysanthemi* and *P. syringae* are likely to be generally important in bacterial plant pathogenicity.

Much research on the molecular basis for pathogenicity in these bacteria has focused on two signature phenomena: maceration and the hypersensitive response (HR). Susceptibility to maceration by pectic enzymes is a common feature of most of the hosts of *E. chrysanthemi*. Key questions are: what is the role of individual enzymes and their mode of deployment in this process? What additional factors are required to be pathogenic? What enables *E. chrysanthemi* to have such a wide host range? The HR, by contrast, is a defense-associated, rapid, local necrosis that is typically elicited when a biotrophic pathogen, such as *P. syringae*, invades the leaves of an incompatible (e.g., resistant) plant. A fundamental enigma of the HR is that the bacterial ability to elicit it in an incompatible plant is related to the ability to be pathogenic in a compatible host plant. Key questions here are: What triggers the HR? How is this related to pathogenicity and the determination of host range?

In the following sections, we will describe the pathogenic nature of *E. chrysanthemi* and *P. syringae* and then focus on the relationship between pectic enzyme production and maceration, the discovery of a class of protein elicitors of the HR, and the pathways by which all of these proteins are secreted. In the concluding sections, we will consider new questions raised by recent discoveries and then compare the modes and roles of virulence protein trafficking in plant and animal pathogens.

2 Contrasting Pathogens and Plant Interactions

2.1 *Erwinia chrysanthemi*: Wide Host Range and Soft Rots

The soft rot erwinias, *E. chrysanthemi* and *E. carotovora*, attack the parenchymatous tissues (which are comprised primarily of living cells without secondary wall thickening) of a wide range of plants. The bacteria are distributed worldwide, with *E. chrysanthemi*, which has a higher optimum temperature for growth, being found primarily in the tropics or in temperate region greenhouses (PEROMBELON and KELMAN 1980). The soft rot erwinias are widespread in surface water (COTHER et al. 1992; COTHER and GILBERT 1990; HARRISON et al. 1987; MCCARTER-ZORNER et al. 1984); they are competitive saprophytes in the rhizosphere (STANGHELLINI 1982); and they can aggressively utilize pectate as a carbon source (BURR and SCHROTH 1977; MENELEY and STANGHELLINI 1976). Pectic enzyme production and sensitivity to desiccation appear to be major factors in the biology of these bacteria.

The soft rot erwinias are particularly devastating as opportunistic pathogens that cause rots in storage organs and fleshy plant tissues that are physiologically compromised by bruising, excess water, or high temperature (PEROMBELON 1982). They also can be true pathogens causing systemic infections, vascular disorders, foliar necroses, and latent infections in many growing plants. Several taxonomic subgroups have been identified within *E. chrysanthemi*, and some strains display a degree of host specificity (BOCCARA et al. 1991; DICKEY 1979, 1981; JANSE and RUISSEN 1988). However, the current taxonomic subgroups do not consistently correspond to hosts of origin, and a given strain of *E. chrysanthemi* is generally capable of attacking many plants. *E. chrysanthemi* and *E. carotovora* are particularly pernicious pests in vegetatively propagated ornamentals because they systemically invade production plants to form latent infections that later become active (often with dramatic effect) when conditions favor the disease.

2.2 *Pseudomonas syringae*: Narrow Host Range and Leaf Spots

Strains of *P. syringae* typically cause watersoaked lesions surrounded by chlorotic halos in the leaves and fruits of a limited range of hosts. The halos are caused by a variety of low molecular weight toxins which are not host-specific and generally are not essential to pathogenicity, but which can contribute significantly to virulence (GROSS 1991; WILLIS et al. 1991a). *P. syringae* strains are classified into pathovars primarily according to their host range (PALLERONI 1984). Thus, *P. syringae* pv. *pisi* is a pathogen of peas, and *P. syringae* pv. *tomato* is a pathogen of tomato. But the pathovar system has many exceptions. For example, some strains of *P. syringae* pv. *tomato* are also pathogenic on *Arabidopsis thaliana*, and *P. syringae* pv. *syringae* contains some strains that cause brown spot of bean, whereas others cause, variously, leaf spots (or cankers) of stone fruit, lilac, or

even tomato seedlings (Hirano and Upper 1990; Jones et al. 1981; Whalen et al. 1991; Cheng et al. 1989). Also, strains within some pathovars can be classified into races on the basis of their limited host range among various cultivars of a given crop species, a phenomenon that has been explored most extensively with *P. syringae* pv. *glycinea* and its host, soybean (Keen 1990), and will be discussed in Sect. 4.3.

There is no evidence that *P. syringae* is prevalent in surface water like *E. chrysanthemi*. In contrast, many *P. syringae* strains are good epiphytes, they multiply and persist on the aerial surfaces of plants, and they can attack healthy plants. Hirano and Upper (1990) have emphasized the importance of epiphytic fitness in the biology of *P. syringae*, and the accompanying chapter by Beattie and Lindow addresses this attribute. Finally, it is important to note that, during pathogenesis, both *E. chrysanthemi* and *P. syringae* colonize the apoplast, which is comprised of the intercellular spaces and water-conducting xylem and therefore are excluded by plant cell walls from the living protoplasts of the host.

3 Maceration and Pectic Enzymes

3.1 The Extracellular Pectic Enzyme Complex of *Erwinia chrysanthemi*

E. chrysanthemi and *E. carotovora* are highly pectolytic bacteria, and their pectic enzymes have been implicated in plant tissue maceration since Jones (1909) observed that cell-free culture fluids of *E. carotovora* possessed both macerating and pectolytic activity. Subsequent research with isolated plant cell wall-degrading enzymes culminated in the finding that the activity of a single, purified pectic enzyme was sufficient for both the maceration and cell killing that is diagnostic of the disease (Basham and Bateman 1975a,b), as discussed further in Sect. 3.2. Since then, the role of pectic enzymes in soft rot pathogenesis has been extensively explored genetically, and other reviews provide a comprehensive background for this work (Collmer and Keen 1986; Kotoujansky 1987; Robert-Baudouy 1991). In the following sections, we will introduce the *E. chrysanthemi* pectic enzyme arsenal in its simplest form and then enlarge the picture to include recent insights into the relationship between enzyme deployment and pathogenesis.

E. chrysanthemi produces diverse pectic enzymes. As summarized in Table 1, these enzymes attack the α-1,4-glycosidic linkages in pectate (polygalacturonate or galacturonan) using different reaction mechanisms and action patterns. The pectate lyase isozymes (discussed individually in Sect. 3.4) cleave internal glycosidic linkages in pectate by β-elimination, have a high pH optimum (ca. 8.5) and a requirement for divalent cations, can attack insoluble polymers in

Table 1. *Erwinia chrysanthemi* EC16 enzymes inducible by pectate and involved in the depolymerization or deesterification of pectic polymers and oligomers

Enzymes	Genes[a]	Reaction mechanism	Substrate and products	Direct role in maceration
Pectate lyase (isozymes PelA-E)	*pelABCE*	β-Elimination of internal glycosidic bonds	Pectate to various oligomers	Yes, depending on isozyme
Exo-poly-α-D-galacturonosidase	*pehX*	Hydrolysis of penultimate glycosidic bond	Pectate to dimers	No
Oligogalacturonide lyase	*ogl*	β-Elimination	Oligomers to monomers	No
Pectin methylesterase	*pem*	Hydrolysis of methylester	Pectin (polymethoxygalacturonide) to pectate	No

[a] The gene designations are based on the reaction mechanism: *pel* (pectic enzyme lyase); *peh* (pectic enzyme hydrolase); *pem* (pectic enzyme methylesterase).

plant cell walls, and generate a 4,5-unsaturated bond in the nonreducing product (COLLMER and KEEN 1986). Exo-poly-α-D-galacturonosidase has a lower pH optimum (6.5), hydrolyzes the penultimate glycosidic bond at the nonreducing end of the pectic polymer, releases digalacturonate (or 4,5-unsaturated digalacturonate from unsaturated substrate), and appears to work in concert with pectate lyase in degrading insoluble pectic polymers to assimilable dimers (COLLMER et al. 1982). Pectate lyase, exo-poly-α-D-galacturonosidase and pectin methylesterase are extracellular, whereas oligogalacturonide lyase is cytoplasmic (HE et al. 1993b). The latter enzyme cleaves the unsaturated dimer to two monomers of 4-deoxy-L-*threo*-5-hexosulose uronate, or the saturated dimer to monomeric galacturonate and 4-deoxy-L-*threo*-5-hexosulose uronate (MORAN et al. 1968). Subsequent catabolism of 4-deoxy-L-*threo*-5-hexosulose uronate leads to the formation of 2,5-diketo-3-deoxygluconate and 2-keto-3-deoxygluconate, inducers of further pectic enzyme synthesis (discussed in Sect. 3.5), and eventually to pyruvate and 3-phosphoglyceraldehyde.

E. chrysanthemi produces several other extracellular degradative enzymes, including two cellulase isozymes (BOYER et al. 1984; GUISEPPI et al. 1991), four protease isozymes (DAHLER et al. 1990; GHIGO and WANDERSMAN 1992; WANDERSMAN et al. 1987), xylanase (BRAUN and RODRIGUES 1993), phospholipase (KEEN et al. 1992), and plant-inducible isozymes of pectate lyase (BEAULIEU et al. 1993; KELEMU and COLLMER 1993; COLLMER et al. 1991). Genetic evidence does not support a major role for protease or phospholipase in virulence (DAHLER et al. 1990; KEEN et al. 1992). Xylanase may be important in diseases involving graminaceous hosts, in which xylans are more prevalent than pectic polymers in plant cell walls, but the role of xylanase has not been tested genetically (BRAUN and RODRIGUES 1993).

E. carotovora produces a somewhat different set of pectic enzymes, although it causes diseases that are often indistinguishable from those caused by *E. chrysanthemi*. For example, *E. carotovora* produces several pectate lyase isozymes but none with an acidic pI like the *E. chrysanthemi* PelA (and it has no *pelADE* homologs), and *E. carotovora* produces endo-cleaving polygalacturonase rather than exo-poly-α-D-galacturonosidase (HINTON et al. 1989a, 1990; RIED and COLLMER 1986; SAARILAHTI et al. 1990).

Pectin lyase, which preferentially cleaves pectin, also is produced by many strains of *E. chrysanthemi* and *E. carotovora* (TSUYUMU and CHATTERJEE 1984). Intriguingly, the enzyme is induced by DNA-damaging agents rather than pectic compounds, and this induction is dependent on RecA and an activator designated Rdg (MCEVOY et al. 1992; ZINK et al. 1985). The complexity of this arsenal leads us to question in the following sections the evidence for the pathogenic role of these enzymes, as determined by physiological effects, mutant phenotypes, and patterns of regulation.

3.2 Pectic Enzymes and Plant Tissue Damage

Most of the endo-cleaving pectic enzymes secreted by the soft rot erwinias can macerate and kill parenchymatous plant tissues (COLLMER and KEEN 1986). The effect is rather dramatic in organs like the potato tuber, which can be liquified by either bacteria or isolated enzymes. No other plant cell wall depolymerases secreted by the soft rot erwinias have this destructive effect, apparently because α-1,4-galacturonosyl linkages in the matrix of the primary cell wall and middle lamella of dicots are unique in being essential for structure but vulnerable to enzymatic attack (MCNEIL et al. 1984). Maceration is attributed to disruption of the middle lamella, which is the intercellular cement that holds plant cells in a tissue. The simplest explanation for the killing effect is osmotic fragility of the protoplast resulting from structural failure of the wall (BASHAM and BATEMAN 1975a,b; STEPHENS and WOOD 1975). However, the sensitivity of turgid (but not plasmolyzed) protoplasts to some cell wall fragments raises the possibility of alternative mechanisms (YAMAZAKI et al. 1983).

Further support for the involvement of *E. chrysanthemi* pectate lyases in damaging susceptible plant cell walls was obtained by immunocytochemically monitoring the distribution of the enzyme and the disorganization of pectate in *Saintpaulia ionantha* leaves infected with *E. chrysanthemi* (TEMSAH et al. 1991). The enzyme was prevalent in the walls of spongy parenchyma cells, which were the cell type most susceptible to degradation during pathogenesis, and the presence of the enzyme, first in the middle lamella and then in an irregular distribution pattern in the cell wall, corresponded with the loss of substrate, as determined with a monoclonal antibody specific for homogalacturonans.

3.3 Pectic Enzymes and Plant Defense Elicitation

Certain products of pectolytic digestion are biologically active as elicitors of defense reactions. These are members of the growing class of "oligosaccharins," which are biologically active oligosaccharides produced by partial degradation of plant cell wall polymers (ALBERSHEIM et al. 1983).

Oligogalacturonides released by *Erwinia* pectic enzymes that have between 10 and 15 galacturonosyl residues and are without methylesterification (which excludes the products of the damage-inducible pectin lyase) elicit the synthesis of phytoalexins (low molecular weight, antimicrobial compounds) and chitinase (representative of a class of "pathogenesis-related" defense proteins) (ALDINGTON et al. 1991; BROEKAERT and PEUMANS 1988; DAVIS et al. 1984; HAHN et al. 1988). The initial response to these oligogalacturonides is rapid, as indicated by measurements of membrane depolarization and increased cytosolic free calcium concentration (MESSIAEN et al. 1993; THAIN et al. 1990). It is not certain whether defense-eliciting oligouronides are actually generated and active during pathogenesis.

It is also important to note that oligogalacturonides do not elicit the HR. In fact, tobacco leaves pretreated with low levels of an *E. chrysanthemi* pectate lyase isozyme or oligogalacturonide products of the enzyme do not undergo the HR when challenged with a strain of *P. syringae* that would normally produce this response, and they are resistant to *P. syringae* pv. *tabaci*, which normally causes wild fire of tobacco (BAKER et al. 1986, 1990). Similarly, pretreatment of tobacco seedlings with *E. carotovora* pectic enzymes induces protection against challenge inoculation with the bacterium (PALVA et al. 1993). Thus, the *Erwinia* pectic enzymes or their oligogalacturonide products can induce resistance in tobacco against diverse bacterial pathogens.

The active resistance of plants against potential pathogens is associated with the de novo synthesis of a variety of putative defense proteins, including phenylalanine ammonia lyase and other enzymes in the phenylpropanoid pathway (which produces precursors for the biosynthesis of phytoalexins and lignin), the pathogenesis-related proteins (which are targeted to the apoplast or the vacuole and include lytic antifungal enzymes like chitinase and β-glucanase), peroxidase (involved in lignin biosynthesis), and hydroxyproline-rich glycoproteins (which strengthen the primary cell wall) (LAMB et al. 1989). Many of these proteins or their encoding mRNAs have been shown to increase after treatment of plant materials with *E. carotovora* pectic enzymes (presumably acting through their oligogalacturonide products) (DAVIS and AUSUBEL 1989; PALVA et al. 1993), and this response can be diminished by particular combinations of enzymes that should prevent the accumulation of larger oligogalacturonides possessing elicitor activity (YANG et al. 1992).

The biochemical basis for resistance to the soft rot erwinias, particularly *E. carotovora*, has been studied most in potato tubers, which are highly susceptible to bacterial soft rot under certain conditions (LYON 1989). This dependency on environmental conditions that impair the host is a hallmark of soft rot patho-

genesis, and wet, hypoxic conditions are particularly important in tuber rot (DE BOER and KELMAN 1978). One explanation for this is that hypoxic stress, which naturally occurs when tuber surfaces are covered with a film of water (BURTON and WIGGINTON 1970), impairs wound healing and active defenses. Wounds enable these bacteria to breach the periderm and enter the underlying, susceptible parenchymatous tissues, but healed wounds are resistant to bacterial penetration (PEROMBELON and KELMAN 1980). Hypoxic stress inhibits multiple aspects of the wound response in potato tubers, including the synthesis of phenylalanine ammonia lyase and hydroxyproline-rich glycoproteins and the oxidative cross-linking of suberin and lignin constituents (BUTLER et al. 1990; RUMEAU et al. 1990; VAYDA et al. 1992; VAYDA and SCHAEFFER 1988). Another explanation for the dependence of tuber rot on hypoxic conditions is that anaerobically incubated whole tubers are far more susceptible to maceration by isolated *E. carotovora* pectic enzymes (MAHER and KELMAN 1983). The diverse physiological effects of pectic enzymes suggest that some enzymes may be particularly adapted for pathogenesis and that deployment of the enzyme complex is carefully regulated.

3.4 Pectic Enzyme Deficient Mutants and *Erwinia chrysanthemi* Virulence

Our knowledge of the genetics of pectic enzyme production, its relationship to virulence, and the relative importance of individual enzymes in the soft rot erwinias is most complete with *E. chrysanthemi* strains EC16 and 3937. Figure 1 depicts the pI profiles of the pectate-inducible, extracellular pectic enzymes of strain EC16 and the arrangement of the encoding genes. All of the pectic enzyme genes have independent promoters despite the fact that *pelB-pelC* and *pelA-pelE-pem* are contained in two (widely separated) clusters (TAMAKI et al. 1988). EC16 differs from 3937 and most strains of *E. chrysanthemi* in not producing PelD because of a natural deletion (TAMAKI et al. 1988). PelB and PelC have 84% amino acid identify, PelA and PelE share 62% identity, and the strain B374 PelD and PelE share 79% identity (TAMAKI et al. 1988; VAN GIJSEGEM 1989). All of these genes in EC16 and 3937 have been cloned, sequenced, and mutated by marker-exchange mutagenesis. The EC16 mutants have been assayed primarily for their ability to macerate whole potato tubers and the 3937 mutants for their ability to systemically invade and rot axenically grown Saintpaulia plants.

Mutations in *E. chrysanthemi* EC16 have revealed that PelE is the most important isozyme in potato tuber maceration: a ΔpelE mutant has half of the potato tuber maceration capacity of the wild type (PAYNE et al. 1987). The potential importance of PelE in virulence has been tested further by subcloning the gene under control of the triple *lac* UV5 promoters in pINK-1, which permits *E. coli* transformants to produce (and leak) high levels of PelE (KEEN and TAMAKI 1986). The *pelE*[+] *E. coli* strain macerates more aggressively than does wild-type *E. chrysanthemi* when injected into whole potato tubers (PAYNE et al. 1987) and is reported to produce typical blackleg (stem rot) symptoms in potato plants (TSROR

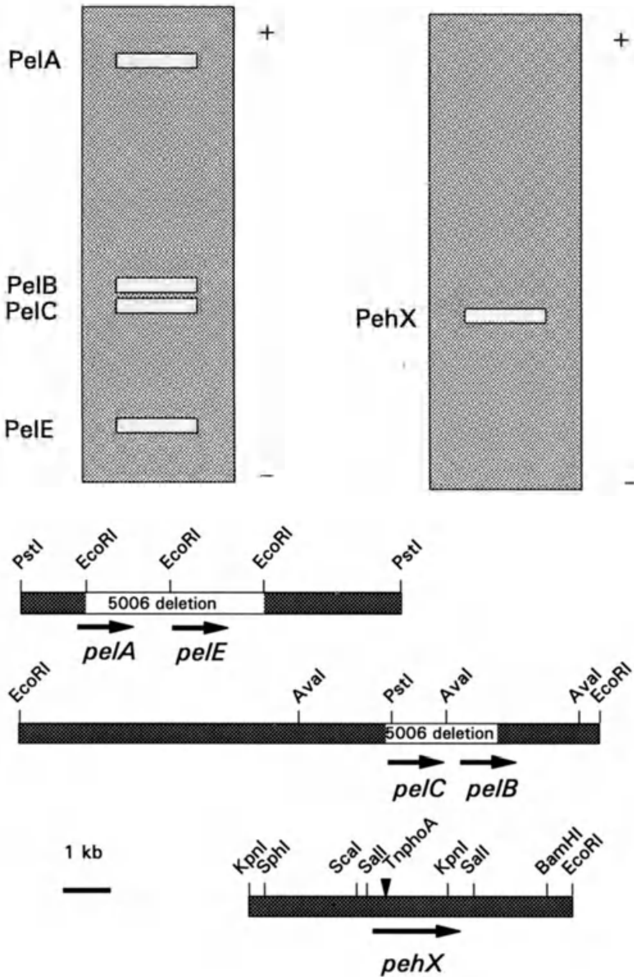

Fig. 1. Diagram of isoelectric focusing (IEF) profiles of the *Erwinia chrysanthemi* EC16 pectate lyase (*Pel*) isozymes and exo-poly-*a*-D-galacturonosidase (*PehX*), the *pel* and *pehX* genes, and relevant mutations. The *Erwinia* pectic enzymes are routinely resolved and detected by activity-staining ultrathin layer IEF gels; *clear zones* in the background of stained polymer in substrate overlays indicate the location of focused enzymes (BERTHEAU et al. 1984; RIED and COLLMER 1985). Unmarked deletions, indicated by the *unshaded regions*, were constructed in the strain EC16 *pel* genes to produce mutant CUCPB5006, which was then used for directed mutagenesis of additional pectic enzyme genes, such as the *pehX*::Tn*phoA* mutation in CUCPB5009. (From HE and COLLMER 1990)

et al. 1991). These results indicate the PelE is sufficient to confer the maceration phenotype to another enterobacterium. Thus, despite the suggestion of DE LORENZO et al. (1991), that the high pH optimum of bacterial pectate lyases is inconsistent with a role in initiating maceration, there seems little doubt that the *E. chrysanthemi* PelE is capable of this when delivered by a living bacterium.

The observation that *E. chrysanthemi* EC16 mutants with deletions in individual *pel* genes are only partially reduced in maceration ability spurred the development of a marker exchange-eviction mutagenesis technique and the construction of *E. chrysanthemi* mutants with multiple *pel* mutations (Collmer et al. 1988; Ried and Collmer 1987, 1988). Surprisingly, mutant UM1005, which has deletions in *pelABCE*, retains significant ability to macerate potato tuber tissue (Ried and Collmer 1988). The *E. chrysanthemi* EC16 *pehX* and *pelX* (encoding exopolygalacturonate lyase) genes have also been cloned, characterized, and mutated, revealing their products to contribute to the utilization of pectate but not to maceration virulence (Brooks et al. 1990; He and Collmer 1990). *E. chrysanthemi* CUCPB5012 (Δ[*pelB pelC*]::28bp, Δ[*pelA pelE*], Δ[*pelX]* Δ4bp, *pehX*::Tn*phoA*) was subsequently constructed and found to produce a second set of Pel isozymes in planta and when grown in the presence of plant extracts (Kelemu and Collmer 1993; Collmer et al. 1991). Culture fluids of CUCBP5012 containing these plant-inducible Pel isozymes are able to macerate chrysanthemum leaves, but the role of the isozymes in the residual maceration ability of the mutant has not been tested genetically.

Many important insights into the role of individual pectic enzymes have been gained by analysis of the pathogenic behavior of mutants of *E. chrysanthemi* 3937 in axenically grown Saintpaulia plantlets. When one leaf is inoculated with strain 3937, most plantlets succumb to systemic invasion and maceration, but some show only maceration of the inoculated leaf, and a few produce just local necrosis or no symptoms (Boccara et al. 1988). Pathogenic behavior in this assay, particularly the capacity for systemic invasion, is substantially altered in *E. chrysanthemi* 3937 strains carrying mutations in certain *pel* genes (Boccara et al. 1988), *pem* (Boccara and Chatain 1989), or multiple *pel* genes (Beaulieu et al. 1993), as summarized in Fig. 2.

In interpreting these results, it is also important to consider the differing action patterns and effects on plant tissues of isolated Pel isozymes. Experiments with strain EC16 Pel isozymes produced by *E. coli* transformants have shown that the relative ability of an isozyme to cause maceration is correlated with its cell killing activity, and both activities increase with the pI of the isozyme (Barras et al. 1987). Thus, PelE is highly destructive, while PelA has little effect. The isozymes also degrade pectate to limit-products in different patterns, although the effect of this on pathogenesis is unclear since none of the isozymes appears to accumulate larger oligomers with elicitor activity (Preston et al. 1992). Thus, with the exception of PelA, the contribution of *E. chrysanthemi* 3937 Pel isozymes to Saintpaulia systemic invasiveness is roughly correlated with the ability of the corresponding EC16 Pel isozymes to damage plant tissues (Fig. 2).

Our lack of understanding of the enzymological basis for the differing actions of the Pel isozymes in pathogenesis is epitomized by the puzzling failure of PelA to macerate potato tuber tissue. It is possible that its low pI causes it to be repelled by the negative charges in the plant cell wall, but the merely intermediate maceration activity of a PelE-PelA hybrid with a pI higher than the expected pH of intercellular fluids argues against this (Tamaki et al. 1988). More knowledge about the enzymology of the Pel isozymes on the diverse and complex pectic polymers

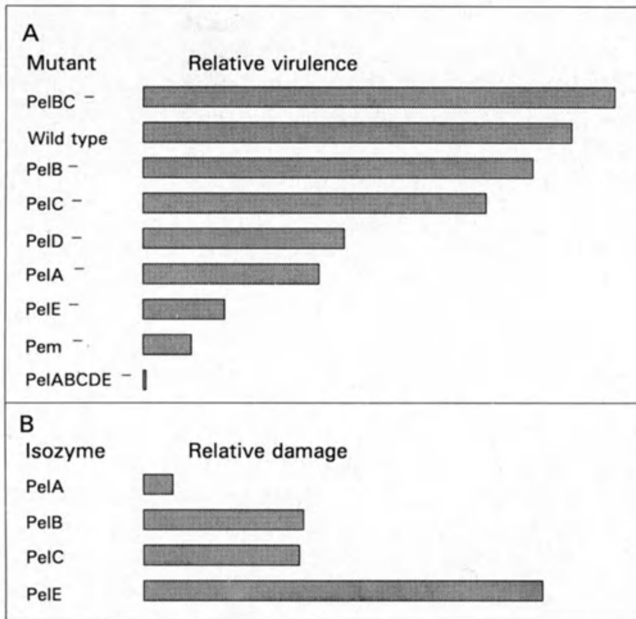

Fig. 2A,B. The relative contributions of *Erwinia chrysanthemi* pectate lyase isozymes to virulence and tissue damage. **A** The relative effect of *pel* and *pem* mutations on the ability of strain 3937 to cause systemic disease in Saintpaulia. **B** The relative permeability changes in potato tuber tissue following incubation with the indicated strain EC16 isozymes for 1 h. (From BEAULIEU et al. 1993; BOCCARA et al. 1988; BARRAS et al. 1987)

in plant tissues is needed (FRY 1988). For example, we do not know if PelA is inactive in tissues that it does not damage, or if instead it preferentially cleaves linkages that have little structural role. Similarly, we do not know what its substrates are in the systemic invasion route. In general, the action of the Pel isozymes on native substrates in the plant cell wall and middle lamella demand further exploration.

The production of multiple Pel isozymes is a conserved feature of *E. chrysanthemi* strains from diverse origins (BERTHEAU et al. 1984; RIED and COLLMER 1986; VAN GIJSEGEM 1986). In a telling experiment, BEAULIEU et al. (1993) have obtained evidence that Pel isozyme multiplicity contributes to the wide host range of the bacterium. *E. chrysanthemi* 3937 mutants with various *pel* mutations were analyzed for their virulence in Saintpaulia plantlets, pea seedlings, potato tubers, and witloof chicory leaves. Many mutations have significantly different effects in different hosts. For example, a *pelE* mutation decreases virulence in Saintpaulia but increases it in potato and chicory, while a *pelBC* mutation does not significantly affect virulence in Saintpaulia but decreases virulence in chicory. Although a *pelABCDE* mutant is significantly reduced in virulence in all plants tested, the level of residual virulence varies substantially. Several new Pel isozymes were detected in extracts from plants infected with the mutant, and these isozymes also appear to differ in their importance in different hosts.

In summary, *E. chrysanthemi* strains EC16 and 3937 produce multiple pectate lyase isozymes (including isozymes additional to PelA-E), each isozyme can enhance (or diminish) virulence quantitatively, and its contribution varies among hosts. All of this suggests complex regulation of enzyme synthesis.

3.5 Pectic Enzyme Regulation in *Erwinia chrysanthemi* and *Erwinia carotovora*

Pectic enzyme production in *E. chrysanthemi* and *E. carotovora* is inducible by pectate and increases dramatically in late log phase (Chatterjee et al. 1979; Collmer and Bateman 1982; Hugouvieux-Cotte-Pattat et al. 1986; Zucker and Hankin 1970; Zucker et al. 1972). The actual inducer during growth of *E. chrysanthemi* on pectate is an intermediate in the ketodeoxyuronate pathway: *ogl* mutants are no longer inducible by pectate or digalacturonates, but pectic enzyme synthesis in *ogl* mutants and wild-type *E. chrysanthemi* can be induced by exogenous 2,5-diketo-3-deoxygluconate, 2-keto-3-deoxygluconate (KDG) and KDG analogs (Chatterjee et al. 1985b; Collmer and Bateman 1981; Nasser et al. 1991).

The focus of recent research in *E. chrysanthemi* has been on the physiological and genetic factors underlying the induction of individual *pel* and *pem* genes, particularly in strain 3937, which has been rigorously studied by Robert-Baudouy and colleagues. The *pelABCDE* genes, *pem*, and all of the genes controlling the intracellular catabolism of 4,5-unsaturated digalacturonate are negatively controlled by the KdgR protein, which in the absence of inducer is expected to interact with conserved KdgR box sequences in the promoter regions of these genes (Condemine 1987; Nasser et al. 1992; Reverchon et al. 1989, 1991). Although *kdgR* mutants produce pectate lyase at a higher level than wild type in the absence of inducer, they still respond to pectate with additional pectate lyase synthesis, indicating the existence of other regulators. Additional loci affecting *pel* expression in *trans* have been identified but not yet characterized (Boccara and Chatain 1989; Hugouvieux-Cotte-Pattat and Robert-Baudouy 1989, 1992).

Several other factors affect the expression of the *E. chrysanthemi pel* genes. Pectate lyase production in *E. chrysanthemi* is subject to cAMP-mediated catabolite repression and self-catabolite repression by 4,5-unsaturated digalacturonate (Chatterjee et al. 1979; Collmer and Bateman 1981), and CAP binding sites are discernible in the regulatory regions of *pel* genes (Gold et al. 1992; Reverchon et al. 1989; Van Gijsegem 1989). Expression of the *pel* genes is reduced by nitrogen starvation and high culture temperatures, and it is stimulated by anaerobiosis, iron limitation, and high osmolarity (Hugouvieux-Cotte-Pattat et al. 1992; Sauvage et al. 1991). The effects of some of these factors are relatively minor and differ among the *pel* genes. For example, high osmolarity stimulates *pelE* but represses *pelD*. However, the end of log phase growth strongly stimulates the expression of

all of the *E. chrysanthemi* 3937 *pel* genes in culture (Hugouvieux-Cotte-Pattat et al. 1992).

Expression of the *E. chrysanthemi* 3937 *pel* genes is also stimulated by a low molecular weight, thermostable, organic compound in carrot extracts but only in the presence of known pectic inducers (Bourson et al. 1993). The *E. chrysanthemi* 3937 *pel* and *pem* genes also have been shown to be expressed during infection of potato tubers, with the *pelA*, *pelB*, *pelC*, and *pem* genes being moderately expressed and the *pelD* and *pelE* genes being strongly expressed (Lojkowska et al. 1993). The relative expression of some of the *pel* genes appears to depend on the tissue. For example, *pelE* and *pem* are expressed at higher levels in growing plants than in tubers. Thus, another factor contributing to the wide host range of *E. chrysanthemi* may be the adaptive regulation of the different pectic enzyme genes.

Work on pectic enzyme regulation in *E. carotovora* has focused on the global regulation of plant cell wall-degrading enzymes because of the prevalence of pleiotropic regulatory mutations effecting virulence and extracellular enzyme production (Beraha and Garber 1971; Hinton et al. 1989b; Murata et al. 1991; Pirhonen et al. 1991). The phenotype of these mutants has been designated Aep (activation of extracellular protein production) (Murata et al. 1991), Exp (exoenzyme production) (Pirhonen et al. 1991), or Rex (regulation of exoproteins) (Jones et al. 1993). For example, *aep* mutants are deficient in production of pectate lyase, polygalacturonase, cellulase, and protease. Extracellular enzyme production and virulence in *aep* mutants of *E. carotovora* subsp. *carotovora* 71 can be restored by the cloned *aepA* gene, which encodes a predicted 51 kDa protein with a signal peptide, several hydrophobic domains, and no homology with known prokaryotic regulatory proteins (Liu et al. 1993; Murata et al. 1991).

Investigation of *exp* mutants in strain SCC3193 and *rex* mutants in strain SCRI193 have revealed that extracellular enzyme production in these bacteria is controlled by *N*-(3-oxohexanoyl) homoserine lactone (HSL), a diffusible autoinducer that also is used by *Vibrio fischeri* to activate *lux* expression and bioluminescence in a cell density-dependent manner (Fuqua et al. 1993; Jones et al. 1993; Pirhonen et al. 1993). The *E. carotovora* SCC3193 *expI* gene encodes a functional analog of the *V. fischeri luxI* gene, whose product directs the biosynthesis of autoinducer (Pirhonen et al. 1993). An *expI* mutant is deficient in virulence and extracellular enzyme production unless exogenous HSL is added. Similarly, some *rex* mutants in strain SCRI193 are dependent on autoinducer for virulence and extracellular enzyme production (Jones et al. 1993). Significantly, an *expI* mutant fails to produce high levels of polygalacturonase as cultures reach the end of log phase, thus indicating that autoinducer is at least one factor controlling growth phase-dependent extracellular enzyme production (Pirhonen et al. 1993). Although density-dependent regulation of extracellular enzyme production has not been proven, it appears likely that autoinducer functions as a quorum sensor (Fuqua et al. 1993), which permits enzyme production during infection only when the pathogen is numerous enough to wage a successful attack (Jones et al. 1993; Pirhonen et al. 1993).

It is not known how similar the pectic enzyme regulatory systems of *E. carotovora* and *E. chrysanthemi* are. A KdgR-like repressor has not been reported in *E. carotovora* and autoinducer-dependent pectic enzyme induction has not been reported for *E. chrysanthemi*, although *E. chrysanthemi* EC16 does produce autoinducer (A. K. Chatterjee, personal communication). In light of the ecological and pathological similarities between these two species, any fundamental differences in regulation of their extracellular enzymes would be surprising.

4 The Hypersensitive Response and Harpins

4.1 *Pseudomonas syringae* and Plant Hypersensitivity

The HR of higher plants is characterized by the rapid, localized death of plant cells at the site of pathogen invasion. It occurs during incompatible interactions, which typically involve a biotrophic, host-specific pathogen that causes disease only in another plant, and it is associated with resistance against many fungi, viruses, and bacteria (Kiraly 1980; Klement 1982). The ability of bacteria to elicit the HR was discovered more than 30 years ago, when Klement infiltrated the intercellular spaces of tobacco leaves with different concentrations of three related bacteria: *P. syringae* pv. *syringae*, *P. syringae* pv. *tabaci*, and *P. fluorescens* (Klement 1963; Klement et al. 1964). The results are summarized in Table 2.

It is important to note that low levels of inoculum are sufficient for *P. syringae* pv. *tabaci* to initiate pathogenesis, but high levels (> 5×10^6 cells/ml) are required for *P. syringae* pv. *syringae* to cause the macroscopic collapse that Klement observed as the HR. We now know that an incompatible pathogen at lower

Table 2. Interactions of three related pseudomonads with tobacco leaf tissue

Bacterium	Relationship with plant	Bacterial population	Plant response	Final outcome
P. syringae pv. *syringae*	Incompatible pathogen	Rises briefly, then declines when HR develops	Rapid (< 24 h) collapse of infiltrated area only	HR (resistance)
P. syringae pv. *tabaci*	Compatible pathogen: "wild fire" of tobacco	Prolonged increase to very high level	Slow (days) necrosis, spreading lesion	Pathogenesis (disease symptoms)
P. fluorescens	Nonpathogen	No increase	None apparent	Null

HR. hypersensitive response. From Klement (1963) and Klement et al. (1964).

concentrations causes the HR only in scattered, individual plant cells, with one bacterium eliciting the death of one plant cell (KLEMENT 1982; TURNER and NOVACKY 1974). The macroscopic HR that is demonstrable in the laboratory is therefore a manifestation of a cellular hypersensitivity that can operate under natural conditions. The HR can be elicited in nonhosts or resistant hosts by most plant pathogenic bacteria, including *Erwinia amylovora* (which causes fire blight of rosaceous plants), *P. solanacearum* (which causes wilts in many solanaceous plants), and *Xanthomonas campestris* (whose many pathovars cause host specific diseases similar to those caused by *P. syringae*). Also, as will be discussed in the next section, *E. chrysanthemi* can be shown to cause the HR.

It is important to emphasize that the HR is not elicited by nonpathogen species like *P. fluorescens*. This implies an underlying relationship between the ability to be a plant pathogen and to elicit the HR. Indeed, KLEMENT (1982) has cogently argued that there are fundamental similarities in the development of the HR and the development of disease. In both cases there is a period of bacterial multiplication that ends with plant cell electrolyte leakage and collapse. A simple interpretation of the difference between the two interactions is that, in the compatible interaction, plant cells are less sensitive to the parasitic growth of the pathogen on their surface. Consequently, plant cell death is delayed until a large bacterial population has developed, spread to surrounding cells, and produced toxins, extracellular polysaccharides, phytohormones and/or other potential virulence factors (COPLIN 1989; EL-BANOBY and RUDOLPH 1979; FETT and DUNN 1989; GROSS 1991; WILLIS et al. 1991a). By contrast, the HR develops rapidly, and the incompatible pathogen is unable to proceed beyond its initial interaction with a few plant cells, presumably because of the production of phytoalexins and other defense molecules associated with the HR (LAMB et al. 1989; LONG et al. 1985; PIERCE and ESSENBERG 1987). Defense gene expression occurs in both interactions but is generally lower or delayed in the compatible interaction (DONG et al. 1991; LAMB et al. 1989; WILLIS et al. 1991b).

The search for early physiological processes occurring in plants inoculated with incompatible *P. syringae* stains has revealed several responses, including a burst of active O_2, membrane lipid peroxidation, Ca^{2+} influx, and a K^+/H^+ exchange (ATKINSON and BAKER 1989; ATKINSON et al. 1985, 1990; CROFT et al. 1990; KEPPLER and BAKER 1989; KEPPLER et al. 1989; KEPPLER and NOVACKY 1986; NOVACKY 1991). The effects of inhibiting Ca^{2+} influx with lanthanum and other channel blockers, ATPase activity with vanadate, and protein synthesis with blasticidin S suggest that these processes are essential for the HR triggered by incompatible *P. syringae* strains and that hypersensitivity is an active response of the plant (ATKINSON and BAKER 1989; ATKINSON et al. 1990; HOLLIDAY et al. 1981). These processes also occur at a slower rate in compatible interactions, and ATKINSON and BAKER (1987a,b) have proposed that the alkalinization of the apoplast resulting from the K^+/H^+ exchange causes leakage of sucrose and other nutrients that permit bacterial growth (discussed further in Sect. 6).

4.2 *hrp* Genes and Harpins

The ability of *P. syringae* strains to elicit the HR or cause disease is controlled by *hrp* (hypersensitive response and pathogenesis) genes, and typical Hrp^- mutants have the null phenotype of a nonpathogen in plants (Lindgren et al. 1986; Niepold et al. 1985; Willis et al. 1991b). The *hrp* genes of phytopathogenic bacteria are discussed in detail in the chapter by Bonas, and here we will review only briefly a few salient points before turning to our discussion of the extracellular protein products of these genes. First, *hrp* genes occur in clusters and are widely conserved among plant pathogens in the genera *Pseudomonas*, *Xanthomonas*, and *Erwinia* (i.e., gram-negative phytopathogens capable of causing necrosis in plants) (Arlat et al. 1991; Fenselau et al. 1992; Gough at al. 1992; Laby and Beer 1992; Lindgren et al. 1988). Second, two cloned *hrp* clusters enable non-pathogens, like *P. fluorescens* or *E. coli*, to elicit the HR (but not cause disease) in tobacco and other plants. These functional *hrp* clusters are carried on cosmids pHIR11 from *P. syringae* pv. *syringae* 61 and pCPP430 from *E. amylovora* Ea321 (Beer et al. 1991; Huang et al. 1988). Third, the pleiotropic phenotype of typical *P. syringae hrp* mutants suggests that the *hrp* genes are involved in the production of factors necessary for the bacterium to initiate a parasitic interaction with the plant (Huang et al. 1991). Fourth, *P. syringae hrp* genes are expressed only in nutrient poor conditions typical of plant intercellular fluids (Huynh et al. 1989; Rahme et al. 1992; Xiao et al. 1992; Yucel et al. 1989). Fifth, DNA sequence analyses have suggested possible functions for some of the *P. syringae hrp* products as positive regulators of gene expression (Grimm and Panopoulos 1989; Miller et al. 1993) or components of a protein secretion pathway (discussed further in Sect. 5.3). In summary, these observations suggest that following inoculation, necrogenic, gram-negative phytopathogens begin to secrete a protein that elicits the HR in nonhosts and is required for pathogenesis in hosts.

The first such protein was discovered by Wei et al. (1992) following the observation that HR elicitor activity was present in cell-free extracts of *E. coli* DH5α(pCPP430), which carries the highly expressed *hrp* cluster of *E. amylovora* Ea321. The elicitor protein was designated harpin (and will be referred to here as $harpin_{Ea}$). $Harpin_{Ea}$ is a glycine-rich, hydrophilic, heat-stable protein with an apparent molecular mass of 44 kDa. Purified $harpin_{Ea}$ can elicit an HR-like necrosis when infiltrated into the leaves of tobacco, tomato, *Arabidopsis thaliana*, and several other plants. Mutations in the $harpin_{Ea}$-encoding *hrpN* gene abolish the ability of *E. amylovora* to elicit the HR in tobacco or cause disease in highly susceptible immature pear fruit. Southern hybridization analysis suggests that homologs of the *hrpN* gene are present in other pathogenic *Erwinia* spp. but lacking in *Pseudomonas* and *Xanthomonas* spp. (Beer et al. 1993).

The *P. syringae* pv. *syringae* 61 harpin gene subsequently was identified when *E. coli* transformants expressing random subclones of the pHIR11 *hrp* genes were lysed in planta, revealing those expressing the *hrpZ* gene to elicit the HR (He et al. 1993a). *hrpZ* encodes a 34.7 kDa protein, $harpin_{Pss}$, which is dissimilar in overall amino acid sequence to $harpin_{Ea}$ but similar in several properties: it is

glycine-rich, cysteine-lacking, hydrophilic, heat stable, and elicits the HR in the same plants that are senstive to harpin$_{Ea}$. Harpin$_{Pss}$ is not produced in rich culture media, and deletion derivatives reveal that the COOH-terminal half of the protein is sufficient for elicitor activity. As will be discussed in Sect. 5.3, harpin$_{Pss}$ is secreted to the bacterial milieu.

The *E. chrysanthemi hrpN* gene was cloned by probing a genomic DNA library with the *E. amylovora hrpN* gene (BAUER et al. 1994b; WEI et al. 1992), and a homolog has been identified also in *E. carotovora* EC71 (A. K. Chatterjee, personal communication). Harpin$_{Ech}$ is a hydrophilic, glycine-rich protein with a molecular mass of 34.3 kDa. A comparison of the amino acid sequence of harpin$_{Ech}$ with that of harpin$_{Ea}$ reveals that the two proteins are highly similar in their COOH-terminal halves. Little sequence similarity is found with harpin$_{Pss}$. However, it is intriguing that all three of these harpins possess a stretch of 22 amino acids near the center of the protein in which 11 amino acids are conserved. This region is not necessary for elicitor activity, but may be involved in secretion.

The production of a harpin by *E. chrysanthemi* demands some comment, since the ability of soft rot erwinias to cause the HR has been considered uncertain because of the broad host range of these bacteria and their production of plant cell-killing pectic enzymes (KLEMENT 1982). *E. chrysanthemi* mutant CUCPB5006, which causes little pectolytic damage to tobacco leaves because of directed mutations in genes encoding the major pectate lyase isozymes, can be seen to elicit a typical HR (BAUER et al. 1994a). The ability of CUCPB5006 to elicit the HR is abolished by marker-exchanged transposon insertions in *hrpN* and restored by complementation with a *hrpN* subclone (BAUER et al. 1994b). Furthermore, marker exchange of the *hrpN* mutation into wild-type *E. chrysanthemi* EC16 results in a substantial reduction in virulence in the witloof chicory maceration assay developed by BEAULIEU and VAN GIJSEGEM (1992; BAUER et al. 1994b). Thus, harpin contributes to the virulence of *E. chrysanthemi*, but it is not required for pathogenicity.

The biochemical activity of harpins is puzzling. It seems unlikely (but not impossible) that harpin$_{Pss}$, for example, is an enzyme because of its heat stability and tolerance of a major deletion. In this regard, it is also noteworthy that harpin$_{Pss}$ shows no pectic enzyme activity, since such an activity could account for its cell killing (HE et al. 1993a). Indeed, pectic enzymes previously have been suggested to be elicitors of the HR, but these reports have not been supported by subsequent genetic analyses (ALLEN et al. 1991; AZAD and KADO 1984; GARDNER and KADO 1976; HUANG et al. 1989). Therefore, the plant apparently responds to elicitor information residing in the harpin structure itself rather than to damage or products resulting from enzymatic activity, as is the case with pectic enzymes.

Another noteworthy difference between harpins and pectic enzymes is that the necrosis resulting from harpin treatment is an active "suicide" response of plant cells. Thus, the HR elicited in tobacco by harpin$_{Pss}$ and harpin$_{Ea}$ is blocked by lanthanum chloride (a calcium channel blocker), sodium vanadate (an ATPase and

phosphatase inhibitor), α-amanitin (a transcription inhibitor), and cycloheximide (a translation inhibitor) (He et al. 1993a, 1994). The necrosis caused by pectic enzymes, by contrast, cannot be prevented by metabolic inhibitors such as lanthanum chloride (D. W. Bauer, unpublished results).

Interestingly, *hrp* mutants still trigger generalized defense responses (e.g., phenylpropanoid pathway gene expression) even though they fail to elicit the HR, indicating that these two defense-associated processes are not coupled (Jakobek and Lindgren 1993). Indeed, phenylpropanoid pathway gene expression is triggered at least weakly by saprophytic bacteria, although the physiological significance of this response is uncertain (Jakobek and Lindgren 1993; Meier and Slusarenko 1993). The availability of harpins should facilitate exploration of the plant signal transduction pathway controlling hypersensitivity and the relationship of this response to other plant defenses.

4.3 Harpins, Avr Proteins and the Puzzle of Host Range Determination

The involvement of harpins in determining the narrow host range of *P. syringae* strains or the broad host range of *E. chrysanthemi* strains is unclear. Limited data indicate that sensitivity to the harpins of *E. amylovora* and *P. syringae* varies among plants without any obvious correlation to host range (He et al. 1993a; Wei et al. 1992). However, such comparisons may not be meaningful because plants appear to be more sensitive to harpin delivered by living Hrp⁺ bacteria than to exogenously applied harpin. Experiments in which the coding sequences for the harpins of different pathovars are switched will definitively test whether information controlling host range in *P. syringae* resides in harpin structure.

If harpins are functionally equivalent among *P. syringae* pathovars, then compatibility may result from a carefully regulated deployment of harpin that avoids triggering the HR or from the production of suppressors of the HR and accompanying defense responses, as has been suggested for *P. syringae* pv. *phaseolicola* on bean (Gnanamanickan and Patil 1977; Jakobek et al. 1993). In *E. chrysanthemi*, rapidly produced pectic enzymes may kill plant cells before their defenses can be mobilized. Furthermore, the necrotrophic interaction of *E. chrysanthemi* with susceptible tissues during maceration argues against the development of a "compatibility" with the host that can be spoiled by the HR. This issue will be discussed in Sect. 6.

In contrast to harpins, there is another class of *P. syringae* proteins whose only known biological effect involves host range. These proteins are encoded by the *avr* genes, and they control the host range of races within certain pathovars for certain cultivars of the host species (see chapter by Dangl, this volume). Various aspects of *avr*-mediated plant-pathogen recognition have been reviewed recently (Dangl 1992; Gabriel and Rolfe 1990; Keen 1990; Long and Staskawicz 1993), and we will discuss here only a few main points that are pertinent to harpins. The *avr* genes are so named because their presence in a strain confers

avirulence (i.e., incompatibility) if the host cultivar carries a corresponding resistance gene. The first *avr* gene was cloned from *P. syringae* pv. *glycinea* on the basis of its ability to render a normally compatible race incompatible on differential cultivars of the host soybean (STASKAWICZ et al. 1984). Such gene-for-gene interactions also control race-cultivar specificity in many important fungal diseases (FLOR 1971). The simplest molecular explanation for this phenomenon is that the interaction of an *avr* "elicitor" protein with a corresponding resistance gene "receptor" protein triggers defense responses (KEEN 1990).

Incompatibility mediated by gene-for-gene interactions involving *P. syringae* strains is marked by the HR. However, typical *avr* proteins do not elicit the HR, they are hydrophilic proteins that appear not to be secreted (and therefore are unlikely to be accessible to postulated plant receptors); and their phenotype is revealed only in Hrp$^+$ pathogenic strains (KEEN 1990). A further puzzle is that the first plant resistance gene that confers *avr*-mediated resistance to be cloned, the tomato *Pto* gene, encodes a putative cytoplasmic serine/threonine kinase with no apparent transmembrane receptor domain (MARTIN et al. 1993).

One Avr protein, AvrD from *P. syringae* pv. *tomato*, directs the synthesis of homologous γ lactones when expressed in various bacteria, including *E. coli*; and syringolides 1 and 2 elicit necrosis in soybean cultivars carrying the *Rpg4* resistance locus (KEEN et al. 1990; MIDLAND et al. 1993). There is no evidence that any of the other *P. syringae* Avr proteins are enzymes, and one bacterial Avr protein, AvrBs3 from *X. campestris* pv. *vesicatoria* (and its homologs in other xanthomonads), appears very unlikely to function as an enzyme because its activity is specified by 17.5 nearly identical, direct repeats of a 34 amino acid sequence (BONAS et al. 1989; HERBERS et al. 1992).

The biochemical puzzles of harpin and Avr protein activity may be interrelated. A key to this interrelationship may reside in the dependency of Avr phenotypes on Hrp$^+$ backgrounds. Although *P. syringae avr* genes are dependent on *hrp* regulatory genes for expression (HUYNH et al. 1989; INNES et al. 1993; SHEN and KEEN 1993), there is likely an additional reason for this Hrp dependency. For example, *P. syringae* pv. *glycinea* race 0 *hrp* mutants carrying an *avrD*$^+$ plasmid fail to elicit an HR on soybean cultivars carrying *Rpg4*, even though they still produce AvrD-elicitor activity in culture (KEEN et al. 1990). Perhaps Avr action is dependent on an initial parasitic interaction that is harpin-dependent. Perhaps harpins and Avr proteins act synergistically to elicit the HR in resistant cultivars of the host. Or perhaps typical, bacterial Avr proteins are dependent on the Hrp secretion pathway, an explanation that is attractive but not supported by available experimental data (BROWN et al. 1993; FENSELAU et al. 1992).

5 Virulence Protein Secretion Pathways

5.1 Alternative Routes

To interact with their targets in the host, the extracellular virulence proteins of gram-negative bacteria must first be translocated across the inner and outer membranes that envelop the cell. Several conserved secretion pathways are used for this purpose. Some bacteria, such as laboratory strains of *E. coli*, possess none of these pathways, whereas others, like *E. chrysanthemi*, use at least three of them. These pathways are categorized in reference to the Sec pathway that is used for the translocation of periplasmic and outer membrane proteins across the inner membrane of all gram-negative bacteria. The hallmark of proteins travelling the Sec pathway is an NH_2-terminal signal peptide that is removed upon translocation to the periplasm (RANDALL and HARDY 1989).

The type I pathway (as classified by SALMOND and REEVES 1993) bypasses the Sec pathway. Proteins using this pathway lack an NH_2-terminal signal peptide and accumulate in the cytoplasm of secretion-deficient cells. *E. chrysanthemi* secretes multiple isozymes of protease by this pathway, which is comprised of the *prtD*, *prtE*, and *prtF* products (DELEPELAIRE and WANDERSMAN 1989, 1991; WANDERSMAN 1989). This pathway is used by several animal pathogens, including *Pseudomonas aeruginosa* and hemolytic strains of *E. coli*, to secrete alkaline protease and hemolysin, respectively (GUZZO et al. 1991; HOLLAND et al. 1990).

The type II pathway is an extension of the Sec pathway and is also known as the Sec-dependent pathway or the main terminal branch of the general secretion pathway (PUGSLEY 1993). Proteins travelling this pathway carry NH_2-terminal signal peptides. Following Sec-mediated translocation across the inner membrane and removal of the signal peptide, these proteins are translocated across the outer membrane by a set of accessory secretion proteins. The *E. chrysanthemi out* genes encode a type II pathway that is used for the secretion of most of the pectic enzymes and cellulase (discussed below). This pathway has been studied most extensively in *Klebsiella oxytoca*, which uses it to secrete pullulanase, and in *P. aeruginosa*, which uses it to secrete exotoxin A, elastase, and other proteins, as reviewed by PUGSLEY (1993) and LORY (1992). It appears to be the primary pathway by which gram-negative bacteria secrete degradative enzymes.

The type III pathway also bypasses the Sec pathway but is distinct from the type I pathway in that internal targeting sequences characteristic of type I exoproteins are absent (MICHIELS and CORNELIS 1991; MICHIELS et al. 1990), and the pathway is formed by a different and more elaborate set of envelope proteins (MICHIELS et al. 1991). The type III pathway is used by *Yersinia* spp., *Salmonella typhimurium*, and *Shigella flexneri* to secrete a variety of virulence proteins, and a related pathway appears to be used by plant pathogens to secrete harpins and possibly other proteins involved in pathogenesis (see reviews by VAN GIJSEGEM et al. 1993 and SALMOND and REEVES 1993).

All three of these pathways permit true secretion of proteins without cell lysis or release of periplasmic or cytoplasmic marker proteins. It is curious that *E. chrysanthemi* and *E. carotovora* may also release pectin lyase by a fourth, colicin-like route, involving partial cell lysis: the RecA-dependent induction of pectin lyase synthesis in *E. carotovora* is accompanied by cell lysis; the sequence of PnlA reveals no NH_2-terminal signal peptide; and the protein is produced by *Erwinia* without removal of any NH_2-terminal sequences (CHATTERJEE et al. 1991; ZINK et al. 1985). Although type I, II, or III pathway mutants have not been tested for any deficiency in pectin lyase secretion, it appears that this represents a fourth route to the cell exterior for soft rot *Erwinia* virulence proteins.

Protein secretion by gram-negative bacteria has been recently treated in reviews that are topical (SALMOND and REEVES 1993), comprehensive (PUGSLEY 1993), or focused on plant pathogens (HE et al. 1993b). The remainder of this section will address recent results with type II and III pathways that are particularly relevant to the secretion of pectic enzymes and harpins.

5.2 The Out Pathway and Pectic Enzymes

Out^- mutants of *E. chrysanthemi* and *E. carotovora* are readily isolated. The synthesis of pectate lyase and several other plant cell wall-degrading enzymes is unaffected in these mutants, but the enzymes accumulate in the periplasm and the mutants are virtually nonpathogenic (ANDRO et al. 1984; CHATTERJEE et al. 1985a; MURATA et al. 1990). Out^- mutants are the only mutants yet demonstrated to have this strong effect on virulence in both *E. chrysanthemi* and *E. carotovora*. The *out* genes of *E. chrysanthemi* and *E. carotovora* are clustered in the genome and are homologous with each other and with accessory secretion genes in a growing list of gram-negative bacteria (HE et al. 1991a; LINDEBERG and COLLMER 1992; MURATA et al. 1990; REEVES et al. 1993). The *out* genes of *E. chrysanthemi* EC16 are unique among all of these bacteria in that they can function in recombinant *E. coli* cells to permit the secretion of several *E. chrysanthemi* pectic enzymes (HE et al. 1991a).

The prototypical type II secretion pathway is used by *K. oxytoca* to secrete pullulanase (PUGSLEY 1993). The *pul* secretion gene cluster also functions in *E. coli*, but in this case to secrete a single protein, the *K. oxytoca* pullulanase (D'ENFERT et al. 1987). Fourteen proteins are required for this process (PUGSLEY 1993). These are the products of the *pulC–O* operon and the *pulS* gene. The *E. carotovora* *outC–O* operon is colinear with *pulC–O* (REEVES et al. 1993). In *E. chrysanthemi*, the *outC–M* genes are arranged in an operon that is colinear with *pulC–M*, but a *pulN* homolog is missing, and *outO* is transcribed independently (LINDEBERG and COLLMER 1992). As with *K. oxytoca*, *E. chrysanthemi* has an *outS* homolog upstream of *outC* (CONDEMINE et al. 1992). *outT* is an additional gene between *outS* and *outC* that is required for secretion (CONDEMINE et al. 1992).

The Out pathway translocates exoproteins from the periplasm to the external milieu. This process is rapid in *E. chrysanthemi*: pulse chase experiments indicate

that pectate lyase isozyme PelE is secreted to the milieu within 2 min of translocation across the inner membrane and periplasmic removal of the signal peptide (He et al. 1991b). A secA^{ts} *E. coli* mutant carrying the functional cluster of *E. chrysanthemi out* genes on pCPP2006 was used to show that PelE secretion is Sec-dependent (He et al. 1991b). Experiments with the pullulanase secretion system in *K. oxytoca* provide further evidence that exoprotein secretion by this pathway is a two-step process in which mature, folded proteins can be recruited from the periplasm for secretion (Pugsley 1992; Pugsley et al. 1991).

The location of many of the Pul secretion proteins has been determined, and surprisingly, most of them are associated with the inner membrane (Pugsley 1993). The biochemical function of only one of these secretion proteins is known. As first discovered with the homologous PilD gene in *P. aeruginosa* (Nunn and Lory 1991), PulO is a type IV prepilin signal peptidase (Pugsley and Dupuy 1992). A function for some of the other secretion proteins can be postulated. PulE is a cytoplasmic protein whose sequence suggests ATPase activity (Possot et al. 1992). PulG, PulH, PulI, and PulJ, which possess type IV prepilin signal peptides, may form a pilus-like assembly structure between the inner and outer membranes. PulD is the only integral outer membrane protein in the complex and shows similarity to outer membrane proteins involved in diverse macromolecular secretion processes in gram-negative bacteria (d'Enfert et al. 1989).

A paramount puzzle with the type II pathway is the basis for its selectivity: only a small subset of the cell's proteins are secreted. This implies the existence of secretion signals in exoproteins and a "gate-keeper" in the pathway that recognizes these signals. However, no secretion signals have been found by sequence comparisons, deletion experiments, or by fusions with periplasmic markers (Pugsley 1993). The available results suggest that a substantial portion of the NH_2-terminal of exoproteins is required and also sufficient for translocation of periplasmic marker proteins (Hamood et al. 1989; Kornacker and Pugsley 1990).

The *Erwinia* Out systems are experimentally attractive for exploring this aspect of secretion because the Out pathways are closely related but species-specific and the tertiary structure of the *E. chrysanthemi* PelC is known (Yoder et al. 1993). Thus, the *E. chrysanthemi* Out pathway does not secrete the cellulase or pectic enzymes of *E. carotovora* (He et al. 1991a; Py et al. 1991), despite the fact that some *E. chrysanthemi* and *E. carotovora* pectate lyase isozymes are quite similar (Hinton et al. 1989a). Deletion and insertion derivatives of the *E. chrysanthemi* cellulase isozyme EGZ (Py et al. 1993) and chimeras between the *E. chrysanthemi* PelC and the *E. carotovora* 71 Pel1 (whose sequence is 70% identical to PelC; A.K. Chatterjee, personal communication) fail to be secreted (M.L. Lindeberg, unpublished), which suggests that tertiary structure is vital for secretion. In summary, the type II pathway is essential for the virulence of pathogens like *P. aeruginosa* and *E. chrysanthemi*, and a better understanding of how this important pathway functions is likely to come from continued exploration of its operation in both animal and plant pathogens.

5.3 The Hrp Pathway and Harpins

The potential importance of protein secretion in the *hrp*-dependent phenotypes of plant pathogenic bacteria was first indicated by the discovery of $harpin_{Ea}$ and the observation that some *hrp* proteins possessed sequence similarities to components of the type III pathway in invasive enterobacterial pathogens (FENSELAU et al. 1992; GOUGH et al. 1992; HUANG et al. 1992; VAN GIJSEGEM et al. 1993; WEI et al. 1992). Because *hrp* homologies and implied functions are being treated in the accompanying chapter by Bonas, we will discuss here only the recent evidence for the role of *hrp* proteins in the secretion of harpins in *Erwinia* spp. and *P. syringae*.

As is characteristic of all proteins secreted by the type III pathway (SALMOND and REEVES 1993), $harpin_{Ea}$, $harpin_{Pss}$, and $harpin_{Ech}$ lack NH_2-terminal signal peptides (BAUER et al. 1994b; HE et al. 1993a; WEI et al. 1992). When *P. syringae* pv. *syringae* 61 is grown in a minimal medium in which the *hrp* genes are expressed, at least half of the $harpin_{Pss}$ is in the medium (HE et al. 1993a). $Harpin_{Pss}$ is the major protein in the medium under these conditions, and its secretion is dependent on HrpH (HE et al. 1993a), an envelope protein with similarity to *Yersinia enterocolitica* YscC and other outer membrane proteins (e.g., the *K. oxytoca* type II pathway component PulC discussed above) that are involved with protein secretion (HUANG et al. 1992). There is indirect evidence that *E. chrysanthemi* secretes harpin by this pathway. Southern hybridization has shown that *E. chrysanthemi* EC16 carries homologs of other *hrp* genes in addition to *hrpN* (LABY and BEER 1992), and transposon insertions in some of these genes indicate that they are involved in $harpin_{Ech}$ export (BAUER et al. 1994a). An important question is whether other proteins are travelling the Hrp pathway in the interactions of *P. syringae* and *E. chrysanthemi* with their hosts.

6 Extracellular Virulence Proteins of Plant Pathogenic Bacteria: New Perspectives and More Questions

Recent discoveries involving extracellular virulence proteins have altered our concepts of how the soft rot erwinias and *P. syringae* cause their signature plant effects, maceration and the HR. The production of autoinducer permits *E. carotovora* to behave socially in its pectolytic attack on plants, and the secretion of a harpin enables *P. syringae* to elicit the HR in tobacco (HE et al. 1993a; JONES et al. 1993; PIRHONEN et al. 1993). Each finding provides answers to some old questions but raises many new ones.

A social attack would explain why the soft rot erwinias do not (in contrast to *P. syringae*) cause numerous, scattered lesions on their hosts. Rather, soft rots typically develop from one or a few foci unless the plant is systemically infected.

It also provides an explanation for the original observations of Jones (1909) on the manner in which lesions in carrot infected with *E. carotovora* develop: "The cells rapidly lose all coherence and always show a sharply defined line of demarkation, indicating that the softening occurs quickly and completely after it begins." And, in turn, this would provide an explanation for how the bacteria can use successfully in pathogenesis a class of enzymes that can elicit plant defenses (as discussed in Sect. 3.3 and 3.5).

The discovery of autoinducer-dependent extracellular enzyme production in *E. carotovora* raises new questions about the operation of this system in pathogenesis. How are the various *aep*, *exp*, or *rex* genes integrated into the regulatory circuitry that controls the complex patterns of enzyme production? Is autoinducer-monitored cell density the limiting factor in high level expression of degradative enzymes by the soft rot erwinias in planta, or is autoinducer production prerequisite for bacterial responsiveness to other controlling stimuli (starvation, plant signals, etc.)? Finally, how do the soft rot erwinias attain the high cell densities in planta that are postulated to precede full induction of the pectic enzymes? We postulate that the answer to the last question resides in harpins, which act in prelude to pectolytic attack.

Our working model is that harpins are involved in establishing the initial parasitic interaction of many gram-negative plant pathogens by raising the pH of apoplastic fluids and fostering bacterial nutrient acquisition. A higher apoplastic pH would favor bacterial multiplication, and it would enhance the activity of the soft rot erwinia pectate lyase isozymes. This postulated role of harpins is supported by several observations: (1) $\text{Harpin}_{\text{Ea}}$ causes alkalinization of the medium of suspension-cultured tobacco cells (Wei et al. 1992). (2) As discussed in Sect. 4.1, apoplast alkalinization and the release of sucrose to the apoplast are correlated with bacterial Hrp activity. Furthermore, raising the pH of intercellular fluids in bean leaves can restore the ability of a *P. syringae* pv. *syringae* 61 *hrp* mutant to multiply (Atkinson and Baker 1987b), (3) *hrp* gene expression is nutritionally regulated (discussed in Sect. 4.2 and in the accompanying chapter by Bonas). (4), early experiments indicate that the growth of a saprophytic bacterium like *P. fluorescens* can be stimulated by coinoculation with *P. syringae*, suggesting that the pathogen alters the apoplastic environment to support bacterial growth (Young 1974). Finally, it is noteworthy that *E. chrysanthemi* produces a catechol siderophore to acquire iron (expected to be a limiting nutrient in the apoplast), and siderophore production is essential for the ability of *E. chrysanthemi* 3937 to systemically invade Saintpaulia plants (Enard et al. 1988; Neema et al. 1993; Persmark et al. 1989, 1992). Thus, there is a precedence in this organism for pathogenesis being dependent on nutrient acquisition.

Although the preceding observations argue for the importance of adaptations involving nutrient acquisition in bacterial plant pathogenesis, there are contrary perspectives. Evidence of nutrient availability in intercellular fluids and leachates and the potential relationship of this to pathogenesis has been presented in previous reviews (Hancock and Huisman 1981; Tukey 1970; Beattie and Lindow, this volume). Furthermore, intercellular fluids obtained from leaves by vacuum

infiltration and subsequent centrifugation have been observed to support the growth of various bacteria (e.g., KLEMENT 1965; NEEMA et al. 1993). However, a bacterium in contact with a thin film of cell wall-associated apoplastic fluid, whose composition is under dynamic control of the plant cell beneath it (GRIGNON and SENTENAC 1991), is likely to be in a rather different environment than a bacterium in a flask containing fluids recovered from many thousands of cells.

Finally, in considering the function of harpins in pathogenesis, it is important to note that the isolated harpins of *E. amylovora* and *P. syringae* have yet to be shown to have any effect on their respective hosts. Furthermore, *P. fluorescens*(pHIR11), although able to elicit the HR in tobacco, does not appear to multiply in planta (although early, transient multiplication may have been missed), and it does not become pathogenic (HUANG et al. 1988). Thus, the available data with *P. syringae* pv. *syringae* 61 indicate that $harpin_{Pss}$ is sufficient for elicitation of the HR, but apparently many factors are required for successful pathogenesis. A logical place to look for additional factors is in other exoproteins, particularly any trafficking the Hrp pathway.

7 Extracellular Virulence Proteins of Plant and Animal Pathogens: Apparent Similarities in Deployment but Potential Differences in Function

Animal pathogenic bacteria also have diverse attack strategies, extra-cellular protein arsenals, and patterns of virulence protein deployment; in some cases, these patterns can be aligned with those of counterpart plant pathogens. For example, the opportunistic animal pathogen *P. aeruginosa* is more similar to the plant pathogen *E. chrysanthemi* in its production of extracellular virulence proteins than it is to its fellow gram-negative animal pathogen *Y. pestis* (or its fellow fluorescent pseudomonad *P. syringae*).

A key component in the arsenals of *E. chrysanthemi* and *P. aeruginosa* are enzymes (pectate lyases and proteases) that are produced by many saprophytic bacteria but are adapted by these organisms for pathogenesis (JONES et al. 1993; PASSADOR et al. 1993 and references therein). Both bacteria are capable of two modes in their attack on eukaryotic hosts. The first is marked by latent or chronic infection, the second by large bacterial populations, massive production of extracellular degradative enzymes, and destruction of host tissues (IGLEWSKI 1989). The second mode typically develops in compromised hosts, and in *P. aeruginosa* and *E. carotovora* (and likely *E. chrysanthemi*) it may also be dependent on a pathogenic quorum of bacteria, as sensed by autoinducer levels (JONES et al. 1993; PASSADOR et al. 1993; PIRHONEN et al. 1993). Thus, as an activated mob, these bacteria may vandalize weakened host defenses.

In contrast, *P. syringae* and *Y. pestis* appear to attack their hosts by "stealth and interdiction" of defense communication lines (BLISKA et al. 1993). The

interdiction of defense signaling is the function of at least three of the *Y. pestis* Yop proteins (STRALEY et al. 1993). Although it appears that parasitic stealth is prerequisite for compatibility and successful pathogenesis by *P. syringae*, any interdiction of defense signaling (and the signaling itself) awaits elucidation. In contrast to the opportunistic pathogens above, *P. syringae* and *Y. pestis* can attack healthy hosts and there are no known quorum requirements for virulence protein deployment. Instead, they seem to sense the environment of target niches in the host, as indicated by factors like nitrogen starvation for *P. syringae* and low Ca^{2+} and high temperature (37°C) for *Y. pestis* (STRALEY et al. 1993). It is also intriguing that in both animal and plant pathogens, proteins associated with stealth and interdiction travel the type III pathway, whereas proteins associated with the mob attack travel the type II pathway.

Despite these similarities in life-style and virulence protein deployment, there may be fundamental differences in the primary functions of extracellular proteins in animal and plant pathogens because of the differing body plans and defense systems of their hosts. Pathogens finding suitable niches in animal hosts may have access to organic nutrients, but they are exposed to a variety of circulating defenses against foreign organisms (FALKOW et al. 1992). Consequently, a primary function of virulence proteins must be protection against host defenses. In contrast, plant pathogens in the intercellular spaces of their hosts find themselves in an environment that is weaker in defenses but possibly poorer in nutrients (as discussed in the previous section). Consequently, a primary need of plant pathogens is more likely to be release of nutrients to the apoplast rather than protection from active host defenses.

As observed in the classic experiments of Klement and coworkers (summarized in Table 2), nonpathogenic bacteria introduced into the intercellular spaces of plants are not rapidly destroyed; they just fail to multiply. In contrast, incompatible pathogens, which do multiply briefly, elicit the HR. Thus, strong defense responses like the HR are not triggered by the simple presence of a microorganism. Rather, it is molecules and activities unique to parasites that elicit effective defense. Whereas higher animals have the capacity to recognize virtually any bacterium as a foreign target for destruction, the active defenses of plants are informed more specifically by the pathogens themselves. Thus, a postulated role for harpins in the initial parasitic growth of necrogenic, gram-negative plant pathogens is consistent with their efficacy in eliciting the HR, and the involvement of *avr* genes in defense recognition suggests that their products also may serve a role (perhaps subtle) in parasitism. Similarly, the activity of pectic enzyme products in eliciting other defense responses is consistent with the expected role of the enzymes in bacterial nutrition.

KELMAN (1979) noted that "Plants have evolved in a virtual microbial jungle surrounded by free-living bacteria with the potential to embrace parasitism. It is remarkable, therefore, that so very few phytopathogenic bacterial species have evolved." We still do not know why saprophytes fail in plants; nor do we know the range of adaptations that enables pathogens to convert the apoplast into a suitable niche for colonization. Further understanding of virulence protein

trafficking should yield insights into the unique needs of bacteria that parasitize plants.

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hrp Genes of Phytopathogenic Bacteria

U. Bonas

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1 Introduction

In nature plants are resistant to the majority of pathogens, and many bacteria live in close contact with the plant without causing any harm (see chapter by Beattie and Lindow in this volume). Among the 1600 different species known in the bacterial kingdom only a small number (about 80) are plant pathogenic and in most cases highly specialized with respect to the plant that can be attacked. Only a few of these species are gram-positive, e.g., *Clavibacter* ssp. and *Streptomyces* ssp. In this review I focus on subspecies of the gram-negative genera *Erwinia, Pseudomonas,* and *Xanthomonas,* which comprise the major bacterial plant pathogens.

To be a successful pathogen the invading bacterium has to overcome the plant's defense. During evolution plant pathogenic bacteria have acquired multiple functions that enable them to colonize and multiply in living plant tissue. In nature, bacteria enter the plant through natural openings (stomata, hydathodes) or

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wounds. The bacterial armory contains a number of weapons that contribute to pathogenicity. Obvious examples include degradative extracellular enzymes such as pectinases, cellulases, and proteases. When the corresponding genes are mutated, bacterial ability to invade plant tissues is more or less affected depending on the pathogen, i.e., these functions contribute to and modulate development and severity of infection to different extents (see chapters by Dow and Daniels, and Collmer and Bauer in this volume).

In addition, phytopathogenic bacteria possess a large number of genes needed for basic pathogenicity. These genes have been operationally defined as *hrp* (hypersensitive reaction and pathogenicity; LINDGREN et al. 1986) based on their mutant phenotype. *hrp* genes are not only essential for pathogenicity on a plant, i.e., the ability to cause disease in a compatible interaction, but also for the incompatible interaction with resistant host varieties or with plants that are not normally a host for the particular pathogen (so called non-host). The incompatible interaction is often associated with the induction of a hypersensitive reaction (HR) in the plant. In contrast to the use of the term hypersensitivity in the animal field, in plants the HR is a rapid defense response involving localized plant cell death, production of phenolics and antimicrobial agents, e.g., phytoalexins, at the site of infection (KLÉMENT 1982; LINDSAY et al. 1993). The HR results in prevention of pathogen multiplication and spread and thus in prevention of disease development. Under natural infection conditions the HR is microscopically small and can be induced by just one bacterial cell. Only when bacteria are introduced into plant tissue at high cell densities in the laboratory (about 10^7 colony forming units or more/ml) is the HR macroscopically visible as confluent necrosis and can be clearly distinguished from typical disease symptoms. It is important to note that saprophytic or nonpathogenic bacteria such as *Escherichia coli* or *Pseudomonas fluorescens* do not induce the HR and are unable to multiply in plant tissue.

2 Isolation of *hrp* Genes and General Features

hrp genes have been isolated from all major gram-negative plant pathogenic bacteria except Agrobacterium. There are excellent reviews that describe the early work or focus more on one particular pathogen (WILLIS et al. 1991; BOUCHER et al. 1992). The majority of *hrp* genes have been identified by complementation of loss-of-function mutants. Mutants obtained by random chemical (e.g., N-methyl-*N'*-nitro-*N*-nitrosoguanidine) or transposon mutagenesis of a pathogenic wild-type strain were inoculated into the host plant and screened for loss of both the ability to cause disease in susceptible plants and to induce the HR in resistant host or non-host plants (often tobacco). The second criterion for the isolation of genes specific for the plant interaction was to ensure that the mutants would still grow in minimal medium. This way mutants affected in genes for basic housekeeping functions were eliminated. A third characteristic of all *hrp* mutants is that they are unable to grow in the plant.

The *hrp* genes were originally described for the bean pathogen *Pseudomonas syringae* pv. *phaseolicola.* LINDGREN and coworkers (1986) isolated Tn*5*-induced mutants of *P.s.* pv. *phaseolicola* that had lost both the ability to induce halo-blight disease on bean and the HR in tobacco. Complementation with cosmid clones from a genomic library of the wild-type strain resulted in isolation of a cluster of *hrp* genes localized in a 20 kb DNA region. This was the first indication that both the ability to cause disease and to induce the HR are mediated by common steps in a "pathway".

Since then *hrp* gene clusters have been cloned from a number of different bacteria. Examples include *Pseudomonas solanacearum* (BOUCHER et al. 1987; Fig. 1B), the *Xanthomonas campestris* pathovars *campestris* and *vitians* (ARLAT et al. 1991), *translucens* (WANEY et al. 1991), and *vesicatoria* (BONAS et al. 1991; Fig.1A), *Erwinia amylovora* (STEINBERGER and BEER 1988; BARNY et al. 1990; WALTERS et al. 1990; BAUER and BEER 1991), and several other pathovars of *P. syringae* (e.g., HUANG et al. 1988; LINDGREN et al. 1988; Fig. 1C). In addition, genes with DNA homology, and in some cases functional homology, have been isolated from other species, e.g., the so-called *wts* genes from *E. stewartii* (COPLIN et al. 1992; LABY and BEER 1992), and a region containing pathogenicity genes from *X.c.* pv. *glycines* that complement *hrp* mutants of *X.c.* pv. *vesicatoria* (HWANG et al. 1992; Bonas, unpublished results). Interestingly, nonpathogenic xanthomonads that were originally isolated from diseased plants as opportunists together with pathogenic bacteria do not contain *hrp*-related DNA sequences (STALL and MINSAVAGE 1990; BONAS et al. 1991). In *Agrobacterium tumefaciens* or in strains of *Rhizobium* ssp. there seem to be no *hrp* gene equivalents present (BONAS et al. 1991; LABY and BEER 1992). This conclusion is based on DNA hybridization experiments and, of course, does not exclude the presence of genes with functional homology to *hrp* genes in these species.

In all of the cases mentioned above, the *hrp* genes are organized in clusters of 22–40 kb, and I will restrict most of this chapter to these large *hrp* clusters. In addition, several smaller *hrp* loci have been described that are not linked to the large cluster present in the same bacterium. These include a region in *P. solanacearum* (HUANG et al. 1990), the *hrpX* locus that is conserved in *X. campestris* pathovars *campestris* (KAMOUN and KADO 1990; KAMOUN et al. 1992) and *oryzae* (KAMDAR et al. 1993), and the *hrpM* locus in *P.s.* pv. *syringae* (NIEPOLD et al. 1985; MUKHOPADHYAY et al. 1988). *hrpM* is functionally conserved in pathovar *phaseolicola* (FELLAY et al. 1991). Besides being nonpathogenic and unable to induce the HR in tobacco, *P. syringae hrpM* mutuants are also affected in mucus production. The *hrpM* locus encodes two putative proteins which are similar and have been shown to be functionally homologous to the products of the *E. coli mdoGH* operon (LOUBENS et al. 1993). The *mdoGH* genes are required for periplasmic membrane-derived oligosaccharide synthesis in *E. coli.* The *hrpQ* and *hrpT* genes from *P.s.* pv. *phaseolicola* (MILLER et al. 1993) will be discussed later in this chapter.

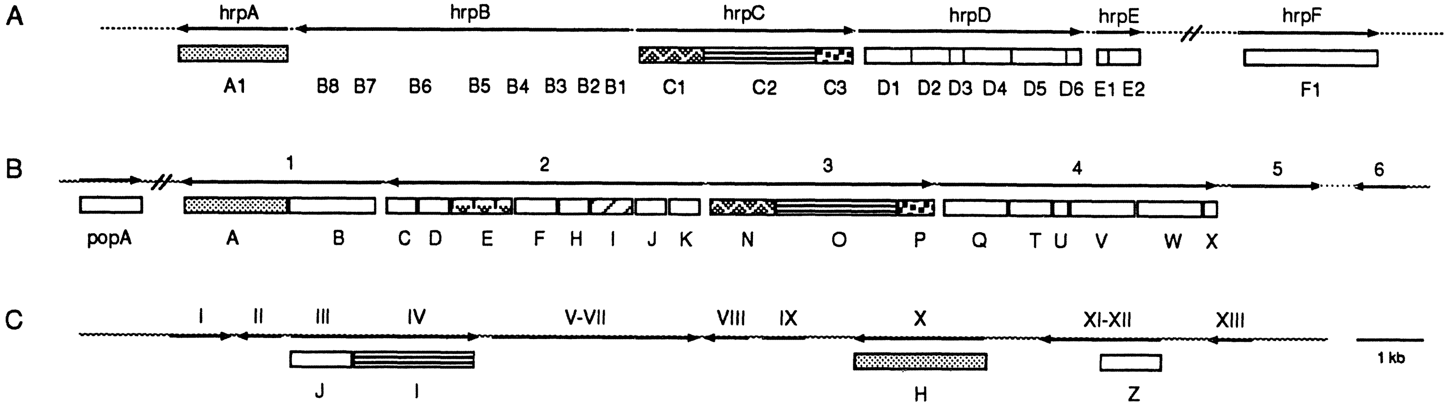

Fig. 1A–C. Genetic and translational organization of the *hrp* gene cluster of different plant pathogenic bacteria. **A** *Xanthomonas campestris* pv. *vesicatoria*; **B** *Pseudomonas solanacearum*; and **C** *Pseudomonas syringae* pv. *syringae*. Arrows represent transcription units as determined by genetic analyses. Boxes correspond to sequences of open reading frames (ORFs) that have been published. In case of sequence similarities between ORFs in different clusters the boxes are filled with the same pattern. For references, see text

3 Structural Organization and Relatedness of *hrp* Clusters

Genetic studies using transposon-induced insertion mutants in the respective bacterial wild-type strains revealed that the *hrp* clusters contain at least six to eight complementation groups (Fig. 1). Some *hrp* gene clusters have clearly been shown to be localized in the chromosome, e.g., in *P.s.* pv. *phaseolicola* (Rahme et al. 1991) and in *X.c.* pv. *vesicatoria* (Bonas et al. 1991), whereas in *P. solanacearum*, the *hrp* cluster is on a megaplasmid (Boucher et al. 1987).

Striking similarities have recently been found between the *hrp* genes of pathogens belonging to different genera. The first indication of homologies came from Southern hybridization studies. DNA homology was observed among different strains of the same pathovar, as well as between pathovars or strains within a species, and in some cases also between species. However, the degree of conservation varies. DNA homology is high within pathovars of a given subspecies, e.g., in *P. syringae* (Lindgren et al. 1988; Huang et al. 1991) and in *X. campestris* (Bonas et al. 1991). The latter studies were recently extended by PCR using primers based on *hrp* sequences from *X.c.* pv. *vesicatoria* (Leite et al. 1994). Furthermore, at least some regions of the *hrp* clusters appear to be conserved on the DNA level between *P. solanacearum* and pathovars of *X. campestris, P. syringae,* and also to *E. amylovora* (Boucher et al. 1987; Arlat et al. 1991; Gough et al. 1992; Laby and Beer 1992). In addition, cross-complementation within a subspecies indicated a high degree of functional conservation of *hrp* genes (e.g., Lindgren et al. 1988; Arlat et al. 1991; Bonas et al. 1991; Laby and Beer 1992). Due to sequence data it is now becoming more and more apparent that several *hrp* genes are conserved in all major gram-negative plant pathogenic bacteria (see below). Whether there are *hrp* genes that are clearly pathovar-specific can only be answered when complete sequence information becomes available for several *hrp* clusters.

4 Function of *hrp* Genes in *Xanthomonas campestris* pv. *vesicatoria* and Other Plant Pathogenic Bacteria

DNA sequence analysis of the *hrp* genes has revealed some important clues to their possible biochemical functions. One of the first genes sequenced was a regulatory gene, *hrpS*, from *P.s.* pv. *phaseolicola* (Grimm and Panopoulos 1989). This gene as well as *hrpB*, a regulatory gene from *P. solanacearum* (Genin et al. 1992), will be discussed below in the context of gene regulation.

Since *hrp* genes are environmentally regulated (see below), it was believed for a while that they would be encoding "alternative" proteins required for adaptation of the bacterium to the plant as the preferred environment. The recently discovered sequence similarities between several putative Hrp proteins and known proteins from other bacteria, however, led to a very different hypothesis, namely, involvement of Hrp proteins in protein secretion. We have

sequenced the entire *hrp* cluster of *X.c.* pv. *vesicatoria.* Since most *hrp* sequences from this and other bacteria are not yet published, I will summarize our results and refer to the other phytopathogenic bacteria as I go along. Based on genetic analyses and the open reading frames (ORFs) with a high coding probability we predict 21 *hrp* genes in the 25 kb *hrp* cluster of *X.c.* pv. *vesicatoria.* Their transcriptional organization is depicted in Fig.1A. The loci *hrpA* and *hrpB* are transcribed from right to left; the other four loci are transcribed from left to right (SCHULTE and BONAS 1992a). According to the locus (*hrpA–hrpF*) we have numbered the ORFs consecutively. The *hrpA* locus appears to contain just one *hrp* gene, *hrpA1*. The *hrpB* operon contains eight ORFs, called *hrpB1–hrpB8*, etc. A region of about 4 kb between *hrpE* and *hrpF* does not seem to be involved in the interaction with the plant because insertions in this region do not lead to a change in phenotype (BONAS et al. 1991).

What are the characteristics of the Hrp proteins? It should be noted that, except for three proteins, expression of the other 18 has yet to be demonstrated in *X.c.* pv. *vesicatoria.* A number of putative Hrp proteins are most likely associated with or localized in the bacterial membrane. For example, the HrpC2 protein sequence contains eight transmembrane domains but lacks a signal sequence, suggesting an inner membrane localization (FENSELAU et al. 1992). Both HrpA1 and HrpB3 contain an NH_2-terminal signal sequence and one (HrpA1) or two (HrpB3) transmembrane domains, suggesting that a part of these proteins might be targeted to the outer membrane. The signal sequence of HrpB3 resembles signal peptidase II sequences which are typical of lipoproteins (FENSELAU et al. 1992). Experiments using radioactively labeled palmitate are underway to test whether HrpB3 is a lipoprotein. Recently, both HrpB3 and HrpA1 were shown to be localized in the *X.c.* pv. *vesicatoria* membrane fraction using polyclonal antibodies (S. Fenselau, C. Marie, and U. Bonas, manuscript in preparation). The HrpB6 protein is a putative ATPase with highly conserved nucleotide and magnesium binding domains. It is more similar to protein traffic ATPases than to proton pump ATPases, and the lack of membrane spanning domains suggests a cytoplasmic location (FENSELAU et al. 1992).

Searches of the database revealed sequence relatedness of more than half of the *X.c.* pv. *vesicatoria* Hrp proteins with putative proteins in other bacteria, including different plant pathogens. High DNA sequence identity (more than 90%) was found to a 2.7 kb fragment carrying pathogenicity genes from *X.c.* pv. *glycines* (HWANG et al. 1992). The authors predicted two ORFs, whereas in *X.c.* pv. *vesicatoria*, this region contains three ORFs corresponding to the *hrpC3, hrpD1* and *hrpD2* genes. Complementation studies indicated that part of the *hrp* region is colinear in the two pathovars of *Xanthomonas* (unpublished).

The deduced amino acid sequences of *hrp* genes published from *P. solanacearum* (GOUGH et al. 1992, 1993; GENIN et al. 1992) show significant similarity to *X.c.* pv. *vesicatoria* proteins (Table 1; Fig.1). One exception is the *hrpB* regulatory gene from *P. solanacearum* which is not present in the 25 kb *hrp* region or in the flanking region of the *X.c.* pv. *vesicatoria hrp* cluster as determined by DNA sequence analysis and hybridization studies (T. Horns and U. Bonas,

unpublished). Furthermore, several of the proteins mentioned are conserved in other species (Fig.1), however, the degree of sequence similarity varies greatly (Table 1). The HrpA1 protein from *X.c.* pv. *vesicatoria* is 48% and 29% identical to proteins from *P. solanacearum* (HrpA; GOUGH et al. 1992) and *P.s.* pv. *syringae* (HrpH; HUANG et al. 1992), respectively. HrpC2 from *X.c.* pv. *vesicatoria* is even more conserved, being 66% idential to the corresponding HrpO protein of *P. solanacearum* (GOUGH et al. 1993), whereas the *hrpI* genes from *E. amylovora* (WEI and BEER 1994) and from *P.s.* pv. *syringae* (HUANG et al. 1993) both show 62% similarity to *hrpC2* from *X.c.* pv. *vesicatoria. P.s.* pv. *syringae* also contains a *hrpB3* related gene, called *hrpY,* and a *hrpD2* related gene, *hrpW* (H.-C. Huang, personal communication). Thus, the high degree of DNA sequence conservation that was reported earlier (see above) is also seen on the protein level. It appears that *hrp* genes in *X.c.* pv. *vesicatoria* are more closely related to *P. solanacearum* than to *P. syringae* and to *Erwinia.* As more and more homologous *hrp* genes are found in other bacteria nomenclature might become confusing. However, as long as the genes have not been shown to be functionally homologous, we will continue to use these names.

Besides genes that are conserved among the major phytopathogenic bacteria some genes are absent in the *hrp* region of more distantly related species. For example, there are no known homologs of the harpin genes *hrpN* (WEI et al. 1992a), and *hrpZ* (HE et al. 1993) (see below), and of *hrpJ* from *P.s.* pv. *syringae* (HUANG et al. 1993) in the *X.c.* pv. *vesicatoria hrp* cluster (unpublished; see Fig. 1).

Similarities of 50%–60% were found recently between HrpA1 and HrpB3 from *X.c.* pv. *vesicatoria* and two putative Nol proteins of *Rhizobium fredii* that are encoded by a cultivar specificity region. NolT and NolW mutants have a wider host range in nodulation of soybean (MEINHARDT et al. 1993). In addition, the authors mention that release of proteins is affected.

Last but not least, Table 1 summarizes the significant sequence similarities which have been found to proteins from animal bacterial pathogens. A number of putative Hrp proteins are related to proteins in animal pathogens such as *Salmonella, Shigella,* and *Yersinia* ssp. Since the first similarities found were to the Ysc, Vir, and Lcr proteins from *Yersinia* ssp, this group of organisms became a "role model" for plant pathologists (FENSELAU et al. 1992; GOUGH et al. 1992; HUANG et al.1992). In *Yersinia,* these proteins are essential for the secretion of virulence factors, called Yops (Yersinia outer protein; MICHIELS et al. 1990, 1991). Since they are described in detail in the chapter by G.R. Cornelis, I will mention only a few important features. The Yops are hydrophilic proteins that lack a typical NH_2-terminal signal peptide, and are secreted by using an entirely different pathway from that previously described for protein secretion. The genes involved in secretion are clustered on a 70 kb virulence plasmid. In case of a mutation, e.g., in *Yscj,* the Yops accumulate in the cytoplasm (MICHIELS et al. 1991). Although their direct role in transport has yet to be demonstrated, it is believed that the Ysc and Lcr proteins mentioned in Table 1 are parts of a special transport apparatus for Yop secretion. Similarly, *Shigella flexneri* secretes virulence factors, called Ipa (invasion plasmid antigens), that are distinct from Yops but share the general

Table 1. Sequence similarities of *Xanthomonas campestris* pv. *vesicatoria* Hrp proteins

Xanthomonas campestris pv. *vesicatoria*	HrpA1[1]	HrpB6[1]	HrpB3[1]	HrpC1[2]	HrpC2[1]	HrpC3[2]	HrpD1[2]	HrpD2[2]
Pseudomonas solanacearum	HrpA[3] (66%)	HrpE[4]	HrpI[3] (70%)	HrpN[5] (74%)	HrpO[3] (81%)	HpaP[5] (54%)	HrpQ[4]	HrpT[4]
Pseudomonas syringae pv. *syringae*	HrpH[6] (52%)				HrpI[7] (62%)			
Yersinia enterocolitica	YscC[8] (55%)		YscJ[8] (56%)					
Yersinia pestis	YscC[9] (55%)				LcrD[10] (70%)		LsaA[11] (52%)	LsaB[11] (72%)
Yersinia pseudotuberculosis		YscN[12] (73%)	LcrKa[13] (56%)					
Shigella flexneri	MxiD[14] (50%)	Spa47[15] (65%)	MxiJ[16] (52%)	Spa40[17] (55%)	MxiA[18] (65%)			Spa24[15] (67%)
Salmonella typhimurium	InvG[19] (52%)	SpaL[20] (70%) FliI[21] (65%)		SpaS[20] (56%)	InvA[22] (67%)			SpaP[20] (64%)
Bacillus subtilis		FlaA-ORF4[23] (68%)		FlhB[24] (62%)	FlhA[25] (63%)			FliP[26] (68%)
Escherichia coli		β-F1[27] (53%)						FliP[28] (65%)
Erwinia carotovora							MopB[29] (49%)	MopC[29] (65%)
Erwinia amylovora					HrpI[30] (62%)			
Rhizobium fredii	NolW[31] (51%)		NolT[31] (61%)					
Caulobacter crescentus					FlbF[32] (55%)			

Similarities between deduced amino acid sequences of Hrp proteins from *X.c.*pv.*vesicatoria* and other proteins include conservative amino acid exchanges. Number in parentheses indicates percent similarity.
Superscript numbers indicate references as follows:
1, FENSELAU et al. 1992; 2,Bonas et al., unpublished; 3, GOUGH et al. 1992; 4, GENIN et al. 1993, sequences unpublished; 5, GOUGH et al. 1993; 6, HUANG et al. 1992; 7, HUANG et al. 1993; 8, MICHIELS et al. 1991; 9, HADDIX and STRALEY 1992; 10, PLANO et al. 1991; 11, Fields et al. unpublished, accession # L22495; 12, Galyov, unpublished, accession # U00998; 13, RIMPILAINEN et al. 1992; 14, ALLAOUI et al. 1993; 15, VENKATESAN et al. 1992; 16, ALLAOUI et al. 1992; 17, SASAKAWA et al. 1993; 18, ANDREWS and MAURELLI 1992; 19, Lodge et al., unpublished, accession # X75302; 20, GROISMAN and OCHMAN 1993; 21, VOGLER et al. 1991; 22, GALÁN et al. 1992; 23, ALBERTINI et al. 1991; 24, Carpenter et al., unpublished, accession # X741212; 25, CARPENTER and ORDAL 1993; 26, BISCHOFF et al. 1992; 27, SARASTE et al. 1981; 28, MALAKOOTI et al. unpublished, accession # L21994; 29, MULHOLLAND et al. 1993; 30, WEI and BEER 1993; 31, MEINHARDT et al. 1993; 32, RAMAKRISHANAN et al. 1991; SANDERS et al. 1992.

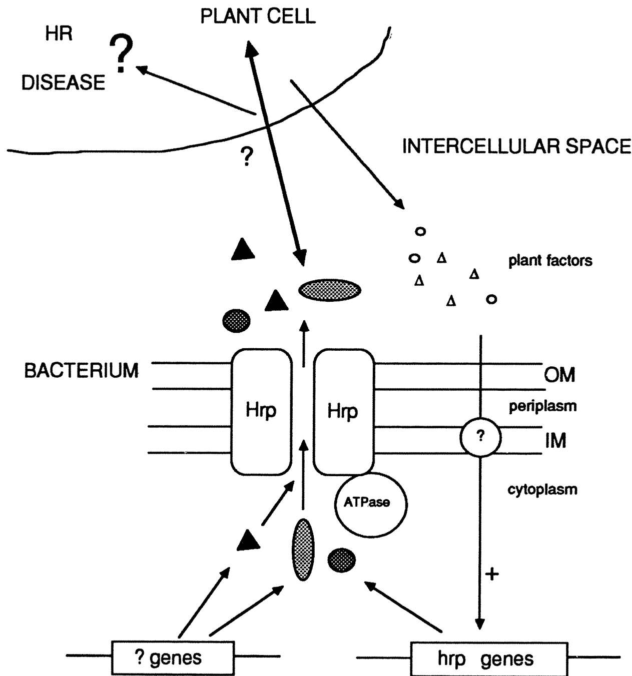

Fig. 2. Hypothetical model of cell signaling between gram-negative bacteria and plants indicating the proposed function of Hrp proteins as an apparatus for protein secretion. The model has been modified after FENSELAU et al. (1992). Hrp proteins may form a tunnel that enables the export of molecules such as virulence factors or avirluence factors leading to either a hypersensitive response (HR) or disease. These factors could be encoded by *hrp* genes or genes unlinked to the large cluster. Both types of genes have been found to encode elicitors of the HR (see text). The secretion of virulence proteins is hypothetical

features mentioned above (HALE 1991; and see chapter by PARSOT, this volume). Although *S. typhimurium* appears to possess a secretion system similar to that in *Shigella*, secreted invasion antigens have not yet been identified (GROISMAN and OCHMAN 1993; see chapter by FINLAY). As unpublished reports indicate that more and more genes in the animal pathogens are conserved, the data shown in Table 1 will soon be out of date. Proteins from other bacteria, e.g., *E. coli, Bacillus, Caulobacter* and from the *mop* region in *E. carotovora* (MULHOLLAND et al. 1993), have also been found to be similar to Hrp proteins (Table 1). Most of these are important for the assembly of the flagella, motility, or chemotaxis, again pointing, in my opinion, to a specialized secretion system rather than an involvement of *hrp* genes in chemotaxis.

These observations led us and others to propose a *hrp*-dependent secretion system in plant pathogenic bacteria (FENSELAU et al. 1992; GOUGH et al. 1992; VAN GIJSEGEM et al. 1993). A model is shown in Fig. 2 and raises certain questions, e.g., if secretion occurs, what is being secreted by plant pathogenic bacteria? So far, a few proteins have been identified as elicitors of the HR but there is no evidence for secretion of virulence factors (see below).

5 *hrp*-dependent Secretion of Hypersensitive Response-Inducing Proteins

5.1 Harpin from *Erwinia amylovora*

An important feature of the isolated *hrp* clusters from both *E. amylovora* and *P.s.* pv. *syringae* is the ability of *E. coli* or *Pseudomonas fluorescens* transformants containing the cloned genes to induce the HR on tobacco (HUANG et al. 1988; BEER et al. 1991; see below). This has prompted to search for the HR-inducing activity within the respective gene clusters.

The first bacterial HR-inducing protein identified, designated harpin, is a cell envelope-associated protein encoded by the *hrpN* gene of *E. amylovora*, a pathogen of pear and apple (WEI et al. 1992a). This $harpin_{Ea}$ is a glycine-rich and heat-stable protein that induces the HR in the non-host, tobacco. The *hrpN* gene is localized within the respective *hrp* cluster and thus has a dual role in also being required for pathogenicity on the normal host plant. Its function in pathogenicity, however, is unknown. BEER et al. (1993) mentioned in a preliminary report that the *hrpN* gene seems to be conserved among *Erwinia* ssp. but that there is no DNA homology between *hrpN* and sequences in the other plant pathogenic bacteria. Although data described below suggest that the $harpin_{Ea}$ protein might be secreted via the Hrp secretory apparatus, there is no published information available that demonstrates this.

5.2 Harpin from *Pseudomonas syringae* pv. *syringae*

Using an elegant approach He and coworkers recently have identified $harpin_{Pss}$, which is encoded by the *hrpZ* gene in the bean pathogen *P.s.* pv. *syringae* (HE et al. 1993; see Fig. 1C and chapter by Collmer and Bauer). Lysates of *E. coli* clones containing an expression library, made using the cloned *P.s.* pv. *syringae hrp* cluster, were directly screened for HR-inducing activity on tobacco leaves. Two proteins were identified, one of which was an NH_2-terminal deletion of $harpin_{Pss}$ with even higher activity than the full size protein. Whether or not processing occurs in natural infection is not clear. Interestingly, two short direct repeats in the COOH-terminus of $harpin_{Pss}$ are essential for elicitor activity. Although the two harpins $harpin_{Ea}$ and $harpin_{Pss}$ differ in their primary sequence, they have several features in common, e.g., a stretch of 22 amino acid that is similar in both proteins (HE et al. 1993). $Harpin_{Pss}$ is also glycine-rich and heat-stable. As with $harpin_{Ea}$ of *E. amylovora,* the function of $harpin_{Pss}$ in pathogenicity is unknown. Its product is secreted by *P.s.* pv. *syringae* in a HrpH-dependent way; HrpH is highly related to proteins involved in secretion in other plant and animal pathogens (HUANG et al. 1992; see Table 1).

5.3 *PopA* from *Pseudomonas solanacearum*

An HR-inducing protein has been identified and characterized from *P. solanacearum* culture supernatants, called Pop (Pseudomonas out protein; ARLAT et al. 1994). PopA1 and two shorter derivatives, PopA2 and PopA3, induce the HR in tobacco and in certain, but not all, *Petunia* lines. Like the harpins, the Pop proteins are also heat-stable and glycine-rich, however, the sequence is entirely different. In contrast to the harpins, the *popA* gene is not a *hrp* gene but is located outside of the large *hrp* cluster. Interestingly, expression of *popA* is *hrpB*-dependent, i.e., the gene is part of the *hrp* regulon. Mutations in *popA* do not affect the HR on tobacco or pathogenicity on tomato suggesting that more than one HR-inducing factor is produced. ARLAT et al. (1994) convincingly showed that secretion of PopA is dependent on other *hrp* genes, such as *hrpA, hrpN,* and *hrpO* (Fig. 1B). If a bacterial strain virulent towards *Petunia* is found it will be interesting to see if PopA acts as an avirulence protein in *Petunia* as has been suggested by the authors.

These exciting findings prove that certain Hrp proteins of *P.s.* pv. *syringae* and *P. solanacearum* play a role in transport of HR elicitors (Fig. 2). They also stimulate more questions. It needs to be shown that harpins and PopA are in fact secreted when the bacteria interact with the plant (the *hrp* genes were induced in vitro). Are harpins conserved among pathovars of *P. syringae*? How many elicitors of the non-host HR in tobacco can be found? Is the mechanism of recognition in tobacco identical with the *Erwinia* and *P.s.* pv. *syringae* harpins and the *P. solanacearum* Pops?

6 Regulation of Expression of *hrp* Genes

Expression of *hrp* genes is controlled by environmental conditions and has been studied on the RNA level as well as using transcriptional fusions to reporter genes such as the *E. coli* genes encoding β-galactosidase or β-glucuronidase. In general, expression of *hrp* loci is not detectable when the bacteria are grown in complex culture media. However, after growth of the bacteria in the plant, *hrp* genes are expressed. Attempts to mimic the conditions that the different bacterial species encounter in the plant tissue resulted in the finding that growth in minimal media without any plant-derived factor was sufficient to induce *hrp* genes. This has led to the speculation that the bacteria have to experience some kind of starvation conditions for full expression of *hrp* genes. One of the first indications for *hrp* gene expression in vitro, and clearly a breakthrough, was a report on the *hrp*-dependent expression of an avirulence gene from the soybean pathogen *P.s.* pv. *glycinea* (HUYNH et al. 1989).

Since the composition of minimal media differs depending on the bacterium studied, the most important findings will be summarized for representative pathogens. Parameters like carbon source, concentration of organic nitrogen and phosphate, osmolarity, and pH have been found to be important. High con-

centration of organic nitrogen generally appears to suppress *hrp* gene activation. Only two regulatory genes have been studied so far (see below). Interestingly, they both belong to different families of regulatory proteins.

6.1 *Pseudomonas syringae*

Expression of all seven *hrp* loci in the large cluster of *P.s.* pv. *phaseolicola* is suppressed in complex medium but induced in the plant. Induction occurs in the susceptible host plant as well as in the non-host, tobacco, suggesting that there is no plant species-specific molecule involved in control of host range (RAHME et al. 1992). Five complementation groups, *hrpAB, hrpC, hrpD,* hrpE and *hrpF*, can also be induced in M9 minimal medium containing sucrose as a carbon source, however, induction is affected by pH, osmolarity, and carbon source, and never reaches the levels obtained in the plant (RAHME et al. 1992). A similar observation was made earlier for the avirulence gene *avrB* in *P.s.* pv. *glycinea.* Induction occurred in a minimal medium containing fructose, mannitol, or sucrose. Expression of *avrB* is dependent on *hrp* genes homologous to *hrpRS* and *hrpL* from *P.s.* pv. *phaseolicola* and was suppressed by TCA cycle intermediates such as citrate and succinate (HUYNH et al. 1989). *hrp* gene expression in *P.s.* pv. *syringae* occurs in the same medium as described by HUYNH et al. (1989); (HUANG et al. 1991; XIAO et al. 1992). The authors showed *hrp* gene induction in the non-host plant, tobacco, but no data for the host plant. The *P.s.* pv. *phaseolicola* loci *hrpL* and *hrpRS* are only expressed to a very low level in M9 minimal medium and are induced at least 1000-fold when the bacteria are inoculated into the plant. This led to the conclusion that, at least for expression of *hrpL* and *hrpRS*, specific plant factors might be necessary (RAHME et al. 1992).

6.2 Regulatory Genes *hrpRS* and *rpoN* of *Pseudomonas syringae* pv. *phaseolicola*

The results on environmental factors inducing or suppressing *hrp* gene expression suggested that specific regulatory genes are involved in the control of *hrp* promoter activities. At least two loci are involved in positive regulation of the other *hrp* loci of *P.s.* pv. *phaseolicola hrp* cluster (FELLAY et al. 1991). While there is no information published for *hrpL, hrpRS* has been sequenced. It contains two genes whose predicted protein products are 60% identical to each other (GRIMM and PANOPOULOS 1989; MILLER et al. 1993). The HrpS protein is similar to members of the NtrC family of regulatory proteins in gram-negative bacteria. Most NtrC-like regulatory proteins are members of two-component systems, with a sensor protein that in turn activates a response element by phosphorylation of a site in the conserved NH_2-terminal domain (ALBRIGHT et al. 1989). The putative sensor component operating in *hrp* gene regulation has not been identified. It is postulated that HrpS is the activating protein, however, direct biochemical data

have not been presented. The lack of a typical NH_2-terminal domain in HrpS could indicate that a different mechanism may be involved in HrpS activation. Apparently, *hrpS*-related sequences are also present in other bacteria, e.g., in *P.s.* pv. *syringae* (HEU and HUTCHESON 1993) and in *Erwinia amylovora* (BEER et al. 1993). *E. stewartii* contains a transcriptional regulator, WtsA, with 52% identity to HrpS of *P.s.* pv. *phaseolicola*. The *hrpS* clone, however, was unable to functionally complement a *wtsA* mutant (FREDERICK et al. 1993).

The structure of the *hrpRS* locus and the finding of –24/–12 consensus sequences upstream of *hrpRS* indicated a possible role in transcriptional activation for transcription factor sigma 54, encoded by *rpoN* (GRIMM and PANOPOULOS 1989). In a preliminary report, FELLEY et al. (1991) demonstrated that *hrp* gene expression in *P.s.* pv. *phaseolicola* is indeed dependent on *rpoN*. A *rpoN* mutant of *P.s.* pv. *phaseolicola* is a glutamine auxotroph and nonpathogenic. Whether *rpoN* is generally involved in regulation of *hrp* gene expression is not clear. In *X.c.* pv. *vesicatoria*, *rpoN* is clearly not involved in *hrp* gene expression and pathogenicity (T. Horns and U. Bonas, manuscript in preparation).

Recently, MILLER et al. (1993) have reported the identification of two new loci, *hrpQ* and *hrpT*, from *P. s.* pv. *phaseolicola* that affect activation of *hrpRS* in *trans*. However, since *hrpRS* is strongly induced in plants while both *hrpQ* and *hrpT* are constitutively expressed, there must be more factors involved in *hrp* gene regulation. Strains carrying mutations in either *hrpQ* or *hrpT* are amino acid auxothrophs (methionine and tryptophan). *hrpQ* and *hrpT* are probably involved in methionine and tryptophan biosynthesis, respectively (MILLER et al. 1993). As stated above, such mutants would normally have been eliminated from the *hrp* mutant analysis.

6.3 Conserved Sequence Boxes in *Pseudomonas syringae*

A conserved sequence, the so-called harp box (TG(A/C)AANC, FELLAY et al. 1991), upstream of four *hrp* loci in *P. s.* pv. *phaseolicola*, was suggested to be involved in positive regulation of expression. Similar motifs were described for the promoter regions of several *P. syringae* avirulence genes, the expression of which is dependent on *hrpRS* and on *rpoN* (HUYNH et al. 1989; SALMERON and STASKAWICZ 1993; INNES et al. 1993; SHEN and KEEN 1993). These studies led to a revised 'harp' box sequence (GGAACCNA). Its significance in protein binding has not been shown but *avrD* promoter constructs lacking the harp box are no longer inducible (SHEN and KEEN 1993). A harp box-related motif was also found upstream of transcription unit 3 in *P. solanacearum* (GOUGH et al. 1993).

There is no harp box sequence in *Xanthomonas hrp* gene promoters. Another sequence motif that occurs in the promoter region of *hrp* loci in *X. c.* pv. *vesicatoria* was recently identified. This "PIP" (plant-inducible promoter) box has the sequence TTCGC-N15-TTCGC and occurs upstream of the –35 consensus sequence in four out of six *hrp* promoters (S. Fenselau and U. Bonas, unpublished). Experiments are underway to test whether this is a protein binding motif.

6.4 *Xanthomonas campestris*

Expression of *hrp* genes in *X. c.* pv. *campestris* was determined after growth in vitro and found to be induced in a minimal medium with sucrose and/or fructose as carbon source. No expression occurred in complex media or with high concentrations of organic nitrogen (ARLAT et al. 1991). In *X. c.* pv. *vesicatoria*, expression of the six *hrp* loci is induced in the plant but cannot be efficiently induced in the synthetic media tested so far. However, culture filtrates of sterile tomato cell suspension cultures (called TCM) induced expression of the six *hrp* loci in *X. c.* pv. *vesicatoria* whereas the basal Murashige-Skoog culture medium did not. The inducing factor(s) could only partially be purified from TCM and was found to be smaller than 1000 dalton, heat-stable, organic, and hydrophilic (SCHULTE and BONAS 1992a). De novo transcription of all *hrp* loci occurs rapidly within 1 h after transfer of the bacteria into TCM (S. Fenselau and U. Bonas, unpublished). A minimal medium was designed which would not suppress *hrp* gene induction. This medium, called XVM1, induces the *hrpF* locus (Fig. 1A) to high levels and differs from the other media described above, in particular by its low concentration in phosphate. Both sucrose and methionine are needed for efficient induction. It is not known whether a plant factor is necessary for activation of the other *hrp* loci, or if the XVM1 medium still lacks components or contains them in suppressing amounts (SCHULTE and BONAS 1992b).

6.5 *Erwinia* and *Pseudomonas solanacearum*

The *hrp* genes of *Erwinia amylovora* are rapidly induced in the non-host, tobacco, and more slowly in the host, pear. Several loci were induced in minimal medium with mannitol as a carbon source. Induction was suppressed by high concentrations of nitrogen and by glucose and was slightly temperature dependent (WEI et al. 1992b).

In *P. solanacearum*, the *hrp* cluster was also induced in host and in non-host plants, as well as in minimal medium. The best carbon sources for induction of four of the six transcription units were pyruvate and glutamate while, as in other bacteria, casamino acids suppressed induction (ARLAT et al. 1992). The two rightmost *hrp* transcription units (5 and 6; Fig. 1B) are constitutively expressed but can be induced under certain conditions (GENIN et al. 1992).

The only other gene reported to regulate *hrp* gene expression is *hrpB* from *P. solanacearum*. The gene is part of the *hrp* cluster and appears to be a member of the AraC family of positive regulatory proteins. Interestingly, *hrpB* is related to *virF* of *Yersinia* (CORNELIS et al. 1989; GENIN et al. 1992). The *hrpB* gene positively regulates four of the six *hrp* loci, as well as the *popA* locus, located outside of the *hrp* cluster which encodes a protein secreted in a Hrp-dependent way (see above; ARLAT et al. 1994). Whether the HrpB protein binds directly to *hrp* promoters is not yet known.

At this time one can only speculate whether the regulatory systems for *hrp* gene expression employed by *P. solanacearum* and *P. syringae* are really different

or whether there is a global regulatory network thus allowing the fine tuning of gene expression in response to environmental cues. In conclusion, most *hrp* loci from different bacteria are inducible in a particular minimal medium. At this time it cannot be ruled out that stimulation of *hrp* gene expression involves specific plant factors as was described for the virulence genes of *Agrobacterium* (WINANS 1992). Since the composition of the nutrients available to the pathogen in the plant is not known one can only speculate that the conditions described above reflect the situation in the plant. It is noteworthy that the in vitro culture will only mimic the dynamic nutritional situation that bacteria experience in their interaction with a plant for a short time. In mammalian bacterial pathogens, the expression of genes involved in virulence is also regulated in response to environmental cues rather than to specific host molecules. This subject has been reviewed recently (MEKALANOS 1992 and in accompanying chapters), and I will only mention some important factors. In *Yersinia,* the *vir* and *lcr* genes are regulated by low calcium (low calcium response genes; STRALEY et al. 1993) and by temperature (CORNELIS et al. 1989; see chapter by CORNELIS). A calcium effect has not been described for any plant bacterium. In our laboratory no effect of calcium on *hrpF* gene expression in XVM1 was observed (Schulte and U. Bonas, unpublished). Expression of *invA* of *S. typhimurium* of the *mxi* and *ipa* genes of *Shigella* is affected by osmolarity and the later genes also by temperature (GALÁN and CURTISS 1990; HALE 1991).

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The Enigmatic Avirulence Genes of Phytopathogenic Bacteria

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1 Action at the "Pathogenic Cusp"

The previous chapters have discussed how phytopathogenic bacteria can sense and respond to conditions present in a variety of microenvironments: soil, water, plant cell surfaces, and intracellular spaces. The switch from epiphyte to pathogen is apparently accompanied by fundamental reprogramming of gene activity and attendant function, as evidenced by induction of *hrp* genes and subsequent production of various virulence and pathogenicity factors, some of which are host-specific, some not. This reprogramming switch between epiphytic and pathogenic growth strategies, "the pathogenic cusp" (DANGL 1994), is the point at which not only the potential pathogen but also the host first sense and respond to each other. A successful plant defense response should be based on surveillance and interdiction before the pathogen has a chance to establish production of the armory of factors which determine successful colonization of that host. It is incumbent on each potential plant host, then, to evolve mechanisms to recognize some factor, preferably one produced at this pathogenic cusp, and to base resistance strategies on early recognition. Thus, an evolutionary tug-of-war is

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begun: plants evolve to "recognize" a particular isolate of a particular pathogen; variants arise in the pathogen population which are no longer recognized; variants arise in the host population which recognize the new pathogen variant, and the familiar game is afoot. This scenario has apparently given rise to systems of plant-pathogen interactions explained genetically by the now well established "gene-for-gene" hypothesis (reviewed in CRUTE 1985; DANGL 1992; ELLINGBOE 1981; FLOR 1971; GABRIEL and ROLFE 1990; KEEN 1990; KEEN and STASKAWICZ 1988). During plant-microbe interactions of this sort, the products, either direct or indirect, of pathogen avirulence (*avr*) genes trigger a successful host resistance reaction through the action of the product of a particular host resistance (*R*) gene. These interactions are thus allele-specific: each partner is defined only by the simultaneous presence of the other. The complexity of resistance loci defined genetically is astounding and the paucity of knowledge regarding their structure and function is beginning to be resolved, as evidenced by the recent cloning of the first plant resistance gene of this class (MARTIN et al. 1993). In contrast, a plethora of avirulence genes from pseudomonads and xanthomonads have been cloned, as discussed in the remainder of this review.

As detailed below, bacterial *avr* genes obviously restrict the number of colonizable plant genotypes within a particular host species. As well, nearly all *avr* genes negatively effect bacterial host range on plant species beyond their nominal host (that from which they were isolated). Since they have a negative effect on pathogen fitness on hosts capable of recognizing their activity, why do *avr* genes persist in bacterial populations? What is the mechanism of *avr* gene action? They must serve a positive function at some stage in the bacterial life strategy, but clear roles as virulence or pathogenicity factors have been ascribed to only a miniority of *avr* genes. Are they required during epiphytic growth? Are they structural triggers of plant resistance, or are they enzymes whose products elicit plant resistance responses? How do they stimulate plant resistance responses, and is that stimulation related to the primary role of the *avr* gene product, or is it a serendipitous function? Although our current answers to these questions are sketchy at best, I will attempt to highlight some key findings bearing on them.

2 Avirulence Genes as Determinants of Host Range Both Within and Across Species

Avirulence genes have been largely defined via their ability to functionally convert a previously virulent bacterial strain to avirulence on a particular genotype (cultivar) of the test plant used in the assay. They are typically identified and cloned by gain of function assays: A conjugative cosmid library is constructed from a bacterial strain proposed to carry a particular *avr* gene (defined via interaction with a particular host cultivar proposed to carry the corresponding resistance gene); single cosmid clones are conjugated into a strain which is, in contrast, virulent on

the same test cultivar; transconjugants are tested for the ability to trigger a resistance reaction on the test cultivar (STASKAWICZ et al. 1984). It is a key tenet of the gene-for-gene hypothesis that the conversion of the recipient strain's phenotype from virulent to avirulent is plant genotype-dependent: an *avr* gene should not effect virulence on other cultivars which lack the corresponding resistance gene. Thus, *avr* genes are defined only in the context of a particular host plant for the virulent recipient bacteria, and in a manner potentially independent of their primary role in the bacteria. Over 30 *avr* genes have been identified in this manner (Tables 1, 2). Other than the two examples discussed in detail below, the deduced amino acid sequences of *avr* genes give no clue as to either their function or to how they trigger resistance on appropriate plant cultivars. Most encode proteins of between 20–100K M_r that are hydrophilic, have no obvious transmembrane or signal sequence (with one potential exception, see PARKER et al. 1993), and are not detected as secreted products in media.

Avirulence genes not only limit bacterial host range within a host plant species; rather, many are also genetically dominant determinants limiting the host range of phytopathogenic bacteria on multiple plant species (Tables1, 2). This was first demonstrated in two sets of seminal experiments (KOBAYASHI et al. 1989; WHALEN et al. 1988). In the first, WHALEN et al. (1988) identified the *avrRxv* gene from *Xanthomonas campestris* pv. *vesicatoria*, a tomato pathogen, that rendered otherwise virulent pathogens of bean, soybean, cowpea, alfalfa, corn cotton, and tomato avirulent on these plant species. The implication is that each of these species contains one or more "novel" *R* gene specificities recognizing this *avr* function. Segregation analysis in bean and tomato, in fact, support this notion (WHALEN et al. 1988, 1993). In the other series of key experiments, three genes, *avrD, avrE,* and a second allele of the previously identified *avrA* gene (STASKAWICZ et al. 1984, 1987), were cloned from a *Pseudomonas syringae* pv. *tomato* isolate via their avirulence in a soybean pathogen (KOBAYASHI et al. 1989). At least *avrD,* and probably *avrE* as well, define novel resistance specificities in soybean (KEEN and BUZZELL 1991; LORANG and KEEN 1994). Subsequent analyses in other bacterial pathovar-plant species combinations strengthen the idea that *avr* gene activity can play a major role in restricting the host range of phytopathogenic bacteria (DANGL et al. 1992; DEBENER et al. 1991; INNES et al. 1993a,b; JENNER et al. 1991; RITTER and DANGL 1994; VIVIAN et al. 1989; WHALEN et al. 1991). These fascinating results suggest that what has been traditionally called "non-host" resistance may be simply the additive effects of many, simultaneously acting, gene-for-gene interactions. This is of potential practical importance, since definition of resistance specificities in model plant species, like Arabidopsis and tomato, may reflect the existence of molecular homologs useful in crop species (reviewed by DANGL 1993a,b). This idea has led to the identification of *avr* genes not in the traditional way of screening plant genotypes within a pathogen host species, but instead by specifically searching for host range restricting genes (WOOD et al. 1994; YUCEL and KEEN 1994b). It is important to note that this finding is not limited to bacterial pathogenes, as has been clearly demonstrated by analyses in two fungal pathosystems (TOSA 1989; VALENT et al. 1990). It has also

Table 1. Avirulence genes from Pseudomonas syringae pathovars

Name[a]	Cloned from	Via interaction with (*R*) gene[b]	Limits host range on (*R*) gene[b]	Cloned alleles from pathovar: (+/-)*avr* activity	Plasmid borne	Virulence function[c]	Reference
avrA (*avrPgyA*)	Glycinea	Soybean; *RPG2*		Tomato (+)		No	STASKAWICZ et al. (1984) STASKAWICZ and NAPOLI (1987)
avrB (*avrPgyB*)	Glycinea	Soybean; *RPG1*	Arabidopsis; RPM1 /RPS3		No	No	STASKAWICZ et al. (1987) TAMAKI et al. (1988, 1991) INNES et al. (1993b)
avrC (*avrPgyC*)	Glycinea	Soybean; *RPG3*		Phaseolicola *avrPphC* (+)	Yes	No	STASKAWICZ et al. (1987) TAMAKI et al. (1988, 1991)
avrD (*avrPtoA*)	Tomato	Soybean; *RPG4*		Glycinea(-) Lachrymans 1(+) Lachrymans 2 (+)	Yes	No	KOBAYASHI et al. (1989, 1990a,b); YUCEL and KEEN (1994a)
avrE (*avrPtoD*)	Tomato	Soybean			No	Yes (tomato[d])	LORANG and KEEN (1994) LORANG et al. (1994)
avrPto (*avrPtoC*)	Tomato	Tomato;*PTO*	Soybean			No	RONALD et al. (1992) SALMERON and STASKAWICZ(1993)
avrRpt2 (*avrPtoB*)	Tomato	Arabidopsis; *RPS2*	Soybean Bean		No	No	WHALEN et al. (1991) INNES et al. (1993a)
avrRpm1 (*avrPmaA1*)	Maculicola	Arabidopsis; *RPM1*	Pea; *R2* Bean; *RN1/RN2* Soybean	Pisi, *avrPpiA1*(+) Maculicola 2(+)	Yes	Yes (Arabidopsis)	DEBENER et al. (1991) DANGL et al. (1992) RITTER and DANGL (1994)
avrPpi2 (*avrPpiA*)	Pisi	Pea; *R2*	Arabidopsis; *RPM1* Bean; *RN1* + *RN2* Soybean	Maculicola; *avrRpm1*(+) Maculicola 2(+)	Yes	No	VIVIAN et al. (1989) FILLINGHAM et al. (1992) DANGL et al. (1992)
avrPpi3 (*avrPpiB*)	Pisi	Pea; *R3*			Yes		BAVAGE et al. (1991)
avrPpiC	Pisi	Bean			No		J. Mansfield, personal communication

avrPph1 (*avrPphA*)	Phaseolicola	Bean; *R1*			No		SHINTAKU et al. (1989) J. Mansfield, personal communication
avrPph2 (*avrPphB*)	Phaseolicola	Bean; *R2*			No	No	Fillingham et al. (1992)
avrPph3 (*avrPphE*)	Phaseolicola	Bean; *R3*	Pea Arabidopsis		No		JENNER et al. (1991) R. Innes, personal communication
avrPphD	Phaseolicola	Pea			Yes		WOOD et al. (1994)
avrPphC	Phaseolicola	Soybean, *RPG3*		Glycinea(+)	Yes		N. T. KEEN, personal communication

[a] Name designation used in publications listed first; designations in parentheses are those suggested by VIVIAN and MANSFIELD (1992) for a "uniform" nomenclature.
[b] Where no resistance gene designation is given, the avirulence gene operates as a genotype-independent, host range restricting element on all tested cultivars of the species in question. (An analogous gene in this class has been cloned from Pseudomonas solanacearum. Alternatively, for several, preliminary evidence suggests the existence of a single *R* gene, but no gene designation has been given pending confirmation).
[c] Host species in which virulence function was demonstrated is shown in parentheses. "No" implies that a marker-exchange mutant in the avr gene led to no obvious change in virulence; this result does not exclude a role in virulence that was not adequately detected in the assay used.
[d] Virulence function in the PT23 strain, but not in the DC3000 strain.

Table 2. Avirulence genes from *Xanthomonas campestris* pathovars

Name	Cloned from	via interaction with (*R*) gene[a]	Limits host range on (*R*) gene[a]	Cloned alleles from pathovar: (+/-)*avr* activity	Plasmid borne?	Virulence function?	Reference
avrBs1	Vesicatoria	Pepper; *Bs1*			Yes		SWANSON et al. (1988) RONALD and STASKAWICZ (1988) KEARNEY et al. (1988)
avrBs2	Vesicatoria	Pepper; *Bs2*			No	Yes (pepper, alfalfa)[a]	MINISAVAGE et al. (1990) KEARNEY and Staskawicz (1990)
avrBs3 (genes in family listed below)	Vesicatoria	Pepper; Bs3	Tomato[b]		Yes	No	MINISAVAGE et al. (1990) BONAS et al. (1989)
avrBsT	Vesicatoria	Pepper			Yes		MINISAVAGE et al. (1990)
avrRxv	Vesicatoria	Bean; *Rxv*	Cowpea, alfalfa, corn, soybean, cotton, tomato				WHALEN et al. (1988, 1993)
avrXca	Raphani	Arabidopsis	Turnip				PARKER et al. (1993)
Other members of the avrBs3 gene family							
avrBs3–2 (*avrBsp*)	Vesicatoria	Tomato			Yes		CANTEROS et al. (1991) BONAS et al. (1993)
avrB4	Malvacearum	Cotton; *AcB1*, *AcB4*			Yes[c]		DE FEYTER and GABRIEL (1991) DE FEYTER et al. (1993)
avrb6	Malvacearum	Cotton, *AcB1*, *Acb6*			Yes	Yes (cotton)	DE FEYTER et al. (1993)
avrb7	Malvacearum	Cotton, *Acb7*			Yes		DE FEYTER et al. (1993)
avrB101	Malvacearum	Cotton			Yes		DE FEYTER et al. (1993)
avrBln	Malvacearum	Cotton, *AcBln*			Yes		DE FEYTER et al. (1993)
avrB102	Malvacearum	Cotton					
pthA	*Citri*	Cotton	Bean alfalfa			Yes (grapefruit)	SWARUP et al. (1991, 1992)
avrXa7	Oryzae	Rice, *Xa-7*				No	HOPKINS et al. (1992)
avrXa10	Oryzae	Rice, *Xa-10*				No	HOPKINS et al. (1992)

[a] Required also for pathogenicity and present in every strain analyzed; see KEARNEY and STASKAWICZ (1990).
[b] Internal deletions of this gene create both new alleles, as assayed on pepper, and novel alleles recognized by tomato, see HERBERS et al. (1992).
[c] All *avrBs3* alleles isolated to date from pv. malvacearum are linked on the same plasmid.

been demonstrated that nod factors can also function as dominant determinants of *Rhizobium* host range (e.g., FAUCHER et al. 1989; see chapter by MCKHANN and HIRSCH, this volume). As HEATH (1991) has discussed, this principle might help to explain the evolution of race-specific resistance within a species, and how those systems can result in race-specific recognition of a particular avr gene by nominal non-host plant species.

3 Virulence Functions of Avirulence Genes

If *avr* genes serve only negative function (restricting host range), why do they persist in bacterial populations? Positive functions in virulence have been described for only a handful of *avr* genes. This is typically accomplished via marker-exchange mutagenesis of the *avr* gene and subsequent analysis of effects on pathogen fitness in vitro and on both previously resistant and susceptible hosts. If there is a loss of fitness on some or all previously susceptible hosts, and no gain of virulence on previously resistant cultivars, the *avr* gene product is also a virulence factor. Alternatively, there is one example of identification of a virulence factor in a gain-of-function assay via conjugation of cosmids from a virulent strain into an attenuated pathogen (the *pthA* gene of *Xanthomonas citri,* see below; SWARUP et al. 1991, 1992).

In light of the previous discussion of *avr* genes as host range determinants, one could also predict that a particular *avr* gene might encode a determinant of virulence on plants within the strain's normal host range (host-specific virulence); or it might encode a function generally required by each strain carrying it on any potential host. The only example of the latter is the *avrBs2* gene from *X. campestris* pv. *vesicatoria* (KEARNEY and STASKAWICZ 1990; MINISAVAGE et al. 1990). This gene is probably required for normal bacterial growth, as it is present in every isolate of *X.c.* pv. *vesicatoria* (over 500 analyzed), and several isolates of other *X. campetris* pathovars (KEARNEY and STASKAWICZ 1990). Both a spontaneous mutant of *avrBs2* in a *X.c.* pv. *vesicatoria* strain (tomato pathogen) and a marker-exchange mutant of this gene in a *X.c.* pv. *alalfae* grew poorly on susceptible hosts. As well, this fitness reduction was overcome by conjugation of the cloned *avrBs2* gene into each mutant (KEARNEY and STASKAWICZ 1990).

Although not directly relevant to an understanding of *avr* gene function, it is important to note that the corresponding resistance gene in pepper, *Bs2,* has never been "overcome." Lack of mutation at *avrBs2,* which would be uncovered in the field as a loss of recognition by the corresponding *Bs2 R* gene and subsequent disease outbreaks, supports the notion that *avrBs2* is indispensible for bacterial fitness. This is in contrast to the *Bs1* gene in pepper, which is rendered irrelevant by frequent insertion of a transposon into the corresponding *avrBs1* gene, leading to virulence of such strains on *Bs1* containing pepper cultivars (KEARNEY et al. 1988; RONALD and STASKAWICZ 1988; SWANSON et al. 1988). Thus, the *avrBs1* gene is apparently dispensible in strains which carry it.

A few examples also exist of genes first identified via *avr* function which in addition also serve as host-specific virulence factors quantitatively affecting pathogen fitness (Tables 1, 2). The examples are the *avrE* gene from *P. syringae* pv. *tomato* (LORANG and KEEN 1994; LORANG et al. 1994) and the *avrRpm1* gene from *P.s.* pv. *maculicola* (RITTER and DANGL 1994), as well as the *pthA* gene from *X. citri* (SWARUP et al. 1991, 1992) and the highly related *avrb6* gene from *X.c.* pv. *malvacearum* (DE FEYTER et al. 1993).

The *avrE* gene (Table 1) was recently identified in *P.s.* pv. *tomato*, and it is noteworthy that this activity is encoded by two tightly linked transcripts. The particular strain from which *avrE* was cloned is also the strain used previously by KEEN and coworkers to isolate *avrA* and *avrD* (KOBAYASHI et al. 1989, 1990), and also contains a functional allele of the *avrPto* gene, originally cloned from another *P.s.* pv. *tomato* strain (RONALD et al. 1992). Each of these *avr* genes is known to interact with a soybean *R* gene (KEEN and BUZZELL 1991). Thus, LORANG et al. (1994) undertook to mutagenize all four of these genes, singly and in combination, in this strain and to ask if the resulting bacteria were still pathogenic on tomato. As well, they assayed the interaction of these mutants with soybean. They found that mutations in *avrE* lowered the ability of low doses (10^4 cfu/ml initial titer) of bacteria to grow and cause disease symptoms on tomato. This reduction in virulence was overcome with higher inoculum (5×10^7 cfu/ml; well above anything that would be encountered under field conditions!). The cloned *avrE* gene transconjugated into the *avrE* deletion mutant strain restored full virulence. A similar, but very slight, effect was reported for *avrA*. These data argue strongly that *avrE* function is required to establish infection in tomato, but that it is not necessary for symptom production if large numbers of bacteria are inoculated into tomato tissue. Moreover, this virulence function is apparently strain-dependent, since mutation of a functional *avrE* allele in another *P.s.* pv. *tomato* strain had no effect on in planta bacterial growth on tomato (LORANG and KEEN 1994; LORANG et al. 1994). It is also worth mention that single mutation of any one of the four *avr* genes did not result in loss of recognition by soybean. Yet construction of a triple mutant lacking *avrA, avrD,* and *avrE* gave rise to a strain incapable of either growth on a single soybean cultivar or triggering a resistance reaction on that same cultivar. This is consistent with the hypothesis that parallel action of multiple gene-for-gene interactions (in this case three) is responsible for at least some non-host resistance reactions in plants. Importantly, however, the *P.s.* pv. *tomato* strain mutated in these three host range determinants is still incapable of growth on the non-host soybean, showing that the absence of host-specific virulence functions also limits host range.

A similar role in virulence has been ascribed to the *avrRpm1* gene from *P.s.* pv. *maculicola* on Arabidopsis (RITTER and DANGL 1994). We showed that a marker-exchange mutant in *avrRpm1* was no longer virulent on any of three susceptible Arabidopsis accessions, and was also incapable of triggering a hypersensitive reaction on resistant accessions. As with *avrE,* the requirement for *avrRpm1* function in virulence seems to be during establishment of infection, as high titer inoculation results in disease symptoms and in planta bacterial growth on

susceptible hosts. Since *avrRpm1* is present in only a few of over 20 tested isolates of *P.s.* pv. *maculicola* (Dangl et al. 1992), and since several of the isolates lacking it are pathogenic on Arabidopsis (Dangl et al. 1992; Debener et al. 1991), it is apparent that combinations of virulence factors, some probably redundant, can operate in a strain on a particular host. This point is reinforced by two findings: first, as discussed above, deletion of *avrE* in one strain affects that strain's fitness, but a similar deletion in a second strain has no effect on virulence; second, mutation of the *avrRpm1* allele *avrPpi2* in *P.s.* pv. *pisi* has no effect on virulence (A. Vivian, personal communication). Since many *avr* genes are plasmid borne (Tables 1, 2), it is not surprising that combinations of *avr* functions (and any attendant fitness or virulence functions) are mixed and matched in the bacterial population. If one assumes a positive role for most or all *avr* genes at some point in the infection process, and if additive or redundant mechanisms govern virulence, then it is also not surprising that some *avr* genes are apparenlty dispensible, or not broadly dispersed.

Two members of the fascinating *avrBs3* family (Table 2; discussed in detail below) have been shown to also act as virulence factors. The *pthA* gene from *X. citri* was, in fact, first identified functionally via its ability to enhance the virulence of a weakly pathogenic *X. citremulo* strain on its nominal host, grapefruit (Swarup et al. 1991, 1992). When *pthA* is mutated in *X. citri,* the resultant strain loses pathogenicity completely on grapefruit. The *pthA* gene also encodes cultivar-specific avirulence when conjugated in *X.c.* pv. *malvacearum* and tested on cotton. Thus, *pthA* encodes a function that acts as an avirulence gene in *X.c.* pv. *malvacearum,* a virulence factor in *X. citrumelo,* and a required pathogenicity factor in *X. citri.* Another member of the highly related *avrBs3* family, the *avrb6* gene from *X.c.* pv. *malvacearum,* acts as a host-specific patho-genicity factor in that it confers the ability to cause disease symptoms (water soaking) on cotton onto strains normally incapable of doing so, and it is required for maximal symptom formation (De Feyter et al. 1993). Oddly, its presence does not render growth capability on the recipient strain tested.

The fact that most *avr* genes seem to have no role in pathogenicity or virulence suggests, among other possibilities, that our infection assay systems might bypass the stage in the normal plant-microbe interaction at which these gene products are required. For example, one could imagine that the *avr* gene products encode functions required at the switch point between epiphytic bacterial growth on the plant surface and intercellular growth as a pathogen. This stage of the interaction is often not assayed during hand or vacuum infiltration experiments. New assay systems for epiphytic fitness, as described in the chapter by Beattie and Lindow, will potentially help to establish when and how *avr* genes exert a positive influence in the infection process.

4 Regulation and Organization of Avirulence Genes

Consistent with the idea that *avr* genes do have an as yet nebulously understood role in virulence, nearly all are transcriptionally induced in environments representative of plant intercellular spaces. Two points are germane to discussions of *avr* gene regulation: First, to date, delivery of all *avr* functions is dependent on structural integrity of the *hrp* secretory apparatus (see chapters by BONAS, and by COLLMER and BAUER, this volume). This supports the idea that *avr*-dependent elicitors of plant response are produced and that they reach the intercellular space via secretion through the *hrp* pathway. Second, it appears that the *hrp* transcriptional regulators *hrpS* and *hrpL* are required for transcription of many, if not all, *P. syringae avr* genes (HUYNH et al. 1989; INNES et al. 1993a; SALMERON and STASKAWICZ 1993; SHEN and KEEN 1993). These analyses have been carried out in *P. syringae* systems, and our understanding of *avr* gene transcription in *X. campestris* is confounded by the fact that no *hrp* gene has been identified with the structural features of a transcriptional activator, and by the finding that *avrBs3* is transcribed in a *hrp*-independent manner (KNOOP et al. 1991).

A shift from rich to minimal media induces transcription of a number of *avr* genes from various *P. syringae* pathovars. The level of induction is variable, but hovers around 30- to 100-fold for several genes. Induction is maximal in minimal media containing sugars such as fructose and sucrose, or sugar alcohols such as mannitol. A further boost in transcriptional activity, again around 30- to 100-fold, has been observed in planta for *avrB, avrPto, avrD,* and *avrRpm1* (HUYNH et al. 1989; INNES et al. 1993a; SALMERON and STASKAWICZ 1993; SHEN and KEEN 1993). RITTER and DANGL (1994), however, showed that only minimal media, without any sugar source may be sufficient to induce transcription of *avRpm1*, but that maximal transcriptional activation requires a sugar such as fructose or sucrose. In all cases, *avr* transcriptional activity is maximal within the first 5 h after infiltration into leaves. As well, there is no differential *avr* expression resistant compared to susceptible plant cultivars. It is noteworthy that these examples are from experiments in soybean, tomato, and Arabidopsis, suggesting that the intercellular milieu of plant leaves, rich in sucrose and with a pH of around 5.5, is generally inductive for *P. syringae avr* gene transcription. It is also important to note that this pH, as well as osmotic conditions, the relative absence of nitrogen sources, and the presence of certain sulfur compounds, is critical to activate *hrp* gene transcription (see chapter by BONAS, this volume). Thus, whether the *hrpS* and *hrpL* requirements for *avr* promoter activity are physically direct or indirect, and whether there may be additional modes of regulation for some *avr* genes, establishment of bacterial growth in leaf intercellular spaces clearly includes early activation of *avr* gene activity.

Sequence comparisons and promoter analysis have defined two *cis*-acting DNA sequences in *avr* genes from *P. syringae* (INNES et al. 1993a; SHEN and KEEN 1993). The so-called "harp box", first noted upstream of *hrp* genes by FELLAY et al. (1991) (see chapter by BONAS, this volume), has been recently expanded

(consensus 5'T/G-G-G-A-A-C-C-$N_{(15-16)}$C-C-A-C) and shown to be essential for *avrD* transcription via block mutation analyses (SHEN and KEEN 1993). Interestingly, these authors also identified a consensus σ^{54} binding site in the *avrD* promoter (no other *avr* gene has been shown to contain this site). Although no evidence for direct DNA-binding activity for *hrpS* on *avr* genes promoters exists yet, it should be remembered that *hrpS* is a member of the σ^{54} requiring *ntrC* family of transcriptional activators. Consistent with a role for a σ^{54}-like factor for at least *avrD* transcription, SHEN and KEEN (1993) also showed that *avrD* expression is abolished in an *ntrA* mutant of *P.s.* pv. *phaseolicola.* Whether this strategy of transcriptional activation is generally applicable for *avr* genes from *P. syringae* awaits further clarification of the function of the HrpL protein, the regulatory and structural relationship between *hrpS* and *hrpL* and demonstration of a direct role for either of these proteins on *avr* gene promoters.

A consistent, though still partially speculative, model regarding early regulatory events at the pathogenic cusp thus begins to emerge. Environmentally mediated induction of *hrp* genes upon migration of bacteria into intercellular spaces in turn leads to activation of a regulon of virulence functions, among them the genes identified through their *avirulence* activity. One could imagine isolation of genes in this regulon through use of DNA probes for the harp box, or via transposon mutagenesis searches for *hrpS*-dependent transcription units and subsequent sequencing. Under the assumption that such functions are *hrp*-linked, the latter approach has already led to the identification of three *hrp*-linked transcription units, one encoding *avrE* (LORANG and KEEN 1994).

Is there a pattern to the physical locations of *avr* genes that is informative for their role during infection? Tables 1 and 2 show that many are plasmid encoded, and now there are examples that are *hrp*-linked on the chromosome. The importance of plasmids carrying virulence factors and other determinants of pathogen fitness is a recurrent theme throughout bacterial pathogenesis and has been demonstrated repeatedly for plant pathogens (for example BOUCHER et al. 1988; KEARNEY et al. 1988; SWANSON et al. 1988; BAVAGE 1991). It is very intriguing that the *avrBs3* family of genes are borne on a self-transmissible plasmid and that some are flanked by insertion sequences (BONAS et al. 1989, 1993; DE FEYTER et al. 1993; HOPKINS et al. 1993). As well, in *X.c.* pv. *malvacearum,* several copies of *avrBs3*-like sequences are tightly linked, suggesting that local transposition and/or recombination can give rise to new family members, encoding potentially new *avr* functions (DE FEYTER and GABRIEL 1991; see also below). For other *avr* genes, no correlation exists between plasmid or chromosomal location and, for example, a demonstrated role in virulence. The variety of plasmid-associated *avr* genes and the conserved regulatory mechanisms of *avr* genes independent of location suggest that their dispersal is probably important in some component of bacterial fitness.

The interplay between various plasmid-borne functions and avirulence functions has recently been highlighted by the findings of Vivian and colleagues (MOULTON et al. 1993). They showed that transfer of RP4 or IncP1 replicons to either of two *P.s.* pv. *pisi* strains resulted in alterations in the structure of resident

plasmids which were clearly associated with changes in interactions with pea plants. Most strikingly, these experiments suggest that the acquisition of an identical novel *avr* gene activity on pea in variants of both strains was dependent on loss of a plasmid-borne suppressor of that *avr* activity. Suppressors of *avr* function have also been demonstrated in several fungal pathogens. These genes would provide a mechanism by which the normal role of an *avr* gene might be retained by the microorganism, but the serendipitous recognition of that function by the plant cell masked.

5 Modes of Avirulence Gene Action

We remain, sadly, very naive regarding not only the mechanism(s) by which *avr* genes function positively in plant-microbe interactions, but also concerning the way in which they trigger plant resistance responses. The "elicitor-receptor model" (Gabriel and Rolfe 1990; Keen 1982) suggested that the *avr* gene product would interact with the *R* gene product to trigger resistance. As discussed below, elegant work in two *avr* gene systems suggests that this is partially correct. The first example for which a clear consensus emerged, the *avrD* gene from *P.s.* pv. *tomato*, necessitated a slight modification of the simplest elicitor-receptor model to allow that an "indirect" product of an *avr* gene could trigger resistance. The second example, less clear, but fascinating specifically because of its fundamental difference to *avrD*, is the gene family of structural homologs related to the *avrBs3* gene from *X.c.* pv. *vesicatoria*. As detailed below, it may be that the different members of this family interact directly with the plant cell, although in an as yet mysterious manner.

5.1 The avrD Elicitor

As alluded to above, the *avrD* gene was isolated from *P.s.* pv. *tomato* via conjugation of cosmids into an isolate of *P.s.* pv. *glycinea* which was virulent on several soybean cultivars (Kobayashi et al. 1989). Subsequently, Keen and Buzzell (1991) showed that the *avrD* gene function defined a new resistance soybean *R* gene specificity, *Rpg4*. Interestingly, overexpression of *avrD* in *Escherichia coli* leads to accumulation of low molecular weight compounds in the culture supernate which is sufficient to trigger a hypersensitive response specifically on soybean cultivars carrying *Rpg4* (Keen et al. 1990). Moreover, response to this "elicitor" compound cosegregates with resistance to *P.s.* pv. *glycinea* strains carrying the *avrD* gene (Keen and Buzzell 1991). Recently, two nearly identical acyl glycoside structures for the avrD elicitor have been proposed, and they have been dubbed syringolides 1 and 2 because of their structural similarity to molecules from *Streptomyces* species known to be inducers of sporulation and antibiotic production (Midland et al. 1993; Smith et al 1993). Thus, the *avrD* gene probably encodes an enzyme, and a product of its enzymatic activity is the avrD elicitor.

Several alleles of *avrD* have been cloned and sequenced in an effort to understand how its structure determines production of the avrD elicitor (KOBAYASHI et al. 1990a,b; YUCEL and KEEN 1994b). These experiments were motivated by the isolation of a highly homologous allele of *avrD* from *P.s.* pv. *glycinea* (86% amino acid identity) that does *not* function as an *avr* gene (KOBAYASHI et al. 1990b). Chimeric avrD proteins were constructed from an active allele cloned from *P.s.* pv. *tomato* and the inactive allele from *P.s.* pv. *glycinea,* and components of avrD protein stability were localized to the carboxyl portion of the protein.

Three further alleles have recently been characterized (Table 1; YUCEL and KEEN 1994b). Two come from one strain of *P.s.* pv. *lachyrmans,* and the third from *P.s.* pv. *phaseolicola.* All *avrD* alleles are plasmid-borne, and the alleles from *P.s.* pv. *lachyrmans* reside on two different plasmids. The deduced amino acid sequences of all five alleles allowed YUCEL et al. (1994a) to propose two homology groups. The two alleles from *P.s.* pv. *lachyrmans* are in different groups, suggesting relatively recent independent acquisition by this strain of the plasmids carrying them. As well, the *avr*-inactive allele is present in a homology group with two different, active alleles. Five amino acid substitutions are all that separate *avr*-inactive from the *avr*-active alleles within this homology group, and YUCEL and KEEN (1994a) recently showed that three of these residues are required for *avrD* function via site-directed mutagenesis. Another contextual motif of six amino acids, differing between homology groups, may influence *avrD* activity in combination with those absolutely required for activity. Finally, YUCEL et al. (1994b) have shown that alleles from the two homology groups produce idiosyncratic syringolides when expressed in *E. coli.* Within one homology group, *avr*-active and *avr*-inactive alleles produce the same HPLC profile of syringolide-like compounds. Two points emerge from this analysis: first, relatively low protein stability and consequent low production of avrD elicitor probably explain the inactivity of the *P. s.* pv. *glycinea avrD* allele, as originally suggested by KOBAYASHI et al. (1990b). Note that this lowered activity may not impinge on the normal role of *avrD* in *P.s.* pv. *glycinea.* Second, slight structural variations are probably allowed in the avrD elicitor with respect to recognition by the soybean *Rpg4* gene. Alternatively, closely linked *R* gene specificites at *Rpg4* may be responsible for recognition of structural variants of the avrD elicitor. At a minimum, these recent analyses establish that the *avrD* gene probably functions as an enzyme and that different alleles of this enzyme discriminate between fatty acid derivatives differing by two carbon atoms (YUCEL et al. 1994b). The parallels here to fatty acid chain decoration of nod factor backbone structures as one determinant of *Rhizobium* host range are striking.

5.2 The Structural Conundrum of the *avrBs3* Gene Family

If the probable role of the *avrD* gene product in triggering plant resistance reactions is enzymatic and indirect, the *avrBs3* family represents an example of an *avr* gene product whose structural features are suggestive of a direct inter-

action with the plant. The paradigmatic member of this family was cloned from *X.c.* pv. *vesicatoria* and shown to encode a bizarre structure of 17 repeats of 34 amino acid residues each (Bonas et al. 1989). Each of the repeat units contains a two amino acid "variable region," and the number of naturally occurring repeat units can vary (from 14 to 23 in the *X. c.* pv. *malvacearum* clones, De Feyter et al. 1993). Homologous sequences are found in many additional *X. campestris* pathovars and in *X. oryzae* (Bonas et al. 1989; De Feyter et al. 1993; Hopkins et al. 1993). A family of six *avr* genes from *X. c.* pv. *malvacearum,* at least three *avr* genes from *X. o.* pv. *oryzae,* and an additional *avr* gene from *X.c.* pv. *vesicatoria* are all highly related to *avrBs3* (Bonas et al. 1993; Canteros et al. 1992; De Feyter and Gabriel 1991; De Feyter et al. 1993; Hopkins et al. 1993). It is not known if all *avrBs3* homologs have avirulence activity, since not all have been cloned and tested. Note, however, that at least two members of this family have been shown to encode virulence functions on susceptible hosts (discussed above; see Table 2).

The most bemusing aspect of this gene family was uncovered by Herbers et al. (1992), who demonstrated that the specificity of plant recognition of *avrBs3* can be changed by altering the number of repeat units. They showed that deletion of different numbers of repeats could, in fact, give rise to *avr* genes now interacting with the "recessive allele" of the *Bs3* resistance gene in pepper! They also created deletion derivatives capable of triggering a plant resistance reaction on another host, tomato. The overall number of repeats does not seem to define the particular *avr* specificity, and it seems that the "variable region" sequences are combinatorially responsible for the new specificities (at least two complete repeats are necessary for any activity of *avrBs3*). The *avrBs3* gene therefore may encode a product which interacts directly with the plant, although other models are possible. Recent comparison of the *avrBs3-2* (or *avrP*) allele, active on tomato, with *avrBs3* derivatives that are also active on tomato did not allow deduction of pepper- or tomato-specific structural motifs (Bonas et al. 1993). In contrast to *avrBs3,* which apparently requires non-repeat unit sequences at both amino and carboxy termini, a series of carboxy truncations of the *avrBs3-2* allele containing more than two complete repeat units retains function. Cellular fractionation studies show that 20%–30% of the protein is membrane-associated, the rest being soluble (Knoop et al. 1991). Recent in situ localization of AvrBs3 protein suggested localization in the bacterial cytoplasm, adding to the whimsical question of how this most interesting structure imparts specific recognition to the plant cell (Brown et al. 1993). An interesting point with respect to this gene, when considering a model of direct interaction with the plant cell, is that its expression, unlike all other *avr* genes analyzed to date, is independent of *hrp* gene function (Knoop et al. 1991; see also above).

6 Other Genes Influencing Host Range

The experimental regime for identification of bacterial *avr* genes obviously identifies a subset of potential host range determinants since it is based on gain-

of-function, namely, dominant triggering of plant resistance response. Yet any gene whose action is required for virulence on a particular host genotype will be identified as a host range determinant when it is mutated, and identified in loss-of-virulence assays. In a broader sense, both regulatory genes, required to sense a particular plant environment and initiate a response cascade, and the effector genes thus activated, also constitute host range determinants. Their absence clearly limits the niches available to bacteria relying on them. Examples of this class are discussed in chapters by Dow and Daniels, and by Collmer and Bauer in this volume, but several recent examples deserve reiteration in the context just described.

One regulatory gene which also is a required host range determinant is the *lemA* gene from *P. s.* pv. *syringae* (HRABAK and WILLIS 1992; WILLIS et al. 1990). This gene is required for symptom formation on bean plant, but is dispensible for in planta bacterial growth. As WILLIS et al. (1990) pointed out, however, definition of these virulence functions is very dependent on assay conditions. They concluded that isolation of the *lemA* mutant, which was accomplished by inoculating bean pods and screening for symptomless mutants, would have been very difficult using leaf assays, as this mutant does trigger dose-dependent necrosis on leaves. The *lemA* gene encodes a "fused" two-component regulatory molecule, molecular homologs of which are present in several *P. syringae* pathovars (HRABAK and WILLIS 1992). Marker-exchange mutagenesis demonstrated that *lemA* is required for lesion formation on bean by several classes of *P. s.* pv. *syringae,* but similar mutation of *lemA* in *P. s.* pv. *phaseolicola* did not diminish lesion formation on bean (RICH et al. 1992). Thus, absence of *lemA* limits *P. syringae* pv. *syringae* host range. It is not known what triggers *lemA* function, but it, in turn, regulates production of toxin and proteases in both *P. s.* pv. *syringae* and *P. s.* pv. *coronafaciens* (BARTA et al. 1992; HRABAK and WILLIS 1993). However, toxin minus and protease minus mutants generated via marker-exchange mutagenesis in a *lemA* wild-type background were still fully pathogenic on bean. This result suggests that although toxin and protease regulation are targets for *lemA* regulation, they are not the downstream targets of *lemA* required for pathogenesis of *P. s.* pv. *syringae* on bean (HRABAK and WILLIS 1993). This consistent with the notion that environmental sensors feed into multiple effector pathways designed to allow the bacterium maximum flexibility during the early phases of pathogenesis.

A clear role in host range limitation was also recently demonstrated for the *hrmA* gene of *P. s.* pv. *syringae* (HEU and HUTCHESON 1993). This locus was identified as a modifier of *hrp* action, as insertions in *hrmA* led to an attenuated hypersensitive response (HR) on tobacco but retained pathogenicity on bean (HUANG et al. 1991). The *hrmA* gene encodes a protein of unknown function and is regulated in an *avr* gene-like manner: induced in sucrose-containing media and in planta, and transcriptionally dependent on *hrpS*. Interestingly, when conjugated into a *P. s.* pv. *glycinea* strain, *hrmA* rendered this strain avirulent on a battery of otherwise susceptible soybean cultivars. Clearly, *hrmA* negatively influences host range in *P. s.* pv. *glycinea*, and is potentially an *avr* gene on soybean. Thus,

it is analogous to those *avr* genes listed in Table 1 which operate, so far, as genotype-independent, host range restricting elements (see also CARNEY and DENNY 1990).

Structural features of the bacterial surface can also effect host range. A recent example is the *opsX* locus of *X. campestris* pv. *citrumelo* (KINGSLEY et al. 1993). This gene encodes an enzyme involved in lippolysaccharide (LPS) synthesis, and mutations in *opsX* pleiotropically effect not only LPS, but also extracellular polysaccharide profiles. *OpsX* mutants are not pathogenic on citrus, but retain pathogenicity on bean. The authors postulate that *opsX* functions to protect bacteria from defense compounds present in citrus, but not in bean.

7 Flexible Adaptation for Opportunistic Infection

Essentially all phytopathogenic bacteria are opportunists in the sense that they switch easily from epiphytic to pathogenic modes of survival when circumstance dictates. Isolation and analysis of *avr* genes, defined by their interactions with plant resistance genes, have allowed us to scratch at the surface of what will undoubtedly be a complex system whereby phytopathogenic bacteria sense and respond to the environmental conditions on and inside the plant surface. Detailed appraisal of *avr* gene function, both in the sense of how it is beneficial to the bacterium and in that it triggers highly specific disease resistance in plants, must address the following questions. What is the normal role of these genes in the life cycle of phytopathogenic bacteria? How do *avr* genes confer a selective advantage to bacteria which potentially suffer narrowing of host range because of their action? How are they recognized by the plant to trigger a resistance response? How is *avr* gene function intertwined with, or mediated via, *hrp* function? And finally, what is the molecular nature of the interacting partners which leads to disease resistance in the plant? Answers to these questions will no doubt be strengthened by recent advances in molecular understanding of bacterial development and interactions with mammalian hosts.

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Note added in proof: Recent sequencing of hrpL provides compelling evidence that this protein is a potential σ-factor. A model for hrp regulation is thus proposed.

The Genetic and Chemical Basis of Recognition in the *Agrobacterium*: Plant Interaction

A.N. Binns and V.R. Howitz

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1 Introduction

Agrobacterium tumefaciens is a gram-negative soil bacterium that causes crown gall tumors on a broad spectrum of dicotyledonous plants (for reviews see: Binns and Thomashow 1988; Winans 1992; Zambryski 1992). This pathogenic response results from the activities of a large tumor-inducing (Ti) plasmid that resides in many but not all agrobacteria found in the rhizosphere. The infection and transformation process is a complex series of interactions between host and pathogen that ultimately leads to the transfer of DNA (the T-DNA) from the Ti plasmid into the plant cell where it is integrated into the nuclear genome. Expression of this T-DNA results in the production of two classes of protein products: (1) enzymes that synthesize plant hormones capable of stimulating continuous cell division in the transformed cells and (2) enzymes that synthesize

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unique amino acid:sugar acid conjugates, termed opines, that are not metabolizable by the host cell but are metabolized by the inciting bacterium, providing it with a dedicated nitrogen and carbon source.

The DNA transfer process requires the activities of *Agrobacterium* chromosomal and Ti plasmid genes referred to as "chromosomal virulence" (*chv*) and "virulence" (*vir*) genes, respectively. Briefly, the bacteria sense an appropriate infection site, generally a wound, through the activities of *chvE*, *virA*, and *virG* and activate transcription of the *vir* genes. Products of the *virC* and *virD* operons release a single-stranded piece of DNA (T-strand) from the T-DNA region of Ti plasmid. The T-strand is capped at its 5' end by VirD2 and coated by the single-stranded DNA binding protein, VirE2, resulting in the formation of the T-complex, which is hypothesized to be the transferred intermediate. Independent of *vir* gene activation and T-DNA processing, the agrobacteria bind to the walls of plant cells at the wound site. Once attachment and T-DNA processing have occurred, the T-complex moves out of the bacterium and into the plant cell in a process that is still not understood. The VirB proteins, most of which are membrane localized and required for virulence, are hypothesized to form a membrane-spanning pore through which the T-complex moves (see chapter by R. Ruppuoli this volume). However, the putative pore and its relationship to the plant cell are undescribed.

This review concerns the mechanisms whereby the host and pathogen interact so as to initiate and execute the process of DNA transfer. Several questions will be addressed. First, what plant-derived cues are being recognized by the bacterium, and why is the plant producing such cues? Second, how does the bacterium sense the plant-derived cues? Third, once the signals are perceived, how are they transduced into biochemical events that lead to *vir* gene transcription? Fourth, what physical cell:cell interactions are necessary for DNA transfer?

2 Signal Production—What's the Plant Doing and Why?

In general, crown gall tumors develop at wound sites on plants only if the site is infected by *Agrobacterium* soon after wounding. In a series of classic experiments Braun and colleagues found that if agrobacteria were added to *Kalanchoë daigremontiana* or *Catharansus* (*Vinca*) *roseus* 7 days after wounding, tumors would not develop (for an interesting early review see Braun and Stonier 1958). Further, they took advantage of the fact that elevated temperature disrupts the transformation process (but not tumor growth after transformation) to show that the bacteria were maximally efficient in tumorigenesis during the period of 48–96 h after wounding (Braun 1947, 1952; Braun and Mandle 1948). More recent experiments utilizing cocultivation procedures in which the bacteria can be eliminated by antibiotic treatment confirms that maximal competence of plant cells for transformation occurs 60–96 h after wounding (Kudirka et al. 1986; Binns 1991).

What processes are occurring at the wound site that make it competent and how does the bacteria recognize and exploit this potential information? At a structural level, it is clear that wounded tissues in most dicots heal their wounds through a combination of cell divisions at the wound site and cell wall strengthening (Kahl 1982). A significant proportion of cells near the wound site are activated and undergo from one to a few rounds of mitosis before returning to the quiescent state. In all cases examined to date the period of maximal competence for transformation correlates with the period of maximal wound cell division (Braun 1952; Lipetz 1966; Kudirka et al. 1986; Binns 1991). Interestingly, most monocots, which are poorly if at all transformed by *Agrobacterium*, do not exhibit wound cell divisions (Kahl 1982).

The most obvious biochemical activities at the wound site are related to wound cell division and/or strengthening of the cell walls near the wound site. Induction of the phenylpropanoid pathway is a general feature of the wound response (Kahl 1982) and is necessary for the production of phenolics involved in cell wall strengthening. For example, ferulic acid dimers are necessary to cross-link pectic and hemicellulosic polymers together (Fry 1983; Tan et al. 1992), and lignin, derived from the peroxidase catalyzed free radical polymerization of phenolic alcohols such as coniferyl and syringyl alcohol, is critical in wall strengthening (Lewis and Yamamoto 1990). In addition to these polymerized molecules, large quantities of soluble phenolics are also present at the wound site. Some of these have antimicrobial activities (Lamb et al. 1989), while others, particularly coniferyl alcohol derivatives, have been implicated in cell division control (Teutonico et al. 1991; Lee et al. 1981). Ultimately, the wound site heals, with cell division and various biochemical activities being shut down.

3 Signal Recognition: How Does the Bacterium Sense That Competent Plant Cells Are Around?

3.1 *vir* Gene Induction is Controlled by a Two-Component Regulatory System

Clearly, the wound site undergoes a complex series of wound healing and defense activities that *A. tumefaciens* must overcome if it is to successfully transform a plant cell. One remarkable feature of this bacterium is that it uses these plant defense responses as both chemoattractants and as activators of the transformation process. The best characterized aspect of signal recognition in the *Agrobacterium*-plant cell interaction is the activation of the *vir* genes by wound exudate. As described above, the *vir* genes encode proteins involved in the processing and export of the T-complex into the plant cell. In order to both identify potential *vir* genes and characterize the control of their transcription, Stachel and co-workers (1985a,b; Stachel and Zambryski 1986) constructed a transposon, Tn*3*HoHol, that carried a promoterless *lacZ* which required translational fusion for

enzyme activity. Insertion into a *vir* gene thus inactivated the gene and could also produce translational fusions with β-galactosidase activity useful as a reporter of promoter activity. Results of these experiments demonstrated that most of the *vir-lacZ* fusions were inactive unless exposed to plant-derived phenolics, such as acetosyringone (AS), that are associated with the wound response (STACHEL et al. 1985b). While the phenolics are critical signals necessary to activate the *vir* genes, subsequent investigations have demonstrated that hexoses and acidic pH are also necessary for maximal induction. For example, phenolic induction of *vir* gene expression is not observed, at least in wild-type bacteria, unless the pH is acidic (e.g., 5.5) (STACHEL et al. 1986). Furthermore, the sensitivity of the phenolic recognition system is increased by nearly two log orders when hexoses such as glucose or arabinose are present in the induction medium (CANGELOSI et al. 1990; SHIMODA et al. 1990).

The genetic basis of signal transduction controlling *vir* gene expression has been extensively studied. *virA* and *virG* are necessary for induction of the other *vir* genes, and, while these two genes are constitutively transcribed, they are also auto-inducible in response to phenolics (STACHEL and ZAMBRYSKI 1986; WINANS et al. 1988). Both sequence and biochemical analysis of the *virA*/*virG* system indicated it is homologous to the family of "two-component" regulatory systems used in a variety of different signal transduction pathways by both eubacteria and archebacteria (STOCK et al. 1990; PARKINSON 1993). As with most two-component systems, the working model is that the plant signals cause the membrane bound "sensor" (VirA) to transfer a phosphate group from itself to the "activator" (VirG), which in turn activates transcription of the *vir* genes (Fig. 1A). Additionally, genetic evidence indicated that one of the chromosomal virulence genes (*chvE*) is required for maximal phenolic sensitivity for *vir* induction, particularly in the presence of hexoses, and encodes a protein homologous to sugar binding proteins found in other systems (CANGELOSI et al. 1990; HUANG et al. 1990.).

3.2 Phenolic Perception

The putative phenolic sensor, VirA, has an approximate molecular weight of 92 kDa with two membrane-spanning domains (MELCHERS et al. 1989b; WINANS et al. 1989). This divides the molecule into three discrete sections; a short, cytoplasmic NH_2-terminal, a 20 kDa periplasmic domain, and a 68 kDa COOH-terminal, also located in the cytoplasm (Fig. 1A). One fundamental question is: what proteins interact with the signals (sugars, phenolics and pH) to provide information that results in phosphate transfer from VirA to VirG? Analysis of other two-component systems indicates the complexity of this issue: the actual ligand binding in these different cases has been documented in only a few instances. Ligands can bind directly to the sensor component or to other proteins which then interact with the sensor, resulting in stimulation. For example, FixL is the sensor in a two-component system that recognizes O_2 and, at low O_2 tension, activates transcription of the nitrogen fixation (*nif*) genes in *Rhizobium meliloti* through the activator FixJ. FixL is a membrane-bound hemoprotein that can directly bind O_2

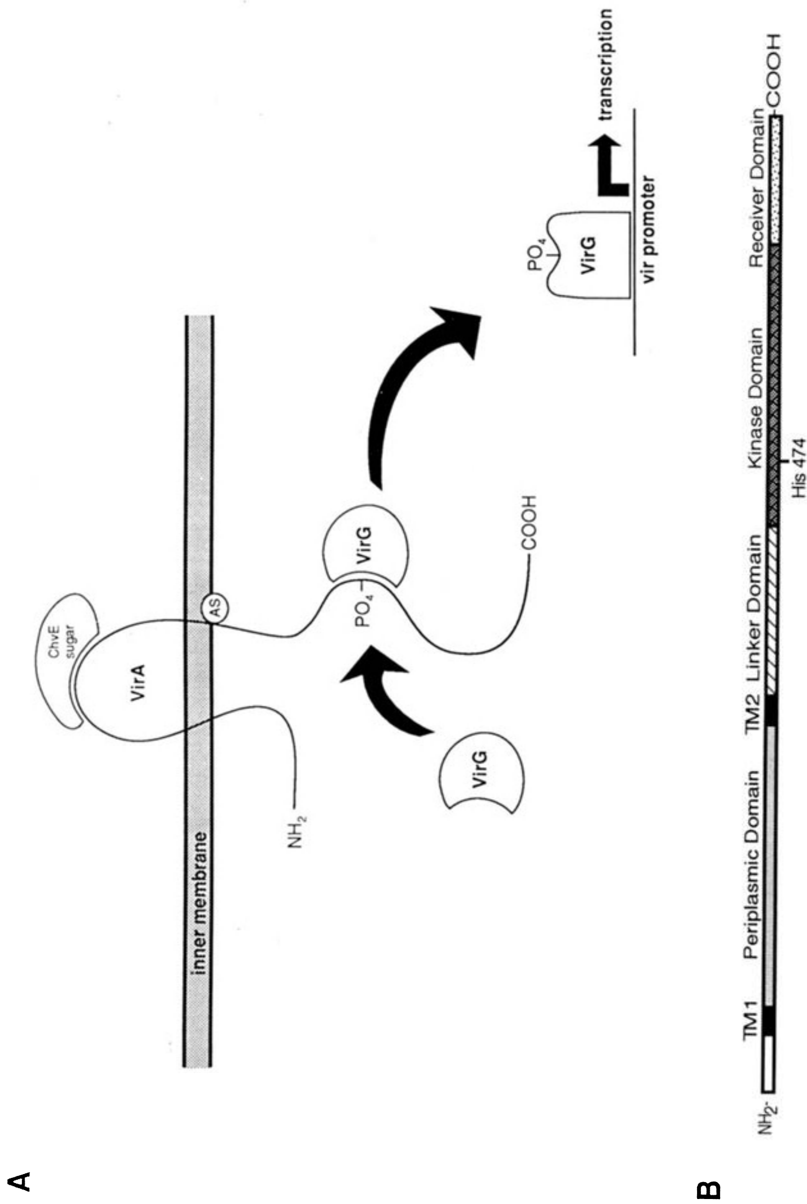

Fig. 1A,B. A The proteins VirA, ChvE and VirG and their interactions in response to sugars and phenolics such as acetosyringone. See text. **B** The VirA protein, delineating its domains as suggested in CHANG and WINANS (1992)

(Lois et al. 1993a,b). Recent studies have shown that FixL/FixJ mediated transcription in response to anaerobic conditions can be demonstrated in vitro (Agron et al. 1993). In contrast, many other ligands appear to bind to receptor proteins other than the sensor, and the interaction of the receptor with the sensor can be direct or indirect. A classic example of indirect signaling is the chemotaxis system in *Escherichia coli*. In this case the sensor kinase (CheA) is actually a soluble, cytoplasmic protein that is fed information from membrane-bound methyl accepting proteins (MCPs, e.g., Tar). These proteins can either bind the ligand directly (aspartate) or interact with other receptor proteins (sugar binding proteins) in order to acquire information about signal concentration (Parkinson 1993). Another well characterized two-component regulatory system in *E. coli* controls phosphatase expression. PhoR is a sensor kinase that responds to the inorganic phosphate (Pi) concentration, controlling alkaline phosphatase expression through the activator PhoB (Wanner 1993). The topology of PhoR is quite similar to that of VirA, having two transmembrane domains, a periplasmic domain and a large cytoplasmic domain. Pi recognition and binding is not mediated directly by PhoR. Rather, Pi recognition requires the activity of *pst1* which encodes a periplasmic protein involved in Pi transport. This protein interacts with other membrane-bound proteins, including PhoU, which ultimately interacts with a cytoplasmic portion of PhoR (Wanner 1993).

The examples described above indicate the flexibility of the two-component regulatory system: signal recognition by the sensor can be either direct or through a series of protein-protein interactions. One strategy to characterize the parts of VirA necessary for phenolic recognition has been to mutagenize the coding sequence either by deleting large portions of the protein or constructing point mutations in particular domains. Most of the mutations that, to date, have been shown to affect phenolic recognition are mutations that actually affect the sugar enhancement effect (see below). Chang and Winans (1992), however, did demonstrate that the minimal VirA molecule necessary to respond to the phenolics does *not* include either of the membrane-spanning domains or the periplasmic domain. These results indicated that the "linker" domain of VirA (Fig. 1B) may be critical to phenolic recognition. This result is reminiscent of PhoB (see above) which requires its cytoplasmic domain just inside the cytoplasmic membrane to interact with other proteins involved in phosphate recognition (Wanner 1993).

Another method used to test the hypothesis that VirA may be the phenolic binding protein has been to reconstruct the VirA/VirG signal control system in heterologous hosts. For example, moving the Ti plasmid into the closely related genus, *Rhizobium*, results in strains that are virulent and will induce the *vir* genes in response to the phenolics (Hooykaas et al. 1977; van Veen 1988). These results indicate that virtually all of the chromosomal virulence genes of *Agrobacterium* have homologues in *Rhizobium*. This has been proven in the case of certain genes necessary for bacterial attachment to plant cells (Cangelosi et al. 1987). It is likely that genes involved in signal perception are also conserved. The fact that the virulence of *Rhizobium* strains carrying Ti plasmids is similar to wild-type *Agro-*

bacterium suggests that they carry a functional equivalent to *chvE*. When the VirA/VirG system is moved to more distantly related bacteria, e.g., *E. coli*, phenolics will not induce transcription from *vir* promoters (Winans, personal communcation). This lack of response may, however, be due to problems other than a lack of phenolic perception. For example, a constitutively active VirG (see below) will not activate transcription from a *vir* promoter in *E. coli* (HAN et al. 1992). This suggests the interactions of the transcriptional machinery with activated VirG at the *vir* promoter may be lacking.

Finally, early genetic studies showed that *virA* of limited host range (LHR) strains of *Agrobacterium* was responsible for their inability to transform many host plants that were susceptible to wide host range (WHR) strains (YANOFSKY et al. 1985). One reason for this was the apparent inability of the LHR VirA to recognize certain phenolics (LEROUX et al. 1987). Later studies, however, demonstrated that the LHR strains were capable of responding to high doses of the same phenolics as the WHR strains (TURK et al. 1991). The molecular basis of the poor response is due to the fact that the LHR *virA* did not exhibit auto-induction in response to phenolics as was observed in case of the WHR *virA*. When the WHR *virA* promoter was used to express the LHR *virA* coding sequence the resultant chimeric gene elicited the WHR phenotype (TURK et al. 1993a). Thus, there is no convincing genetic evidence which shows that VirA is the phenolic receptor.

An alternative approach to understanding phenolic recognition has been through chemical analysis of the phenolic specificity of the system. An extremely broad range of active phenolics, in terms of structure, has been noted by several laboratories (SPENCER and TOWERS 1988; MELCHERS et al. 1989a; DUBAN et al. 1993). This wide specificity is unusual for most ligand-receptor interactions. The relationship between structure and *vir* inducing activity has been analyzed in detail by LYNN and coworkers, who tested a model suggesting that certain structural features were necessary for phenol binding (DUBAN et al. 1993). The results of these studies indicated that, in addition to such structural features, the most active phenolics were those having the greatest hydrolytic reactivity. A model was presented suggesting that the ability of the phenolic to transfer a proton to the receptor is crucial (HESS et al. 1991; DUBAN et al. 1993) (Fig. 2). This model predicts that a proton is transferred from one region of the receptor to the oxygen para to the phenolic hydroxyl group and that ionization of the resonance-conjugated phenolic hydroxyl group causes proton transfer to a basic component of the receptor. The protonation of this residue is predicted to result in conformation changes in the receptor which then affects the downstream signaling activities (see below).

The proton transfer model was examined by HESS et al. (1991), who tested for the appearance of a strong nucleophilic residue (e.g., the carboxylate that had donated the proton to the phenolic) by preparing and testing α-bromoacetosyringone (ASBr). Such α-haloketones had been used earlier to characterize acid-mediated enzymatic activities. They formed covalent bonds with the carboxylate residue at the active site the enzyme, irreversibly inhibiting its activity (HARTMAN 1971). Similarly, ASBr was shown to be an irreversible

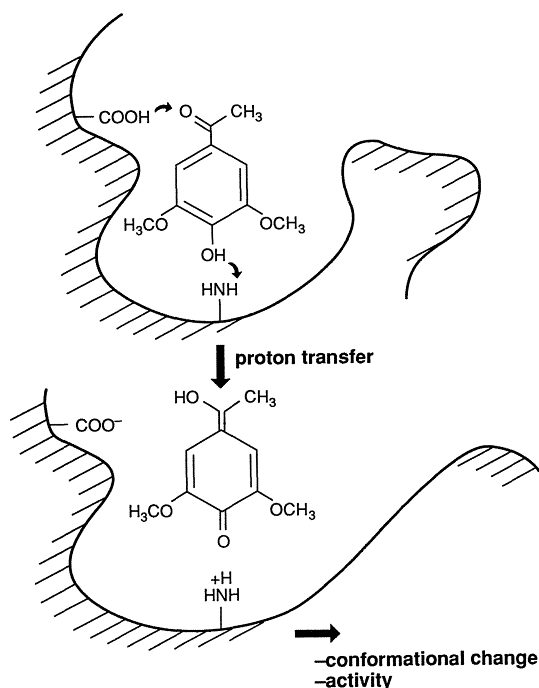

Fig. 2. The proton transfer model of phenolic activation at the phenolic binding protein

inhibitor of the *vir* induction process, but not of other inducible enzyme activities or of growth (HESS et al. 1991). An analog of ASBr, 5-iodo α-bromo acetovanillone (IAVBr), was subsequently shown to be an irreversible inhibitor of *vir* induction and could be prepared to high specific activity for use as an affinity label. Unexpectedly, the results of such studies indicated that VirA did not incorporate radiolabel in vivo or in vitro, even when VirA was overexpressed in *Agrobacterium* (LEE et al. 1992). Rather, two low molecular weight proteins, p10 and p21, were labeled by IAVBr. These proteins were found in the soluble fraction prepared from agrobacteria either with or without the Ti plasmid.

The affinity labeling results suggest that proteins other than VirA may be the phenolic receptor in the *vir* induction system. One problem with this possibility is that searches for genes that are required for phenolic-dependent *vir* gene expression have yielded only *virA*, *virG* and *chvE*. Certainly it is possible that the genes encoding the putative phenol binding proteins have not been mutagenized: genes required for virulence continue to be discovered, e.g. *chvI*, *chvG* (CHARLES and NESTER 1993; MANTIS and WINANS 1993) (see below). However, the fact that LEE et al. (1992) observed two candidate phenolic binding proteins raises the possibility that there is more than one phenolic receptor. Finally, it is possible that the hypothetical phenolic binding protein has not been identified by genetic means because it encodes an essential function.

3.3 Sugar Perception

As described above, phenolic compounds alone are insufficient for maximal induction of the *vir* genes in *Agrobacterium*. Various hexoses that are known to be involved in plant cell wall synthesis (and, hence, wound repair) can enhance sensitivity to the phenolics by at least two log orders in terms of concentration (Shimoda et al. 1990). ChvE, a 31 kDa, chromosomally encoded, periplasmic protein, has been shown to be responsible for this sugar effect (Cangelosi et al. 1990a; Huang et al. 1990a). Since it is strongly homologous to *E. coli* periplasmic ribose binding and galactose binding proteins and almost identical to a periplasmic galactose/glucose binding protein from *A. radiobacter*, ChvE is likely to be the sugar perceiving element in this control system. Cangelosi et al. (1990a) showed that in the presence of low levels of AS, many sugars enhanced *vir* gene induction by wild-type strains of *A. tumefaciens*, whereas those strains with mutated *chvE* did not induce at these AS concentrations.

The periplasmic domain of VirA mediates two types of responses related to the sugar effect: phenolic sensitivity and maximal levels of induction. Sequence analysis indicated that the VirA periplasmic domain contains a region homologous to the sugar transporter protein binding domains of the transmembrane protein, Trg, that is involved in *E. coli* sugar chemotaxis (Cangelosi et al. 1990). The enhancement of phenolic sensitivity by sugars was eliminated when a mutated form of VirA, missing the periplasmic domain, was used (Cangelosi et al. 1990a; Shimoda et al. 1990; Lee et al. 1992). These results indicate that ChvE interacts with this domain of VirA to impart the sugar enhancement of *vir* induction. Interestingly, in the absence of sugar, these mutants were: (1) more sensitive to phenolics than wild type and (2) had significantly higher levels of maximal vir induction than wild type (Cangelosi et al. 1990a; Lee et al. 1992; Machida et al. 1993; Binns et al. 1993). Thus, these changes in the VirA molecule seem to lock the protein in a conformation responsive to AS activation in the absence of inducing sugars. Turk et al. (1993b) have also seen this effect when the periplasmic domain of VirA is replaced with the periplasmic domain of the *E. coli* chemoreceptor, Tar.

Machida et al. (1993) continued this line of investigation by comparing a series of point and deletion mutations in the perplasmic domain of VirA. Several point mutations resulted in forms of VirA that were insensitive to sugar. For example, the single changes of Glu-210 to Val or Gly-211 to Asp were enough to make VirA unresponsive to glucose. Point mutations closer to the second periplasmic domain, Gln-229 to Leu or Glu-235 to Val still allowed VirA to be sugar responsive. Work in this laboratory demonstrated the importance of the Glu-255, a residue conserved in the *virA* gene from four different Ti plasmids (Binns et al. 1993; Banta et al. 1994). When this glutamate was mutated to either glutamine or leucine all sugar enhancement was abolished. In contrast, the mutation Glu-255 to Asp resulted in a wild-type phenotype suggesting that the acidic nature of the residue at this position is critical for activity. This glutamate residue lies outside the predicted site for ChvE interaction and thus may be necessary

for conformational shifts in VirA after it interacts with ChvE. Two further points can be made about the sugar insensitive point mutants. First, contrasting with the large periplasmic deletions, in the absence of inducing sugar these point mutations have a phenolic sensitivity near that of the wild type. Second, in the absence of inducing sugar they induce maximal levels of *vir* gene expression that are significantly higher than wild type (MACHIDA et al. 1993; BINNS et al. 1993). These results suggest that in the absence of sugar some there is negative regulation of VirA and this is observed only in forms of VirA that are responsive to sugar. Certainly this warrants further investigation. Finally, MACHIDA et al. (1993) provided strong evidence for the direct interaction of VirA and ChvE by selecting for point mutations in *chvE* that suppressed the effect of the point mutations in the periplasmic domain of VirA. These suppressor mutations restored sugar enhancement of AS induction in strains carrying the Glu-210 to Val point mutation in VirA.

3.4 pH Perception

A pH of 5.5 or below is necessary for maximal *vir* induction in response to phenolics (STACHEL et al. 1986). Given that wounding and active growth at the wound site would each be expected to acidify the extracellular environment (KAHL 1982; WINANS 1992), the ability of the bacterium to utilize this as a signal is not surprising. Both VirG and VirA are involved in regulation of *vir* induction system by pH. First, pH has a specific effect on *virG* expression. P2, one of two promoters for *virG*, was originally thought to allow its constitutive expression. Work by WINANS and colleagues (WINANS 1990; MANTIS and WINANS 1992) showed that this promoter is primarily induced by acidic growth conditions. Since strains expressing *virG* from a constitutive promoter still exhibit low pH dependence for *vir* induction, it is unlikely that the pH effect on *virG* expression is the sole mechanism by which pH exerts its effect. There is evidence for a direct effect of pH on the VirG molecule. Three groups (HAN et al. 1992; PAZOUR et al. 1992; JIN et al. 1993) have reported that a VirG point mutation, Asn54 to Asp, results in constitutive activation of *vir* gene expression in the presence or absence of VirA. Interestingly, *vir* gene expression in strains carrying this mutant form of VirG is greatly enhanced at low pH. The mechanism by which external pH affects the cytoplasmic VirG protein is unknown.

VirA also plays a role in the pH effect. TURK et al. (1991) demonstrated that strains with *virA* genes from different Ti plasmids (octopine, nopaline, agropine, leucinopine), exhibited varying pH optima and these were dependent on the source of VirA. For example, when the *virA* gene from an octopine type Ti plasmid was moved into a strain carrying a *virA* deficient nopaline type Ti plasmid, the pH optimum was that originally seen in the octopine type Ti plasmid. Substitution of the periplasmic domain of VirA with the periplasmic domain of the *E. coli* chemosensory protein Tar resulted in partial desensitization to pH (MELCHERS et al. 1989b). This data indicates that some, but not all, of the pH effect is generated in

the periplasmic domain. However, a recent report from CHANG and WINANS (1992) demonstrated that the entire periplasmic domain of VirA plus both trans-membrane regions could be deleted without changing the pH effect on *vir* gene expression. Perhaps the Tar portion of the chimeric VirA-Tar hybrid described above is capable of providing an effect on VirA that is normally dependent on pH.

3.5 Other Influences on *vir* Gene Expression

In addition to the signals described above, there are several other factors that appear to influence *vir* gene expression. Mutation in the chromosomal *ros* gene results in constitutive expression of *virC* and *virD*, whereas other *vir* genes are unaffected (CLOSE et al. 1985, 1987; COOLEY et al. 1991). These experiments suggest that the *ros* gene product represses transcription from promoter regions of *virC* and *virD* that would otherwise be active, even in the absence of phenolic inducer. The relationship of this control to virulence is not known, except that *ros* mutants are, in fact, virulent. Several molecules other than AS appear to have positive effects on phenolic mediated *vir* transcription. For example, the opines, produced by the transformed plant cells, can facilitate *vir* induction, though the mechanism of this enhancement is not known (VELUTHAMBI et al. 1989).

An intriguing example of the complexity involved in *vir* expression is derived from the observation that low phosphate can stimulate *virG* transcription even in the absence of inducing phenolic (WINANS et al. 1988; WINANS 1990). Given that the *phoR/phoB* two-component regulatory system is critical in sensing phosphate status in *E. coli* (WANNER 1993) and that the PhoB protein can bind to a portion of the *virG* promoter (AOYAMA et al. 1991), MANTIS and WINANS (1993) searched for *Agrobacterium* phosphate regulatory genes. They screened an *A. tumefaciens* cosmid library for a clone that would restore alkaline phosphatase activity in an *E. coli* strain carrying a *phoB* mutation. An *A. tumefaciens* chromosomal gene, *chvI*, was identified in this screen and shown to have 35% homology to *phoB* of *E. coli*. *A. tumefaciens* strains carrying a *chvI* mutation were avirulent, but, surprisingly, still exhibited low phosphate induction of *virG*. These strains exhibited reduced AS induction of *vir* gene expression and were susceptible to severe growth inhibition by wound sap. The sensor of this two component system, *chvG*, was independently isolated through an entirely different strategy (CHARLES and NESTER 1993). This study showed that *chvG* and *chvI* were both required for virulence, and mutations in either of them caused reduced *vir* gene expression and severely constrained growth characteristics. Neither the genes regulated by the ChvG/ChvI system nor the inducing signals have been identified. Nevertheless, these experiments demonstrate the complexity of the *vir* induction process and the fact that genes affecting it continue to be discovered.

4 *vir* Gene Transcription Activation

Once the various *vir*-inducing signals are perceived, it is necessary to transduce that information in a fashion that will activate *vir* gene transcription. Here the activities of the cytoplasmic domain of VirA and VirG are critical. The COOH-terminal cytoplasmic domain of VirA can be divided into three regions: the linker domain, which is next to the second transmembrane domain; the kinase domain; and the receiver domain (Fig. 1B) (CHANG and WINANS 1992). The linker domain, as described above, appears to be either directly or indirectly involved in phenolic recognition. Similar to all sensor proteins of the two-component signal transduction family, VirA has been found to have an autophosphorylation activity. When provided with ATP, purified VirA readily phosphorylates itself in vitro at a histidine residue conserved in sensor proteins (HUANG et al. 1990b; JIN et al. 1990b; PARKINSON 1993). The phosphate on this histidine, His-474, can then be transferred to a conserved aspartate in VirG, thereby activating it (JIN et al. 1990a).

Sequence analysis has shown that the COOH-terminal "receiver" domain of VirA is highly homologous to the region of the NH_2-terminal domain of VirG that is phosphorylated. This feature is not unique to VirA. Other receiver domains have been found among the sensor proteins in the two-component signal transduction protein family (ARICO et al. 1989; MCCLEARY and ZUSMAN 1990; STOUT and GOTTESMAN 1990; HRABAK and WILLIS 1992). ENDOH and OKA (1993) overproduced and purified two mutants forms of VirA in *E. coli*: one contained the kinase domain only and the other contained the kinase and receiver domains. Both purified proteins, when incubated with [γ-^{32}P]ATP, became phosphorylated, showing that the kinase domain, alone, can autophosphorylate, and this in vitro activity is not affected by the receiver domain. A possible function of the VirA receiver domain, based on its homology to VirG, is that it acts as a competitive inhibitor of the phosphorylation of VirG by the kinase domain of VirA. Induction of VirA with AS would then act by disrupting the receiver domain's interaction with the kinase domain. Removal of the receiver domain of VirA may accomplish the same effect. CHANG and WINANS (1992) showed that by deleting the receiver domain they produced a mutant VirA whose activity was high and AS-independent, if conditions were optimized for induction (pH 5.5, 5 mM glucose). However, ENDOH and OKA (1993) reported a similar experiment that gave different results. Their VirA molecule, without its receiver domain, was unable to induce *vir* gene expression at all. Before we can understand and interpret this result it is imperative to know that this version of VirA was, in fact, stable and present in the bacteria.

Point mutations at four different sites in VirA have been found to cause *vir* gene expression in the absence of AS (PAZOUR et al. 1991; ANKENBAUER et al. 1991). The mutations were located in the first transmembrane domain, the kinase active site, the putative nucleotide binding site and the COOH-terminal receiver domain. In the case of the receiver domain, a mutation might result in a loss of inhibition of VirA phosphorylation activity similar to that proposed for the deletion of COOH-terminal domain (CHANG and WINANS 1992). This might also be true of the mutations in the kinase domain and the putative nucleotide binding site.

However, phosphate can be transferred in vitro from VirA to VirG when the receiver domain of VirA is present (JIN et al. 1990a; ENDOH and OKA 1993), leaving the role of the receiver domain still unresolved. The point mutations in the first transmembrane domain could result in a conformational change that mimics the conformation of wild-type VirA when it is induced with AS.

Before transcriptional activation can occur, VirG must first be phosphorylated by VirA (JIN et al. 1990a). As discussed above, VirA transfers a phosphate from His-474, in its kinase domain, to Asp-52 in the NH_2-terminal receiver domain of VirG. Asp-52 is conserved in all regulators in the protein family of two-component regulatory system and acts as the phosphate acceptor in all systems thus far studied (STOCK et al. 1990). If this aspartate is changed to asparagine through mutagenesis, VirG no longer becomes phosphorylated and loses its ability to activate *vir* gene transcription (JIN et al. 1990a). The NH_2-terminal region of VirG is homologous to CheY, one of the best characterized regulators. The crystal structure of CheY has been determined and it shows that this conserved aspartate is involved in a series of hydrogen bonds or salt bridges with other residues. Phosphorylation disrupts this network causing conformational changes. Mutations that disrupt these interactions would be expected to affect VirG's activity. This is seen in the creation of VirA-independent constitutive mutations of VirG. A single change of either Asn-54 to Asp or Ile-106 to Leu is enough to cause this effect (HAN et al. 1992; PAZOUR et al. 1992; JIN et al. 1993). These mutations might act by forcing the VirG molecule into the same active conformation caused by the phosphorylation of Asp-52.

Activated VirG is thought to initiate *vir* gene transcription as a result of sequence specific interactions with the *vir* gene promoters. It binds to a *cis*-acting regulatory sequence (TNCAATTGAAAPy) called the *vir* box which is found in the 5'-noncoding region of *vir* genes (DAS et al. 1986; POWELL et al. 1989; JIN et al. 1990c). The COOH-terminal domain of the 26 kDa VirG protein specifically binds to this sequence (JIN et al. 1990c). Of interest to the present discussion is that nonphosphorylated forms of VirG can bind to the *vir* box (JIN et al. 1990c; PAZOUR and DAS 1990), but nonphosphorylated VirG, even when overexpressed in *Agrobacterium*, does not activate *vir* gene expression to high levels (PAZOUR and DAS 1990). This suggests that only phosphorylated VirG can interact and activate other components of the transcriptional machinery.

5 Systems Necessary for Physical Interaction Between Plant and Bacterial Cells

5.1 Chemotaxis

The abilities to sense and then move toward a wound site are properties that vastly increase the chance of *Agrobacterium* to physically associate with competent plant cells. *Agrobacterium* has peritrichous flagella and is capable of swimming towards a variety of substances, including sugars and phenolics (PARKE et al. 1987; ASHBY et al. 1988; LOAKE et al. 1988; CANGELOSI et al. 1990a), both of

which are indicative of a wound site. Mutants lacking motility or chemotactic capabilities inoculated into the soil were unable to colonize and transform pea plants, though they were capable of transforming the plant if supplied directly to the wound (HAWES and SMITH 1989). There is evidence that chemotaxis towards sugars occurs in Ti plasmidless strains (LOAKE et al 1988) and may be at least partially mediated through the ChvE protein already discussed in terms of its role in *vir* gene expression (CANGELOSI et al. 1990a; PALMER and SHAW 1992). The ChvE homologs in *E. coli* are sugar receptors involved in chemotaxis as well as sugar uptake. CANGELOSI et al. (1990a) showed that strains which carried Tn*5* mutagenized *chvE* were deficient in chemotaxis toward D-galactose, D-glucose, L-arabinsose, D-fucose, and D-xylose, when compared to wild type. The ability to move toward other sugars remained intact. It is not known whether ChvE mediates it chemotactic effects through the VirA/VirG or other systems, analogous the MCP-CheA-CheY system of *E. coli.*

SHAW and colleagues (ASHBY et al. 1988; SHAW et al. 1988; PALMER and SHAW 1992) showed that strains cured of their Ti plasmid, but carrying *virA*, *virG*, *virB*, and *virC*, showed a chemotactic response to AS at concentrations that do not induce virulence. Interestingly, these experiments were carried out at a pH (7.0) at which *vir* gene expression cannot be induced. If either *virA* or *virG* were not present, no chemotaxis towards AS was observed. In addition, if the residues which become phosphorylated during virulence induction, His-474 on VirA and Asp-52 in VirG, were mutated to Gln and Asn, respectively, the chemotactic response was lost. Taken together, these observations imply that low levels of AS are sufficient to cause phosphorylation of VirA and VirG, that the process mediated has a different pH dependency than that of *vir* gene induction, and that phosphorylation is necessary for this response to occur. One case has been published suggesting that chemotaxis towards some phenolics is chromosomally encoded (PARKE et al. 1987), but these investigators noted no movement towards AS. While experimental protocols and bacterial strains varied slightly, the reasons for the apparent discrepancies are not clear.

5.2 Attachment of Bacteria to the Plant Cell

Once the bacteria reach the wound site, the next physical step necessary for transformation is the attachment of the bacteria to the plant cell. Mutant bacteria unable to bind to plant cells are avirulent (DOUGLAS et al. 1982, 1985; CANGELOSI et al. 1987; MATTHYSSE 1987; THOMASHOW et al. 1987). The products of three *A. tumefaciens* chromosomal loci, *chvA*, *chvB*, and *exoC* (*pscA*) involved in the synthesis and transport of β-1,2- glucans are required for attachment (ZORREGUIETA et al. 1988; CANGELOSI et al. 1989; UTTARO et al. 1990). Mutations at these loci also drastically reduce virulence, though occasional tumors do develop. Interestingly, functional equivalents of *chvA* and *chvB* exist in *R. meliloti*: the *Rhizobium* versions of these genes complement mutations in *Agrobacterium* (CANGELOSI et al. 1987). While the production and export of β-1,2-glucan is necessary for

attachment of *A. tumefaciens* to plant cells, its role in the attachment process is unclear. One known role of periplasmic β-1,2-glucan is osmotic protection, suggesting that loss of the glucan might indirectly affect virulence by reducing the activity of cell surface virulence proteins under suboptimal osmotic conditions (Cangelosi et al. 1990b). This seems unlikely since these *chv* mutants continue to exhibit the avirulent phenotype under high osmolarity conditions in either leaf explant or protoplast cocultivation transformation assays (Cangelosi et al. 1990b; Binns 1991).

Since the *chv* mutants do bind, albeit inefficiently, to plant cells, one possibility is that the β-1,2-glucan in the periplasm helps other proteins (or polysaccharides) involved in attachment find or interact with the plant attachment site. Several other attachment deficient mutants (*att*) have been identified by Mathysse (1987). The mutations identified to date map to a single 12 kilobase chromosomal *EcoRI* fragment distinct from the *chvA*, *chvB* and *exoC* genes. The *att* mutants exhibit greatly reduced attachment and are avirulent on carrot cells. In contrast to the *chvA* and *chvB* mutants the membrane protein profiles of the *att* mutants differs from wild-type cells only in the loss of only a few distinct membrane proteins (Mathysse 1987). The role these proteins have in bacterial attachment to plant cells is unknown.

Several studies have shown that attachment of bacteria to plant cells is saturable (Neff and Binns 1985; Gurlitz et al. 1987) suggesting that specific plant cell wall components are involved. The attachment process occurs in two steps: a reversible interaction between bacterium and plant cell is followed by an irreversible attachment. After the second phase is complete, bacteria cannot be easily washed off the plant cell (Neff and Binns 1985). Bacteria also often aggregate to each other at and around the attachment site. The plant components involved in these processes are not known, although cellulose microfibrils from the bacteria appear to be important (Matthysse 1987). In certain cases, treatment of bacteria with various plant cell wall fractions will inhibit their ability to attach, resulting in an inability to transform the plant cells (Neff and Binns 1985; Neff et al. 1987). Recently, evidence has been presented indicating that vitronectin-like protein in the plant cell wall may play an important role in attachment. Treatment of bacteria with vitronectin blocks their ability to attach and transform, and treatment of plant cells with antivitronectin renders them incapable of attaching bacteria (Wagner and Matthysse 1992). The bacterial components with which vitronectin may interact and the role vitronectin plays in the attachment process are unknown.

One of the problems confronted by agrobacteria is that the extracellular environment of the wound site is expected to result in *vir* induction whether or not the bacteria are attached to the plant cells. This results in the production of both the T complex and the membrane bound components of what is proposed to be the T complex transport apparatus. Yet several workers have not been able to detect any T complexes, or components thereof, in vitro in induction media (J. Ward; E. Dale; P. Christie, personal communications). These results suggest that as yet undescribed signals, possibly generated at the attachment site, are critical

for the initiation of transport or for the construction of competent transport complexes at the attachment site.

6 Concluding Remarks

The signaling activities necessary for *Agrobacterium* to initiate and complete the DNA transfer process are complex and only partially understood. While much of the signaling necessary to initiate the expression of the *vir* genes revolves around conserved two-component regulatory systems, the mechanism of signal recognition and transduction are vibrant areas of inquiry. The recent studies concerning signal perception indicate that the concept of a two-component regulatory scheme may be confining. Rather, it appears that these represent ancient and conserved components in a regulatory scheme that can be integrated through a series of other proteins into a system that recognizes and responds to the environmental stiumuli in question. Analysis of the physical interaction of bacterium with the plant cells and the signaling activities generated by bacterial attachment to the plant cell are less advanced, yet represent a critical aspect of the pathogenic interaction that is under discussion. Clearly, the combination of chemical, biochemical and molecular genetic approaches will play a critical role in unraveling these issues.

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Does *Rhizobium* Avoid the Host Response?

H. I. McKHANN[1] and A. M. HIRSCH

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1 Overview

Nodule development begins when the first cell division of a mitotically quiescent cortical cell is induced by *Rhizobium* (*sensu lato*) in the root of its host plant. This cell division brings about a change in cortical cell fate; the derivatives of these cell divisions form a new organ, the nodule. Prior to the initial anticlinal cell division in the root cortex, numerous interactions between the host and symbiont have already taken place, including the chemotaxis of rhizobia to the legume root, the induction of rhizobial *nod* genes by plant-derived flavonoids, the production of Nod factor—a substituted lipo-oligosaccharide—root hair deformation, and shepherd's crook formation (the formation of a 360° curl). The latter two responses are the first visible signs of the interaction. The bacteria enter the curled root hair, presumably by degrading the plant cell wall (CALLAHAM and TORREY

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1981; see also DART 1977), and evoke the formation of an infection thread, in which the bacteria are contained. The infection thread, formed from an invagination of the plant membrane and the deposition of cell wall material, extends into the root cells toward the nodule primordium. The nodule primordium originates from cortical cell divisions that take place in advance of the extending infection thread (NEWCOMB et al. 1979). Eventually, bacteria, which have multiplied within the matrix of the infection thread, are released from its end, and become enclosed within a host-derived membrane, designated the peribacteroid membrane. The bacteria, enclosed within this membrane, differentiate into novel forms, bacteroids capable of nitrogen fixation. They remain surrounded by host cell membrane until either the plant or bacteroid cells senesce. In response to the interaction with rhizobia, the plant expresses nodule-specific proteins (nodulins; LEGOCKI and VERMA 1980), which have been classed as early (those involved in nodule morphogenesis) and late (those involved in nodule maintenance and function) (GOVERS et al. 1987). *Rhizobium* infection and nodule development (Fig. 1) have been recently reviewed by BREWIN (1991), KIJNE (1992), and HIRSCH (1992).

The interaction of rhizobia and the host during nodule development and the similarity of some of the host responses have given rise to the idea that *Rhizobium* evolved from a parasitic or pathogenic microorganism (see reviews by

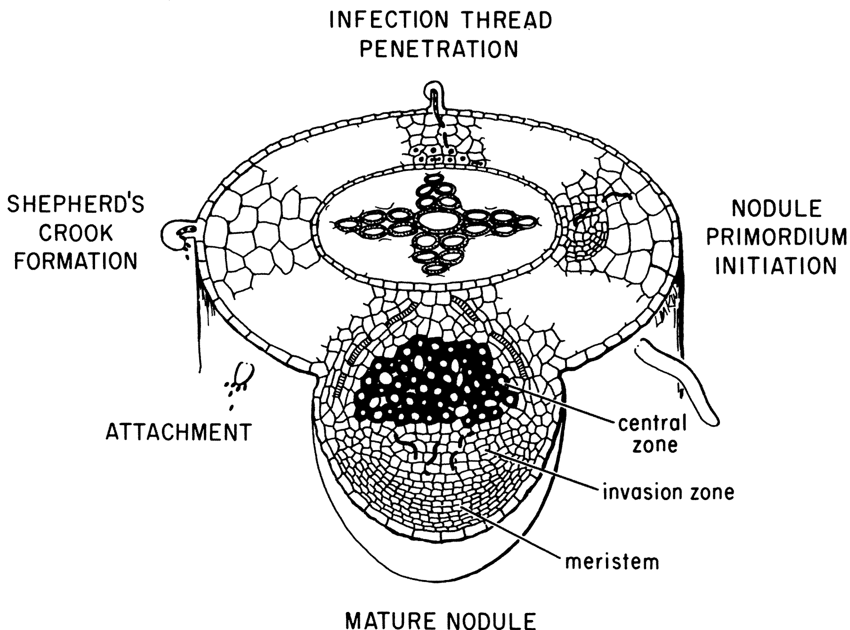

Fig. 1. The major stages of alfalfa nodule development illustrated for convenience at the same level in the root. Note that the stele, here shown as tetrarch, is normally triarch

VANCE 1983; DJORDJEVIC et al. 1987; LONG and STASKAWICZ 1993). During the initial stages of the interaction, when they are invading the plant and before nitrogen fixation takes place, rhizobia colonize their hosts, but in a restricted manner. This colonization is considered to be similar to the ancestral condition in which bacteria lived saprophytically and nonspecifically on plant exudates or remains (LONG and STASKAWICZ 1993). However, other manifestations of the interaction are reminiscent of a pathological interaction. These include the infection thread, which resembles the appositional wall structures formed during penetration by fungal pathogens (see references in VANCE 1983), and the induction of the early nodulins, many of which are proline-rich proteins (FRANSSEN et al. 1987; SCHERES et al. 1990; LÖBLER and HIRSCH 1993), along with the enzymes of the phenylpropanoid biosynthetic pathway. Furthermore, when the symbiosis fails, as when infection threads abort in effective associations or when ineffective (non-nitrogen-fixing) nodules are formed, a "hypersensitive response," a term normally applied to plant-pathogen interactions, has been said to occur (DJORDJEVIC et al. 1988; VASSE et al. 1993).

The closest relative of *Rhizobium* and *Bradyrhizobium* is the plant pathogen *Agrobacterium,* suggesting that rhizobia have similar characteristics. However, *Agrobacterium* is a unique pathogen. Although crown gall and hairy root syndromes are classified as diseases (see LONG and STASKAWICZ 1993), agrobacteria, unlike typical plant bacterial pathogens, do not cause extensive host necrosis. Like their symbiotic cousins, *A. tumefaciens* and *A. rhizogenes* elicit cell divisions that result in hyperplasias. And, in contrast to other plant diseases, neither the tumors nor the hairy roots are inhabited by agrobacteria. The *Agrobacterium*-induced hyperplasias are transformed. A segment of bacterial DNA, the T-DNA, is transferred to the host cell nucleus where it resides. The details of the plant-*Agrobacterium* interaction can be found in reviews by WINANS 1992; ZAMBRYSKI 1992; LONG and STASKAWICZ 1993; and the chapter by BINNS and HOWITZ in this volume.

A major difficulty with the hypothesis that *Rhizobium* is a highly sophisticated pathogen is that it assumes that modern-day plant pathogens serve as the ancestral paradigm. Instead, prokaryotic plant pathogens today represent highly derived groups that are evolutionarily distinct from the Rhizobiaceae (Fig. 2). We believe that the plant-pathogen interaction is an unsatisfactory model for describing the *Rhizobium*-legume association even though ineffective rhizobia provoke a host response that superficially resembles encounters with an incompatible pathogen. As we shall see, many of the host responses are consistent with the occurrence of a nonspecific biotic interaction or with abiotic stresses such as wounding or senescence.

Before we review the evidence for and against *Rhizobium's* ability to provoke a response resembling that elicited by a plant pathogen, we will define the gene-for-gene recognition system and what is meant by the term hypersensitive response. We will then apply these concepts to the symbiotic association, focusing on two major points in nodule development (1) the initial stages (the first 5 days); and (2) the mature nodule (2–3 weeks postinoculation). We will

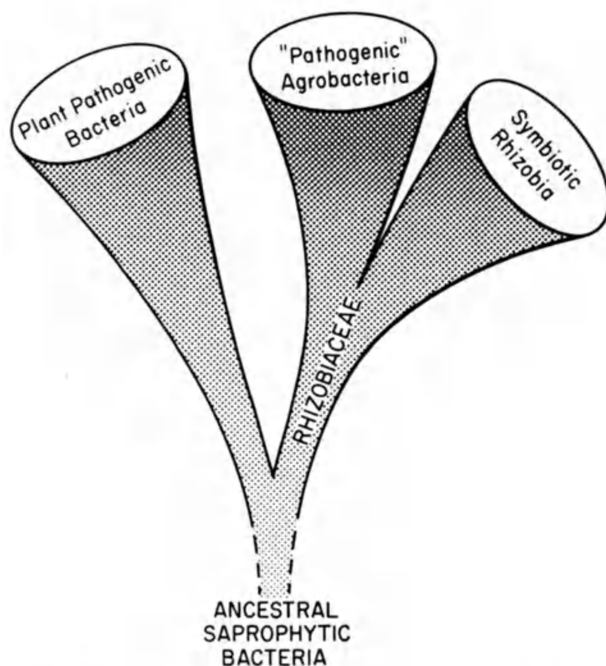

Fig. 2. Evolutionary relationships of modern day plant-interacting bacteria and their presumed saprophytic ancestor

concentrate, for the sake of convenience, on the association between fast-growing rhizobia (*Rhizobium sensu stricto*) and legumes that form indeterminate nodules; however, we will bring in examples of determinate nodules where appropriate. We will pay particular attention to the role of the phenylpropanoid biosynthetic pathway in nodulation because its products are typically involved in host defense. Finally, we will suggest some other ways of visualizing the *Rhizobium*-legume tête-à-tête.

2 The Hypersensitive Response

The conceptual framework for plant–pathogen interactions rests on the pioneering work of Flor (1955) on race-specific resistance to fungal pathogens and the presentation of evidence for the gene-for-gene hypothesis. This concept can be applied to viral, bacterial, and fungal pathogens. In brief, in an incompatible interaction, if a host has a single dominant resistance (*R*) gene and the pathogen has a single dominant avirulence (*A* or *avr*) gene, the pathogen is recognized by the host and elicits a defense reaction, part of which is characterized by the hypersensitive response (HR), a localized, relatively rapid (less than 24 h) death of host cells at the point of infection (Klement 1982). Potential product(s) of *R* are currently being investigated; most are assumed to be receptors for or part of the

signal elicitors encoded by transduction chain avirulence genes (see review by KEEN 1992). In a compatible reaction, if the susceptible host lacks an *R* gene or if the pathogen lacks an avirulence gene, the virulent pathogen is not recognized by the host and causes a disease.

The HR is thought to be an important part of disease resistance; its outcome is the formation of water-soaked lesions that undergo necrosis. Changes in respiration and membrane permeability resulting in electrolyte leakage occur within hours after infection (or within minutes after addition of elicitor) (KLEMENT 1982; LAMB et al. 1989). The water-soaked lesions become lignified, impeding the progression of the pathogen into plant tissues. Phytoalexins, low molecular weight antimicrobial compounds, are also produced in the infected tissue. These latter responses are often called defense responses and involve induction of mRNAs for enzymes of the phenylpropanoid biosynthetic pathway. Polymers with structural roles such as callose and hydroxyproline-rich glycoproteins (HRGPs) as well as enzymes like peroxidases for increasing the cross-linking of cell walls and PR (pathogenesis-related) proteins such as glucanses and chitinases that damage the pathogen are also produced (see reviews by BOWLES 1990; KEEN 1992). The responses of the host to elicitors, incompatible, and compatible bacteria as well as the approximate timing of appearance of the various host reactions are illustrated in Fig. 3.

Bacterial genes involved in the hypersensitive reaction and pathogenicity (*hrp* genes) control the ability of pathogens to elicit an HR on non-host and resistant cultivars, as well as to generate disease symptoms on susceptible cultivars (see chapters by COLLMER and BAUER, and BONAS in this volume and reviews by WILLIS et al. 1991; LONG and STASKAWICZ 1993). Mutations in *hrp* genes result in bacteria that can no longer induce these host responses (LINDGREN et al. 1986, 1988). Recently, JAKOBEK and LINDGREN (1993) reported that the HR can be separated from defense gene transcription. Using a Hrp^- mutant of *Pseudomonas syringae* pv. *tabaci,* these investigators found that transcripts for enzymes of the phenylpropanoid biosynthetic pathway, phenylalanine ammonia lyase (PAL), chalcone synthase (CHS), and chalcone isomerase (CHI), as well as transcripts for chitinase, accumulated in infiltrated bean leaves even though an HR did not occur. The timing of transcript accumulation was similar to that seen with a wild-type strain in which the HR took place. Furthermore, inoculation with various bacteria—*Escherichia coli* (nonpathogenic bacteria, like *E. coli*, do not elicit an HR), *P. fluorescens*, heat-killed *P. syringae* pv. *tabaci*, and *P. syringae* pv. *tabaci* that had been treated with protein synthesis inhibitors—led to accumulation of defense transcripts and phytoalexin production without the ensuing HR. Treatment with water or *P. syringae* pv. *phaseolicola*, a compatible pathogen, did not lead to transcript accumulation (JAKOBEK and LINDGREN 1993). These results suggest that defense transcripts are induced nonspecifically, while the HR involves a different specific mechanism. Moreover, these findings imply a strict definition for the HR: the HR is limited to the host responses brought about by infection with incompatible pathogens that have either avirulence or *hrp* genes (JAKOBEK and LINDGREN 1993).

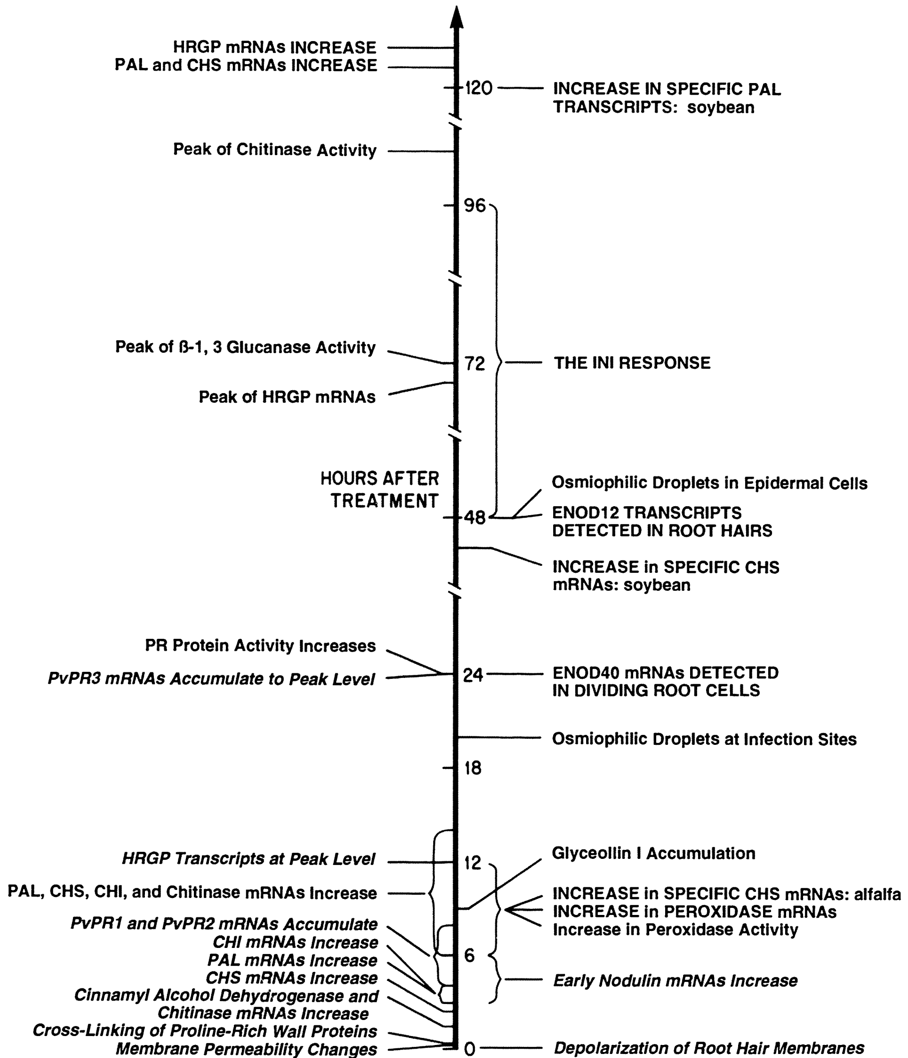

Fig. 3. Timetable of a plant's response to elicitor (*italics*), incompatible (*lower case*), and compatible (*capital letters*) pathogens, including viruses, bacteria, and fungi (*left*). Timetable of the response of the host legume to Nod factor (*italics*), ineffective (*lower case*), and effective (*capital letters*) rhizobia (*right*). *HRGP*, hydroxyproline-rich glycoproteins; *PAL*, phenylalanine ammonia lyase; *CHS*, chalcone synthase; *CHI*, chalcone isomerase; *PR*, pathogenesis-related; *PvPr*, *Phaseolus vulgaris* pathogenesis-related. References for the hypersensitive response are: BRADLEY et al. 1992; CRAMER et al. 1985; EDWARDS et al. 1985; HEDRICK et al. 1988; JAKOBEK and LINDGREN 1993; KOMBRINK et al. 1988; LEGRAND et al. 1987; LOW and HEINSTEIN 1986; RYDER et al. 1984; SHARMA et al. 1992; SHOWALTER et al. 1985; TEMPLETON and LAMB 1988; and WALTER et al. 1990. For the *Rhizobium*-legume symbiosis, the references are: DJORDJEVIC et al. 1988; EHRHARDT et al. 1992; ESTABROOK and SENGUPTA-GOPALAN 1991; Fang, Asad, and Hirsch, unpublished results; McKhann, Fang Paiva, Dixon, and Hirsch, submitted; PICHON et al. 1992; RECOURT et al. 1992a; SCHERES et al. 1990, and YANG et al. 1993.

3 The Initial Stages of the Symbiotic Interaction—Bacterial Point of View

3.1 Rhizobial *nod* Genes

The relative ease of performing genetic studies on rhizobia has led to a much greater understanding of the bacterial genes involved in nodulation compared to the plant genes. Early events in nodulation are mediated by the nodulation (*nod*) genes. These are categorized as "common *nod* genes" (for example, *nodABC* genes, which can be complemented by genes of heterologous rhizobia) and host-specific *nod* genes (for example, *nodEFGH* genes in *R. meliloti,* which cannot be complemented by genes of heterologous rhizobia) (DOWNIE and JOHNSTON 1986). In fast-growing *Rhizobium* species, the *nod* genes are present on plasmids called symbiotic plasmids (pSym), and in slow-growing *Bradyrhizobium* species, the *nod* genes are on the chromosome.

Analysis of how the products of the *nod* genes mediate nodulation events has focused on studying the phenotypes of rhizobial mutants blocked at different steps in nodule development (see review by LONG 1989). The *nod* genes were initially identified by mutations that eliminated nodulation. Other mutations were subsequently identified that either delayed nodulation or changed the host range of the bacterium. Nodulation genes are present in all rhizobial species and are clustered in several operons that share common promoter features, including a consensus sequence called the "nod box."

The *nodD* gene regulates the other known *nod* genes via NodD binding to the nod boxes in the promoters of the *nod* gene operons. Some rhizobial species have a single *nodD* gene, for example, *R. leguminosarum* bv. *trifolii* and *R. leguminosarum* bv. *viciae* (DJORDJEVIC et al. 1985; DOWNIE et al. 1985), whereas others have more than one *nodD* and may have a complex regulatory circuit for controlling *nod* gene expression (MULLIGAN and LONG 1989). *R. meliloti,* with three *nodD* genes, *nodD1, nodD2,* and *nodD3* (GOTTFERT et al. 1986; HONMA and AUSUBEL 1987), as well as the *nodD* homolog *syrM* (MULLIGAN and LONG 1989), has been thoroughly studied. Each copy of *nodD* appears to be regulated independently (HONMA et al. 1990; MULLIGAN and LONG 1989). NodD1 activates *nodABC* in the presence of crude seed exudates, plant washes, or the flavonoid luteolin (see review by ROLFE 1988). The major NodD2-activating compounds have been identified as the betaines, trigonelline and stachydrine (PHILLIPS et al. 1993). These NodDs are hypothesized to regulate transcription by undergoing a conformational change brought about by the inducers (SCHLAMAN et al. 1992). By contrast NodD3 is unaffected by inducers and causes a basal level of *nodABC* gene expression.

3.2 Nod Factor Triggers the Host's Response—Is It an Elicitor?

Early studies on the nodulation genes supposed that the *nod* gene products were themselves involved in initiating the symbiosis. Now, it has been shown that most of the known *nod* genes contribute to the synthesis of a complex signal molecule, a lipo-oligosaccharide known generically as Nod factor (Lerouge et al. 1990; Roche et al. 1991a,b; Spaink et al. 1991; Schulze et al. 1992; Sanjuan et al. 1992; Price et al. 1992; Martinez et al. 1993; Mergaert et al. 1993). Each rhizobial species synthesizes a family of related molecules, based on a $(\text{glucosamine})_n$ backbone with a fatty acid side chain on one end and various substitutions on the reducing end. The major *R. meliloti* Nod factor, called NodRm-IV(S), is a sulfated β-1,4-tetraglucosamine with three amino groups acetylated and one acylated with a C_{16} bi-unsaturated fatty acid (Lerouge et al. 1990). Some investigators have noted the resemblance of the glucosamine backbone to chitin elicitors produced by fungal pathogens (Ehrhardt et al. 1992; Niehaus et al. 1993). However, fungal elicitors are nonspecific and can often induce an HR on a large number of hosts, whereas Nod factor is extremely host-specific, affecting only the legume that the particular *Rhizobium* species nodulates. Therefore, Nod factor is not an elicitor in the same sense as the elicitors derived from plant pathogens.

4 The Initial States of the Symbiotic Interaction—Plant Point of View

4.1 Is There an *R* Gene in the Legume Host?

If *Rhizobium* evolved from a pathogen, one might expect to find a system analogous to the gene-for-gene interaction in the legume-*Rhizobium* symbiosis. The resemblance of Nod factor to chitin elicitors has already been noted, so it seems reasonable to assume that there is a receptor or other plant gene product that interacts with the Nod factor. Genetic analysis indicates that a potential *R* gene is dominant. The Nod^- genotype in legumes results from a recessive mutation. Some 25–30 loci may be involved in producing a Nod^+ pea plant (see review by Phillips and Teuber 1992), making it difficult to predict the identity of the gene product of each locus. However, only one mutant gene could produce a Nod^- phenotype.

There are several examples of what appears to be a gene-for-gene interaction in the *Rhizobium*-legume symbiosis. *Trifolium subterraneum* (subclover) cv. *Woogenellup* is not nodulated by several *R. leguminosarum* bv, *trifolii* strains, including TA1, which effectively nodulates other *T. subterraneum* cultivars. Mutations in the *nodMNX* operon on pSym or in the gene *csn-1*, located on the chromosome, restore nodulation on *T. subterraneum* cv. *Woogenellup* to TA1, indicating that these genes function as negatively acting host-range determinants (Lewis-Henderson and Djordjevic 1991a). In contrast, *nodT* from strain ANU843 is

a positively acting determinant, enabling TA1 to nodulate *T. subterraneum* cv. *Woogenellup*. A single recessive host gene, termed *rwt1*, confers the inability to be nodulated by TA1 (LEWIS-HENDERSON and DJORDJEVIC 1991b).

In soybean, single dominant genes, the *Rj2-4* genes, are involved in limiting nodulation by specific rhizobial strains (see review by TRIPLETT and SADOWSKY 1992). A single gene has also been found in plant introduction (PI) genotypes of soybean that specifically exclude nodulation by certain competitive members of *B. japonicum* serocluster 123 (KEYSER and CREGAN 1987). On the bacterial side, *nolA* has been identified as a gene, the product of which enables members of serocluster 123 to nodulate the PI genotypes (SADOWSKY et al. 1991). Thus, gene-specific nodulation (GSN) in soybean parallels the gene-for-gene recognition system in that dominant genes are present in both the plant and the restricted bradyrhizobia. However, defense transcripts did not accumulate after infection of *Rj4* soybeans by USDA 61, the restricted strain (STOKKERMANS et al. 1992). Moreover, bradyrhizobial strains that nodulate *Rj4* soybeans have a different profile of Nod factors than the restricted strains (STOKKERMANS et al. 1992).

One of the best known examples of the gene-for-gene interaction in symbiosis is the *R. leguminosarum* bv. *viciae* TOM-*Pisum sativum* cv. *Afghanistan* interaction (LIE 1971). Afghanistan pea is not nodulated by European strains or *R. leguminosarum* bv. *viciae* that lack the *nodX* gene found in strain TOM (DAVIS et al. 1988). FIRMAN et al. (1993) have shown that NodX is an *O*-acetyl transferase responsible for the production (along with other *nod* operon gene products) of a novel nodulation factor—NodRlv-V (Ac, Ac C18:4). The inability to nodulate on the plant side is conditioned by a gene *sym2*. Peas with the *sym2* allele can no longer be nodulated by western *R. leguminosarum* bv. *viciae* strains unless they contain *nodX*. The TOM-Afghanistan pea symbiosis, like the others described above, is suggestive of a gene-for-gene recognition system and implies that the novel Nod factor produced by TOM is recognized by a *sym2*-encoded receptor. However, as FIRMAN et al. (1993) have pointed out, the genetic data do not fit this model because *sym2* is recessive, meaning that there has been a loss of gene function rather than a modification of a potential receptor for strain TOM's Nod factor.

In summary, the gene-for-gene recognition system in plant-pathogen interactions is not the same as the symbiotic recognition system. Although dominant genes are involved, *nod* for the rhizobia and an unknown *"R"* for the plant, the outcome upon recognition is nodule formation and *not* failure to nodulate. Thus, as RECOURT et al. (1992a) have observed, the *nod* genes can be thought of as being involved in a compatible reaction and represent virulence genes. This differs from the plant-pathogen interaction, in which elicitors, which trigger an HR—an incompatible reaction—are encoded by avirulence genes. Also, although recognition of the symbiont involves recognition of Nod factor, a "compatible" and highly host-specific elicitor, it is likely that other cell surface molecules are recognized by the plant as well (see below).

4.2 Host Responses from Zero to Twenty-Four Hours

One of the earliest responses to Nod factor is the initial depolarization of root hair cell membranes (EHRHARDT et al. 1992). Addition of 10^{-7} *M* Nod factor results in a rise in proton efflux from the root hair cell within 5 min (COX et al. 1993). These early changes in membrane dynamics in response to Nod factor are in some ways similar to an HR. However, as stated earlier, in contrast to elicitor, Nod factor, which generates the change in the host's membrane potential, results from an effective or "compatible" reaction (RECOURT et al. 1992a).

Other early responses to Nod factor and *Rhizobium* inoculation include the expression of the early nodulin genes, particularly ENOD12 (PICHON et al. 1992; VIJN et al. 1993; Fang, Asad, and Hirsch, unpublished results) and ENOD40 (VIJN et al. 1993; Fang, Asad, and Hirsch, unpublished results). Even though the ENOD genes are induced within 3–6 h after addition of rhizobia or Nod factor, their expression is not correlated with the "pathogenic" nature of *Rhizobium*: ENOD gene expression does not increase following inoculation with ineffective rhizobia. Moreover, not only do wild-type and mutant bacteria elicit the expression of ENOD12 and ENOD40, but cytokinin does also (Fang, Asad, and Hirsch, unpublished results), suggesting that these early nodulins are related primarily to nodule development and secondarily to the interaction with rhizobia.

Induction of mRNAs for the enzymes of the phenylpropanoid biosynthetic pathway is, by contrast, correlated with the presence of bacteria. Using gene-specific probes and sensitive RNAse protection assays, we found that transcripts for two members of the large CHS gene family in alfalfa increased in abundance within 6 h after inoculating alfalfa roots with wild-type *R. meliloti* compared to uninoculated plants (McKhann, Fang, Paiva, Dixon, and Hirsch, submitted). A similar increase was seen for CHI transcripts, but unlike CHS, CHI is encoded by only one or two genes in alfalfa (MCKHANN and HIRSCH, 1994). RECOURT et al. (1992a) found that CHS is expressed at higher levels in vetch roots 12 h after inoculation, with a peak at 24 h, using wild-type *R. leguminosarum* bv. *viciae,* and LAWSON et al. (1994), using PCR to analyze total CHS gene expression in clover, detected elevated levels of transcripts within 6 h after inoculation with *R. leguminosarum* bv. *trifolii.*

In contrast to RECOURT et al. (1992a), we found that the 6 h symbiotically enhanced CHS peak also appeared following inoculation with rhizobia that are defective in nodulation. Exo^- *R. meliloti* mutants as well as heat-killed wild-type rhizobia induced the accumulation of CHS transcripts over the uninoculated controls (McKhann, Fang, Paiva, Dixon, and Hirsch, submitted). The induction of CHS gene expression by mutant and heat-killed rhizobia is reminiscent of the experiments of JAKOBEK and LINDGREN (1993) and suggests that the initial 6 h burst in CHS gene expression is a nonspecific plant response.

Another approach to determine whether *Rhizobium* elicits "defense" transcripts is to infiltrate leaves with rhizobia as is typically done for a plant pathogen. This experiment resulted in the observation that very little cell necrosis occurred (McKhann, unpublished results; R. Esnault, personal communication). In addition, no fundamental difference was detected between rhizobia or nonpathogenic

bacteria in the levels of defense transcripts accumulated in response to infiltration (R. Esnault, personal communication). The kinetics of transcript accumulation in response to rhizobia and nonpathogenic bacteria also differed significantly from the kinetics exhibited in response to incompatible bacteria (ESNAULT et al. 1993), further indicating that the plant responds to rhizobia and nonpathogenic bacteria in the same, nonspecific way.

Early changes in peroxidase gene expression and activity also take place after inoculation. Northern blot analysis with a gene-specific probe showed that mRNA levels of a specific peroxidase from *Medicago truncatula* increased within 3 h, with maximal expression at 4 h, after inoculation with *R. meliloti* (K. Vandenbosch, personal communication). Total peroxidase activity was also found to increase in clover root hairs 6 h after inoculation. However, SALZWEDEL and DAZZO (1993) observed a greater increase following inoculation with the heterologous *R. leguminosarum* bv. *viciae* than with the homologous *R. leguminosarum* bv. *trifolii.* In the latter interaction, the onset of peroxidase activity was delayed. Also, in the heterologous relationship, staining for peroxidase was localized over the entire deformed root hair, especially the tip, whereas in the homologous combination, staining was found only where the infection thread was initiated, even 5 days after inoculation. Similarly, total peroxidase staining increased significantly in subclover root hairs inoculated with strain TA1, whereas strain ANU843 caused a little staining at the point of entry (M. de Boer and M.J. Djordjevic, personal communication).

Although no difference was found between restricted and nonrestricted *B. japonicum* strains in the level of CHS transcript accumulation in *Rj4* soybean, STOKKERMANS et al. (1992) observed that there was an increase over the controls 6 h after inoculation. Using a sensitive radioimmunoassay, SCHMIDT et al. (1992) found that inoculation of soybean with a wild-type strain of *B. japonicum* caused an increase of up to 50-fold in glyceollin I, a soybean phytoalexin, in root exudates as compared to root exudates of uninoculated seedlings. Maximum levels were reached within 10 h after inoculation. A lower level of glyceollin I was observed in root hairs at the same period. However, the levels detected in root exudates were much lower than those observed in pathogen-inoculated root exudates, leading the authors to suggest that wild-type *B. japonicum* suppresses the host's reaction (see below). Glyceollin I accumulation was also seen in response to the addition of supernatant, a suspension of autoclaved cells, or the supernatant of broken cells of *B. japonicum.* Cell extracts of *R. meliloti* and *R. fredii* induced glyceollin I accumulation in treated seedlings to relatively high levels after 20 h of incubation, but *R. leguminosarum* cell extracts did not. No correlation was found between glyceollin I accumulation and ability to nodulate soybean. SCHMIDT et al. (1992) also found that inhibition of flavonoid synthesis using an inhibitor of PAL, (*R*)-(1 amino-2-phenylethyl) phosphonic acid, during the first 20 h of the interaction led to a decrease in nodule number, contrary to what would be predicted if the glyceollin I accumulation were related to a defense response.

In summary, some of the earliest responses of the plant to rhizobial inoculation mimic a plant-pathogen interaction in that defense-related genes are transcribed. However, the accumulation of defense transcripts soon after

Rhizobium inoculation appears to be a nonspecific response. The earliest (6 h) induction of the genes for the enzymes of the phenylpropanoid biosynthetic pathway is elicited by either live or dead rhizobia, and the slightly later (10–20 h) increase in glyceollin I production is also induced by autoclaved or broken bacterial cells. In any case, defense transcripts accumulate to low levels compared to inoculation with an incompatible pathogen. We propose that the earliest expression of genes for enzymes of the phenylpropanoid biosynthetic pathway occurs as a nonspecific response similar to what JAKOBEK and LINDGREN (1993) reported for Hrp⁻ mutants of *P. syringae*. Furthermore, we propose that the response to rhizobia is not related to defense. A similar conclusion has already been reached by RECOURT et al. (1992a). However, the situation for peroxidase is still equivocal. Heterologous rhizobia or ineffective strains appear to elicit more peroxidase activity than homologous wild-type strains. However, as is the case in CHS gene expression, inoculation with the homologous strain results in an early (< 6 h) elicitation of specific peroxidase gene expression.

4.3 Forty-Eight Hours to Five Days: The Ini Response

In addition to the flavonoids that activate the expression of rhizobial *nod* genes, there is evidence that a positive feedback occurs so that inoculation of legumes with *Rhizobium* leads to increased flavonoid excretion from the root. This is termed the Ini—increase in nod gene inducing activity—response, which was originally observed in the root exudate of *Vicia sativa* subsp. *nigra* after inoculation with *R. leguminosarum* bv. *viciae*, but not in response to inoculation with heterologous rhizobial strains (VAN BRUSSEL et al. 1990). The Ini response has also been reported to occur in reponse to rhizobial Nod factor (RECOURT et al. 1992a). In some cases, flavonoids other than the *nod* gene-inducing flavonoids are also produced as part of the Ini response. These are thought to result from de novo synthesis because inoculation with *R. leguminosarum* bv. *viciae* leads to 1.5- to 2.0-fold increases in PAL activity, CHS mRNA level, and eriodictyol methyltransferase activity (RECOURT et al. 1992a,b).

An Ini response also appears in alfalfa after inoculation with wild-type *R. meliloti* (DAKORA et al. 1993; McKhann, Fang, Paiva, Dixon and Hirsch, submitted). DAKORA et al. (1993) identified formononetin 7-*O*-(6"-*O*-malonyl-glycoside) (FGM) as well as aglycone and glycoside forms of the alfalfa phytoalexin medicarpin in root exudates from inoculated alfalfa. These compounds were not found in uninoculated alfalfa root exudates. However, alfalfa root exudate from plants inoculated with a heterologous *Rhizobium* strain, *R. leguminosarum* bv. *phaseoli*, also showed that an Ini response had occurred, although at lower levels than when exudates from *R. meliloti*-inoculated plants were examined (65% vs 200% increase in *nod* gene inducing activity; DAKORA et al. 1993). Correlated with the Ini response is an increase in CHS and CHI gene expression that occurs at the same time as the release of flavonoids, 2–4 days after inoculation (McKhann, Fang, Paiva, Dixon, and Hirsch, submitted). Using in situ hybridization, we

observed that CHS transcripts accumulated in epidermal and root hairs cells of spot-inoculated roots (McKhann, Fang, Paiva, Dixon and Hirsch, submitted). Moreover, we found that alfalfa roots inoculated with exo-mutants produced both medicarpin and FGM, which were not secreted into the exudate.

In soybean, there is conflicting evidence for an increase in flavonoids or for differences in gene expression following inoculation with *B. japonicum*. GRAHAM (1991) studied the *nod* gene-inducing activity of root exudates from uninoculated roots and found that conjugates of the *nod* gene inducers daidzein and genistein were selectively excreted from roots as well as from seeds in a continuous, saturable process. CHO and HARPER (1991) found similar levels of isoflavonoids in root extracts from uninoculated wild-type, hypernodulating, and non-nodulating soybean seedlings. This is consistent with the results of MATTHEWS et al. (1989), who found that there was no difference in *nod* gene-inducing flavonoids among root exudates obtained from 3-day-old uninoculated wild-type, non-nodulating, or supernodulating plants. SUTHERLAND et al. (1990) demonstrated that root extracts from uninoculated 12-day-old seedlings of non-nodulating, supernodulating, or wild-type plants had *nod* gene-inducing activity that was similar to the activity of extracts from inoculated wild-type plants. In contrast, CHO and HARPER (1991) found increased concentrations of isoflavonoids, some of which were Ini flavonoids, 9–12 days after inoculation in root extracts of hypernodulating soybean mutants compared to the wild-type soybean. Moreover, the non-nodulating cultivar, when inoculated, was shown to contain more flavonoids than the wild-type control (CHO and HARPER 1991). These differences in results may be related to the fact that two cultivars—*Bragg* vs *Williams*—were studied. In any case, rhizobial inoculation provokes the production of flavonoids in roots that form determinate nodules, but the flavonoids do not appear to be excreted into the root exudate. Also, they are produced later during development of determinate nodules than of indeterminate nodules.

WINGENDER et al. (1989) examined CHS expression in soybean and found that *B. japonicum* inoculation did not cause a change in CHS transcript levels up to 10 h after inoculation. Inoculation with *Agrobacterium tumefaciens*, however, induced CHS expression in soybean roots and cell cultures within 2 h. Elevated levels of CHS transcripts were observed 16 and 28 days postinoculation in both uninoculated and inoculated roots. CHS expression was not enhanced in the roots exclusive of nodules until 19–30 days after inoculation and in nodules 19–23 days after inoculation. In contrast, ESTABROOK and SENGUPTA-GOPALAN (1991) examined gene expression leading to flavonoid biosynthesis in soybean roots using specific probes for PAL and CHS and found evidence for enhanced expression of specific gene family members of PAL and CHS at 4 days and 1–2 days, respectively, in response to *B. japonicum* inoculation. This effect was enhanced in a supernodulating mutant of soybean.

In summary, these results indicate that in indeterminate nodules the increase in defense-related transcripts is related to the Ini response. New flavonoids do not appear to be secreted into the medium by inoculated soybean seedlings, but are detected in root extracts. In addition, the plant responds to inoculation by turning

on genes, and in the two cases studied, a specific subset of genes, for enzymes of the phenylpropanoid pathway, suggesting that this is a normal response of alfalfa and soybean to rhizobial inoculation. Although phytoalexins are detected in the exudate, the levels are significantly below those present after pathogen infection. These results imply that a defense response does not occur during this stage of the symbiosis, at least in response to inoculation with wild-type rhizobia. Not enough studies have been done to generate conclusions about the consequences of inoculating with mutant or heterologous rhizobia.

5 What Happens When Nodulation Fails?

Infection threads are not visible within root hairs until more than 12 h after inoculation. Moreover, because not all infections associated with cortical cell divisions develop into mature nodules, regulation must occur at some step prior to nodule primordium formation. Examination of the number of infection threads associated with cell division in alfalfa indicate that only a small percentage of infections are successful (Wood and Newcomb 1989). In soybean, the number of infections is also much greater than the number of nodules eventually formed. Development was found to be arrested prior to the formation of nodule primordia. Furthermore, a large proportion of potential primordia had localized regions of cortical cell division, but lacked infection threads and were designated "pseudoinfections" (Calvert et al. 1984). The mechanism for this control is unknown, but a defense-type response may be involved.

Vasse et al. (1993) reported the presence of a hypersensitive reaction in alfalfa in unsuccessful infection events—those which do not lead to nodule formation. Between 1 and 2 weeks after inoculation with wild-type *R. meliloti*, certain cortical cells become pigmented, turning light yellow to black. Approximately 90% of the pigmented cells were found to contain the end of an infection thread. These investigators hypothesized that the host plant may restrict development of some infection threads by undergoing a hypersensitive-like reaction. Immunolocalization of plant defense proteins, including PAL and CHS, showed that these proteins were localized in the necrotic and adjacent cortical cells. Moreover, the infection threads that terminated in a pigmented cell were lined by wall appositions. The bacteria within these threads underwent necrosis, further suggesting that an HR had occurred.

In ineffective associations, the expression patterns of genes involved in flavonoid biosynthesis as well as the products of the pathway have also been investigated. Elevation of CHS transcript levels during ineffective interactions has been used to support the hypothesis that inoculation with certain mutant *Rhizobium* strains leads to a defense-related response or even an HR. Estabrook and Sengupta-Gopalan (1991) showed in soybean that specific PAL and CHS gene family members are induced during the symbiotic interaction. They also determined that inoculation with a Fix$^-$ mutant of *B. japonicum* led to an increase in transcripts of PAL and CHS gene family members different from those induced by

wild-type *B. japonicum*. However, induction began 12 days postinoculation, at a point when nodules are already developed. Moreover, it was not determined whether the transcripts of PAL and CHS expressed during this ineffective association eventually led to phytoalexin production in the nodules.

An early response to inoculation with mutant rhizobia was observed in siratro (*Macroptilium atropurpureum*) (DJORDJEVIC et al. 1988). Here, a mutant strain, with pleiotropic effects (it overproduces polysaccharide, is an adenine auxotroph, and is Nod^- on siratro), of the broad-host-range *Rhizobium* NGR 234 induced the rapid (within 20 h) accumulation of osmiophilic droplets at potential sites of infection. By 48 h, the droplets were observed in the epidermal cells near the site of infection; eventually these cells died. These authors stated that this reaction was similar to an HR, but it is not known which of the many defects of this *Rhizobium* strain are responsible for the elicitation of the defense symptoms.

WERNER et al. (1985) were among the first to report that phytoalexin synthesis could occur in response to an ineffective strain of *B. japonicum*. Ineffective soybean nodules contained ten-fold higher levels of glyceollin I than did control root tissue or nodules elicited by wild-type *B. japonicum*. The ineffective nodules were distinguished by premature peribacteroid membrane breakdown. The level of glyceollin I in these mature nodules was similar to that in roots of soybean 24 h after inoculation with the pathogen *Phytophthora megasperma* f. sp. *glycinea*. Another Fix^- mutant and two Fix^+ strains did not lead to glyceollin I accumulation, leading the authors to conclude that an intact peribacteroid membrane was necessary for preventing the host plant defense response. A *nifA* mutant was also found to lead to glyceollin I accumulation (PARNISKE et al. 1991). Nodules induced by *nifA* mutants at first develop normally, but early senescence, complete loss of cellular compartmentalization, and death of cells at the infection site take place, leading the authors to suggest that an HR had occurred. A so-called HR was also observed in the interaction between *Glycine soja* and *B. japonicum* (PARNISKE et al. 1990). In this interaction, there is an enhanced accumulation of glyceollin I in the 30-day-old nodules.

An increase in CHS transcript levels was observed in alfalfa nodules by GROSSKOPF et al. (1993) in response to the Fix^- mutant AK1540 of *R. meliloti*. Using a CHS probe from soybean, these researchers examined CHS gene expression in several different nodule types by in situ hybridization and northern analysis. Roots and nodules induced by wild-type *R. meliloti* or the Fix^- strain TF178 showed similar, but low levels, of CHS. In contrast, AK1540-induced nodules had increased levels of CHS, but only when the nodules were devoid of bacteria. When nodules were partially invaded, CHS transcript levels also dropped. The transcripts were located primarily in the outermost cells of the empty nodules and were present at maximum level at 18 days postinoculation. Five times more phenolic compounds were present in the empty nodules than in nodules induced by wild-type *R. meliloti*; however, the phytoalexin medicarpin was not detected.

We examined CHS and CHI expression in alfalfa and determined that transcript levels are elevated in nodules induced by a broad spectrum of defined Fix^- mutants of *R. meliloti*, including those defective in exopolysaccharide

synthesis (*exoB*) and nitrogenase regulation (*nifA*) (McKhann, Paiva, Dixon and Hirsch, unpublished). We also examined the effects of inoculation with *A. tumefaciens* transconjugants carrying either one or both *R. meliloti* symbiotic plasmids (FINAN et al. 1986; HIRSCH et al. 1992) and found that CHS transcript accumulation was also increased in these nodules. Associated with the elevated transcript levels is an increase in the level of FGM. Also, low levels of medicarpin and medicarpin glycosides were detected in these ineffective nodules (McKhann, Paiva, Dixon and Hirsch, unpublished).

Nevertheless, CHS gene expression was not elevated in "spontaneous" nodules—the Nar$^+$ (nodulation in the absence of *Rhizobium*) phenotype (TRUCHET et al. 1989; CAETANO-ANOLLÉS et al. 1992), nor in NPA-induced nodule-like structures (HIRSCH et al. 1989). CHS transcript levels were comparable to wild-type *R. meliloti*-induced nodules, indicating that the increase in CHS mRNA accumulation is not correlated with the ineffective state of the nodule. We believe that the induction of CHS mRNAs in the bacterial-induced nodules is related in part to nodule senescence, which occurs prematurely in these nodules and also to the fact that these nodules are at least partially infected. Evidence for the importance of senescence in flavonoid accumulation is provided by the study of VANCE (1978), who examined 60–65-day-old wild-type *R. meliloti*-induced alfalfa nodules. Nodule tissue was found to contain 120% more total phenolics than root tissue and 70% greater PAL activity. But what makes senescence in the presence of *Rhizobium* different from senescence that occurs in the nodules of plants with the Nar$^+$ phenotype or in the NPA-induced structures? why does infection, even partial infection, lead to the accumulation of defense-related transcripts?

Under normal conditions, rhizobia are retained outside the plant cell cytoplasm by being either encapsulated within an infection thread or surrounded by peribacteroid membrane. An analogy to nodules and *Rhizobium* exists in humans through our relationship with *E. coli* and other commensal microbes that live extracellularly in our guts. These organisms normally remain in their respective places in the body and do not harm their host unless the host becomes immuno-compromised (like AIDS sufferers) or dies. When this happens, the microorganisms are released from confinement and rapidly overrun their host's barriers to cytoplasmic invasion. This model can be applied to the *Rhizobium*-legume interaction, with the caveat that only the nodule dies and not the entire host. Maintaining rhizobia in a nodule is an energetically expensive undertaking for the plant. This can be seen in ineffective nodules that accumulate massive quantities of starch. If nodulation fails, the rhizobia become a liability. Nodule senescence takes place, and probably at the same time, defense-related transcript accumulate. Eventually, both nodule and rhizobial cells die. The trigger for the plant's defense-related response may be increased ethylene production. Ethylene is known to induce PAL, 4-coumarate CoA ligase, and CHS gene expression (ECKER and DAVIS 1987). In any case, the later stages of an ineffective symbiosis can be considered as most similar to a host defense response. It is at this stage in the process that rhizobia may be recognized as intruders. However, such possibilities do not explain the fact that not all *Rhizobium* mutants elicit a defense-type response (WERNER et al. 1985; GROSSKOPF et al. 1993).

In summary, infection thread abortions in effective symbioses and ineffective associations appear to share features of a plant's response to incompatible bacteria in that defense-related transcripts are elevated and phytoalexins accumulate. However, the timing of the failed symbiotic interaction is significantly different from that in response to an incompatible pathogen (Fig. 3). Furthermore, based on the definition established earlier in this review, the failed interaction cannot be properly called an HR. Although one could argue that the difference between the plant's response to plant pathogens vs rhizobia is merely due to differences in timing, we think this is unlikely. Effective rhizobia provoke a number of host reactions early in the nodule development pathway in contrast to compatible plant pathogens which do not elicit any response from a susceptible host until more than 120 h after infection (Fig. 3). These interactions suggest that the plant continually communicates with its symbiont. However, this does not preclude the idea that wild-type rhizobia are intruders and have evolved mechanisms to avoid recognition by the host.

6 Do Effective Rhizobia Suppress or Evade the Host Responses as Nodules Develop?

In many host-pathogen interactions, defense gene transcription and phytoalexin accumulation occur in both compatible and incompatible interactions, but with altered timing: phytoalexin accumulation is delayed in the compatible interaction compared to the incompatible interaction (Bell et al. 1986; Bonhoff et al. 1986; Ebel 1986). Some evidence suggests that differences in the timing of phytoalexin synthesis are the result of suppression of defense gene activation by pathogen-produced suppressors. For fungi, these suppressors have been identified as low molecular weight glycopeptides (Yamada et al. 1989), glucans (Doke et al. 1980), or glycoproteins (Kessmann and Barz 1986; Ziegler and Pontzen 1982). The lack of induction of PAL, CHS, and CHI (up to 120 h) in bean after infiltration with *P. syringae* pv. *phaseolicola*, the compatible pathogen, also appears to be due to an active suppression mechanism by the bacteria (Jakobek et al. 1993). This suppression requires active metabolism, but so far the identify of the suppressor is unknown.

As we have seen so far, wild-type and mutant *Rhizobium* inoculation elicits an early (6 h) burst in CHS expression. Some 2–5 days after inoculation, the Ini response takes place and with it, another increase in CHS gene expression. Neither of these host responses can be classified as a defense reaction. Moreover, once mature nodules develop, effective nodules accumulate very low levels of defense-related transcripts compared to ineffective nodules. Are the host's responses suppressed?

Exopolysaccharide (EPS I) has been proposed to act as a suppressor of the plant defense responses (Niehaus et al. 1993). Exo$^-$ mutants of *R. meliloti* induce the formation of bacteria-free nodules in which infection threads abort in the peripheral cells (Finan et al. 1985). Niehaus et al. (1993) have shown that these

nodules accumulate phenolic compounds and that the host cell walls are thick and encrusted with autofluorescent material as well as callose. After prolonged incubation, however, some normal, nitrogen-fixing nodule lobes formed from the bacteria-free nodule, but the rhizobia maintained their Exo$^-$ genotype (NIEHAUS et al. 1993). It is not known, however, how the plant's defense responses were suppressed in these nodule lobes. Interestingly, addition of low molecular weight oligosaccharides of EPS to *R. meliloti* exoA mutants restored their ability to induce normal, nitrogen-fixing nodules (BATTISTI et al. 1992). This suggests that some component of EPS may function as a suppressor.

An important question is how the distinction between "friend or foe" vs "no recognition" is made in plant-microbe interactions. If there is a suppressor produced by wild-type *Rhizobium*, what is it? If no suppressors are produced and yet *Rhizobium* avoids the host response, is it because recognition is delayed? If so, are there determinants, as postulated by DAZZO and HUBBELL (1975), on the rhizobial surface that are similar either in structure or confirmation to the plant cell surface? If they are either lipopolysaccharide (LPS) or EPS, then some explanation is required as to why neither seems to be universally required for complete nodule development. LPS is essential for normal invasion of *Bradyrhizobium* into hosts that form determinate nodules, whereas EPS is required for proper *Rhizobium* invasion of hosts that form indeterminate nodules (see references in HIRSCH 1992). However, perhaps a part of LPS or EPS serves as a signal. Some cell surface component or released molecule must be involved in triggering the earliest, 6–10 h, nonspecific host responses because both living and dead rhizobia are able to induce them. Alternatively, enclosure by the infection thread and later the peribacteroid membrane may protect the rhizobia from being recognized by the host (SMITH 1979).

7 Conclusions

Although there have been proposals that the *Rhizobium*-legume symbiosis represents a modified pathogenic interaction (VANCE 1983; DJORDJEVIC et al. 1987), as we have pointed out, there are some major conceptual differences between the symbiotic and plant-pathogen interactions. The induction of defense transcripts and the accumulation of phytoalexins during the *Rhizobium*-legume symbiosis has been highlighted as evidence to consider *Rhizobium* as a controlled pathogen. We believe that the earliest increases in PAL, CHS, and CHI transcripts (those coincident with an HR) that occur after inoculation with rhizobia result from a general, nonspecific plant response, which could be brought about by wounding, various abiotic stresses, or the presence of ethylene. In contrast, the Ini response appears to be specifically related to effective rhizobia and the correct Nod factor. However, more studies using mutant or heterologous rhizobia at this stage in the interaction are required. Following infection by wild-type

rhizobia, phytoalexin production by the plant's nodules is low, indicating that a classic host defense response does not occur. However, the increases in CHS and CHI transcripts and phytoalexin production observed in ineffective nodules are more likely to be defense-related. Thus, we propose that the plant's earliest responses to *Rhizobium* should not be thought of as a defense reaction, but rather as part of the host's generalized program of response to mechanical stress or to nonspecific microbial interactions. Moreover, the term HR should not be applied to the *Rhizobium*-legume symbiosis and should be restricted to the responses elicited by incompatible plant pathogens. The later stages of an ineffective relationship may be considered in the broad sense as a host defense reaction.

A reader could argue that these are just "words, words, words" (SHAKESPEARE, Hamlet II: 2), but words set the framework for ideas. By concentrating on the similarities between rhizobia and plant pathogens and attempting to fit symbiosis into an inappropriate model, our focus has veered away from the fact that *Rhizobium* invades the plant root, induces cell divisions so that a new organ is formed, and then takes up residence within host cells where the bacterial cells remain until they or the nodule senesces. So far, the mechanistic details of how *Rhizobium* invades the plant cell remain elusive. Nod factor is probably not the sole *Rhizobium* molecule required for the invasion process, but yet few clues exist as to how many molecules are necessary and what they are. Is the rhizobial surface cloaked in molecules that mimic plant cell wall components? Do EPS and LPS perform these roles or is there some other surface component(s) required? In addition, how does *Rhizobium* manipulate the host to form an infection thread? Do the bacterial (or plant) cells actually produce hydrolytic enzymes to degrade the cell wall in order to enter the root hair cell? Or does *Rhizobium* subvert the host cell wall synthesizing machinery and cytoskeleton to invade much like a *Yersinia* cell invades a mammalian cell (see chapters by Cornelis and by Parsot, this volume; BLISKA et al. 1993)? Do rhizobia bind to integrin-like proteins or other plasma membrane proteins like mammalian bacterial pathogens to initiate invasion? These questions get at the foundation of the interaction between microbe and host, and so far we have too few answers. To understand root hair invasion by rhizobia, we should reconsider the plant-pathogen paradigm for the *Rhizobium*-legume symbiosis and replace it with a new model, perhaps looking to the mammalian cell pathogens, as reviewed in this volume, for inspiration.

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Molecular and Cellular Mechanisms of *Salmonella* Pathogenesis

B.B. Finlay

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1 Introduction

"Salmonellae are among the most resourceful and successful of human pathogens, and as such have long beguiled microbiologists, epidemiologists, and clinicians" (Rubin et al. 1977). And they continue to do so! However, we have begun to gain insight into some of the mechanisms used by these pathogens to cause disease. These mechanisms reveal that the bacterium must possess

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several traits which are needed for each stage of the infection, allowing the salmonellae to establish an intimate relationship with the host. As a result, whenever *Salmonella* pathogenesis is being studied, the contribution of the host also needs to be considered. Thus, throughout this review emphasis will be placed on both the bacterial components and host contributions to these interactions. Work in this field has revealed several unexpected findings about bacterial and host cell mechanisms that are applicable to other systems. Much of the basic bacterial machinery is conserved between many plant and animal pathogens. However, despite this enthusiasm, it has also reinforced how little we really know about these interactions and the bacterial and host processes involved.

2 Pathology and Clinical Manifestations of *Salmonella* Infections

A brief introductory overview of the pathology and clinical manifestations of *Salmonella* infections is presented here, since this knowledge is essential in understanding the basic mechanisms of pathogenicity used by *Salmonella* species. This subject is reviewed in detail elsewhere (RUBIN et al. 1977; GOLDBERG and RUBIN 1988).

2.1 Pathology

Given that there are a wide variety of serotypes of *Salmonella*, it is not surprising that there are also several syndromes caused by salmonellae in humans. However, as a rule, particular clinical manifestations are usually associated with particular *Salmonella* species. Nearly all *Salmonella* infections occur from oral ingestion of bacteria. A sufficient dose is required to overcome host degenses such as gastric acidity, normal flora, and peristaltic movements. The infectious dose in humans ranges from approximately 10^6 to 10^9 organisms for most serotypes, including *S. typhi*. This dose is significantly decreased if the stomach acidity is buffered or the transit time through the stomach is decreased, indicating that these organisms are sensitive to the low pH found in the stomach. *Salmonella* species do not appear to colonize the stomach, but instead move to the lumen of the small intestine.

The bacteria multiply in the lumen of the small intestine, in apparent competition with the normal flora. *Salmonella* penetrate the mucosa at the distal ileum of the small intestine and the proximal large bowel. It is thought that the bacteria penetrate through specialized ileal epithelial cells called M cells which are found overlaying Peyer's patches. The mechanism and site of *Salmonella* invasion are discussed in detail later. The organisms rapidly penetrate the intestinal mucosa

and reach the mesenteric lymph follicles where they multiply. Most infections do not proceed beyond the local lymph nodes. However, more invasive strains such as *S. typhi* and *S. choleraesuis* spread to deeper tissue over a period of a few days. They infect the thoracic lymph, thereby spreading into the circulatory system. This bacteremic phase leads to infection of the liver, spleen, gall bladder, and bile. Infected bile can cause a secondary intestinal infection approximately 2 weeks after the initial ingestion, especially with *S. typhi*. They may also infect other distant sites such as bone and other tissues.

There is marked hyperplasia and hypertrophy of the reticuloendothelial system, indicating an active involvement of the phagocytic cells in this system. The intestinal lymphoid tissues, liver, and spleen all become enlarged. Salmonellae are actively phagocytosed by host cells, including macrophages and polymorphonuclear leukocytes, which presumably leads to the prominent enlargement of the reticuloendothelial system. There is ileal inflammation which may cause intestinal bleeding and perforation later in the infection, especially with *S. typhi*.

2.2 Clinical Manifestations

2.2.1 Gastroenteritis

There are four main clinical syndromes caused by *Salmonella* species. Although each species can cause any one of the four manifestations, particular species usually cause a given syndrome. Gastroenteritis is usually caused by *S. enteritidis* and most of its subspecies such as *S. typhimurium*. Gastroenteritis (food poisoning) is usually a non-life threatening disease characterized by nausea and vomiting 8–48 h after bacterial ingestion. Diarrhea, abdominal pain, and often fever follow later in the infection. Immunocompromised individuals often have a more severe and longer course of diarrhea.

2.2.2 Enteric Fever

Although any serotype of *Salmonella* can cause enteric fever, this disease is usually caused by *S. typhi* or *S. paratyphi*. Enteric fever is characterized by prolonged fever, sustained bacteria in the bloodstream (bacteremia), activation of the reticuloendothelial system, and multiple organ dysfunction. The incubation period for this disease is longer than that for gastroenteritis (usually 1–2 weeks), and the disease lasts longer.

2.2.3 Bacteremia

Once *Salmonella* have penetrated the intestinal barrier, they can enter the bloodstream, resulting in a sustained bacteremia which can then affect many body sites. *S. choleraesuis* is the most common *Salmonella* species which

causes bacteremia. Blood infections with this species often occur without any apparent intestinal manifestations.

2.2.4 Carrier State

The fourth symptom caused by *Salmonella* species is the chronic carrier state. *Salmonella* can persist in stool samples (10^6–10^9 organisms/gram) for periods exceeding a year. The carrier state may occur after a symptomatic disease, or it may establish without any symptoms. It usually occurs after ingestion of a small innoculum of bacteria. The site of bacterial multiplication and persistence is usually the bile duct, although other chronic sites have been described.

3 *Salmonella* in the Stomach

The low pH of the stomach plays a critical role in determining the outcome of a *Salmonella* infection. As mentioned above, if the pH of the stomach is increased (due to achlorhydria, antacid buffering, or gastric resection), a much lower dose of *Salmonella* can cause an infection. If the transit time through the stomach is decreased (as is often seen with waterborne outbreaks), again the infectious dose is lowered. Given this critical role of acidic pH, one would suppose that the bacteria should possess mechanisms to enhance its survival at low pH.

Both *S. typhimurium* and *E. coli* encode an adaptive acid tolerance response (ATR) that enables the organisms to survive at a decreased pH after exposure to low pH (FOSTER 1991). Despite the effectiveness of this system for enhancing survival in vitro, the role of the ATR in virulence remains questionable. For example, *S. typhimurium* carrying mutations in *atp*, which encodes an ATPase needed for the ATR, are avirulent in the mouse typhoid model (GARCIA DEL PORTILLO et al. 1993). However, if the stomach is buffered with bicarbonate, the *atp* mutants still remain avirulent when delivered orally, suggesting that stomach pH is not the major reason for the attenuation. Fur is an Fe^{2+}-binding regulatory protein which also regulates many proteins involved in the ATR. Mutants in *fur* were attenuated orally, yet were completely virulent by the intraperitoneal route. Addition of bicarbonate prior to oral infection of the *fur* mutant caused a 1 log decrease in its LD_{50}. Collectively, this work indicates that separate ATR genes may have different roles in *S. typhimurium* virulence.

It is possible that *Salmonella* does not possess special genes that are needed for survival in the stomach and instead rely on small numbers of organisms from a large dose to survive and proceed to the intestine. Alternatively, they may have some genes that enhance survival in the stomach slightly. Whatever the case, *Salmonella* species are unlike *Shigella* and enteroinvasive *E. coli* as these bacteria appear to be able to withstand the low pH of the stomach and a very small infectious dose is needed.

4 *Salmonella*-Induced Diarrhea

The mechanisms whereby *Salmonella* species cause diarrhea are poorly understood. It is generally believed that salmonellae must interact with the intestinal mucosa and trigger an influx of polymorphonuclear leukocytes (WALLIS et al. 1986). In neutropenic animals, or in infections with bacterial strains which do not trigger this influx, fluid secretion does not occur (WALLIS et al. 1989). This infiltration then presumably triggers the production of prostaglandins, since indomethacin, an inhibitor of prostaglandin synthesis, blocks fluid secretion (GIANNELLA et al. 1975). Prostaglandin synthesis then leads to activation of adenylate cyclase and increase in fluid secretion (GIANNELLA et al. 1975). Bacterial invasion is not correlated with diarrhea, since there are invasive strains which do not trigger fluid secretion (GIANNELLA et al. 1975), and invasion precedes fluid secretion by several hours (WALLIS et al. 1986).

The bacterial factors responsible for diarrhea also remain poorly characterized. It has been established that *S. typhimurium* encodes a cholera-like enterotoxin. (PRASAD et al. 1990; CHOPRA et al. 1991). When cloned into *E. coli* this enterotoxin exhibits enterotoxin-like activity (PRASAD et al. 1992). However, the role of this toxin in *Salmonella* diarrhea remains to be established. *S. typhimurium* also produces an outer membrane protein that inhibits host protein synthesis and cytotoxic activity (REITMEYER et al. 1986). Additionally, given the inflammatory nature of diarrhea, bacterial lipopolysaccharide (endotoxin) may contribute to diarrhea. It has been demonstrated that *Salmonella* can disrupt tight junctions (FINLAY et al. 1988). It is possible that such disruptions in the gut could contribute to ionic imbalance and diarrhea. Finally, as discussed below, *S. typhimurium* may trigger the production of arachadonic acid and other prostaglandins as it invades epithelial cells (PACE et al. 1993). Although extremely speculative, it is possible that these inflammatory mediators may contribute to fluid secretion.

5 *Salmonella* Interactions with Nonphagocytic Cells

5.1 The Intestinal Barrier

The intestinal barrier is a relatively impermeable barrier comprised mainly of epithelial cells tightly linked to each other by tight junctions. There are several types of epithelial cells exposed to the lumen of the intestine, including columnar epithelial cells, goblet secreting cells, and M cells. *Salmonella* species appear to have the capacity to penetrate this barrier, although the precise portal of entry into their host is not certain. There is mounting evidence that M cells within Peyer's patches are probably the site that are preferentially invaded by *Salmonella* species, although the model system that is used appears to influence this

conclusion. For example, in a classic descriptive study, TAKEUCHI (1967) found that *S. typhimurium* uniformly penetrated the intestinal epithelium, including both columnar epithelial cells and M cells of the guinea pig. However, KOHBATA et al. (1986) found that *S. typhi* preferentially invaded and destroyed M cells in mouse ligated ileal loops. POPISCHIL et al. (1990) found that *S. typhimurium* did not have any predilection for intestinal epithelial type in infected swine, yet *S. choleraesuis* was located preferentially in ileal M cells within Peyer's patches. Collectively this data would suggest that although *Salmonella* species have the capacity to invade any intestinal epithelial type, larger numbers enter through M cells within Peyer's patches. The contribution to disease of each of these sites remains to be determined. The differences in the intestinal invasion sites between *Salmonella* species and host types may contribute to the observed host specificity of different salmonellae.

More recently, polarized epithelial monolayers have been used to examine *S. typhimurium* and *S. choleraesuis* interactions with epithelial cells as a model for intestinal penetration (FINLAY et al. 1988; FINLAY and FALKOW 1990). These cell lines (of canine kidney and human intestinal origin) form well developed microvilli and tight junctions, have defined apical and basolateral domains, and mimic a columnar epithelial cell barrier. When salmonellae are added to the apical (top) surface of these cells, they cause morphological alterations identical to that observed with infected intact intestinal epithelium (described below). The bacteria also invade these cells and penetrate through the monolayers. The bacteria depolarize these barriers by disrupting tight junctions and subsequently cause significant cytotoxic effects on these epithelial cells. Thus these systems provide a defined in vitro system to study *Salmonella* penetration of columnar epithelial cells. Unfortunately, at present there are no M cell lines to further examine bacterial interactions with this cell type.

5.2 Invasion

5.2.1 Morphological Description

In 1967, TAKEUCHI described a detailed morphological description of *S. typhimurium* interacting with and invading guinea pig ileal intestinal epithelial cells. This description is representative of *Salmonella* interactions with most nonphagocytic cells and has been documented by other workers (for examples see POPIEL and TURNBULL 1985; KOHBATA et al. 1986; FINLAY and FALKOW 1990; FRANCIS et al. 1992). Prior to initial bacterial contact with the intestinal epithelium, the brush border remains intact. However, when the bacterium comes close to the epithelial surface (less than 350 Å), microvilli in the immediate vicinity begin to degenerate through elongation, swelling, and budding. There are often long fibrous structures linking the organism with the apical surface, although the organism always retains space between the bacterial and host surfaces. As this process progresses, the apical cytoplasm close to the organism begins to bleb and swell, distorting outwards. As the cytoplasm is distorted, the organism is

internalized within a membrane bound vesicle, often surrounded by the cytoplasmic extrusion (Fig. 1). Accompanying this extrusion is a marked increase of localized endocytic activity, resulting in internalization of many vesicular structures. For unknown reasons, bacteria preferentially infect certain cells of the monolayer. It is common to see several bacteria following another organism into one cell, yet the neighboring cells (and even distant areas of the same cell) remain unaffected. TAKEUCHI also reported organisms passing through tight junctions between cells, apparently resealing after bacterial entry. Initially, each invading organism is internalized within an individual vacuole, although at later times these vacuoles may fuse. Although several regulatory factors are involved in *Salmonella*

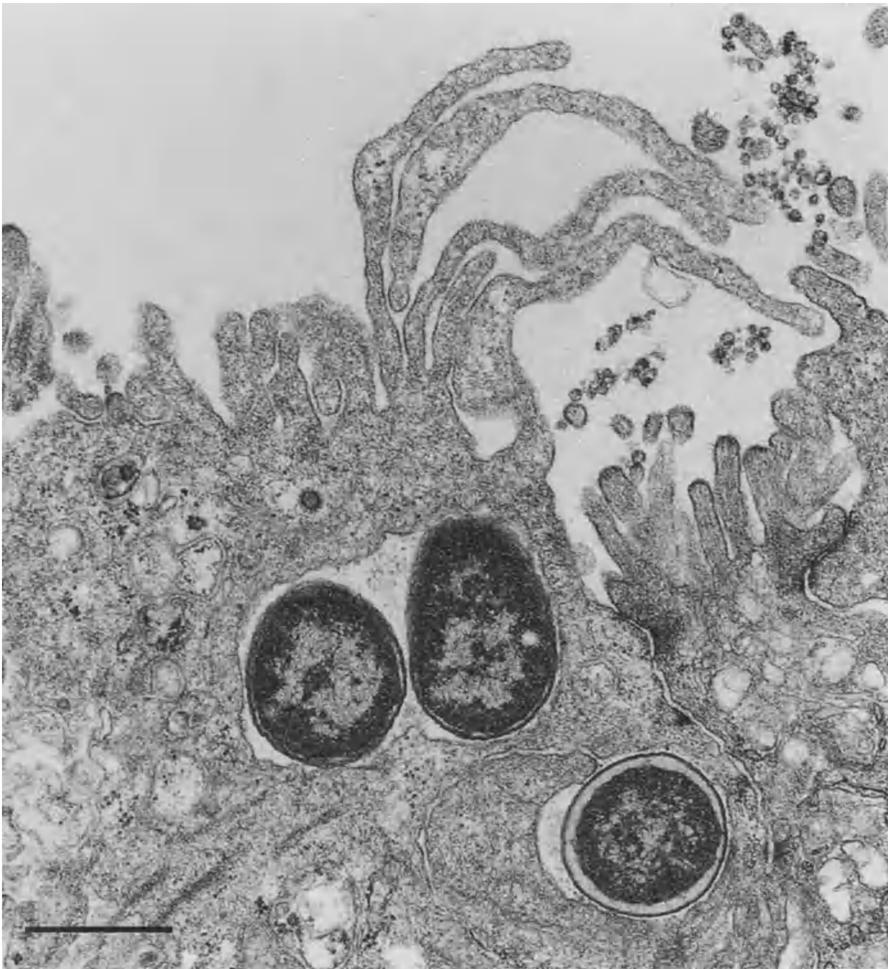

Fig. 1. Transmission electron micrograph of *Salmonella typhimurium* entering polarized Caco-2 human intestinal epithelial cells. Note the microvilli distortions and that the bacteria are localized within membrane-bound inclusions. Bar, 1 μm

invasion (see below), once the bacteria are committed to invade, the entire invasion process occurs within minutes (FRANCIS et al. 1992).

5.2.2 Bacterial Factors Involved in Invasion

Several invasiveness loci have been identified in various *Salmonella* species by several groups. Unfortunately, of those characterized, nearly all loci are involved in regulation of invasion, secretion of products to the bacterial surface, or motility. A large region of *S. typhi* DNA has been cloned into *E. coli* and shown to facilitate a low level of invasiveness by this normally noninvasive bacterium (ELSINGHORST et al. 1989). However, homologous sequences from *S. typhimurium* are unable to confer invasiveness to *E. coli*, and this locus remains uncharacterized. Tn5 mutagenesis of *S. typhi* resulted in the identification of four nonmotile mutants which are unable to invade cultured epithelial cells (LIU et al. 1988). Mutations resulting in defects in lipopolysaccharide (LPS) decrease *S. typhi* and *S. choleraesuis* invasiveness (MROCZENSKI-WILDEY et al. 1989; FINLAY et al. 1988), but not *S. typhimurium*. Tn*phoA* mutagenesis of *S. choleraesuis* led to the identification of six classes of mutants with decreased invasiveness that are unable to penetrate polarized epithelial monolayers (FINLAY et al. 1988) although no motility mutants were detected. Two of the four invasiveness mutants which did not affect LPS structure were avirulent in mice, while the other two remained virulent. Tn*phoA* mutagenesis of *S. enteritidis* resulted in the identification of six classes of noninvasive mutants which had different invasion phenotypes with different cell lines (STONE et al. 1992). Similarly, Tn*phoA* mutagenesis of *S. abortusovis* identified several mutants that were defective for adherence, but had varying degrees of virulence (RUBINO et al. 1993). One Tn*10* mutant in *S. typhimurium* has been identified that has reduced adherence and invasiveness in both cultured epithelial (Caco-2) and cultured macrophage (J774) cells (GAHRING et al. 1990). Four classes of mutants in *S. typhimurium* were identified that had decreased invasion levels, although three classes were still virulent in mice (BETTS and FINLAY 1992). Several other nonmotile noninvasive mutants were also identified in that study. It has been suggested that *S. typhimurium* invasion is affected by the direction of flagellar rotation and the physical orientation of flagella around the bacteria (JONES et al. 1992) and the requirement of motility in invasion can be bypassed by centrifuging bacteria onto the monolayer (FINLAY and FALKOW 1989).

There is currently only one well characterized invasion locus from *Salmonella*. This region (*inv*) has been cloned and characterized from *S. typhimurium* (GALAN and CURTISS 1989) and maps at approximately 59 minutes on the chromosome. Lesions in these genes result in a slight decrease in virulence when the bacteria are delivered orally, but not when they are administered intraperitoneally. There appear to be several genes in this locus that are required for adherence and/or invasion. Initially, three sequential genes, *invA-C*, were identified that were involved in invasion (GALAN and CURTISS 1989), and *invA* has been further characterized (GALAN et al. 1992). Immediately upstream of *invA* is *invE*, another

invasion locus that is required for triggering the uptake of *S. typhimurium* into epithelial cells (GINOCCHIO et al. 1992), and two other loci, *invF and invG* (ALTMEYER et al. 1993). Upstream of *invF*, and transcribed in the opposite direction, is *invH*, a locus that is involved in adherence and is strongly conserved between *Salmonella* species (ALTMEYER et al. 1993). However, this locus is not needed for virulence, since it was originally identified as a virulent class 6 Tn*phoA* mutant in *S. choleraesuis* (FINLAY et al. 1988). It was subsequently also disrupted in three noninvasive Tn*phoA* mutants of *S. enteritidis* (class I) (STONE et al. 1992).

The number of genes needed for *Salmonella* invasion is large. However, recent results indicate that the bacterial products needed for invasion are similar to virulence factors in other pathogens, thereby suggesting roles for some of these products. The first indication of such homology came from *invA*. This gene encodes a predicted protein that shared homology with LcrD from *Yersinia*, MxiA from *Shigella flexneri*, and other proteins (GALAN et al. 1992; VAN GIJSEGEM et al. 1993). LcrD is a membrane-bound calcium regulator involved in *Yersinia* pathogenesis, while MxiA is involved in secreting *Shigella* invasion antigens to the bacterial surface (see chapters by Cornelius and Parsot in this volume). Furthermore, it has recently been shown that several plant pathogens have similar secretory systems, including HrpO from *Pseudomonas solanacearum* and HrpC2 from *Xanthomonas campestris pv. vesicatoria* (VAN GIJSEGEM et al. 1993; related chapter in the volume). A recent paper by GROISMAN and OCHMAN (1993) has extended this homology for most of the *inv* locus of *S. typhimurium*. They sequenced the region downstream of *invB* and found at least nine predicted open reading frames. Furthermore, these genes share significant homology to the *S. flexneri spa* genes which, like *mxiA*, are needed for secretion of *Shigella* invasion antigens to the bacterial surface (Table 1). They also found that at least one of the *Shigella* genes (*spa24*) could complement noninvasive mutants containing mutations in the homologous gene of *S. typhimurium* (*spaP*). Collectively, these results suggest that the complex machinery needed to transport virulence proteins to bacterial surfaces is conserved in *Yersinia*, *Shigella*, and *Salmonella*, and is also conserved in flagella export machinery and in various plant pathogens. However, the actual antigens that are transported and the regulatory mechanisms that control these systems vary. These results help reconcile the numerous invasion genes that are found in *Salmonella*. Unfortunately, they also imply that the actual *Salmonella* invasins that are being exported by this machinery remain to be discovered.

S. typhimurium invasiveness is regulated by several factors, including anaerobic growth, growth state, and calcium concentration (ERNST et al. 1990; SCHIEMANN and SHOPE 1991; LEE and FALKOW 1990; NIESEL and PETERSON 1987). The *inv* locus in *S. typhimurium* is regulated by changes in DNA supercoiling affected by osmolarity (GALAN and CURTISS, 1990). Additionally, a "hyperinvasive" locus has been identified, and mutations in this locus appear to uncouple invasion from its traditional regulators, yielding constitutive invadors (LEE et al. 1992). This locus (*hil*) maps to 59.5 minutes, which is very near the *inv* region. Interestingly, unlike *Shigella* and *Yersinia*, *Salmonella* invasion is not regulated by temperature.

Table 1. Examples of homologous loci between pathogenic bacteria[a]

Yersinia enterocolitica	Shigella flexneri	Salmonella typhimurium	Pseudomonas solanacearum
YscL			HrpF
YscJ	MxiJ		HrpI
YscC	MxiD		HrpH
VirF			HrpB
LcrE	MxiC	InvE	
LcrD	MxiA	InvA	HrpO
	Spa15	InvB	
Orf6	Spa47	InvC/SpaL	HrpE
	SpaM	SpaM	
	Spa32	SpaN	
	Spa33	SpaO	HrpQ
	Spa24[b]	SpaP[b]	HrpT

[a] For details see text, GROISMAN and OCHMAN (1993), and VAN GIJSEGEM et al. (1993).
[b] Spa24 from S. flexneri can complement a SpaP⁻ S. typhimurium mutant for invasion (GROISMAN and OCHMAN 1993).

5.2.3 Host Factors Involved in Invasion

Uptake of *Salmonella* into epithelial cells requires host cell metabolism and energy (KIHLSTROM and NILSSON 1977). This observation suggests that *Salmonella* uptake into non-phagocytic cells is an active process, and, given the morphological alterations that occur, that the bacteria is capable of transmitting a localized signal at the host cell surface which mediates bacterial uptake. Several lines of evidence suggest that such a process occurs.

It is becoming increasingly clear that host actin containing micro-filaments are required for *Salmonella* uptake. Treatment of cultured cells with cytochalasins, which disrupt actin filaments, blocks *Salmonella* uptake in several systems (for example, KIHLSTROM and NILSSON 1977; FINLAY and FALKOW 1988), although inhibitors of microtubules do not affect bacterial invasion. Additionally, it has been shown that *S. typhimurium* triggers rearrangement of polymerized actin and other micro-filament related proteins including α-actinin, tropomyosin, talin, and ezrin (FINLAY et al. 1991). This rearrangement consists of loose "strings" of actin filaments accumulating in the vicinity of the invading organism. This rearrangement is also closely correlated with invasion, and, once the bacterium is internalized, the cytoskeleton returns to its normal distribution (FINLAY et al. 1991). This rearrangement again suggests signals are being transmitted through the host membrane to mediate cyto-skeletal rearrangement.

As with most invasive enterics, there appear to be several signals that are transduced in the host cell that are involved in *Salmonella* uptake (reviewed in ROSENSHINE and FINLAY 1993; BLISKA et al. 1993). Given the marked cytoskeletal rearrangement triggered by *S. typhimurium* and the role intracellular Ca^{2+} plays in cytoskeletal rearrangements, it was not unexpected to find that *S. typhimurium*

triggers a Ca^{2+} flux in cultured epithelial cells (GINOCCHIO et al. 1992). Mutations in *invE* (which encodes a putative secretory machinery product) were unable to trigger the intracellular Ca^{2+} flux or actin rearrangement, although they could be rescued in *trans* by adding the parental strain. Additionally, chelators of intracellular Ca^{2+}, but not extracellular Ca^{2+}, block *S. typhimurium* entry into cultured epithelial cells (RUSCHKOWSKI et al. 1992). Release of intracellular Ca^{2+} is often mediated by fluxes in the inositol phosphate IP_3, and it has been shown that *S. typhimurium* also triggers fluxes in inositol phosphates, and this closely correlates with invasion (RUSCHKOWSKI et al. 1992).

Further information about the signal(s) used by *S. typhimurium* to enter cultured cells came from work by GALAN et al. (1992) that described the activation of the epidermal growth factor receptor (EGFR) by invading *S. typhimurium* in Henle-407 cells. These workers showed that *S. typhimurium* triggered tyrosine phosphorylation of the EGFR, and *invA* mutants (another putative secretory machinery product) were unable to induce such activation. Addition of EGF to cells increased the invasiveness of the *invA* mutation. These workers then extended their findings and proposed a complex sequence of events that mediate *S. typhimurium* invasion (PACE et al. 1993). This sequence order was: activation of the EGFR; EGFR activates MAP kinase; MAP kinase activates phospholipase A_2 (PLA_2); PLA_2 generates arachidonic acid; arachidonic acid is converted into leukotriene LTD_4 by 5-lipoxygenase; LTD_4 opens Ca^{2+} channels; and influx of extracellular Ca^{2+} causes membrane ruffling, cytoskeletal rearrangements, and bacterial uptake.

This model has several appealing features. EGFR activation is known to trigger membrane events similar to those seen with *S. typhimurium*. It also causes Ca^{2+} fluxes and several other signals. However, despite the appeal of this pathway, there are several unexplained and contradictory results that have arisen. For example, this pathway does not invoke any role for intracellular Ca^{2+}, nor is there any involvement of phospholipase C, which presumably generates the inositol phosphates seen during *S. typhimurium* invasion (RUSCHKOWSKI et al. 1992). Additionally, cells which are lacking the EGFR are still invaded efficiently (GALAN et al. 1992), and cells treated with potent inhibitors of the EGFR tyrosine kinase do not affect *S. typhimurium* invasion (ROSENSHINE et al. 1992), although they block EGF mediated signal transduction. Furthermore, another study of EGFR activation by *S. typhimurium* indicated that the EGFR was not activated by *S. typhimurium* in Henle-407 cells, or other cells expressing various amounts of EGFR, although receptor activation was observed with EGF (I. Rosenshine et al., unpublished observations). Further supporting evidence that the EGFR does not participate in *S. typhimurium* invasion comes from two recent studies. The first demonstrated that inhibition of rac and rho, two host proteins that are essential in EGF mediated cytoskeletal ruffling, are not involved in *S. typhimurium* mediated ruffling (JONES et al. 1993). These workers also found that complete down-regulation of the EGFR had no effect on *S. typhimurium* invasion. In another study, this group found that *Salmonella* promoted ruffling and bacterial uptake via an EGF-independent mechanism, and *S. typhimurium* could trigger ruffling in cell

lines that do not possess the EGFR (FRANCIS et al. 1993). They concluded that *S. typhimurium* invades via an EGFR-independent pathway.

The role of the bacteria in invasion is also controversial. It has been reported that addition of bacterial protein or RNA synthesis inhibitors blocks *Salmonella* adherence and invasion (FINLAY and FALKOW et al. 1989). However, if bacteria are grown under appropraite inducing conditions, it has been suggested that *S. typhimurium* can invade cells in the presence of chloramphenicol (LEE and FALKOW 1990). Prolonged inhibition of bacterial protein synthesis inhibits *S. typhimurium* invasion, indicating that the bacterial products may be rapidly turned over (MACBETH and LEE 1993). However, addition of chloramphenicol after bacterial invasion has commenced blocks further bacterial invasion, suggesting that invasion may indeed be an active process. It has been reported that invading *Shigella* must be metabolically active to enter cultured epithelial cells (HALE and BOVENTRE 1979). Given the conservation of invasion secretion machinery between *Salmonella* and *Shigella* species (see above), one can speculate that energy may be required for these invasion/secretion systems to function for both these pathogens, despite differences in invasins and receptors.

5.3 The Intracellular Environment

Nonphagocytic cells such as epithelial cells are inefficient at killing intracellular bacteria. However, since *Salmonella* spends at least some time within such cells as it penetrates the intestinal mucosa, it is worth examining some of the features of this environment, and how this impacts intracellular *Salmonella*. A discussion of *Salmonella* inside phagocytic cells occurs later in this review.

There is general agreement that *Salmonella* species reside within a membrane-bound vacuole within both phagocytic and nonphagocytic cells. However, the targeting of this vacuole has only recently been examined. We have examined the targeting of *S. typhimurium* within cultured epithelial cells and found that the vacuole containing *S. typhimurium* contains the lysosomal markers lysosomal glycoprotein (lgp) and lysosomal acid phosphatase (Garcia del Protillo et al. 1993, unpublished). It also contains MHC class I heavy chain and β_2 microglobulin. However, it does not contain any mannose-6-phosphate receptor which is necessary for transport of certain lysosomal enzymes to lysosomes. Fluid phase markers such as lucifer yellow and the lysosomal marker rhodamine ovalbumin rarely colocalize with intracellular *S. typhimurium* (Garcia del Portillo and Finlay, unpublished). Collectively these data suggest that *S. typhimurium* enters into a vacuole which contains cell surface molecules with low internalization rates and bypasses the late endosomal pathway, proceeding directly to lysosomal fusion.

There have been no bacterial factors identified that are needed for enhanced survival within epithelial cells, although this is not surprising, since even laboratory strains of *E. coli* are capable of surviving within this environment (LEUNG et al. 1992). However, some attempts have been made to further characterize the

intracellular environment by using bacterial reporter genes (GARCIA DEL PORTILLO et al. 1992). Measurement of β-galactosidase activity of various *lacZ* fusions using a fluorescent substrate led to the conclusion that the concentrations of free Fe^{2+} and Mg^{2+} in the vacuole of epithelial cells are low, that the vacuole has a mild acidic pH, and that lysine and oxygen are present within the intracellular environment. This work demonstrates the utility of using bacterial gene fusions to measure genes that are expressed intracellularly. Improvement of reporter systems (such as by using chemiluminescence) will enhance this technique.

5.4 Intracellular Replication

Salmonella species have the capacity to multiply within vacuoles in nonphagocytic cells after an initial lag of approximately 4 h (FINLAY and FALKOW 1988; GAHRING et al. 1990; YOKOYAMA et al. 1987). This lag period would indicate that some process must occur prior to bacterial replication, and specific bacterial genes may be required for replication in this unique niche, since nonvirulent *E. coli* does not replicate within epithelial cells. The intracellular replication process is not dependent on vacuole acidification, since endosome acidification inhibitors have no effect on intracellular replication (FINLAY and FALKOW 1988).

Several mutants in *Salmonella* have been identified which are unable to replicate intracellularly. One mutant of *S. choleraesuis* survived within macrophages yet was unable to grow in epithelial cells, although vacuoles containing this mutant fused (FINLAY et al. 1991). This mutant was not an auxotroph and was completely avirulent when administered orally or intravenously to mice. Unfortunately, the transposon used to generate this mutant was not linked to this phenotype and the locus involved remains uncharacterized. Additional nonreplicating mutants in *S. typhimurium* were obtained by treating epithelial monolayers infected with pools of transposon mutants with a β-lactam, cefotaxime (LEUNG and FINLAY 1991). This treatment identified 13 mutants, ten of which were auxotrophic (purine, pyrimidine, purine/ methionine, and valine/isoleucine). The auxotrophic mutants could be complemented by adding the appropriate nutrients to the tissue culture media. The three prototrophic mutants were highly attenuated for virulence in mice, yet persisted within livers and spleens for at least 3 weeks. Collectively, the identification of these three mutants and the *S. choleraesuis* mutant suggests that there are genes that are needed for intracellular replication and these are essential for virulence.

A possible function for these genes has recently been reported (GARCIA DEL PORTILLO et al. 1993). As mentioned above, *S. typhimurium* is localized within vacuoles that contain lysosomal glycoproteins. However, 4–6 h after invasion, intracellular *Salmonella* induce the formation of stable filamentous structures that contain lgps that are connected to the vacuoles containing bacteria (Fig. 2) (GARCIA DEL PORTILLO et al. 1993). The kinetics of formation of these lgp-rich structures paralleled closely the kinetics of intracellular replication. Filament formation

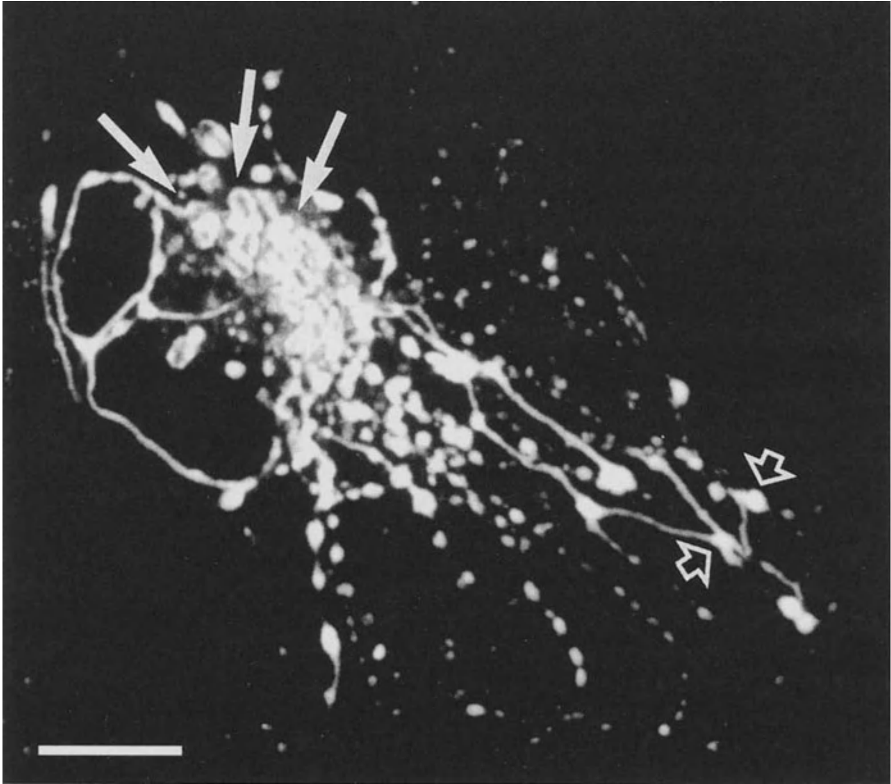

Fig. 2. Fluorescent micrograph of filamentous structures in HeLa epithelial cells infected with Salmonella typhimurium. Cells were infected for 6 h, then fixed and labelled with antibodies to a lysosomal glycoprotein (lgp). Bacteria within lgp-rich vacuoles are marked by solid arrows, and open arrows mark blebs often associated with the filamentous structures. Bar, 10 μm

requires viable intracellular bacteria and is blocked by endosome acidification inhibitors or microtubule disrupting agents. These unique structures are never present in uninfected cells, nor those infected with *Yersinia* species, although all *Salmonella* species tested trigger their formation. All four of the prototrophic avirulent *Salmonella* mutants (three from *S. typhimurium* and one from *S. choleraesuis*) that are unable to multiply inside cells are also completely defective for triggering formation of these filamentous structures. Thus it appears that *Salmonella* have specific loci which are responsible for triggering filament formation, and these same loci are needed for intracellular replication. The role of these filaments in intracellular replication has yet to be defined.

6 *Salmonella* Interactions with Phagocytic Cells

6.1 The Intracellular Environment

Salmonellae are rapidly taken up by macrophages underlying the intestinal mucosa, presumably following penetration of the intestinal epithelial barrier. Bacterial invasins appear to significantly enhance uptake into phagocytic cells such as macrophages, since noninvasive *S. typhimurium* mutants have decreased levels of invasion into cultured macrophages (GAHRING et al. 1990; BETTS and FINLAY 1992). Whether entering via an invasin mediated pathway or a classical phagocytic pathway affects the intracellular targeting of the organism remains to be determined. Once inside macrophages, salmonellae remain within membrane-bound vacuoles, similar to non-phagocytic cells. It also remains to be determined if *Salmonella* trigger extensive membrane ruffling and cytoplasmic extrusion in macrophages.

Several investigators have begun to characterize the intracellular targeting and environment of *S. typhimurium* inside macrophages. A previous report indicated that *S. typhimurium* resides within phagosomes that have fused with lysosomes (CARROL et al. 1979). Other workers recently concluded that *S. typhimurium* inhibited phagosome-lysosome fusion within several types of mouse derived macrophages (BUCHMEIER and HEFFRON 1991; ISHIBASHI and ARIA 1990). It was also suggested that viable intracellular bacteria are needed for this inhibition (BUCHMEIER and HEFFRON 1991). It has been reported that phagosomes containing *S. typhimurium* are acidified slowly, and it takes 4–5 h before the pH drops below 5.0 (ALPUCHE et al. 1992). In contrast, vacuoles containing killed organisms were rapidly acidified (pH < 4.5 within 1 h). This data would suggest that viable organisms are either needed for inhibition of acidification, or are needed to invade via a bacterial mediated pathway which delivers the organism to an intracellular location which is acidified slower than a phagocytic pathway. It was also found that fluid phase markers fused with internalized bacteria, which is different for that seen with epithelial cells. ALPUCHE et al. (1992) concluded that *S. typhimurium* resides within a fused lysosome, yet is capable of blocking endosome acidification. It is also possible that the organisms reside within an intracellular environment which contains some of the lysosomal markers, yet this environment is not a "classical" phagolysosome.

Although there is conflicting data, it appears that *Salmonella*, especially *S. typhimurium*, does not actively grow within most macrophages (CARROL et al. 1979; BUCHMEIER and HEFFRON 1989). Mutant strains that are unable to grow within epithelial cells are unaffected within macrophages (LEUNG and FINLAY 1991). However, it has recently been proposed that two populations of *S. typhimurium* exist within macrophages: one which is static, and the other which is rapidly growing (ABSHIRE and NEIDHARDT 1993). The existence of these two pools may perhaps explain the conflicting data regarding lysosome fusion and intracellular growth within phagocytic cells.

6.2 Bacterial Factors

The past few years have seen the identification of several bacterial factors that enhance *S. typhimurium* survival within macrophages. In a classic study of microbial pathogenesis, FIELDS et al. (1986) screened transposon mutants in *S. typhimurium* for survival in macrophages. They found numerous mutants that had decreased capacity to survive within macrophages, and these mutants were avirulent in the mouse model, suggesting that the capacity to survive within macrophages is essential for *S. typhimurium* virulence. Further characterization of these loci has led to the identification of several factors that enhance intracellular survival in macrophages. These survival factors are macrophage-specific, since these mutants survive equally well within non-phagocytic cells (GAHRING et al. 1990).

The best characterized of these loci is the PhoP/PhoQ system. This is a two component regulatory system that activates at least five bacterial products (*pag*) and represses others (*prg*) (reviewed in MILLER 1991). One of the phenotypes that PhoP/PhoQ regulates is the capacity to survive bacterial cationic peptides which are thought to be involved in killing intracellular bacteria (FIELDS et al. 1989; MILLER et al. 1989), although resistance to such antimicrobial peptides appears to have a broader role in *S. typhimurium* pathogenesis (GROISMAN et al. 1992). It has recently been demonstrated directly (by measuring β-galactosidase fusions) that the PhoP/PhoQ system is induced by low pH within macrophages, and inhibition of endosome acidification blocks activation of the PhoP/PhoQ system. One of the PhoP/PhoQ activated genes, *pagC*, encodes a protein which is homologous to a *Yersinia enterocolitica* invasin, Ail, and appears to enhance resistance to complement, although it does not directly mediate invasion or resistance to cationic peptides (MILLER et al. 1992; PULKKINEN and MILLER 1991).

Other bacterial products may also contribute to intracellular survival within phagocytic cells. For example, a 59 kDa outer membrane from *S. typhimurium* has been reported to provide protection from oxidative killing within polymorphonuclear leukocytes (STINAVAGE et al. 1990). Additionally, mutations in *recA* and *recBC* in *S. typhimurium* are avirulent, and are sensitive to the oxidative burst of macrophages, indicating that the ability to repair DNA damage is essential for survival within macrophages and virulence (BUCHMEIER et al. 1993). Recently a Tn*5* mutant of *S. typhimurium* has been described that lacks the ability to block phagosome-lysosome fusion (ISHIBASHI et al. 1992). Interestingly, although this mutant was susceptible to intracellular killing, it was still virulent in mice.

6.3 The Virulence Plasmid

It has been well established that species of *Salmonella* other than *S. typhi* contain a plasmid that is essential for virulence (reviewed by GULIG et al. 1993). Further work by many investigators has shown that a region of the plasmid encoding five genes (*spvA–D* and *spvR*) is sufficient to restore virulence to plasmidless

Salmonella strains (see GULIG et al. 1993 for details). However, the virulence functions of these genes remains undefined: currently it is thought that these genes enhance growth within the host, probably within host cells (GULIG and DOYLE 1993), although no in vitro assay has been developed that measures the function of these genes.

The regulation of the spv genes has been characterized and provides clues about their function. SpvR is a regulator of the other four *spv* genes and is regulated by growth phase and starvation conditions (GULIG et al. 1993), with maximal expression occurring during stationary phase. FANG et al. (1992) have demonstrated that the alternate σ factor KatF regulates the *spv* operon. KatF regulates many genes that are induced during starvation and the stationary phase of bacterial growth. They also showed that KatF mutants of *S. typhimurium* are avirulent in mice. Regulation by these factors has led to the suggestion that the *spv* genes are be needed for survival within phagocytic cells. Recent evidence indicates that these genes are expressed inside macrophages (J. Fierer et al., submitted). Thus it seems that these genes may be needed for some mechanism which enhances intracellular survival in some host cell which is related to *Salmonella* virulence.

7 Is Salmonella an Intracellular Pathogen?

Most sources consider Salmonella species to be facultative intracellular pathogens. This assumption is based on two concepts: (1) the induction of cell mediated immunity and (2) microscopic observation of bacteria "surviving" within phagocytic cells. However, HSU (1989) has presented an analysis of the information leading to this assumption, and, in the murine model of salmonellosis, it was concluded that *S. typhimurium* should not be considered an intracellular pathogen. Although the role of cell mediated immunity in salmonellosis is beyond the scope of this review, there is increasing evidence to suggest that salmonellae must spend at least some of their life within host cells during an infection.

Perhaps the most persuasive argument in favor of *Salmonella* being classified as a facultative intracellular pathogen comes from the phenotypes of specific *Salmonella* mutants. For example, as discussed above, mutants in both *S. choleraesuis* and *S. typhimurium* that are unable to replicate within epithelial cells are completely avirulent in the mouse model. Other evidence comes from the generation of mutants in *S. typhimurium* that are defective for survival within macrophages (see above). Again, all of these mutants are avirulent, suggesting that the capacity to survive within macrophages is critical for *S. typhimurium* virulence. There is also a report that *S. typhimurium* resides within an intracellular "safe site" in the liver and spleen, protected from antibiotics that are unable to penetrate host cells (DUNLAP et al. 1991), again suggesting that *S. typhimurium* survives within phagocytic cells.

A collective overview describing *Salmonella* (at least *S. typhimurium*) could be as follows: (a) it has the capacity to replicate within nonphagocytic cells such as epithelial cells; (b) it has the capacity to survive (but not replicate) within phagocytic cells such as macrophages, and (c) it is doubtful that it has the capacity to survive within polymorphonuclear leukocytes. If the definition of a facultative intracellular pathogen includes replication within macrophages, *S. typhimurium* does not fit that description. However, if a more liberal definition which only specifies that a facultative intracellular pathogen must pass through (i.e., survive) inside a host cell is used, there is ample evidence to suggest that salmonellae belong to such a classification. It is this author's opinion that the more liberal definition be used.

8 Regulation of *Salmonella* Virulence Factors

When one considers the pathogenesis of salmonellae, it is immediately apparent that, during the course of infection, the organism will pass through many different environments, including the stomach, intestinal lumen, inside epithelial cells, inside macrophages, inside polymorphonuclear leukocytes, and perhaps even free in the blood. One of the fundamental tenants emerging in microbial pathogenesis is the strict regulation of bacterial virulence factors such that they are expressed only in certain defined environments (reviewed by MEKALANOS 1992). This theme is exemplified clearly by *S. typhimurium*.

The profile of proteins produced by *S. typhimurium* within macrophages is, not surprisingly, quite different than that of extracellular bacteria. For example, BUCHMEIER and HEFFRON (1990) found that at least 30 proteins are induced when *S. typhimurium* infects macrophage, including the heat shock proteins GroEL and DnaK. A similar analysis by another group saw the intracellular expression of at least 40 bacterial proteins induced and approximately 100 repressed when compared to extracellular bacteria (ABSHIRE and NEIDHARDT 1993). Although they did not see the above heat shock proteins being induced, they found some overlap with other proteins associated with various environmental stresses. However, the macrophage induced response in *S. typhimurium* was not a collective sum of individual stress response proteins. There is a significant amount of information regarding regulation of various extracellular stresses on *S. typhimurium*, including various starvation conditions, anaerobiosis, heat shock, and acid shock (for example, see SPECTOR et al. 1986). However, application of this knowledge to the stresses encountered in the host is just beginning. For example, as discussed earlier, the role of the ATR in virulence is complex.

Differential regulation of specific *Salmonella* virulence factors is critical for pathogenesis. Continuing with the example of *S. typhimurium* inside macrophages, as discussed above, the PhoP/PhoQ system is a regulatory mechanism that appears specific for bacterial survival inside macrophages.

However, if this system is uncoupled by making a constitutive mutation in *phoP*, the virulence of *S. typhimurium* is attenuated (MILLER and MEKALANOS 1990). This work emphasizes the strict control of virulence factors needed as the bacterium moves between environments

Another example of such regulation can be found with *S. typhimurium* invasion. As discussed above, invasion is regulated by many environmental parameters including growth phase, osmotic levels, and oxygen levels. A similar theme is found with KatF regulation of the virulence plasmid genes (see above). The capacity of *Salmonella* to sense a specific environment and convey this information leading to expression of certain virulence factors is the hallmark of a successful pathogen.

Recent progress has been made in measuring *S. typhimurium* gene transcription inside a host cell by measuring reporter gene fusions. The utility of this technique was demonstrated by determining a few environmental parameters of the *S. typhimurium* containing vacuole in epithelial cells (GARCIA DEL PORTILLO et al. 1992) and by measuring PhoP expression inside macrophages (ALPUCHE ARANDA et al. 1992). Although these studies used *lacZ* fusions, other workers have begun to use *lux* fusions such that light production can be measured (FRANCIS and GALLAGHER 1993). The advantage of this system is that the host cells do not need to be disrupted, and measurements can be obtained with the same sample over a prolonged time. Applications such as these will further enhance our knowledge of the parameters that affect regulation inside host cells.

An alternate approach has recently been taken to identify *S. typhimurium* genes that are expressed within a host but that are not expressed when grown in vitro (MAHAN et al. 1993). This powerful and elegant technique relies upon the mouse to provide selective pressure to enrich for cloned promoters that are induced inside a mouse, thereby enhancing recovery from the mouse. These workers showed that defects in all of these induced genes are attenuated for virulence, again emphasizing the necessity for regulation of virulence factors.

9 Concluding Remarks

Salmonella pathogenesis is complex. Although many bacterial virulence factors have been identified for several stages of infection, most of these factors have yet to be characterized. The recognition that regulation plays a key role in virulence and the development of several new techniques to study genes induced inside host cells and the host will facilitate this work greatly. As is painfully obvious, nearly all of the basic work on *Salmonella* pathogenesis has been done on *S. typhimurium*, presumably because there is a well developed animal model and the molecular genetic tools are available. However, key insights can be made by studying other *Salmonella* species. Fundamental questions such as what

determines host specificity and what factors determine which disease await investigation. Molecular pathogenic studies with *S. typhi* are surprisingly few for such a major pathogen. As exemplified throughout this book, conservation of basic mechanisms between pathogens and how they interact with their host provides many clues for future lines of investigation. The future is bright indeed for the study of *Salmonella* pathogenesis!

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Molecular and Genetic Determinants of the *Listeria monocytogenes* Infectious Process

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1 Introduction

Listeria monocytogenes was first characterized in 1926 following an outbreak of listeriosis in laboratory animals (MURRAY et al. 1926). However, it was not until the 1980s that an unambiguous link was established between the human disease and the consumption of *Listeria*-contaminated foodstuffs (SCHLECH et al. 1983). Immunosuppressed individuals, pregnant women, foetuses and neonates are most susceptible to *Listeria* infection. Human listeriosis is characterized by a high mortality rate, with clinical features including meningitis or meningo-encephalitis, septicemia, abortion, and perinatal infections (GRAY and KILLINGER 1966). If diagnosed early, listeriosis can be successfully treated by the administration of high doses of antibiotics, most frequently ampicillin or penicillin, either alone or in combination with aminoglycosides.

L. monocytogenes is a gram-positive, non-spore-forming, facultative intracellular bacterium. It is the best characterized of the six species of the genus *Listeria*, which is closely related to the genera *Brochothrix* and *Bacillus* (COLLINS et

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al. 1991). These six species are considered to represent two closely related, but distinct, lines of descent, with *L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. seeligeri*, and *L. welshimeri* forming one grouping and *L. grayi* the other (Collins et al. 1991; Jones 1992). Only *L. monocytogenes* and *L. ivanovii* are pathogenic. However, unlike *L. monocytogenes*, *L. ivanovii* is exclusively an animal pathogen, accounting for approximately 10% of listerial infections in animals, and is not associated with human disease (Cooper and Dennis 1978).

Following the pioneering work of Mackaness in the early 1960s (Mackaness 1962), murine infection by *L. monocytogenes* has been used as a model to study T cell-mediated immunity (reviewed by Kaufmann 1993). Resistance to *L. monocytogenes* infection is independent of humoral defense mechanisms as the passive transfer of immune serum fails to protect the host against listerial infection. Recovery from a primary *Listeria* infection, resistance to reinfection, and protective immunity are mediated by anti-*Listeria*-specific T cells. The activation, clonal expansion and mobilization of anti-*Listeria*-specific T cells (CD8 and CD4) is due to the ability of *L. monocytogenes* to survive and replicate in resident macrophages (Kaufmann 1993). Survival in phagocytic cells is considered to be a major virulence determinant of *L. monocytogenes* pathogenesis and a prerequisite to successful infection by this pathogen. Recently, the ability to reproduce certain aspects of the human disease in the mouse model of infection after inoculation by a variety of routes (including conjunctival, nasal, respiratory, gastrointestinal, intravenous and intra-peritoneal) has facilitated the molecular genetic analysis of *L. monocytogenes* virulence determinants (Cossart and Mengaud 1989).

Recent outbreaks of listeriosis following the ingestion of *Listeria*-contaminated foodstuffs have emphasized the importance of the oral route in natural infections and a number of studies have sought to identify the site of entry of *L. monocytogenes* into the host organism following oral or intragastric inoculation. MacDonald and Carter (1980) have suggested that *L. monocytogenes* specifically penetrates the specialized epithelial cells (M cells) overlying the Peyer's patches. They observed that *L. monocytogenes* could be cultured from the Peyer's patch-containing intestinal mucosa of mice infected by the intragastric route, but not from intestinal mucosa from which Peyer's patches had been removed (MacDonald and Carter 1980; Marco et al. 1992). The penetration of *L. monocytogenes* into intestinal epithelial cells in vivo was observed by electron microscopy (Racz et al. 1970, 1972, 1973). Thus, entry into the host may occur via different cell types including epithelial cells and the M cells covering the Peyer's patches. Indeed, in vitro studies have shown that *L. monocytogenes* can penetrate and multiply within various epithelial and fibroblast-like cells (see Cossart and Mengaud 1989).

Following translocation across the intestinal barrier, bacteria can be observed in phagocytic cells present in the underlying lamina propria (Racz et al. 1972, 1973). In murine infections the bacteria spread via the bloodstream, with considerable accumulation occurring in the liver and in the spleen. In the liver the bacteria are rapidly phagocytosed by resident macrophages and most of the

inoculum is destroyed during the first 6 h. Thereafter, the survivors grow logarithmically, reaching a maximum 2–3 days after infection. In mice, the hepatocyte is the major site of intracellular bacterial multiplication (Conlan and North 1991; Rosen et al. 1989). These infected cells appear to be the target of neutrophils in the early stages of infection and later of mononuclear phagocytes, both cell types expressing complement receptor type 3 (CR3). This early killing in the liver serves to reduce infection to a level which may be resolved by subsequently acquired resistance mechanisms (Conlan and North 1991, 1993; reviewed by Portnoy 1992). Depending on the immune response of the host, the bacteria will either be eliminated, or they will undergo further hematogenous dissemination to the brain (or placenta). Macrophages appear to be very heterogeneous with regard to listericidal activity. In listericidal macrophages, CR3 acts as the major receptor of *Listeria* by binding to serum complement component C3 deposited on the bacterial surface. In macrophages unable to kill *Listeria*, receptors other than CR3 seem to mediate most phagocytosis (Drevets and Campbell 1991; Drevets et al. 1992).

Detailed analysis of *Listeria*-infected cell cultures has revealed a complex series of host-pathogen interactions culminating in the direct dissemination of *L. monocytogenes* from one infected cell to another (Gaillard et al. 1987; Mounier et al. 1990; Tilney and Portnoy 1989; Tilney and Tilney 1993 and references therein). Host cell infection (Fig. 1 and summarized in Fig. 2) begins with the internalization of the bacteria either by phagocytosis, in the case of macrophages, or by induced phagocytosis, in the case of nonphagocytic cells. The bacteria are rapidly incorporated into a membrane-bound vacuole which they lyse after about 30 min. In the cytoplasm, the bacteria multiply with a doubling time of approximately 1 h (Gaillard et al. 1987) and become associated with actin filaments. After about 2 h, these filaments are rearranged into tails which mediate bacterial movement through the cytoplasm to the cell periphery. This movement is rapid, reaching speeds of about 1 µm/s (Dabiri et al. 1990), and is independent of known cellular motor molecules like myosin II. Measurements of the rate of actin tail formation suggest that the force for propulsion is provided by the actin polymerization itself (Sanger et al. 1992; Theriot et al. 1992). When moving bacteria contact the plasma membrane they induce the formation of pseudopod-like protrusions of the membrane. Contact between these protrusions and neighboring cells results in the internalization of the bacteria-containing protrusion. In the newly infected cell the bacterium is surrounded by two plasma membranes which must be lysed to initiate a new cycle of multiplication and movement. Thus, once *Listeria* has entered the cytoplasm it can disseminate directly from cell to cell circumventing such host defenses as circulating antibody and complement. This ability to disseminate in tissues by cell-to-cell spreading provides an explanation for the early observation that antibody (although induced and abundant) is not protective (Mackaness 1962) and that anti-*Listeria* immunity is T cell-mediated (reviewed by Kaufmann 1993).

Since the first two early reviews on this subject (Chakraborty and Goebel 1988; Cossart and Mengaud 1989), considerable progress has been made in the

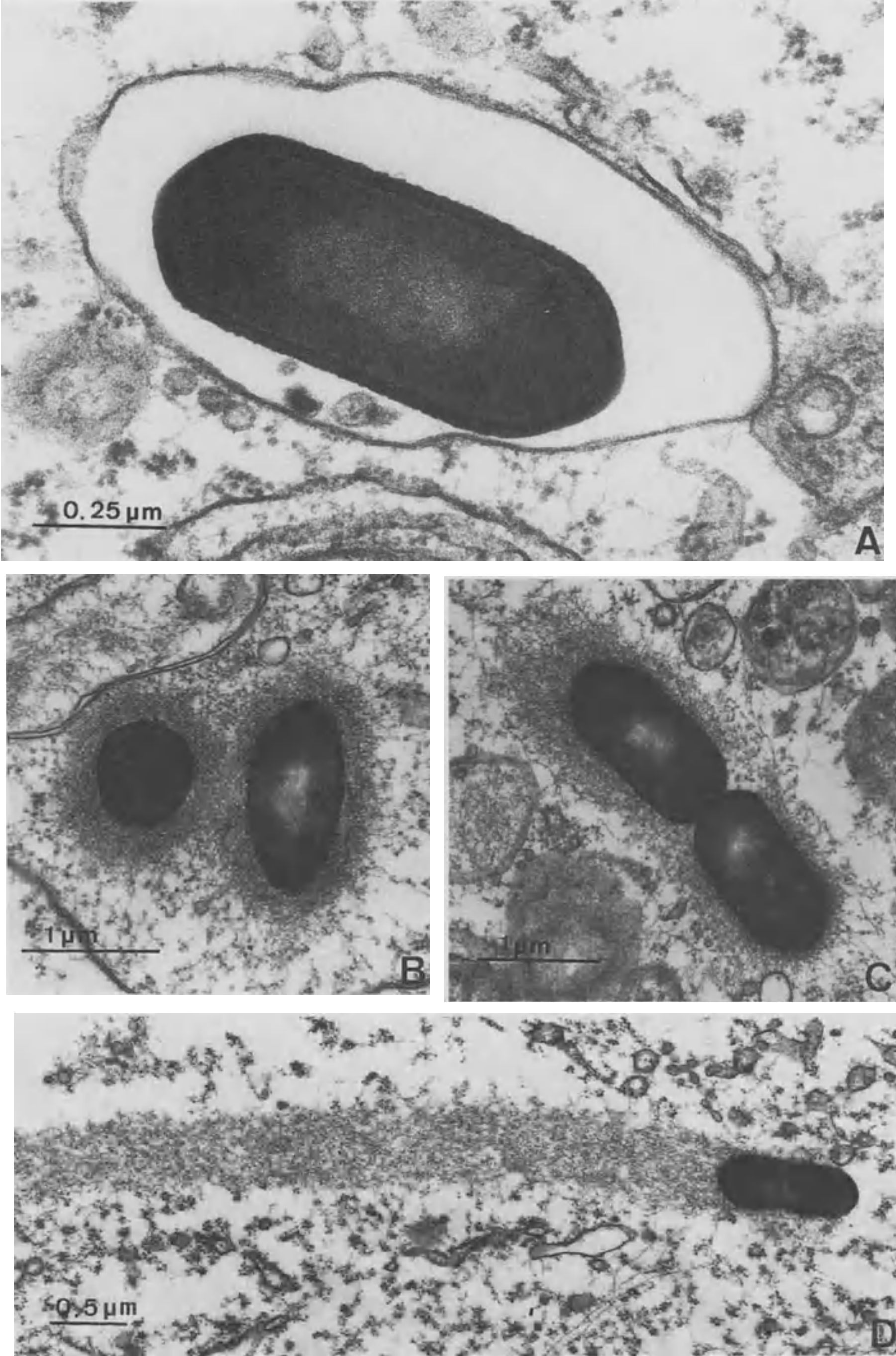
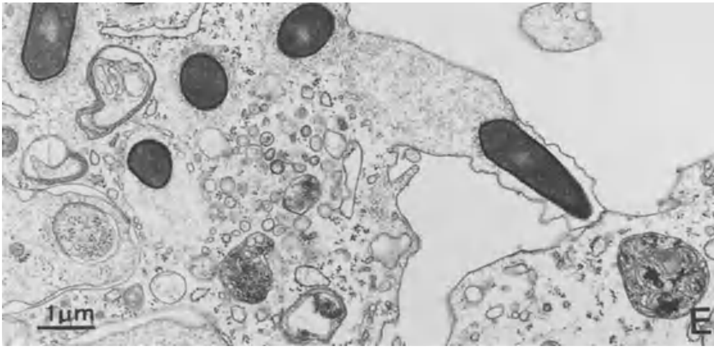


Fig. 1A–H. For caption see p. 192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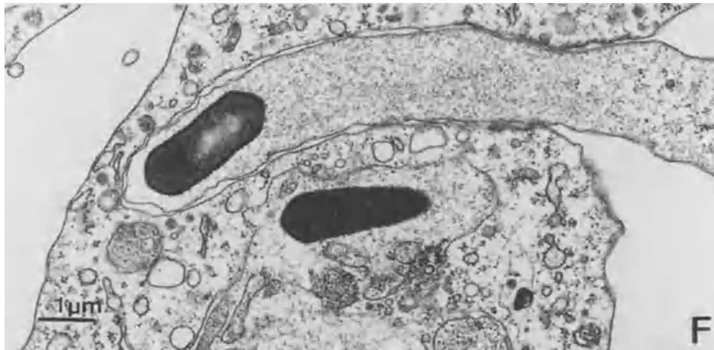
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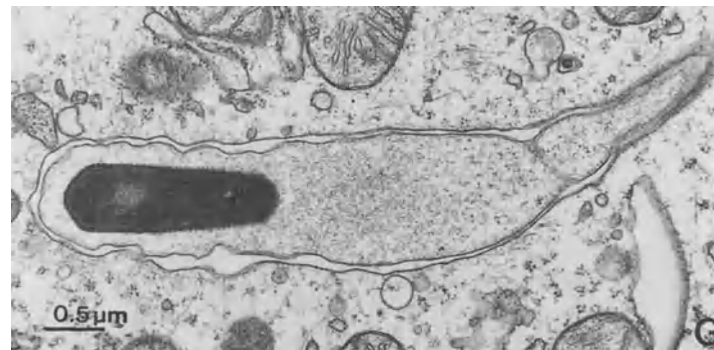
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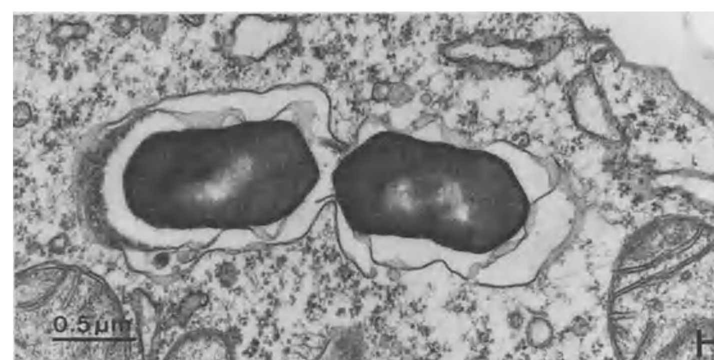
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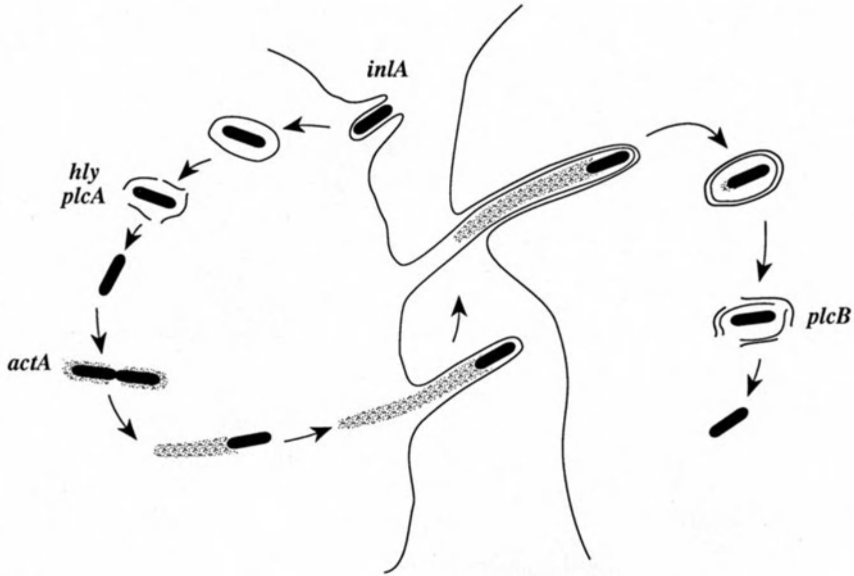

Fig. 2. The infectious process. The genes required at each step of the process are indicated. (Adapted from TILNEY and PORTNOY 1989)

identification and characterization of the bacterial factors required at each stage of the infectious process (Fig. 2). Recently, a common nomenclature for the identified *L. monocytogenes* virulence genes has been adopted by the various groups working in the field (PORTNOY et al. 1992b). The purpose of this review is to summarize the molecular genetic analysis of these bacterial virulence factors. (See also COSSART 1994 and COSSART and KOCKS 1994).

2 Host Cell Infection

2.1 Entry

Epithelial cell invasion is a key virulence mechanism of many bacterial pathogens and has been extensively studied in gram-negative bacilli, including members of the genera *Shigella*, *Salmonella*, and *Yersinia*. The genetic determinants

Fig. 1A–H. Thin sections of macrophage-like J774 cells at 0.5–5.5 h of infection with Listeria monocytogenes strain LO28. **A** Intracellular bacterium surrounded by a vacuolar membrane after uptake by the host cell. **B, C** Cross- and longitudinal sections through dividing bacteria which are covered by host-cell derived actin filaments (electron-dense filamentous material on the bacterial surface). **D** Moving bacterium with an actin "comet" tail. **E, F** Bacteria incorporated into cell surface protrusions that are invading neighboring cells. **G** Protrusion with a bacterium in the cytoplasm of the new host cell. The bacterium is completely surrounded by two cytoplasmic membranes. **H** Lysis of the two plasma membranes in the cytoplasm of the new host cell. (Electron micrographs were obtained in collaboration with Pierre Gounon and Héléne Ohayon, Station Centrale de Microscopie Electronique, Institut Pasteur)

promoting bacterial penetration vary considerably among these pathogens and have been reviewed recently (BLISKA et al. 1993; FALKOW et al. 1992; SANSONETTI 1992; see chapters by PARSOT, FINLAY, and CORNELIS, this volume). As suggested by the electron microscopic studies of RACZ et al. (1970, 1972, 1973) invasion of epithelial cells by *L. monocytogenes* may constitute an important early step in infection. To date, two surface proteins, internalin and p60, have been implicated in the induced internalization of *L. monocytogenes* by nonprofessional phagocytic cells.

Entry into epithelial cells is mediated by internalin, a surface protein of approximately 90 kDa, encoded by the gene *inlA* (GAILLARD et al. 1991). Internalin was identified by screening a library of Tn*1545* mutants of *L. monocytogenes* for loss of invasiveness into the intestinal epithelial cell line Caco-2. Three such mutants were obtained. These mutants were unable to adhere to Caco-2 cells and were defective for entry in a variety of epithelial cell lines. In all mutants the transposon had inserted into a region upstream from two open reading frames, *inlA* and *inlB*. Transcription of these two genes was abolished in the noninvasive mutants. *inlA* encodes an 800 amino acid protein whose characteristic features include a signal sequence (recently recognized after the detection of a sequencing error, see DRAMSI et al. 1993b), two regions of repeats (one of which is rich in leucine residues), and a COOH-terminal hydrophobic region (Fig. 3) which may be a membrane anchor. The first region of repeats (region A, see Fig. 3) is made up of 15 highly conserved successive repeats of a string of 22 amino acids which display a periodicity of hydrophobic residues and have the consensus sequence 1-NQISDITPL..LTNL..L.L..-22 (where dots represent any amino acids). The second region of repeats (region B, Fig. 3) is formed by three successive repeats, the first two of 70 amino acids each and the third of 49 amino acids. In the region common to the three repeats, the same amino acid is found in 27 out of 49 positions. Region B contains no periodicity of nonpolar residues and is dissimilar

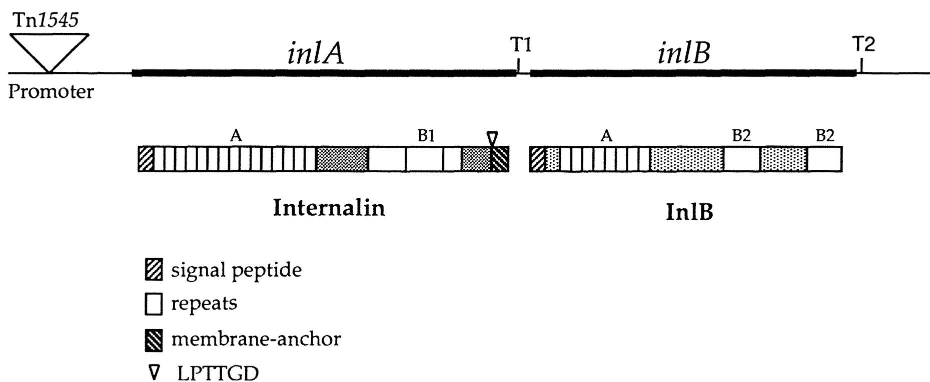

Fig. 3. Structural organization of the inlAB locus. Transcription of the locus proceeds from left to right. The transcriptional termination signals (T1, T2) are indicated as is the position of the Tn*1545* insertion in the promoter region of the operon. The major structural features of the proteins internalin and InlB are as described in the legend

to region A. The putative membrane anchor is preceded by the hexapeptide LPTTGD, considered to be a signature of gram-positive surface proteins (FISCHETTI et al. 1990). When *inlA* is expressed in *L. innocua* it confers invasiveness on this otherwise noninvasive, nonpathogenic *Listeria* species. Thus, in the genetic background of a closely related *Listeria* species, internalin expression is sufficient to promote the entry into epithelial cells. Recent results indicate that internalin can be released into the culture supernatant (DRAMSI et al. 1993b). Maximal invasivity is obtained with exponential cultures, corresponding to the growth phase during which the cell-wall associated form of internalin is most strongly expressed. This finding suggests that the cell-wall associated form of internalin plays a crucial role in invasion, but cannot rule out an involvement of the released form. *InlB*, located downstream from and cotranscribed with *inlA*, encodes a 630 amino acid protein which is structurally analogous to internalin, except for the absence of a hydrophobic COOH-terminal region. The *inlB* gene product contains eight leucine-rich regions with homology to those of internalin. *InlB* seems to play no role in epithelial cell invasion (Dramsi and Cossart, unpublished).

Low stringency Southern blot analysis demonstrated that several other DNA sequences with homology to *inlA* are present on the chromosome of *L. monocytogenes* and all *Listeria* species (GAILLARD et al. 1991). Five other genes with homology to *inlA* (in addition to *inlB*) have been cloned and sequenced (S.D., P.D., and P.C., unpublished results). The role of this multigene family is currently under investigation with the hypothesis that the *inl* repertoire may encode surface proteins with different cellular tropisms. Interestingly, internalin and the proteins of the internalin family are structurally analogous to a number of repeat proteins from gram-positive bacteria (including the F and M proteins of *Streptococcus pyogenes* and the fibronectin-binding proteins of *Staphylococcus aureus*) which are involved in cell contact or cell recognition (DRAMSI et al. 1993a; WESTERLUND and KORHONEN 1993).

p60, a major extracellular protein, has been suggested to play a role in the invasion process. Spontaneously occurring mutants of *L. monocytogenes* which produce greatly reduced levels of p60 display a rough colony morphology and reduced adherence and invasiveness into certain cell types (KUHN and GOEBEL 1989). Such mutants form long chains in which the bacteria are separated by double septa. The gene coding for p60, *iap*, has been cloned. It encodes a protein of 484 amino acids with a signal sequence but contains no further hydrophobic sequences which might serve as membrane spanning domains (BUBERT 1992; KÖHLER et al. 1990, 1991). About 75% of the protein is found in the culture supernantant and the remaining 25% is associated with the cell wall (RUHLAND et al. 1993). p60 has bacteriolytic activity and, on the basis of amino acid sequence homologies, is thought to possess a murein hydrolase activity required for a late step in cell division (WUENSCHER et al. 1993). p60 is an essential protein and deletions of *iap* are lethal. It remains to be shown whether p60 plays a direct role in adhesion and invasion, or whether the effect of its reduced expression on adherence and invasion can be explained by the altered morphology of the p60 defective mutants.

Among invasive bacteria, the invasin of *Yersinia pseudotuberculosis* is the only protein demonstrated to mediate (by itself) entry of bacteria into mammalian cells (FALKOW et al. 1992). In the case of *Salmonella* and *Shigella*, the entry process is clearly multifactoral (FALKOW et al. 1992; SANSONETTI 1992). Internalin is the first invasion factor identified in gram-positive bacteria. It remains to be established whether internalin can mediate entry by itself or whether it forms complexes with other bacterial components such as the *inlB* gene product or the proteins encoded by the genes of the internalin superfamily.

2.2 Escape from the Vacuole and Intracellular Multiplication

Subsequent to internalization, *L. monocytogenes* escapes from host vacuoles and enters the cytoplasm, where rapid growth ensues. Convergent studies have shown that listeriolysin O (LLO), the first identified virulence factor of *L. monocytogenes*, plays a crucial role in this step (for review see COSSART and MENGAUD 1989). This 58.6 kDa secreted protein is a member of a family of pore-forming thiol-activated cytolysins, of which streptolysin O is the prototype (SMYTH and DUNCAN 1978). These hemolysins share immunological cross-reactivity and are irreversibly inactivated by cholesterol, their putative membrane receptor. Their lytic activity in vitro involves two steps: a temperature-independent binding of the toxin to the membrane followed by an oligomerization process leading to pore formation and membrane lysis.

The role of LLO in lysis of the phagosomal membrane was established by genetic analysis of nonhemolytic mutants. In these mutants, insertion of various transposons into the LLO structural gene (*hly*) resulted in production of inactive truncated proteins (COSSART et al. 1989; GAILLARD et al. 1986; KUHN et al. 1988; PORTNOY et al. 1988). The *hly* gene was shown to be a monocistronic unit that, when introduced into such LLO defective strains, restored the wild-type phenotype (COSSART et al. 1989; MENGAUD et al. 1989). Electron microscopic studies revealed that the mutants were not affected in their capacity to enter human intestinal epithelial cells (Caco-2 cells) but remained within the phagosomal vacuole, could not gain access to the cytoplasm and were thus unable to grow intracellularly (GAILLARD et al. 1987). In all cases, virulence of the mutants was strongly affected with an increase in the LD_{50} of about five orders of magnitude. In the cases in which revertants were obtained, recovery of the hemolytic phenotype correlated with the recovery of virulence.

Further evidence for the role of LLO in the lysis of the phagosomal membrane was obtained from an experiment in which *hly* was cloned into the noninvasive soil bacterium *Bacillus subtilis* and expressed under the control of an IPTG-inducible promoter (BIELECKI et al. 1990). In the presence of IPTG, this strain exhibited hemolytic activity and, following internalization by a macrophage-like cell line, lysed the phagosomal membrane and grew rapidly and extensively in the host cell cytoplasm. In the absence of IPTG the bacteria stayed trapped in the host vacuoles where they could survive for several hours but could not replicate.

All thiol-activated cytolysins contain a unique cysteine residue in the COOH-terminal part of the proteins (with the exception of that produced by *L. ivanovii*, ivanolysin, which contains a second cysteine residue; HAAS et al. 1992). This unique cysteine is contained within an undecapeptide ECTGLAWEWWR which is conserved in all thiol-activated cytolysins sequenced so far, except for a single amino acid change in seeligerolysin (an LLO-like cytolysin produced by *L. seeligeri*; HAAS et al. 1992). Mutational analysis studies showed that conservation of the undecapeptide is important for hemolytic activity, although surprisingly, it is not the cysteine residue but the surrounding tryptophane residues that seem to be required for activity (MICHEL et al. 1990). The study of isogenic mutants affected in single amino acid positions in LLO established a direct correlation between hemolytic activity and virulence. However, there seems to be no correlation between the hemolytic activity of various *L. monocytogenes* strains and their virulence (KATHARIOU et al. 1988).

LLO and the related toxin produced by *L. ivanovii* are the only examples of thiol-activated toxins produced by intracellular bacteria. In addition, unlike other thiol-activated hemolysins, LLO has an acidic pH optimum and is relatively inactive at neutral pHs (GEOFFROY et al. 1987). These properties led to the suggestion that the low pH optimum of LLO represented a *Listeria*-specific adaptation to maximize cytolysin activity within acidified phagolysosomes. However, the IPTG-regulated expression of perfringolysin O in *B. subtilis* allowed this organism to escape from the host cell vacuole and to replicate intracellularly (PORTNOY et al. 1992c). Such bacteria were observed to cause greater damage to the host cells than *B. subtilis* expressing LLO. This may suggest that the low activity of LLO at neutral pH may provide a mechanism whereby the host cell is protected fom the potentially deleterious effects of this protein in the cytoplasm.

Nonhemolytic mutants of *L. monocytogenes* are still able to grow (although to a lesser extent) inside the human epithelial cell line Henle 407 and the human fibroblast cell line WS1 (PORTNOY et al. 1988). These results suggest that factors other than LLO may be involved in escape from the phagosomal compartment. *L. monocytogenes* produce a phosphatidylinositol-specific phospholipase C (PI-PLC; (GOLDFINE and KNOB 1992; LEIMEISTER-WÄCHTER et al. 1991; MENGAUD et al. 1991a). This enzyme is a 36.3 kDa secreted protein encoded by the *plcA* gene and homologous with the *B. cereus*, *B. thuringiensis*, and eukaryotic PI-PLCs. It is a type II PI-PLC as it is a soluble enzyme that hydrolyses phosphatidylinositol (PI) and glycosyl phosphatidylinositol (GPI) moieties, by which many eukaryotic membrane proteins are anchored to the membrane, but it is unable to hydrolyse PI-4-phosphate (PIP) or PI-4,5-bisphosphate (PIP_2) (GOLDFINE and KNOB 1992).

The role of PI-PLC in phagosomal membrane lysis and virulence could not be clearly defined by analyzing transposon insertion mutants as insertions into *plcA* had a polar effect on the downstream regulatory gene *prfA* (MENGAUD et al. 1991a,b). Recently, an in-frame deletion mutant in the *plcA* gene was constructed (CAMILLI et al. 1993). This mutant was only slightly affected in virulence on the basis of its LD_{50} in mice, but the absence of PI-PLC clearly correlated with decreased efficiency of host vacuole lysis in primary cultures of murine

macrophages (but not in the macrophage-like cell line J774; CAMILLI et al. 1993). The PI-PLC displays a relatively broad pH optimum, ranging from pH 5.5 to pH 7 depending on the assay conditions (GOLDFINE and KNOB 1992). It is possible that the PI-PLC acts in concert with LLO within the acidified phagosome to mediate lysis of the vacuolar membrane: the PI-PLC, by hydrolysing PI and GPI-anchored proteins present in the extracytoplasmic leaflet of the phagosomal membrane, may facilitate access to, and lysis of, the membrane by LLO.

Two other genera of bacterial pathogens, *Rickettsiae* and *Shigellae*, follow the same general pathway as *Listeria* through the host cell, i.e., escape from the phagosome and intracellular multiplication in the cytoplasm. In both cases a hemolytic activity has been associated with the ability to gain access to the host cytoplasm. It has been proposed that a phospholipase A of rickettsial origin is responsible for entry of *Rickettsiae* into the cytoplasm (SILVERMAN et al. 1992; WINKLER 1990). In the case of *Shigella* the protein IpaB plays a key role, both in triggering cytoskeletal rearrangements to induce phagocytosis and in the lysis of the phagosomal vacuole (HIGH et al. 1992). This protein exhibits contact hemolytic activity but has no sequence homology with any of the known hemolysins. In common with LLO, its hemolytic activity is higher at low pH (5.5) than at neutral pH. It has been hypothesized that IpaB would undergo a conformational change in the phagolysosome which would allow full expression of the hemolytic activity.

L. monocytogenes mutants which are incapable of phagolysosomal lysis do not replicate intracellularly, suggesting that the phagolysosomal milieu is inherently nonpermissive for *Listeria* growth. In contrast, several lines of evidence suggest that the cytoplasm of eukaryotic cells readily supports bacterial multiplication. In a recent study, MARQUIS et al. (1993) demonstrated that a variety of auxotrophic mutants of *L. monocytogenes* replicate in the cytoplasm of J774 macrophages and Henle 407 human epithelial cells. For most of these mutants, the doubling times of the auxotrophic strains were similar to that of the wild-type strain, although there was some variation depending on the cell line used, with the Henle 407 cells being more restrictive for auxotrophic growth than the J774 macrophage-like cells. In the case of *S. flexneri*, purine and aromatic amino acid auxotrophies do not affect the ability of the bacteria to grow intracellularly or to spread from cell to cell (FORMAL et al. 1971; LINDBERG et al. 1988). In addition, following phagolysosomal lysis, *B. subtilis* expressing LLO (BIELECKI et al. 1990) and *E. coli* harboring the *Shigella* virulence plasmid (SANSONETTI et al. 1986) grow and multiply intracellularly, suggesting that survival in the eukaryotic cytoplasm does not require the expression of genes or functions specific to intracellular bacteria.

To identify *L. monocytogenes* genes induced by the intracellular environment, a library of Tn*917-lac* transposon fusion mutants was screened for fusions which were preferentially expressed during intracellular growth. Five such genes were identified, displaying up to 100-fold higher expression of β-galactosidase during growth in J774 macrophages than during growth in rich laboratory broth (BHI; KLARSFELD et al., in press). Four of these genes encode proteins involved in purine (*purH* and *purD*) and pyrimidine biosynthesis (*pyrE*),

and in the transport of arginine acids via an ABC-type system (HIGGINS 1992) which we have called Arp for *A*BC a*r*ginine *p*ermease. The isolation of these genes suggests that nutrients such as nucleotides, although not limiting for intracellular bacterial growth (see below), are at sufficiently low concentrations to induce bacterial genes which are repressed in laboratory media. In addition, a transcriptional *lacZ* fusion to the *plcA* promoter (CDI5; MENGAUD et al. 1991b) was found to be preferentially expressed during intracellular growth. The PurH, PyrE and Arp mutants were tested in the mouse model (following intravenous injection) and were shown to be less affected in virulence than CD15. Only the Arp mutant was affected in its LD_{50} (a two-fold increase relative to wild-type). Nonetheless at 48 hours postinfection, Arp and PurH, but not PyrE mutants, displayed 10–20-fold reduced bacterial loads in the liver with respect to wild type bacteria. Although the inactivation of the *pur* and *pyr* genes results in purine or pyrimidine auxotrophy, respectively, all mutants grow intracellularly reinforcing the view that the eukaryotic cytoplasm is permissive for bacterial growth.

2.3 Intracellular Movement and Cell-to-Cell Spread

Cell-to-cell spreading by *L. monocytogenes* is a complex biological process. It requires the induction and regulation of fundamental host cell-derived functions like actin assembly coupled to intracellular movement, association with the plasma membrane, formation of pseudopod-like protrusions, recognition and phagocytosis of these protrusions by the neighboring cell, and breakdown of the two membranes that surround the invading bacteria after uptake by the new host cell (MOUNIER et al. 1990; TILNEY and PORTNOY 1989). The ability of *L. monocytogenes* to spread within host tissues by direct cell-to-cell spreading constitutes an essential pathogenicity determinant. It is reflected by the pathogen's ability to form plaques, i.e., zones of destroyed cells on fibroblast monolayers that are covered by a bactericidal overlay to prevent extracellular multiplication (HAVELL 1986). *L. monocytogenes* mutants which are unable to form plaques display strongly attenuated virulence in mice, although these mutants are still able to invade cells, to multiply in the cytoplasm, and even to replicate transiently in spleen and liver of infected mice (GOOSSENS and MILON 1992; KOCKS et al. 1992; DOMANN et al. 1992; KUHN et al. 1990). To date, three genes, *mpl*, *actA* and *plcB*, have been implicated in cell-to-cell spreading. These genes are part of the lecithinase operon which is located downstream from *hly* on the chromosome and includes, downstream of *plcB*, three small open reading frames of unknown function (VAZQUEZ-BOLAND et al. 1992; see Fig. 4).

Lecithinase production is, like LLO secretion, a characteristic phenotype of *L. monocytogenes* (FUZI and PILLIS 1962). To understand the role of lecithinase in the pathogenesis of *Listeria*, transposon-induced, lecithinase-negative mutants were analyzed. One such mutant was strongly affected in virulence and defective in plaque formation due to the inability to polymerize actin (KOCKS et al. 1992). The transposon insertion in this mutant mapped to *actA*, the second gene of the

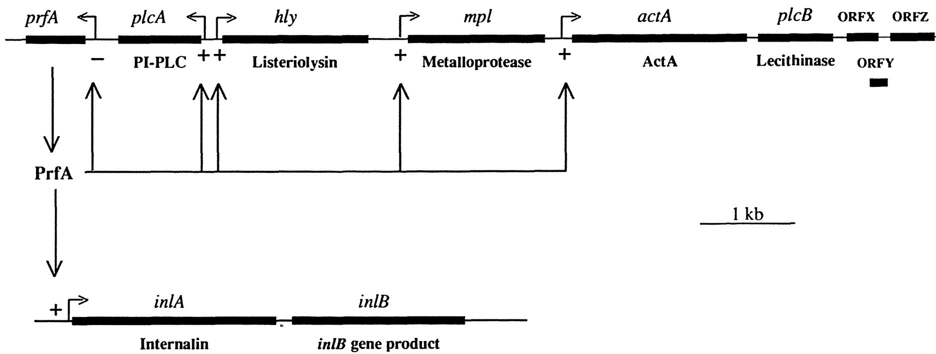

Fig. 4. The coordinate regulation of virulence gene expression by PrfA in *Listeria mono-cytogenes*. The principle features of this model are described in the text. (Adapted from DRAMSI et al. 1993b)

lecithinase operon. Plasmid insertion mutagenesis in the downstream genes showed that the insertion in *actA* was solely responsible for the lack of actin assembly (KOCKS et al. 1992) and that the lecithinase-negative phenotype of the *actA* mutant was due to a polar effect of transposon insertion on transcription of *plcB*. Actin polymerization by *L. monocytogenes* is thus dependent on the expression of the *actA* gene (DOMANN et al. 1992; KOCKS et al. 1992). The lecithinase encoded by *plcB* also plays a role in cell-to-cell spread (see below).

actA encodes a surface protein with an apparent molecular weight of 90 kDa which migrates aberrantly on SDS-PAGE gels. The protein is thought to be anchored in the bacterial membrane by its COOH-terminal end, since insertion mutations in the coding region lead to secretion of nonfunctional truncated molecules that are not associated with the cell wall (DOMANN et al. 1992; KOCKS et al. 1992). At least two thirds of the protein molecule protrudes from the cell wall with the potential to interact with components of the cytoskeleton (KOCKS et al. 1993). In addition to the membrane-bound form, some ActA can be detected in the culture medium (DOMANN et al. 1992; NIEBUHR et al. 1993).

Sequence data bank searches have not revealed striking similarities with known proteins, but weak similarity could be detected to the actin-binding protein caldesmon (20% identity in the NH_2-terminal 243 amino acids) and to human microtubule-associated protein 4 (20% identity in 196 amino acids; for references see KOCKS et al. 1992, 1993). However, the sequence similarity to caldesmon was in the spacer region, separating the known actin and tropomyosin-binding domain. The NH_2-terminal domain of ActA contains a motif (64-LKEKAE-70) similar to the presumptive actin binding site of caldesmon (498-LKEKQQ-503) and to hexapeptide LKEAET which can induce actin polymerization in vitro (VANCOMPERNOLLE et al. 1992). A short motif present twice in the ActA protein (237-PPPTDEEELRLAL-250 and 272–283) is similar to a region in mouse elongation factor 1a (237-PPRPTDKPLRLPL). This similarity may be of interest as *Dictyostelium* ABP-50, a protein that cross-links actin filaments, has recently been identified as elongation factor 1a. In addition, ActA shares short regions of

similarity with the cytoskeleton protein vinculin. One such region is motif 394-DRLADLRDRGTG-405 similar to residues 333–344 of chicken vinculin (DQLADLRARGQG). The central part of the ActA contains several proline- and glutamic acid-rich repeats which share similarity to a region of vinculin which is rich in proline, aspartic and glutamic acid. The ActA repeat motif harbors several consensus phosphorylation sites for a cellular protein kinase, casein kinase II.

The mechanism by which ActA mediates actin assembly is not known (see review by COSSART and KOCKS 1994). In order to generate forward propulsion, one would expect that actin filament formation should be initiated on the bacterial body in an asymmetric way. ActA is indeed asymmetrically expressed during cell division, resulting—after division—in absence from one end (the newly formed end) and abundance towards the other (older) end (KOCKS et al. 1993). In the cytoplasm of infected cells, the protein localizes precisely to the site of actin filament formation on the bacterial surface (KOCKS et al. 1993). It cannot be detected in the actin tails (KOCKS et al. 1993; NIEBUHR et al. 1993). This localization pattern suggests that ActA somehow triggers the actin polymerization process, either by directly interacting with actin or indirectly by inducing actin polymerization through a cellular nucleator (THERIOT and MITCHISON 1992; THERIOT et al. 1994). The activity of ActA may be modulated by phosphorylation, since in the cytoplasm ActA gets phosphorylated by an as yet unidentified host cell-derived kinase (BRUNDAGE et al. 1993). In addition to ActA, comet tail formation and movement may require further bacterial factors. Evidence for the latter possibility comes from the phenotype of a nonmotile mutant that is severely affected in its capacity to form comet tails, but still induces actin polymerization and becomes surrounded by actin filaments (KUHN et al. 1990). In this mutant, both the level of expression and the distribution of ActA are indistinguishable from wild type (C.K, P.C. unpublished data).

The third gene of the lecithinase operon, *plcB*, encodes the *L. monocytogenes* lecithinase (PC-PLC; VAZQUEZ-BOLAND et al. 1992). Lecithinase is a secreted PLC with a pH optimum between 5.5 and 8. This enzyme catalyzes the hydrolysis of a broad spectrum of phospholipids. It is active on phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, to a lesser extent on sphingomyelin and very weakly on phosphatidylinositol (GEOFFROY et al. 1991; GOLDFINE et al. 1993). Through the analysis of a *plcB* insertion mutant, evidence has been obtained that this lecithinase contributes to the breakdown of the two plasma membranes that surround *L. monocytogenes* after cell-to-cell spread (VAZQUEZ-BOLAND et al. 1992). On fibroblast monolayers *plcB* mutants form significantly smaller plaques than wild-type bacteria, and in an electron microscopic study such mutants were found to accumulate in two membrane vacuoles in the cytoplasm of the new host cell. The broad spectrum specificity of the purified *L. monocytogenes* PC-PLC is consistent with a role in lysis of the two leaflets of the plasma membrane, which are known to display phospholipid asymmetry. However, that such mutants remain capable of making plaques suggests that lecithinase is not the only factor involved in the escape from this compartment and it remains possible that LLO may also play a role.

Lecithinase is secreted in an inactive form that can be detected as a 33 kDa doublet band on western blots (GEOFFROY et al. 1991; NIEBUHR et al. 1993). Genetic evidence indicates that the predicted *mpl* gene product, a 57-kDa polypeptide with a signal sequence and propeptide sharing homologies with metalloproteases from other bacterial pathogens (DOMANN et al. 1991; MENGAUD et al. 1991a), cleaves the PC-PLC proenzyme in *L. monocytogenes* broth cultures, resulting in the mature, active 29 kDa form of the phospholipase (NIEBUHR et al. 1993; POYART et al. 1993; RAVENEAU et al. 1992). It is not clear whether cleavage occurs at the bacterial surface or in solution. The *mpl* product has been detected in *L. monocytogenes* culture supernatants as a 60 kDa polypeptide, corresponding to an immature, presumably inactive, proform of the enzyme. In only one strain (NCTC 7973) could the mature form of the protein and protease activity be detected (DOMANN et al. 1991).

The analysis of the roles that *mpl*, *actA* and *plcB* play in virulence has been hampered by their genetic organization in an operon. Transcription in this operon is complex and is initiated from two promoters (CHAKRABORTY et al. 1992; DOMANN et al. 1991; LEIMEISTER-WÄCHTER et al. 1992; MENGAUD et al. 1989, 1991c; VAZQUEZ-BOLAND et al. 1992). One promoter is situated in front of *mpl*, giving rise to either a long polycistronic transcript spanning all genes of the operon or to a small transcript covering only the *mpl* gene. A second promoter lies in front of *actA*; the transcripts from this promoter are less well characterized. Insertion mutations into *mpl* exert a partial polar effect on the transcription of *actA* and *plcB* (MENGAUD et al. 1991c; POYART et al. 1993; RAVENEAU et al. 1992), and insertion mutations into *actA* eliminate *plcB* expression (KOCKS et al. 1992; see above). Therefore, precise assignment of the roles of these genes in virulence requires the construction of in-frame deletions of individual genes, creating mutants in which transcription of the other genes of the operon is not affected. Thus far, only an *actA* in-frame deletion mutant has been constructed. Its LD_{50} in mice is more than three orders of magnitude increased when compared to wild type (BRUNDAGE et al. 1993).

Shigellae and the spotted fever group (SFG) *Rickettsiae* also use actin polymerization and direct cell-to-cell spreading to within infected tissues (BERNARDINI et al. 1989; HEINZEN et al. 1993; TEYSSEIRE et al. 1992). Although the three parasites cause different clinical syndromes (*L. monocytogenes*: meningoencephalitis and abortions; *Shigella*: dysentry; SFG *Rickettsia*: spotted fever), they probably use similar strategies to exploit the various host cell functions involved. While a molecular genetic analysis of the mechanism of *Rickettsiae* pathogenesis has not been performed, two of the genes directly involved in cell-to-cell spreading have been isolated in *Shigella*. *icsA* (also called *virG*; LETT et al. 1989) is a 120 kDa outer membrane protein which is exposed on the *Shigella* surface (GOLDBERG et al. 1993). It is partially released owing to COOH-terminal cleavage giving rise to a 95 kDa form. In contrast to ActA, the protein can be detected in the bacterially induced actin tails. A weak ATPase activity has been demonstrated for IcsA, but the significance of these findings for the actin assembly process is not known. IcsA harbors a phosphorylation site for cAMP-dependent protein kinase which down-regulates the plaque-forming capacity of

S. flexneri. It has been proposed that phosphorylation of IcsA by this host cell kinase represents a defense mechanism of the host cell (D'HAUTEVILLE and SANSONETTI 1992). *icsB* (ALLAOUI et al. 1992) is a protein of 54 kDa whose molecular activities are unknown. It is necessary for efficient escape from two membrane vacuoles after spreading. IcsA and IcsB have no sequence similarities to the *Listeria* proteins ActA and PC-PLC and it is not clear whether they are functionally equivalent.

3 Regulation of Virulence Factors

As detailed above, the successful penetration of, survival within, and dissemination throughout the host requires the elaboration of a variety of *Listeria* proteins. The coordinate regulation of these virulence determinants was first hypothesized following the identification of conserved palindromic sequences in the *hly* promoter and in the promoter regions of the flanking virulence-related operons (MENGAUD et al. 1989). In addition, several mutations with pleiotropic effects on virulence gene expression were isolated (KATHARIOU et al. 1990; MENGAUD et al. 1991b; SUN et al. 1990). Subsequently, molecular genetic analysis of the type strain SLCC-53, which is nonhemolytic and avirulent, demonstrated that the strain contained an intact, but transcriptionally silent, *hly* gene (LEIMEISTER-WÄCHTER et al. 1990). The defect in *hly* expression in this strain coincided with the presence of a small deletion located immediately downstream of the *plcA* gene (GORMLEY et al. 1989; LEIMEISTER-WÄCHTER et al. 1989). Cloning and sequence analysis of the corresponding DNA from wild-type strains resulted in the identification of an open reading frame, designated *prfA*, which encodes a protein of 27 kDa (LEIMEISTER-WÄCHTER et al. 1990; MENGAUD et al. 1991b). Introduction of the cloned *prfA* gene into strain SLCC53 restored hemolysin production by this strain, thus providing the first direct evidence that *prfA* regulates *hly* expression in *L. monocytogenes*. Subsequently, detailed complementation analysis and the study of transposon or insertion mutants demonstrated that *prfA* is a pleiotropic regulator of virulence gene expression (Fig. 4) and activates transcription from the *hly*, *plcA*, *actA*, and *mpl* promoters (CHAKRABORTY et al. 1992; MENGAUD et al. 1991b). Most recently, *prfA* has been shown to be required for expression of the invasion-associated, *inlAB* locus (DRAMSI et al. 1993b).

Until recently, the deduced amino acid sequence of the protein encoded by *prfA* was believed to display little overall homology to other prokaryotic regulatory proteins (LEIMEISTER-WÄCHTER et al. 1990; MENGAUD et al. 1991b). However, recent data bank searches (R. EBRIGHT, personal communication) have shown that PrfA exhibits similarity throughout its length to the *Escherichia coli* cAMP receptor protein, CAP (20% identical residues; 39% similar residues; AIBA et al. 1982; COSSART and GICQUEL-SANZEY 1982) and to CAP-related proteins from the gram positive bacterium *Lactobacillus casei*, FLP (26% identical residues; 44% similar residues; IRVINE and GUEST 1993) and the cyanobacterium *Synechococcus*, NtcA (20% identical residues; 35% similar residues; VEGA-PALAS et al. 1992). If this

PrfA-binding site (−35)	−10	
ATAACATAAGTTAA	TTCTTTTTTTTGGAAAAATAGTTATTATTATTT**A**-397bp-GTG	P2*inlA*
TTAACAAATGTTAA	TGCCTCAACATAAAAGTCACTTTAAGATAGGAAT**A**-24bp-TTG	P*plcA*
TTAACATTTGTTAA	CGACGATAAAGGGACAGCAGGACTAGAATAAAGCT**AT**-130bp-ATG	P2*hly*
TTAACAAATGTAAA	AGAATATCTGACTGTTTATCCATATAATATAAGC**A**-150bp-ATG	P*mpl*
TTAACAAATGTTAG	AGAAAAATTAATTCTCCAAGTGATATTCTTAAAA**T**-148bp-GTG	P*actA*

PrfA-binding site

Fig. 5. Comparison of the 14 bp palindromes present in the –35 regions of the *prfA*-regulated virulence genes. The transcriptional start points (*bold letters*) and the –10 regions (*underlined*) of the various promoters are shown. Nucleotide substitutions with respect to the *hly* palindrome are indicated. (Adapted from DRAMSI et al. 1993b)

similarity is functionally significant, then PrfA would be predicted to have a helix-turn-helix motif at amino acid 171–191 (with a possible 1 amino acid insertion in the turn) or from 173–191 (with a possible 1 amino acid deletion in the turn). Recently, FREITAG et al. (1993) have used gel retardation analysis with purified PrfA to demonstrate that PrfA is a site-specific DNA-binding protein. The PrfA protein specifically retarded the mobility of DNA fragments containing the 14 bp palindromic sequence located between the divergent *hly* and *plcA* promoters. Significantly, PrfA-dependent activation of *hly* transcription in *B. subtilis* is abolished by point mutations in this palindrome (FREITAG et al. 1992). Similar sequences of dyad symmetry are found in the –35 regions of the *mpl* (MENGAUD et al. 1989), *actA* (VAZQUEZ-BOLAND et al. 1992), and the internalin locus promoters (DRAMSI et al. 1993b) (Fig. 5), suggesting that PrfA activates gene expression by binding these target sequences and interacting directly with RNA polymerase. The *actA* and *mpl* palindromes each contain a single base change relative to the *hly* sequence while that preceding the *inlA* promoter contains two base differences relative to the *hly* palindrome (Fig. 5). These observations and evidence that the activation of *actA*, *inlA* and *mpl* expression by PrfA in *B. subtilis* is less efficient than that observed for *hly* and *plcA* (SHEEHAN and COSSART, unpublished) suggests that a hierarchy of palindrome binding affinities by PrfA does exists. Such a system could facilitate the temporal activation of virulence genes, with high affinity promoters being activated at low PrfA concentrations, whereas low affinity promoters would require relatively higher levels of PrfA (FREITAG et al. 1992; MENGAUD et al. 1989).

Transcription of *prfA* is not constitutive and there are two maxima of *prfA* transcription during the growth cycle in vitro; these peaks coincide with increased LLO expression in the culture supernatant (MENGAUD et al. 1991b). The first occurs in early exponential phase when *prfA* is cotranscribed with *plcA* as part of a 2.2 kb transcript. Production of this bicistronic transcript requires transcription through a putative rho-independent terminator-like structure at the 3' end of the *plcA* gene. The second peak of *prfA* transcription occurs at the end of exponential growth when the major *prfA*-specific transcript is 1 kb and originates from a promoter region located immediately 5' to the *prfA* gene. Both transcripts appear to be

required to ensure sufficient PrfA production for wild-type plaque formation in monolayers of L2 cells (CAMILLI et al. 1993). As *prfA* stimulates transcription of *plcA*, *prfA* activates its own synthesis, at least in early exponential phase. During the stationary phase of growth, transcription of *prfA* and other genes of the *prfA* regulon decreases suggesting that PrfA may also play a role in negatively regulating its own synthesis (MENGAUD et al. 1991b).

Recent studies have confirmed a role for PrfA in the repression of transcription initiation at the *prfA* structural gene promoters. Transcripts initiated from the *prfA*-specific promoters prfAp1 and prfAp2 were more abundant in a *prfA* mutant strain than in the isogenic wild-type strain 10403S (FREITAG et al. 1993). The palindrome which may serve as a recognition sequence for PrfA binding is not present in the *prfA*-specific promoter region. Thus, repression of transcription initiation at these promoters may result from the direct interaction of the PrfA protein with other sites in or near the promoter, or through the activation of a repressor by PrfA. It is worth noting here that there appears to be a degree of strain-dependent variability in the number and positioning of the *prfA* promoters which lie in the 273 bp intergenic region between the *plcA* and the *prfA* structural genes. Strain LO28 contains two *prfA*-specific promoters, P1 and P2, located at positions –113 bp and –143 bp, respectively, from the AUG initiation codon of *prfA* (MENGAUD et al. 1991b). In strain 10403S, two promoters prfAp1 and prfAp2 are found at –113 bp and –30 bp, respectively, while strain EGD appears to contain only the promoter at –113 bp (FREITAG et al. 1993; LEIMEISTER-WÄCHTER et al. 1992).

Virulence determinants of many pathogenic bacteria are subject to environmental modulation and evidence suggests that similar strategies are employed by *Listeria* to optimize gene expression within the host. The transition from ambient temperature to that of the human body induces pleiotropic alterations in gene expression in a variety of pathogens including *Escherichia coli*, *Bordetella pertussis*, *Shigella* and *Yersinia species*, and *Vibrio cholera* (reviewed by MEKALANOS 1992). In *L. monocytogenes* optimum virulence gene expression occurs at 37°C (LEIMEISTER-WÄCHTER et al. 1992). Northern blot analysis has demonstrated that this thermoregulation is effected at the level of transcription with fewer transcripts corresponding to the virulence-related genes *hly*, *plcA*, *mpl*, and *inlA* occurring at temperatures below 37°C. Interestingly, transcription of the smaller *prfA*-specific mRNA appears to be unaffected by the growth temperature, suggesting that PrfA may act in concert with other regulatory proteins (that are themselves thermoregulated) or that PrfA may require posttranscriptional modification for activity (LEIMEISTER-WÄCHTER et al. 1992).

Recently, PARK and KROLL (1993) have described a negative effector molecule of virulence gene expression in *L. monocytogenes*. Using transcriptional fusions between the *hly* and *plcA* promoters and the *luxAB* reporter genes, the authors observed that the expression of luciferase by these fusions was specifically repressed by the plant-derived disaccharide cellobiose. This molecule is considered to be present in significant quantities in the soil and decaying vegetation that constitute the primary habitats of *L. monocytogenes*. Cellobiose may thus act to repress virulence gene expression in the saprophytic

environment. Although the low, residual level expression of *hly* and *plcA* in *prfA* mutants was not greatly affected by cellobiose, it is not yet clear if this molecule exerts its effect via the *prfA* regulatory system. Interestingly, like the effect of temperature described above, regulation of *hly* and *plcA* transcription in the presence of cellobiose is not coupled to the levels of monocistronic *prfA* mRNA (KLARSFELD et al. 1994).

Stress conditions such as heat shock and nutrient stress result in the preferential synthesis of *prfA*-regulated proteins in vitro. LLO and at least four other *prfA*-dependent proteins (which may include ActA and PI-PLC) are preferentially synthesized when bacteria are grown at 48°C (heat shock conditions; SOKOLOVIC and GOEBEL 1989; SOKOLOVIC et al. 1990, 1993). Moreover, at least 12 proteins are specifically labeled by [^{35}S] methionine in wild-type, but not PrfA-defective, strains, when bacteria were incubated in a medium (MEM) containing low concentrations of essential amino acids and iron and which did not support bacterial growth (SOKOLOVIC et al. 1993). Included among these stress-induced proteins are a number of previously characterized *prfA*-regulated proteins (ActA, LLO, Mpl, PI-PLC, and PC-PLC) and their proteolytically processed derivatives. The five remaining stress-induced, PrfA-regulated, proteins represent novel surface-associated products whose role in virulence, if any, remains to be determined. Similar experiments revealed the presence of a 64 kDa surface-located protein, the expression of which is repressed by PrfA, suggesting that PrfA, in common with many other bacterial regulators, may act both as an activator and as a repressor of target gene expression.

While the *prfA* regulation of virulence gene expression occurs primarily at the level of transcript initiation, there is evidence that other regulatory controls exist to modulate expression of *prfA*-dependent genes. Gene regulation by transcriptional antitermination has been documented in other gram-positive organisms including *B. subtilis* (see review by KLIER et al. 1992 and references therein). In *L. monocytogenes*, transcriptional antitermination has been suggested to occur at the putative terminater located in the intergenic region between the *plcA* and *prfA* genes, resulting in the production of the bicistronic mRNA during the early stages of growth (MENGAUD et al. 1991b) and in the intergenic region between the *inlA* and *inlB* genes (DRAMSI et al. 1993b). It remains to be determined if the same mechanism of antitermination operates in both operons.

4 Other Potential Virulence Determinants

In addition to those described above, a number of other *Listeria* genes have been identified which may contribute to the infectious process. These include genes encoding a superoxide dismutase (BREHM et al. 1992), a protein homologous to the cholera toxin of *Vibrio cholerae* (GARCIA DEL PORTILLO et al. 1992; VICENTE et al. 1989) and the *L. monocytogenes* flagellin protein (DONS et al. 1992). Furthermore, regulatory genes which may contribute to the virulence of *L. monocytogenes* have been identified (WREN et al. 1992).

Oxidizing agents such as the superoxide radical and hydrogen peroxide exhibit potent antimicrobial activity and the ability to counteract these products may contribute to the intracellular survival of *L. monocytogenes*. The *L. monocytogenes* superoxide dismutase-encoding gene, *lmsod*, has recently been cloned and sequenced (BREHM et al. 1992). Superoxide dismutase (SOD) converts superoxide radicals to hydrogen peroxide as the first step in the elimination of these toxic metabolites from the cell. While a role for SOD in the pathogenicity of faculative intracellular bacteria such as *Nocardia asteroides* (BEAMAN and BEAMAN 1990) and *Shigella flexneri* (FRANZON et al. 1990) has been proposed, the role of the *Listeria* SOD in intracellular survival has yet to be demonstrated. Similarly, catalase production by *L. monocytogenes* has been suggested as a virulence factor which contributes to bacterial defense against the products of oxidative metabolism. However, transposon-induced, catalase-deficient mutants of *L. monocytogenes* were not affected in virulence when tested in mouse models of infection (LEBLOND-FRANCILLARD et al. 1989).

Recently, a fragment of a *L. monocytogenes* gene with homology to the *ctxA* gene of the gram-negative pathogen *Vibrio cholerae* has been cloned and sequenced (GARCIA DEL PORTILLO et al. 1992; VICENTE et al. 1989). The *V. cholerae ctxAB* operon encodes the A and B subunits of the ADP-ribosylating enzyme cholera toxin, which is largely responsible for the diarrheal syndrome associated with *V. cholerae* colonization of the intestinal mucosa. The role of the *L. monocytogenes ctxAB* homologue in virulence is currently unknown. It should be noted that sequences hybridizing to a *ctxAB* probe are present in all, including nonpathogenic, *Listeria* species (Gouin and Cossart, unpublished).

A relationship between bacterial motility and virulence has been reported for several pathogens including *Helicobacter pylori*, *Salmonella typhi* and *Vibrio cholerae* (EATON et al. 1992; LIU et al. 1988; RICHARDSON 1991). In all cases, loss of motility correlated with decreased virulence in in vitro or in vivo models of infection. Strains of *L. monocytogenes* grown at low temperatures (20°–25°C) possess flagella and are motile. In contrast, *Listeria* grown at 37°C are only poorly flagellated and non-motile. The flagellin-encoding *flaA* gene has recently been cloned and sequenced and the NH_2-and COOH-terminal regions of flagellin show considerable homology to other flagellin proteins (DONS et al. 1992). In northern blot experiments the *flaA*-specific mRNA was undetectable at 37°C, suggesting that the thermoregulation of flagellin production occurs at the level of transcription. Southern blot analysis with a *flaA*-specific probe has demonstrated that *flaA* homologues are present in all *Listeria* species (DONS et al. 1992). The availability of the cloned *flaA* gene should allow the construction of isogenic *flaA*-defective strains and the analysis of the role of flagellar expression and motility on *Listeria* virulence.

A wide variety of bacterial adaptive responses, including the coordinate regulation of virulence determinants in pathogenic microorganisms, are mediated by the well characterized, two component, signal transduction systems (for reviews see PARKINSON 1993; STOCK et al. 1989, 1990). These systems contain two classes of well conserved proteins; a sensor (generally a histidine protein kinase),

which is involved in environmental signal detection, and a response regulator, frequently a cytoplasmic transcriptional regulator. All response regulators contain a conserved domain of approximately 100 amino acids which extends from the NH_2-terminal. Degenerate oligonucleotide primers which hybridize to this conserved region have been used to amplify by PCR *L. monocytogenes* DNA fragments with sequence homology to response regulator proteins (WREN et al. 1992).

5 Genetic Map of *Listeria monocytogenes*

Until recently, most genes identified in *L. monocytogenes* encode proteins involved in the pathogenesis of this organism. The majority of these are clustered at a single 10 kb locus containing the *hly* gene and the flanking *plcA-prfA* and lecithinase operons. As transducing phages are not available in *Listeria*, pulse field gel electrophoresis was used to establish the current physical and genetic map of *L. monocytogenes* (Fig. 6; MICHEL and COSSART 1992). Included on this map are a number of recently identified listerial genes including *cheR*, (43% identity to

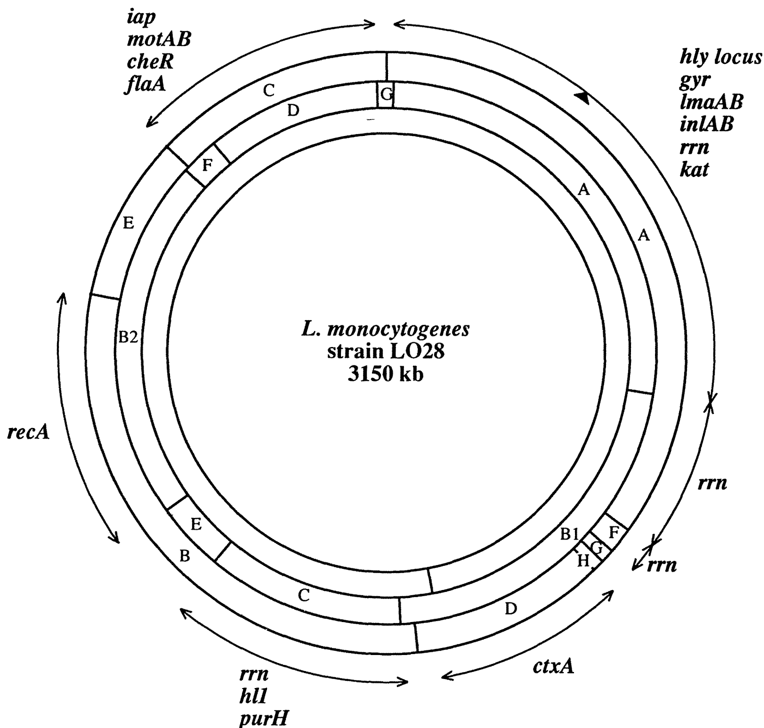

Fig. 6. Physical and genetic map of the *Listeria monocytogenes* chromosome. The *outer intervals* correspond to *NotI* fragments, the *intermediate* to *Sse*8387I fragments, and the *inner* indicates the location of the single *SfiI* site. The intervals where genes have been located are indicated by *arcs* outside the circular map. The arrow indicates the precise location of the *hly* locus.

the *B. subtilis* chemotaxis methyltransferase); *motA* and *motB*, (45% identity to *B. subtilis* flagellar motor proteins) (Eric Michel, Sally Galsworthy, Jérome Mengaud, Pascale Cossart, manuscript in preparation); *flaA*, (encoding the *L. monocytogenes* flagellin protein; Dons et al. 1992), *hl1*, (homology to bacterial histone-like protein-encoding genes; M. Sanchez-Campillo, J. M. Gomez, and J.C. Perez-Diaz, personal communication), and *purH*, (de novo purine biosynthesis; Klarsfeld et al.1994). The location of the *L. monocytogenes* catalase gene, *kat* has been determined by hydridization using sequences derived from the cloned *L. seeligeri kat* gene (Haas et al. 1991).

6 Genetic Analysis of Other *Listeria* Species

Of the other members of the genus *Listeria*, only *L. ivanovii* is considered to be pathogenic but, unlike *L. monocytogenes*, it is a pathogen of animals and is not associated with human disease (Cooper and Dennis 1978). *L. ivanovii* infects a variety of domestic animals causing abortion, neonatal sepsis, and enteritis. Recent results obtained in in vitro tissue culture models of infection suggest that *L. ivanovii* follows the same general pathway of entry, phagolysosomal lysis, intracellular multiplication and cell-to-cell spreading as *L. monocytogenes. L. ivanovii* can induce its uptake by epithelial cells and fibroblasts (Gaillard et al. 1987; Karunasagar et al. 1993; Kuhn et al. 1988). Entry into the host cell is followed by lysis of the phagolysosome. *L. ivanovii* produces a thiol-activated cytolysin, ivanolysin (ILO), which is closely related to LLO and which, by analogy with LLO, may be required for phagolysosomal lysis (Leimeister-Wachter and Chakraborty 1989; Haas et al. 1992). Once in the cytoplasm, *L. ivanovii* becomes associated with host cell actin filaments which are reorganized to form comet tails similar to those observed with *L. monocytogenes* (Karunasagar et al. 1993). The ability of *L. ivanovii* to induce actin polymerization suggests the existence of an ActA homologue in this species, but several early attempts to detect sequences similar to *actA* in *L. ivanovii* have been unsuccessful (Karunasagar et al. 1993; Vazquez-Boland et al. 1992). However, using different probes, Southern blot analysis under low stringency conditions has now demonstrated that the virulence locus of *hly*, *plcA-prfA* and the lecithinase operon, including the gene *actA*, is present in the three hemolytic species of *Listeria*, *L. monocytogenes*, *L. ivanovii*, and *L. seeligeri* (see Fig. 7; Gouin et al. 1994). In contrast, genes with putative "housekeeping" functions such as *ldh* and *prs* (Fig. 7), which encode proteins with homology to lactate dehydrogenase, and to bacterial and eukaryotic phosphoribosyl-pyrophosphate synthetases, respectively, and which flank the virulence locus, are found in all *Listeria* species. The chromosomal arrangement of *plcA*, *prfA* and the ivanolysin gene is highly reminiscent of the arrangement of the corresponding genes in *L. monocytogenes* (Goebel et al. 1994).

Interestingly, although electron microscopic observations suggest that *L. ivanovii* is capable of cell-to-cell spreading, it does not cause plaques in fibroblast monolayers (Karunasagar et al. 1993). While the mechanism of cytotoxity in *Listeria* species is not yet known, Karunasagar et al. (1993) have suggested that

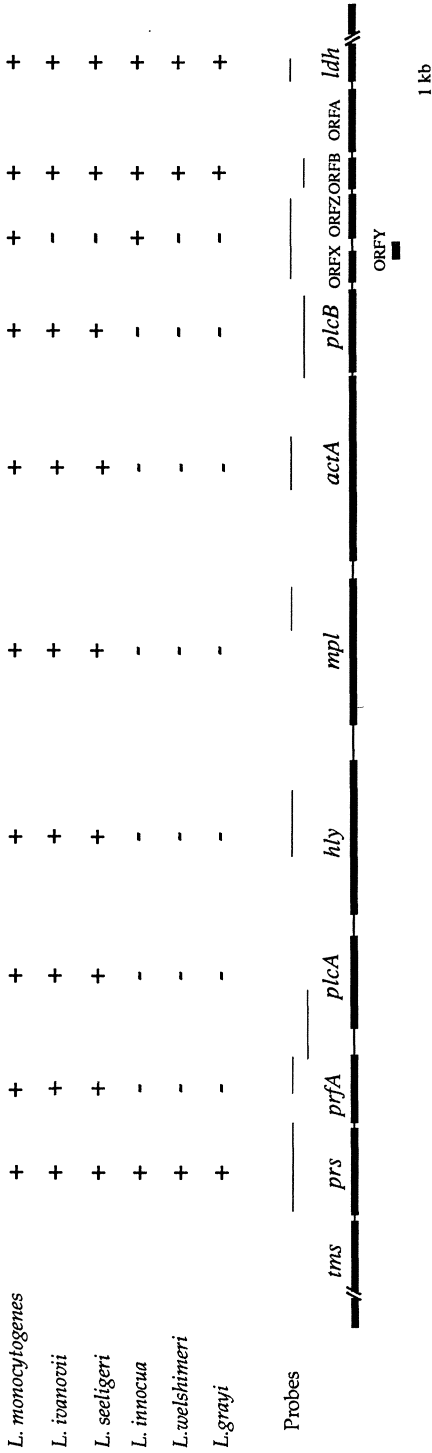

Fig. 7. Distribution of the genes of the 10 kb virulence locus and flanking regions within the genus Listeria. The DNA probes used in the Southern hybridization analysis are indicated by the horizontal lines above the genes

L. ivanovii lacks a cytolytic activity which leads to the destruction of host cells by *L. monocytogenes* and that this lack of cytotoxicity may account for lower degree of virulence of *L. ivanovii* compared to *L. monocytogenes*.

L. seeligeri, a nonpathogenic *Listeria* species, displays a weak hemolytic activity on blood agar plates (Geoffroy et al. 1989). These bacteria are very weakly invasive for epithelial cells and rarely escape from the phagosome (Gaillard et al. 1987). It is possible that this species lacks some virulence loci required for *L. monocytogenes* and *L. ivanovii* virulence or, more likely, that some of the genes hybridizing to the known virulence cluster, although present, may be not functional or not sufficiently well expressed to confer virulence (Gouin et al. 1994).

7 Concluding Remarks

Considerable advances in our understanding of the infection of host tissues by *L. monocytogenes* have occurred during the past decade. These have been made possible by the development of techniques that allow *L. monocytogenes* to be genetically manipulated and by the availability of in vitro and in vivo models of infection. Beginning with the identification of *hly* as an essential virulence determinant, many genes required for successful penetration, multiplication and dissemination of this pathogen within host tissues have been described. However, these genes are unlikely to represent the entire spectrum of *L. monocytogenes* requirements for intracellular parasitism. For example, it is to be expected that genes other than *inlA* are required for entry. In addition, bacterial factors inducing the efficient phagocytosis of bacteria-containing protrusions by neighboring cells during cell-to-cell spreading remain to be identified. While it is becoming clear that intracellular *Listeria* do not express many specialized metabolic functions for survival and multiplication in the host cell cytoplasm, evidence suggests that these bacteria have evolved sophisticated strategies to exploit components of the host cell cytoskeleton. Bacterially induced phagocytosis and intracellular actin-based motility provide elegant examples of this type of host pathogen interaction. Future research will undoubtedly focus on the identification of the host cell receptor for internalin, on bacterially induced signaling events and on the nature of the eukaryotic proteins recruited by *Listeria* for actin tail formation and movement. The recent development of an in vitro system for *Listeria* motility based on *Xenopus* oocyte extracts promises to make *Listeria* an exciting tool for the investigation of actin-based motility phenomena in general (Theriot and Mitchison 1992; Theriot et al. 1994).

Finally, although similarities can be observed between the behavior of the gram-negative pathogen *S. flexneri* and *L. monocytogenes* in tissue culture models of infection (i.e., cytoplasmic multiplication, actin-based movement, and direct cell-to-cell spread), the clinical features of shigellosis and listeriosis are quite different. This disparity between host cell infection at the cellular level and the clinical manifestations of the resultant disease emphasize the need to complement the in vitro analysis of bacterial pathogenesis with relevant in vivo studies.

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Notes added in proof

Since this review was written a number of papers have been published with relevance to intracellular movement and the regulation of virulence factors.

Pistor et al. (1994) have demonstrated that the expression of ActA in eukaryotic cells (in the absence of other bacterial factors) is sufficient to induce actin nucleation. Southwick and Purich (1994) have shown that microinjection of a synthetic peptide analogous to one of ActA's oligoproline repeats into Listeria-infected cells blocks the formation of actin-filament tails and arrests bacterial movement. Injection of this peptide also resulted in host cell membrane retraction.

Bohne et al. (1994) have shown that ActA, PC-PLC and LLO are the major PdPs synthesized when *L. monocytogenes* is shifted from BHI to MEM. The preferential synthesis of PdPs in MEM requires de novo transcription. Freitag and Portnoy (1994) have demonstrated that the two *prfA* promoters which occur in the intergenic region between *plcA* and *prfA* are functionally redundant in vivo. Finally, Lampidis et al. (1994 in press) have cloned sequenced the *prfA* homologue from *L. ivanovii*. They have independently identified the homology between the *Listeria* PrfA proteins and the Crp-Fnr family of transcriptional regulators.

Shigella flexneri: Genetics of Entry and Intercellular Dissemination in Epithelial Cells

C. PARSOT

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1 Introduction

A century ago, Shiga isolated the etiologic agent of bacillary dysentery (SHIGA 1898). The genus *Shigella* is now divided into four species, *S. boydii*, *S. dysenteriae*, *S. flexneri*, and *S. sonnei*. Shigellosis, whose clinical signs range from mild diarrhea to severe dysentery with blood, mucus, and pus in the stool, remains endemic throughout the world. Epidemiological studies indicate that *Shigella* is transmitted by the fecal-oral route and sometimes by contaminated food (WHARTON et al. 1990). *Shigella* are highly infectious organisms for humans, since only a few hundred bacteria administered orally caused disease in 50% of volunteers (DUPONT et al. 1989).

Shigellosis is caused by penetration of invasive bacteria into the intestinal mucosa of the colon, where degeneration of the epithelium and a strong inflammatory reaction indicate the sites of *Shigella* infection (LABREC et al. 1964). Most of our knowledge on the pathogenesis of the disease is derived from

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studies using experimentally infected monkeys (TAKEUCHY et al. 1968). Injection of bacteria into rabbit ligated ileal loops, which elicits fluid accumulation and mucosal destruction (GOTS et al. 1974), and infection of the corneal epithelium of guinea pigs, which provokes keratoconjunctivitis (SERENY 1957), are sometimes used to assess *Shigella* virulence.

Since *Shigella* can invade epithelial and nonepithelial cells in vitro (GERBER and WATKINS 1961; LABREC et al. 1964), the invasive process has been mainly studied on cultured cell lines. In vitro, this process consists of the integration of two steps, entry and intercellular dissemination, and eventually leads to a cytopathic effect that can be visualized by the formation of plaques on a confluent cell monolayer (OAKS et al. 1985). The cellular biology and genetics of entry and intercellular dissemination (reviewed by HALE 1991 and by SANSONETTI 1991) have been performed mainly using *S. flexneri*, but most conclusions derived from these studies apply to the other *Shigella* species as well as to enteroinvasive *Escherichia coli* (EIEC) strains that cause a dysentery-like syndrome similar to shigellosis (DUPONT et al. 1971).

The various aspects of *Shigella* pathogenicity were the focus of an issue of this series (SANSONETTI 1992).

2 Cellular Biology of Entry and Intercellular Dissemination

Following adhesion to the cell, a step that is quite elusive in the case of *Shigella*, the bacteria are internalized by epithelial cells in a process similar to phagocytosis, in that actin polymerization and myosin accumulation at the site of entry are required (HALE et al. 1979; CLERC and SANSONETTI 1987). Infection of polarized cells, which were differentiated from the human colonic epithelial cell line Caco-2, indicated that *S. flexneri* enters through the basolateral pole rather than the apical pole of epithelial cells (MOUNIER et al. 1992).

Within a few minutes after entry, *Shigella* lyses the membrane of the phagocytic vacuole and gains access to the cytoplasm of the cell where it multiplies with a generation time of about 40 min (SANSONETTI et al. 1986). Protein synthesis of the infected cell is rapidly blocked (HALE and FORMAL 1981). Invasion by *S. flexneri* of a murine macrophage cell line, J774, resulted in rapid killing of the host cell (SANSONETTI and MOUNIER 1987). Wild-type *Shigella* induced apoptosis, i.e., programmed cell death, in infected macrophages (ZYCHLINSKY et al. 1992).

While extracellular *Shigella* are nonmotile organisms, intracellular bacteria move to occupy the entire cytoplasm of the infected cell and to spread from cell to cell. Two apparently independent movements have been described in different cell lines. In chicken embryo fibroblasts, which have a highly organized cytoskeleton, intracellular bacteria interact with and progress along stress fibers, a movement that was designated Olm (for organelle-like movement) (VASSELON et al. 1991). A movement of the bacteria along the actin filament ring of the peri-junctional area has also been observed in infected Caco-2 cells (VASSELON et al. 1992).

The initial observation that *Shigella* moves into the cytoplasm of infected cells came from phase contrast microcinematography studies; the movement of the bacteria was random and sometimes led to the formation of structures protruding from the cell surface and containing bacteria at their tip (OGAWA et al. 1968). This intracellular movement can be reversibly inhibited by treatment with cytochalasin D which prevents polymerization of monomeric actin (G-actin) into filaments (PAL et al. 1989; BERNARDINI et al. 1989). The use of *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) phallacidin, which specifically binds to polymerized actin (F-actin), showed labeling of intracellular bacteria and trails of F-actin at one pole of the bacteria, confirming the involvement of actin in the movement of bacteria (BERNARDINI et al. 1989).

Electron microscopy studies have allowed a detailed ultrastructural analysis of these protrusions, which have a diameter of about 0.5 μm and a length up to 20 μm (KADURUGAMUWA et al. 1991; PRÉVOST et al. 1992; SANSONETTI et al. 1993). Bacteria in these protrusions are located at the top of tightly packed actin filaments which, in some instances, appeared to form a cylinder. Pictures showing a protrusion extending from one cell and penetrating into the adjacent cell indicated that these protrusions can allow passage of *Shigella* from cell to cell without release of the bacteria into the extracellular medium. Genetic studies (see below) confirmed the importance of this movement, designated Ics (for intra- and intercellular spread), in the dissemination of bacteria from the primary infected cell to the adjacent cells. A movement based on actin polymerization and bundling of actin filaments at one pole of the bacteria has similarly been described for *Listeria monocytogenes*, an invasive bacteria that, like *Shigella*, is able to lyse the membrane of the phagocytic vacuole (TILNEY and PORTNOY 1989, 1990; MOUNIER et al. 1990; see the review by Sheehan et al. on *L. monocytogenes* in this volume).

In addition to actin, several cellular proteins, such as vinculin and plastin, but not myosin, are associated with the polymerized structure that trails behind the intracellular bacteria (KADURUGAMUWA et al. 1991; PRÉVOST et al. 1992). Using a cell line which does not produce cell adhesion molecules and transfectants expressing either L-CAM or N-cadherin, SANSONETTI et al. (1994) have shown that cell adhesion molecules are required for cell-to-cell spread of *Shigella*. Cadherin was important for both the structural organization of the protrusion and the internalization of a protrusion by an adjacent cell. Moreover, L-CAM, α-actinin, vinculin, and α- and β-catenins were found to be associated with the protrusions that initiated at the intermediate junctions.

3 Plasmid Genes Involved in Entry

Evidence for an essential role of plasmid determinants in invasion came from the observation that a large plasmid of about 200 kb was present in all invasive isolates of *Shigella* and EIEC strains and that deletions within or loss of this

plasmid resulted in avirulence (SANSONETTI et al. 1981, 1982; HARRIS et al. 1982). Hybridization studies indicated a high degree of relatedness between the large plasmids carried by *Shigella* and EIEC strains (SANSONETTI et al. 1983a). Moreover, mobilization of the large plasmid from *S. flexneri* to *E. coli* K 12 gave rise to a recombinant strain that was able to invade HeLa cells (SANSONETTI et al. 1983b).

The use of minicell-producing strains of *S. flexneri*, *S. sonnei*, and EIEC led to the characterization of 15–20 proteins encoded by the virulence plasmid (HALE et al. 1983). Seven proteins common to *S. flexneri* and enteroinvasive *E. coli* O143 were identified by two-dimensional gel electrophoresis and designated a–g (HALE et al. 1985). Four of these polypeptides, a (78 kDa), b (62 kDa), c (43 kDa), and d (39 kDa), were found to be the predominant antigens recognized by the sera from humans convalescing from shigellosis, as well as by those of monkeys experimentally infected with *S. flexneri* (HALE et al. 1985; OAKS et al. 1986). The generic term Ipa (for invasion plasmid antigen) was subsequently used to designate these proteins and the corresponding genes (BUYSSE et al. 1987).

Two strategies, cloning into a cosmid and transposon mutagenesis, were used to identify genes responsible for the invasive phenotype. A library of cosmids containing 45 kb DNA fragments of the pWR100 plasmid from *S. flexneri* 5 was introduced into a *Shigella* strain lacking the virulence plasmid and the recombinant strains were then screened for their ability to enter HeLa cells. The inserts present in the cosmids that conferred entry contained a common region of about 37 kb and allowed the expression of the IpaA, IpaB, IpaC, and IpaD antigens (MAURELLI et al. 1985). A similar strategy was used to isolate the invasion region of the *S. sonnei* virulence plasmid (KATO et al. 1989). Using a different approach, SASAKAWA et al. (1986) isolated over 300 independent Tn*5* insertions in pMYSH6000, the virulence plasmid of *S. flexneri* 2a, and screened the mutants for their ability to invade LLC-MK2 cells. This led to the identification of a 30 kb fragment, the integrity of which was required for invasion. The restriction map of this fragment was very similar to that of the entry region characterized in *S. flexneri* 5 and in *S. sonnei*. Since then, the combined effort of several laboratories has resulted in the elucidation of the complete nucleotide sequence of the 30.5 kb region that is necessary and apparently sufficient for entry of *S. flexneri* into epithelial cells in vitro (ADLER et al. 1989; ALLAOUI et al. 1992a, b, 1993a, b, and unpublished data; ANDREWS and MAURELLI 1992; BAUDRY et al. 1988; BUYSSE et al. 1990; SASAKAWA et al. 1989, 1993; VENKATESAN et al. 1988, 1992; VENKATESAN and BUYSSE 1991). As shown in Fig. 1, this fragment contains 33 genes clustered in two regions which are transcribed in opposite orientation.

3.1 The *mxi* and *spa* Secretion Genes

The initial characterization of the proteins expressed by the virulence plasmid indicated that several of them, including the IpaA, IpaB, IpaC, and IpaD antigens, were associated with the outer membrane of the bacterium (HALE et al. 1983). The reactivity of monoclonal antibodies directed against IpaB and IpaC with whole

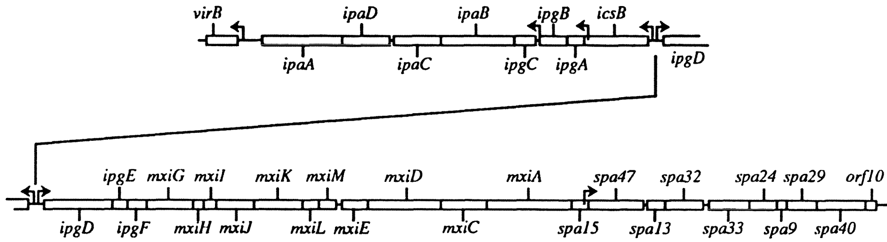

Fig. 1. The *Shigella flexneri* invasion region

bacteria in an ELISA confirmed that these proteins were exposed on the surface of virulent shigellae (MILLS et al. 1988; HROMOCKYJ and MAURELLI 1989). Secretion of IpaB and IpaC into the culture medium was demonstrated by ANDREWS et al. (1991), and analysis of concentrated culture supernatant fluids by SDS-PAGE and Coomassie blue staining indicated that wild-type *S. flexneri* secretes about ten polypeptides into the growth medium (ALLAOUI et al. 1992b). In addition to IpaB and IpaC, the two other Ipa antigens, IpaA and IpaD, have been detected in the culture medium of *S. flexneri* (MÉNARD et al. 1993 and unpublished data).

Characterization of the phenotype of noninvasive mutants, obtained after transposon mutagenesis or constructed by allelic replacement, led to the identification of a locus that contains many genes whose products are involved in the surface presentation and secretion of the Ipa antigens (ANDREWS et al. 1991; ALLAOUI et al. 1992b, 1993a, and unpublished data; VENKATESAN et al. 1992; SASAKAWA et al. 1993). These genes were designated *mxi* (for membrane excretion of invasion plasmid antigens) and *spa* (for surface presentation of Ipa antigens). All the *mxi* and *spa* noninvasive mutants are probably defective in both surface presentation and secretion of the four Ipa antigens as well as at least four other proteins (ALLAOUI et al. 1992b). Since the IpaB, IpaC and IpaD proteins are necessary for entry (see below), the noninvasive phenotype of the *mxi* and *spa* mutants is most likely a consequence of their inability to secrete these antigens.

The entire region containing the *mxi* and *spa* genes is designated the *mxi-spa* secretion locus. This locus contains 24 genes which are organized in two operons, one extending from *ipgD* to *spa15* and the other from *spa47* to ORF10 (ANDREWS et al. 1991; TOBE et al.1991; ALLAOUI et al. 1993a; SASAKAWA et al. 1993). Complementation studies and construction of nonpolar mutants have indicated that a few genes located in these operons, such as *ipgD* (*ipg* for invasion plasmid gene), *ipgF*, *spa15*, and ORF10, were neither involved in secretion of the Ipa proteins nor in invasion of HeLa cells (ALLAOUI et al. 1993a; SASAKAWA et al. 1993).

Preliminary studies and sequence analysis have indicated the location of some of the Mxi and Spa proteins. For example, the NH_2-terminal sequences of IpgF, MxiD, MxiJ, and MxiM exhibit features characteristic of a signal sequence. MxiJ and MxiM were shown to be lipoproteins and proposed to be anchored in the outer membrane by their NH_2-terminal lipid moiety (ALLAOUI et al. 1992b).The COOH-terminal moiety of MxiD, which has significant sequence similarities with

the COOH-terminal domain of the *Klebsiella oxytoca* PulD protein (D'ENFERT et al. 1989) and of protein IV of filamentous bacteriophages (PETERS et al. 1985; LUITEN et al. 1985), may be involved in the targeting of MxiD to the outer membrane (ALLAOUI et al. 1993b). MxiA has been detected in the inner membrane (ANDREWS et al. 1991) and sequence analysis suggests that this protein contains two domains, a NH_2-terminal domain composed of six transmembrane spanning segments and a COOH-terminal domain located in the cytoplasm (ANDREWS and MAURELLI 1992). Similarly, the presence of internal, hydrophobic segments in Spa9, Spa15, Spa24, Spa29, and Spa40 suggests that these proteins might also be located in the inner membrane (VENKATESAN et al. 1992; SASAKAWA et al. 1993).

Sequence comparisons have revealed extensive similarties between some Mxi and Spa proteins and *Yersinia* proteins involved in the secretion of the Yop proteins. For example, MxiD, MxiJ, and MxiH are homologuous to YscC, YscJ, and YscF, respectively, three proteins encoded by the *virC* operon of *Yersinia enterocolotica* (MICHIELS et al. 1991). As indicated in Table 1, several Spa proteins also have homologues encoded by the *Yersinia* virulence plasmid (H. Wolf-Watz, personal communication). Representatives of the *Shigella* Mxi and Spa proteins are also present in *Salmonella typhimurium* where they are required for invasion (GALÀN et al. 1992; GROISMAN and OCHMAN 1993), in plant pathogens such as *Erwinia carotovora* (MULHOLLAND et al. 1993), *Xanthomonas campestris* (FENSELAU et al. 1992; HWANG et al. 1992), and *Pseudomonas solanacearum* (ARLAT et al. 1992; GOUGH et al. 1992, 1993) where they are involved in pathogenicity, and in *E. coli* and *Bacillus subtilis* where they are involved in flagellar assembly (MALAKOOTI et al. 1989; ALBERTINI et al. 1991; VOGLER et al. 1991; BISCHOFF and ORDAL 1992; BISCHOFF et al. 1992; CARPENTER and ORDAL 1993). The phenotype of the *Shigella mxi* and *spa* mutants, as well as that of the *Yersinia ysc* mutants, suggests that these proteins form a secretion apparatus for the *Shigella* Ipa and *Yersinia* Yop proteins, whose NH_2-terminal amino acid sequences do not exhibit the features characteristic of a signal sequence and which do not appear to be processed during secretion.

3.2 The *ipaB, ipaC,* and *ipaD* Entry Genes

The genes encoding the IpaA, IpaB, IpaC, and IpaD antigens are clustered in a locus that comprises eight genes in the order *icsB, ipgA, ipgB, ipgC, ipaB, ipaC, ipaD*, and *ipaA* (Fig. 1). The transcriptional organization of this locus was analyzed by northern blotting and S1 nuclease protection experiments, cloning into a promoter probe vector, and by studies of the effect of polar insertions on the expression of downstream genes (BAUDRY et al. 1987; VENKATESAN et al. 1988; SASAKAWA et al. 1989; ALLAOUI et al. 1992a). In addition to the *icsB* promoter, a promoter necessary for the full expression of the *ipa* genes was identified upstream from *ipgB*, and internal, weaker promoters have been detected upstream from *ipgA* and *ipaD*.

Table 1. Homologues of the *Shigella flexneri* Mxi and Spa proteins in other bacterial species

Shigella flexneri	MxiH	MxiJ	MxiD	MxiA	Spa15	Spa47	Spa13	Spa32	Spa33	Spa24	Spa9	Spa29	Spa40
Salmonella typhimurium				InvA	InvB	SpaL	SpaM	SpaN	SpaO	SpaP	SpaQ	SpaR	SpaS
Yersinia spp	YscF	YscJ	YscC	LcrD		YscN			YscR	YscS	YscT	YscU	
Pseudomonas solanacearum		HrpI	HrpA	HrpO		HrpE				HrpT			
Xanthomonas campestris		HrpB3	HrpA1	HrpC2		HrpB6				Orf2			
Escherichia coli/ Salmonella typhimurium				FlhA		FliI			FliN	FliP	FliQ	FliR	
Bacillus subtilis				FlhA		FliI				FliP	FliQ	FliR	
Erwinia caratovora									MopA	MopC	MopD	MopE	

Data compiled by VAN GIJSEGEM et al. (1993), GROISMAN and OCHMAN (1993), and H. Wolf-Watz (personal communication).

The role of these genes in entry was first investigated using transposon insertion mutants constructed either on the large virulence plasmid or on cosmids that conferred entry (MAURELLI et al. 1985; SASAKAWA et al. 1986; BAUDRY et al. 1987; WATANABE et al. 1990). Transposon insertions in *ipgA, ipgB, ipgC, ipaB, ipaC, and ipaD,* but not in *ipaA,* abolished invasion of epithelial cells. Complementation analysis using various recombinant plasmids indicated that *ipaB, ipaC,* and *ipaD,* but not the upstream *ipg* genes, were involved in virulence, as evaluated by the formation of plaques on a cell monolayer (SASAKAWA et al. 1989). Recently, each of the *ipaB, ipaC,* and *ipaD* genes carried by the large virulence plasmid of *S. flexneri* 5 was inactivated by allelic replacement with a gene mutagenized in vitro by insertion of a nonpolar cassette (HIGH et al. 1992; MÉNARD et al. 1993). The *ipaB, ipaC,* and *ipaD* mutants were each unable to enter into Hela cells, a phenotype that could be complemented by recombinant plasmids carrying only a wild-type copy of the mutated gene. Although the three mutants were not impaired in adhesion to the cells, they were unable to induce actin polymerization at the site of attachment of the bacteria to the cellular membrane.

Shortly after entry into the cell, *Shigella* lyses the membrane of the phagocytic vacuole and gains access to the cytoplasm of the cell. This membranolytic activity is reflected by the contact hemolytic activity expressed by invasive *Shigella* (SANSONETTI et al. 1986). The *ipaB, ipaC,* and *ipaD* mutants were nonhemolytic, suggesting that the three Ipa proteins were also involved in the escape from the phagosome (HIGH et al. 1992; MÉNARD et al. 1993). The role of each of the IpaB, IpaC, and IpaD proteins in the lysis of the membrane of the phagocytic vacuole has been confirmed using a macrophage cell line which allows internalization of noninvasive bacteria; the *ipa* mutants remained trapped in the phagosome and were not cytotoxic (ZYCHLINSKY et al. 1994).

Since the *ipa* mutants were defective in invasion but not in secretion of the remaining Ipa proteins, which differentiated them from the *mxi* and *spa* mutants, the IpaB, IpaC, and IpaD proteins appear to be potential effectors of *Shigella* entry into epithelial cells (MÉNARD et al. 1993). The similar phonotype of the *ipa* mutants suggests that IpaB, IpaC, and IpaD might act together in induction of phagocytosis as well as in escape from the phagosome. An association of IpaB and IpaC has indeed been detected in the culture supernatant of wild-type *S. flexneri* (R. Ménard et al., in preparation). However, the mechanism of action of the Ipa proteins in these processes remains to be elucided.

3.3 The *virB* and *virF* Regulator Genes

The invasive phenotype of *Shigella* is regulated by the temperature of growth; strains which are invasive when grown at 37°C become noninvasive when grown at 30°C (MAURELLI et al. 1984). The temperature-regulated expression of the *ipa, mxi,* and *spa* operons is under the control of a regulatory cascade that involves two transcriptional activators, VirB and VirF, which are encoded by the large plasmid, and the product of a chromosomal gene, designated *virR* (see below).

Transposon insertions in a gene located immediately downstream from the *ipa* operon (Fig. 1) led to a noninvasive phenotype (MAURELLI et al. 1985; SASAKAWA et al. 1988). This gene, which was designed *virB* in *S. flexneri* 2a (ADLER et al. 1989), *ipaR* in *S. flexneri* 5 (BUYSSE et al. 1990), and *invE* in *S. sonnei* (WATANABE et al. 1990), encodes a 36 kDa protein homologous to ParB of plasmid P1 and SopB of plasmid F, two DNA-binding proteins involved in plasmid partioning. The *virB* (*ipaR, invE*) mutants, which did not produce the Ipa antigens, were altered in the transcription of the *ipa, mxi,* and *spa* operons. Overproduction of VirB from recombinant plasmids led to an increased transcription of the invasion operons even at 30°C (WATANABE et al. 1990; TOBE et al. 1991). The VirB binding site(s) on the promoters of the *ipa, mxi,* and *spa* operons have not yet been identified. Expression of the virB gene is itself positively regulated by VirF.

The *virF* gene is located about 40 kb away from the invasion region of the virulence plasmid pMYSH6000 of *S. flexneri* 2a (SAKAI et al. 1986a,b). Sequence analysis indicates that the 30 kDa VirF protein belongs to the AraC family of transcriptional activators. Transposon insertions in *virF* abolished transcription of the *virB* gene, which resulted in the lack of expression of the invasion genes (SAKAI et al. 1988; ADLER et al. 1989; TOBE et al. 1991). Deletion analysis indicated that activation of the *virB* promoter by VirF requires a DNA segment extending 110 bp upstream from the *virB* transcription start site (TOBE et al. 1993). The *virF* gene is expressed at 30°C, in contrast to *virB* which is transcribed only at 37°C. At 30°C, overexpression of *virF* from recombinant plasmids did not enhance transcription of *virB* (TOBE et al. 1991); this is likely to be due to the binding of the chromosomally encoded VirR protein (see below) on the *virB* promoter at 30°C (TOBE et al. 1993).

4 Plasmid Genes Involved in Intercellular Dissemination

Once they have reached the cytoplasm of the infected cell, i.e., after entry and escape from the phagosome, wild-type shigellae are able to move and to occupy the entire cytoplasm of the cell. Moreover, bacteria induce the formation of long protrusions which consist of cellular membrane extensions that are filled with actin filaments and contain a dividing bacterium or two bacteria at their tip. These protrusions are involved in the dissemination of bacteria from cell to cell without release into the extracellular medium, a phenotype that is reflected in vitro by the formation of plaques on a confluent cell monolayer (the plaque assay) (OAKS et al. 1985) and in vivo by the induction of a keratoconjunctivitis in guinea pigs (the Sereny test) (SERENY 1957).

4.1 The *icsA* (*virG*) and *virK* Genes

Transposon insertions in the *virG* gene, which is located 40 kb away from the invasion region on the virulence plasmid pMYSH6000 of *S. flexneri* 2a, did not

reduce the rate of invasion of cultured cells but abolished the ability of *Shigella* to spread from cell to cell (MAKINO et al. 1986; LETT et al. 1989). A similar gene, designated *icsA* has been characterized on the virulence plasmid pWR100 of *S. flexneri* 5; the *icsA* mutant, which was unable to induce the formation of protrusions, did not elicit the polymerization of actin filaments at the poles of the bacteria which is seen with the wild-type strain (BERNARDINI et al. 1989). For the sake of clarity, this gene will be hereafter referred to only as *icsA*.

The *icsA* gene, whose expression is regulated by VirF (SAKAI et al. 1988; ADLER et al. 1989), encodes a 130 kDa protein which was first detected in the bacterial outer membrane (LETT et al. 1989; BERNARDINI et al. 1989; D'HAUTEVILLE and SANSONETTI 1992). Recently, it has been shown that about 50% of IcsA was released into the culture medium as a 95 kDa molecular species (GOLDEBERG et al. 1993). Sequencing the NH_2-terminal extremity of the 95 kDa secreted protein indicated that the protein is cleaved after residue Ala-52, downstream from a region that has the characteristics of a signal sequence. The difference in size between the 95 kDa secreted protein and the 130 kDa gene product indicates that a second cleavage should occur in the COOH-terminal portion of the 130-kDa precursor. A polypeptide of 37 kDa, corresponding to the COOH-terminal part of IcsA, has indeed been detected by western analysis of *Shigella* whole cell extracts (NAKATA et al. 1992, 1993).

Since IcsA is secreted by *mxi* and *spa* mutants (ALLAOUI et al. 1992b, 1993b; VENKATESAN et al. 1992) and by an *E. coli* strain carrying a recombinant plasmid expressing the *icsA* gene (GOLDBERG et al. 1993), secretion of IcsA is independent of the Mxi secretion apparatus and may not require determinants carried by the virulence plasmid. Cleavage of the COOH-terminal portion of the precursor prior to the release of the mature protein into the culture medium is reminiscent of the secretion mechanism of *Nesseria gonorrhoeae* and *Haemophilus influenzae* IgA1 proteases. The COOH-terminal domain of the IgA1 protease is responsible for the translocation of the 160 kDa periplasmic precursor across the outer membrane and autoproteolytic cleavage allows the release of the 100 kDa mature protein (POHLNER et al. 1987; GRUNDY et al. 1987). The role of the COOH-terminal moiety of IcsA in the translocation of a periplasmic intermediate and the nature of the protein involved in the cleavage remain to be determined. A possible function of the COOH-terminal part of IscA in targeting to the outer membrane is supported by sequence similarities detected between this region of IcsA and the COOH-terminal part of AIDA-I, the adhesin involved in the diffuse adherence phenotype of diarrhoeagenic *E. coli* (BENZ and SCHMIDT 1992). AIDA-I is synthetized as a 132 kDa precursor whose COOH-terminal portion is cleaved to give rise to a 100 kDa mature protein. Whether mature AIDA-I is secreted is not known, but, in contrast to the mature form of IcsA which is released in the culture medium, at least a significant amount of the processed form of AIDA-I remains in the outer membrane.

Mutations in the *virK* gene, which is also located on the virulence plasmid, decreased the amount of the cell-associated 130 kDa IcsA protein, but not that of the 37 kDa COOH-terminal fragment (NAKATA et al. 1992). Like the *icsA* mutants,

the *virK* mutants were unable to spread from cell to cell, probably as a consequence of their reduced ability to induce actin polymerization (NAKATA et al. 1992). The same phenotype has been recently described for *Shigella* strains harboring a recombinant plasmid expressing the *E. coli* surface protease OmpT (NAKATA et al. 1993). As in the case of the *virK* mutants, the *Shigella* strains carrying the *E. coli ompT* gene had a decreased amount of the cell-associated 130 kDa IcsA protein, but not of the 37 kDa COOH-terminal fragment. However, since the amount of mature IcsA in the culture supernatant of the *virK* mutants, or in the culture supernatant of the *Shigella* strains expressing the *E. coli* OmpT protease, has not been determined, surface-exposed IcsA could be either degraded or more efficiently processed and released in these strains. The latter hypothesis is supported by the observation that IcsA expressed from a recombinant plasmid in *E. coli* was not present in the outermembrane but released into the culture medium as a 95 kDa species (GOLDBERG et al. 1993).

In bacteria grown in vitro, surface-bound IcsA is located at the distal poles of dividing bacteria (GOLDBERG et al. 1993). This unipolar distribution was also observed in intracellular bacteria after infection of HeLa cells (GOLDBERG et al. 1993) and correlates wih the unipolar reorganization of F-actin seen at the surface of dividing bacteria (PRÉVOST et al. 1992). Moreover, labeling with an anti-IcsA antiserum indicated that IcsA was also present in the actin tail that trails the bacteria in the cytoplasm and in the protrusions extending from the cell surface (GOLDBERG et al. 1993). This indicates that both the surface-bound and the secreted form of IcsA interact with elements within the tail, possibly F-actin or some actin-associated protein(s). Association of IcsA with actin or with an actin-bundling protein was hypothesized from the phenotype of the *icsA* mutants which were unable to elicit accumulation of polymerized actin on the bacterial surface (BERNARDINI et al. 1989). The *L. monocytogenes* ActA protein, which, like the *S. flexneri* IcsA protein, is involved in the actin-based movement of intracellular bacteria (KOCKS et al. 1992), has also been located at one pole of the bacteria (KOCKS et al. 1993). Despite the polarized localization of both IcsA and ActA and the similar phenotype of the *S. flexneri icsA* and *L. monocytogenes actA* mutants, there is no sequence similarity between the *icsA* and *actA* gene products. The purified 130 kDa IcsA precursor bound and hydrolyzed ATP, which suggests that ATP hydrolysis might be involved in bacterial movement (GOLDBERG et al. 1993).

4.2 The *icsB* Gene

Once the protrusion extending from an infected cell is engulfed by an adjacent cell, the bacteria within this protrusion are surrounded by two cellular membranes, that of the protrusion itself and that of the cell into which the protrusion enters. Lysis of these two membranes allows the bacteria to access the cytoplasm of the newly infected cell, thereby completing the process of intercellular dissemination. Inactivation of the *icsB* gene, which is located in the

invasion region of the virulence plasmid (Fig.1), gave rise to a *S. flexneri* strain that did not spread from cell to cell although, in contrast to the *icsA* and *virK* mutants described above, it was still able to induce actin polymerization and the formation of protrusions (ALLAOUI et al. 1992a). The *icsB* mutant remained trapped in vacuoles surrounded by two membranes. That the *icsB* mutant was able to lyse the membrane of the phagocytic vacuole during the entry process indicated that different membranolytic activities are required fo escape from the phagosome and from the protrusion. A phenotype similar to that of the *icsB* mutant has been described for a *L. monocytogenes* strain in which the *plcB* gene, encoding a lecithinase, had been inactivated (VASQUEZ-BOLAND et al. 1992). However, no lecithinase activity has been detected in *S. flexneri* and there is no sequence similarity between the *S. flexneri icsB* and the *L. monocytogenes plcB* gene products.

5 Other Plasmid Genes

The sizes of the *Shigella* virulence plasmids range from 180 to 230 kb (SANSONETTI et al. 1981, 1982; HARRIS et al. 1982; SASAKAWA et al. 1986), of which "only" 40 kb or so have been implicated in either invasion (the *ipa, mxi*, and *spa* genes, *virB* and *virF*) or intercellular dissemination (*icsA*, *icsB*, and *virK*). Although there is considerable variations between the restriction patterns of the plasmids extracted from different *Shigella* species, hybridization studies have shown a high degree of relatedness between these plasmids (SANSONETTI et al. 1983a). This, as well as the conservation of the size of the virulence plasmids, suggest that other regions of the plasmid might play a role in virulence, even though they have not yet been identified as such by the tests that have been used in the laboratory. For example, with the exception of the *virF* regulatory gene, no determinant required to induce a positive Sereny test in the mouse model was detected on a 90 kb fragment of the virulence plasmid pMYSH6000 of *S. flexneri* 2a (SASAKAWA et al. 1986). In addition, 142 mutants carrying a transposon insertion in any of ten different *Sal* I fragments of pMYSH600 did not show any defect in invasion or in the Sereny test (SASAKAWA et al. 1986).

The specific inactivation of such genes as *ipaA* (SASAKAWA et al. 1989; MÉNARD et al. 1993) and *ipgD* (ALLAOUI et al. 1993a), which are likely to be relevant to virulence in as much as these genes are present in the invasion region (Fig. 1) and the encoded proteins are secreted by the Mxi-Spa secretion apparatus, did not result in an inability to invade HeLa cells or induce keratoconjunctivitis. This suggests that these tests might not be sensitive enough to explore the full spectrum of *Shigella* pathogenicity, which is not unexpected since shigellosis is restricted to human beings and primates.

Inactivation of *ipgF*, which is also located in the invasion region (Fig. 1), did not affect the invasion rate (ALLAOUI et al. 1993a); the *ipgF* gene product, a presumably periplasmic protein, is homologous to a protein encoded by a gene located within the leader region of the F, R1 and R100 conjugative plasmids. The

leader region is the first portion of these plasmids to enter recipient bacteria during conjugation but is not itself essential for conjugative transfer (LOH et al. 1989). Likewise, the products of *spa15* and ORF10, two genes of the *mxi-spa* secretion locus (see Fig. 1), are not required for invasion (SASAKAWA et al. 1993).

The *ipaH* gene was first identified by screening a library of λgt11 recombinant phages using a polyclonal serum raised against a protein fraction enriched for the virulence plasmid-encoded proteins (BUYSSE et al. 1987). An *ipaH* probe hybridized to five different *Hin*dIII fragments on the DNA of pWR100, suggesting that multiple copies of this gene are present on the virulence plasmid of *S. flexneri* 5 (HARTMAN et al. 1990). Moreover, multiple copies of *ipaH* have also been detected on the chromosome (VENKATESAN et al. 1989). Sequencing four of the five plasmid-borne *ipaH* copies revealed a puzzling arrangement of variable and constant regions (HARTMAN et al. 1990; VENKATESAN et al. 1991a). Two adjacent copies, designated, *ipaH7.8* and *ipaH4.5*, are located between the invasion region and *icsA* and should encode 532 and 574 amino acid polypeptides, respectively. These polypeptides have indeed been detected by immunoblot analysis. The two other copies, *ipaH1.4* and *ipaH2.5*, have not yet been mapped on the virulence plasmid and appear to correspond to truncated genes which might not be expressed. Analysis of the sequence deduced from *ipaH7.8* and *ipaH4.5* revealed the presence of six and eight repeated regions, respectively; each of these repeats consists of the 20 residue long motif L-X-X-L-P-X-L-P-X-X-L-X-X-L-X-I/ V/A-X-X-N-X (where X represents any amino acid residue) (VENKATESAN et al. 1991a). This motif, designated LPX, is very similar to the consensus sequence derived from the nine repeats detected in the sequence of the YopM protein from *Y. pestis* (LEUNG and STRALEY 1989). YopM was shown to inhibit thrombin-induced platelet aggregation and to be involved in the virulence of *Y. pestis* in a mouse model (LEUNG et al. 1990). Whether IpaH is secreted and its role on the virulence of *Shigella* have not yet been determined.

6 Chromosomal Genes Involved in Virulence

As indicated by the invasive phenotype of *E. coli* transconjugants which have received the virulence plasmid of *S. flexneri*, there is no *Shigella* chromosomal gene required for invasion that does not have its counterpart in *E. coli* (SANSONETTI et al. 1983b). However, the full spectrum of *Shigella* virulence in animal models was not expressed by the *E. coli* transconjugants, in that they failed to elicit fluid accumulation in rabbit ileal loops and provoke keratoconjunctivitis in guinea pigs. Conjugal transfer of chromosomal material between *E. coli* and *Shigella* and, later on, construction or transduction of defined mutations as well as characterization of transposon-induced mutants have allowed the identification of chromosomal genes which are involved in virulence.

6.1 Modulation of Gene Expression and Protein Stability

The *virR* gene was identified following transposon mutagenesis of a *S. flexneri* strain carrying a transcriptional *mxi-lac* fusion (Maurelli and Sansonetti 1988). Since the invasion genes are expressed at 37°C but not at 30°C (Maurelli et al. 1984), the parental strain could grow on lactose only at 37°C. A mutant carrying a Tn*10* insertion in a gene that was designated *virR* was selected for a Lac$^+$ phenotype at 30°C. Transduction of the *virR*::Tn*10* mutation to wild-type *Shigella* resulted in a strain that was invasive at both 30°C and 37°C, confirming that the *virR* gene was involved in the temperature-regulated expression of the invasion phenotype. The *virR* mutation was shown to be allelic to the *osmZ, drdX, bglY,* and *pilG* mutations identified in *E. coli* (Dorman et al. 1990; Göransson et al. 1990; Hulton et al. 1990; May et al. 1990). The corresponding wild-type gene encodes the histone-like protein H1 (H-NS) which may induce local change in DNA supercoiling, thereby modulating gene expression (Hulton et al. 1990). This protein was recently shown to bind the *virB* promoter and repress its activity at 30°C (Tobe et al. 1993).

In *E. coli,* the *envZ* and *ompR* genes encode a two-component regulatory system that controls the transcription of the *ompF* and *ompC* genes in response to change in osmolarity of the growth medium (Comeau et al. 1985). Modulation of *Shigella* invasion gene expression by osmolarity was investigated using a *mxi-lac* transcriptional fusion (Bernardini et al. 1990). Expression of this fusion was enhanced three- to fourfold in high osmolarity conditions and reduced in *envZ* and Δ*(ompR-envZ)* mutants. Transduction of these mutations to wild-type *Shigella* gave rise to mutants that were less invasive than the wild-type strain and unable to form plaques on confluent HeLa cell monolayers (Bernardini et al. 1990). The respective role of the OmpC and OmpF porins in entry was then investigated after transduction of *ompC* and *ompF* mutations from *E. coli* to *S. flexneri* (Bernardini et al. 1993). Whereas the resulting *S. flexneri ompF* mutant behaved like the wild-type strain, the *ompC* mutant showed a reduced rate of invasion and was unable to spread from cell to cell. Moreover, the Δ(*envZ-ompR*) mutant was restored to virulence by complementation with a plasmid expressing the *E. coli* OmpC protein. Whether the phenotype of the *ompC* mutant is due to the lack of direct interaction between OmpC and some cellular structure(s), or to an indirect effect on the conformation or stability of other bacterial outer membrane protein(s), because of the absence of OmpC, remains to be determined.

The *kcpA* locus was originally defined following replacement of the *Shigella purE* region by the homologous region from *E. coli.* The resultant hybrid was unable to provoke keratoconjunctivitis (Formal et al. 1971), spread from cell to cell, and showed a decreased production of IcsA (Pal et al. 1989). It has been recently shown that the *kcpA* locus corresponds to the *ompT* gene carried by a remnant lambdoid phage that is present in *E. coli* but not in *Shigella* (Nakata et al. 1993). Expression of the OmpT outer membrane protease resulted in the degradation (or perhaps the release, as discussed above) of IcsA, which probably accounts for the intercellular spreading defect of *Shigella* strains carrying the *E. coli kcpA* locus.

A transposon insertion in the *vacB* gene (for virulence associated chromosomal gene), which is located downstream from *purA*, resulted in a tenfold decrease in invasion and an inability to spread from cell to cell (Tobe et al. 1992). These two phenotypes can be correlated with the decreased amounts of IcsA and IpaB detected in the *vacB* mutants. Since the transcription of the *ipa* and *icsA* genes was not affected in this mutant, VacB might act at a posttranscriptional level, such as on the stability of these and perhaps other proteins.

6.2 Bacterial Metabolism

The respective contributions to virulence of superoxide dismutase and catalase activities, both of which may protect bacteria from oxygen toxicity, were examined after transduction into *S. flexneri* of *sodB* and *katF-katG* mutations constructed in *E. coli* (Franzon et al. 1990). The *sodB* and, to a lesser extent, the *katFG* mutants were more sensitive than the wild-type strain to killing by phagocytes. The *katFG* mutant was still able to elicit a positive Sereny test and, when tested in the rabbit ligated ileal loop mode, caused damage to intestinal villi similar to that induced by the wild-type strain. In contrast, the *sodB* mutant was negative in the Sereny test and produced little detectable damage in ligated loops, which indicates that the superoxide dismutase activity encoded by *sodB* may play a critical role in pathogenesis.

S. flexneri utilizes a hydroxamate siderophore, aerobactin, for the transport of iron. Synthesis of aerobactin and of its outer membrane receptor are specified by the *iucABCD* and the *iutA* genes, respectively (Lawlor and Payne 1984; Griffiths et al. 1985). The role of aerobactin in the virulence of *S. flexneri* was studied in transposon-induced mutants (Lawlor et al. 1987; Nassif et al. 1987). The mutants did not show any alteration in their ability to invade HeLa cells, grow intracellularly, or kill infected cells, indicating that sufficient amounts of iron were present in the cytoplasm of infected cells to sustain bacterial growth in the absence of siderophore production. However, an inoculum-dependent effect was observed with the *iuc* mutant in the Sereny test and in the rabbit ligated ileal loop model, in that 10- to 100-fold more mutant bacteria were required to cause alterations which were qualitatively similar to those caused by the wild-type strain. This suggested that aerobactin was important for growth wihin tissues rather than in the intracellular compartment.

The *S. flexneri* loci involved in the biosynthesis of lipopolysaccharide (LPS) are alleles of the *E. coli rfb* and *rfa* loci which participate in the synthesis of the LPS basal core and group somatic antigen, respectively (Formal et al. 1970; Sansonetti et al. 1983b; Okada et al. 1991a; for a review see Schnaitman and Klena 1993). In contrast to *S. flexneri, S. dysenteriae* and *S. sonnei* require some plasmid genes for LPS biosynthesis (Sansonetti et al. 1981; Watanabe and Timmis 1984; Watanabe et al. 1984). Rough *S. flex*neri strains are still invasive but do not spread to adjacent cells and are negative in the Sereny test (Sansonetti et al. 1983b; Okamura et al. 1983; Okada et al. 1991b). How changes in the LPS affect the

behavior of bacteria in the cytoplasm of infected cells remains to be elucidated. The effect may be indirect, since, in *E. coli*, changes in the LPS core modify the conformaion of outer membrane proteins, including the OmpF and OmpC porins (Reid et al. 1990). Modifications of the *Shigella* LPS might thus affect the conformation, stability, or localization of IcsA (or of OmpC, see above), thereby accounting for the inability of the *Shigella* rough mutants to spread from cell to cell.

To identify chromosomal genes involved in virulence, each clone of a bank of over 9000 Tn*5*-induced mutants of *S. flexneri* 2a was screened for their ability to induce the formation of plaques on a cell monolayer (Okada et al. 1991b). Among the mutants thus identified, 50 had a Tn*5* insertion in the chromosome and most of them were affected in the structure of the core or of the LPS side chains, which confirmed the importance of the integrity of the LPS for intercellular spread. Determination of the site of insertion of Tn*5* in the other mutants allowed the identification of seven loci required for virulence; these loci were subsequently located on a *Not*I physical map of the *Shigella* chromosome (Okada et al. 1991a). Although the nature of the genes, designated *vac* (for virulence associated chromosomal gene), which have been inactivated in these mutants remains to be elucidated, one mutant, which showed reduced intracellular survival, was found to be a thymine auxotroph (Okada et al. 1991b). Similarly, *thyA* and *aroD* mutants were shown to be negative in the Sereny test (Ahmed et al. 1990; Lindberg et al. 1988).

6.3 Production of Shiga Toxin

In contrast to the other *Shigella* species, *S. dysenteriae* expresses a potent cytotoxin, Shiga toxin, that cleaves the 28s rRNA of eukaryotic cells (Endo et al. 1988). The two subunits of this toxin are encoded by the *stxA* and *stxB* genes which are almost identical to the genes specifying the Shiga-like toxin of *E. coli* (Kozlov et al. 1988; Stockbine et al. 1988). To evaluate the contribution of Shiga toxin in pathogenesis, a *S. dysenteriae* Tox$^-$ strain was constructed by allelic replacement with a *stx* locus which had been inactivated by insertion of a selectable fragment into the *stxA* gene (Fontaine et al. 1988). The Tox$^-$ strain did not show any reduction in the rate of intracellular growth or in the killing of the infected cells, and no significant differences were observed between the wild-type and the Tox$^-$ strains in the Sereny test or after infection of rabbit ligated ileal loops. Moreover, following intragastric inoculation of macaque monkeys, both strains induced diarhea with pus and mucus in the stools. However, the presence of blood in the stools was detected only in animals infected by the toxigenic strain. The nature of the histopathological alterations, such as destruction of capillary vessels within the connective tissue of the colonic mucosa observed with the wild-type strain but not with the isogenic Tox$^-$ strain, suggests that Shiga toxin influences the severity of bacillary dysentery by inducing colonic vascular damage.

7 Conclusion

In vitro, infection of an epithelial cell monolayer by *Shigella* is a multistep process involving; (1) adhesion of the bacteria to the cell surface, (2) entry by induced phagocytosis, (3) escape from the phagosome, which completes the process of entry, (4) intracellular multiplication, (5) polymerization of actin filaments and reorganization of these filaments at one pole of the dividing bacteria to generate a movement leading to the formation of protrusions, and (6) lysis of the two cellular membranes surrounding the bacteria once the protrusions have entered into adjacent cells, thus completing the process of intercellular dissemination. A systematic genetic analysis has already shown that most of these steps can be dissociated.

The protein(s) involved in adhesion have not yet been identified, but they are probably encoded by the virulence plasmid, since a *S. flexneri* mutant that had lost the virulence plasmid was tenfold less adherent to HeLa cells than the wild-type strain (PAL and HALE 1989). The *mxi* and *spa* mutants are not invasive, a phenotype that can be correlated with their inability to secrete the Ipa and a few other proteins. The Ipa proteins do not have a signal sequence and do not appear to be processed during secretion. Although several Mxi proteins have a characteristic signal sequence, secretion of the Ipa proteins can be considered as sec-independent. In contrast, the NH_2-terminal portion of IcsA is cleaved, presumably during export to the periplasm. Recent studies indicate that, like the Yop proteins of *Yersinia* (MICHIELS and CORNELIS 1991), the determinant(s) required for secretion of IpaB and IpaC are located in the NH_2-terminal extremity of these proteins (R. Ménard et al., in preparation). Elucidation of the function of each of the proteins encoded by the *mxi-spa* locus is a challenge for future research and will certainly have implications beyond the field of *Shigella* pathogenesis, since several of these proteins have homologues in other animal and plant pathogens. The striking conservation of the genetic organization of the *Shigella* and *Salmonella spa* loci (GROISMAN and OCHMAN 1993) suggests that these regions were derived from a common ancestor, although their G+C content is quite dissimilar, 35% G+C for the *mxi-spa* genes of *Shigella* vs 47% G+C for the *inv-spa* genes of *Salmonella*.

The IpaB, IpaC, and IpaD proteins now appear as prime candidates for having an effector role in the entry process. The *ipaB, ipaC,* and *ipaD* mutants are unable to induce entry into HeLa cells and, when internalized by macrophages, escape from the phagosome. That these two activities, along with the contact hemolytic activity, have not yet been genetically dissociated may indicate that they have the same molecular basis. It is conceivable that insertion of bacterial proteins, such as some of the Ipa proteins, into the cellular membrane might be involved first in the induction of phagocytosis and then in the disruption of the phagosome, as these proteins accumulate in the membrane of the phagocytic vacuole.

The Ipa proteins have been detected on the surface of the bacteria and in the culture supernatant, but the bulk of these antigens is located in the cytoplasm of bacteria grown in vitro (ANDREWS et al. 1991). Although the Mxi-Spa secretion

apparatus is required for invasion, a direct role in entry of the fraction of the Ipa present in the culture supernatant has not yet been determined; these secreted proteins may represent some leakage of the Mxi-Spa secretion apparatus which appears to be poorly active in bacteria grown in vitro. It is tempting to speculate that the activity of the Mxi-Spa secretion apparatus might be turned on upon contact of the bacteria with the cell surface, thereby allowing the delivery of the Ipa proteins directly onto their target, the cellular membrane or a membrane receptor.

The G-C content of the invasion region (30% G+C) is very different from that of the *Shigella* chromosome (50% G+C), which suggests that this region came from another genus. Accordingly, the involvment of chromosomal genes (*virR*, *ompR/envZ*) in the modulation of invasion gene expression probably reflects the adaptation to regulatory networks responsive to environmental changes that already existed when the invasion genes were acquired by *Shigella*. From the similarity between the *Shigella* and *E. coli* chromosomes (BRENNER et al. 1973; OKADA et al. 1991a), the invasive phenotype of *E. coli* strains that have artificially acquired the *Shigella* virulence plasmid, and the natural occurence of EIEC strains whose virulence plasmids are related to those of *Shigella*, *E. coli* appears as a likely origin for *Shigella*. One may caricature a *Shigella* strain as a smooth *E. coli* that has received the invasion plasmid and lost the *ompT* gene.

A striking feature of *Shigella*, which is shared with *L. monocytogenes*, is its ability to use some of the cytoskeletal components, and especially to reorganize actin, to move intracellularly and disseminate from cell to cell. The unipolar localization of IcsA, which correlates with the reorganization of F-actin filaments at one pole of the dividing bacteria during the onset of the bacterial movement, and the detection of IcsA within the tail of actin that trails behind bacteria in the protrusions strongly suggest that there is a direct interaction between IcsA and some cellular protein(s). The recent demonstration that cellular adhesion molecules are required in the cell-to-cell dissemination process, not only for the proper structure of the protrusions but also for the internalization of the protrusions by the adjacent cells, suggests that the protrusions might be actively endocytosed during intercellular spread (SANSONETTI et al. 1994). The nature of bacterial components that may be involved in this other type of induced phagocytosis remains to be determined.

To what extent do the data obtained on the mechanism of invasion of cultured cells apply to the "real life" situation, i.e., the pathogenesis of shigellosis? For obvious reasons, the effect of each mutation that has been shown to alter virulence in vitro cannot be evaluated in volunteers or even in monkeys. Noninvasive mutants appear avirulent in animal models such as the Sereny test or the rabbit ligeated ileal loop model. Moreover, a *S. flexneri* 2a strain, whose virulence plasmid had suffered deletions eliminating both the invasion region and *icsA* (VENKATESAN et al. 1991b), was found to be safe in large vaccine trials (MEITERT et al. 1984). The invasive phenotype and the ability to escape from the phagosome are correlated with the ability of *Shigella* to induce apoptosis in infected macrophages (ZYCHLINSKY et al. 1992, 1994), a mechanism

that may be responsible for the death of inflammatory cells in the colonic mucosa observed during shigellosis. The importance of the ability to spread from cell to cell is illustrated by the very important decrease in virulence of *icsA* mutants administered by intragastric inoculation in macaque monkeys. In addition, the combination of an *icsA* mutation with either an *iuc* or an Δ(*envZ-ompR*) mutation led to strains that no longer produced diarrhea or dysentery symptoms (Sansonetti and Arondel 1989; Sansonetti et al. 1991). It thus appears that factors which have been identified in the laboratory as important for either invasion, intercellular dissemination, bacterial metabolism, or Shiga toxin production in the case of *S. dysenteriae*, have a key role in the pathogenicity of *Shigella*.

An intriguing observation of the in vitro studies was that *Shigella* binds and enters into the enterocyte-like Caco-2 cells by the basolateral rather than the apical pole of the cell (Mounier et al. 1992). The definition of the site of entry of *Shigella* is of importance, considering that mucosal destruction and presumably invasion occur only in the colon. Using the rabbit intestinal loop model, Wassef et al. (1989) have shown that both invasive and noninvasive *Shigella* were phagocytosed by M cells over lymphoid follicles of Peyer's patches. The invasive strain appeared to escape from the phagocytic vacuole and replicate intracellularly. The M cell may thus serve as a preferential site of entry for *Shigella*, from which the bacteria could either disseminate to the adjacent enterocytes by expressing the Ics phenotype or, following transcytosis through or lysis of the M cells, invade the enterocytes by their baselateral pole. This scheme is supported by the observation that the small nodular abscesses, which were induced by the *icsA* mutant in macaque monkeys, were located over lymphoid follicles (Sansonetti et al. 1991). Since the *icsA* is unable to spread from cell to cell, the sites of the tiny ulcerations should correspond to the sites of entry into the epithelium. Transmigration of polymorphonuclear leukocytes across the epithelium has recently been proposed as another means by which *Shigella* could gain access to the basolateral pole of enterocytes (Perdromo et al. 1994). *S. flexneri* was shown to induce migration of polymorphonuclear leukocytes through a confluent monolayer of T-84 cells, a migration that appeared to be necessary for the bacteria to enter into the monolayer and invade the T 84 cells. That complex interactions between bacteria and two different cell populations can be reconstituted (or mimicked) in vitro opens new perspectives for the dissection of the *Shigella* infectious process.

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1 Introduction

Among the many species of the *Yersinia* genus, only *Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica* are adapted to multiply at the expense of a host that is still alive. *Y. pestis*, the agent of plague, and *Y. pseudotuberculosis* are essentially rodent pathogens. *Y. enterocolitica* is a common human pathogen which causes gastrointestinal syndromes of varying severities, ranging from mild self-limited diarrhea to mesenteric adenitis evoking an appendicitis. Systemic involvement is unusual with *Y. enterocolitica* but reactive arthritis and erythema nodosum are common complications (Cover and Aber 1989). In Europe, pork is a current source of *Y. enterocolitica* contamination (Tauxe et al. 1987).

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Although all three yersiniae infect their host via different routes and cause diseases of very different severity, they share a common tropism for lymphoid tissue and a remarkable ability to resist the nonspecific immune response of the host. Their main strategy seems to consist of: (1) avoiding lysis by complement; (2) avoiding phagocytosis by polymorphonuclear leukocytes and macrophages; and (3) forming extracellular microcolonies in the infected tissues (LIAN et al. 1987; HANSKI et al. 1989, 1991; SIMONET et al. 1990). The three yersiniae have common basic virulence functions. For the sake of clarity, we will essentially deal with *Y. enterocolitica* and the differences with the other species will be mentioned throughout. This review will focus essentially on the pathogenicity functions which are the hallmark of yersiniae, and specifically the Yop proteins. It will pay special attention to the yersiniae secretion system, the archetype of a new pathway encountered so far only in pathogenic bacteria. Due to space limitations, many contributions could not be paid the tribute they deserved. A short review focusing on regulation was published recently by STRALEY et al. (1993). For a complete overview of yersiniae, the reader should refer to broader reviews such as BUTLER 1983; BRUBAKER 1991; CORNELIS 1992.

2 The Chromosome-Encoded Virulence Functions of *Yersinia enterocolitica*

2.1 The Enterotoxin Yst

The chromosome of *Y. enterocolitica*, but not of *Y. pseudotuberculosis* and *Y. pestis*, encodes a heat-stable enterotoxin, Yst, detectable in broth culture supernatant by the infant mouse test (PAI and MORS 1978). It is a 30-amino acid peptide (TAKAO et al. 1984; ROBINS-BROWNE et al. 1979) which resembles both the heat-stable enterotoxin ST_a (also called STI) of *Escherichia coli* and guanylin, an endogenous activator of intestinal guanylate cyclase (Fig. 1) (CURRIE et al. 1992). The enterotoxin Yst is synthesized as a 71 amino acid polypeptide (DELOR et al. 1990). The COOH-terminal 30 amino acids correspond to the toxin extracted from culture supernatants, the NH_2-terminal 18 amino acids have the properties of a signal sequence and the central 22 residues are removed during or after the secretion process. This organization in three domains (Pre, Pro and mature Yst) also resembles that of guanylin and ST_a but Yst is larger than these two peptides. In view of the close resemblance between Yst and ST_a and the association with pathogenic serotypes, it is tempting to speculate that the production of Yst is responsible for the diarrheal manifestation associated with yersiniosis. A study conducted with isogenic Yst+ and Yst– strains in the young rabbit concluded that, at least in this model, Yst was indeed responsible for the diarrhea (DELOR and CORNELIS 1992).

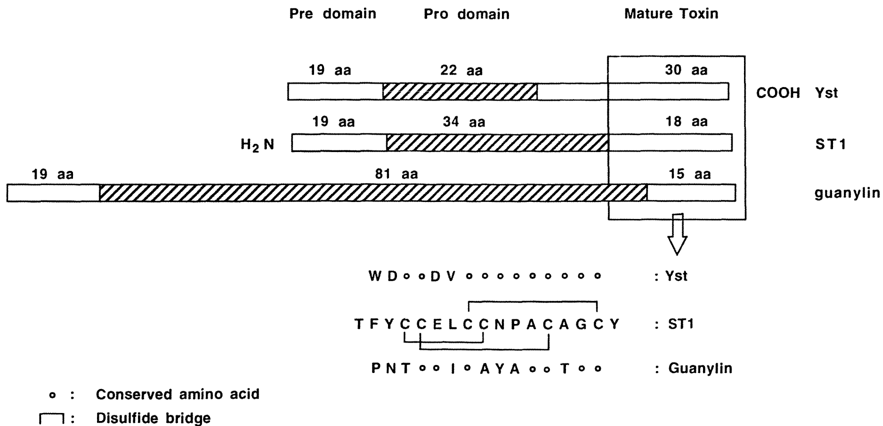


Fig. 1. The enterotoxins Yst and ST1 compared to guanylin

2.2 The Myf Fibrillae

When grown at 37°C in acidic conditions, *Y. enterocolitica* synthesizes a fibrillar structure known as Myf and resembling the CS3 pili of human enterotoxigenic. *E. coli* (IRIARTE et al. 1993). As for the enterotoxin Yst, the presence of Myf is restricted to the pathogenic serotypes of *Y. enterocolitica* (IRIARTE et al. 1993). The assembly of Myf requires the classical components of the pili assembly systems, namely a periplasmic chaperone called MyfB and a channel-forming outer membrane protein called MyfC (IRIARTE et al. 1993). The counterpart of the *myf* operon in the *Y. pestis* and *Y. pseudotuberculosis* chromosomes (IRIARTE et al. 1993; LINDLER and TALL 1993) encodes a structure known since the mid-1950s as pH6 antigen (pH6 Ag). The degree of identity between the major subunit MyfA and pH6 Ag is only 44%, which is significantly lower than that observed for the proteins encoded by the pYV plasmid described below. This suggests that Myf and pH6 could have diverged during evolution to serve different functions related to the different invasion routes of *Y. pestis* and *Y. enterocolitica*. *Y. pestis* expresses pH6 Ag after 10 h survival in cultured RAW264.7 macrophages but, surprisingly, this capacity does not contribute to the survival of bacteria within the macrophage (LINDLER and TALL 1993). pH6 Ag does nevertheless contribute to the virulence of *Y. pestis* intravenously inoculated to mice (LINDLER et al. 1990). In *Y. enterocolitica*, we hypothesize that Myf could fulfill the role of a colonization factor for the human or porcine intestine, reinforcing the action of Yst, but this remains totally speculative (IRIARTE et al. 1993). Both Myf and Yst are produced during the stationary phase (IRIARTE and CORNELIS, in preparation; A. MIKULSKIS and G. R. CORNELIS 1994). This observation could be taken as an argument in favor of a joint function, but the definite answer can only be given by adhesion experiments, presently being carried out.

2.3 The Internalins Inv and Ail

The chromosome of enteropathogenic *Y. enterocolitica* encodes two independent pathways for attachment to, and entry into, cultured mammalian cells (MILLER and FALKOW 1988; ISBERG 1990). The signal transduction events occurring during these steps have been recently reviewed by BLISKA et al. (1993). In vivo, the internalin Inv appears to play a vital role in promoting entry into intestinal tissue during the initial stage of infection (PEPE and MILLER 1993). An histopathological analysis of the infected mouse ileum suggests that the crossing probably occurs through the phagocytic M cells that overlay the Peyer's patches lining the gastrointestinal tract (GRÜTZKAU et al. 1990). Surprisingly, the concomitant loss of both Inv and YadA (see below) by *Y. pseudotuberculosis* results in a severe increase (and not decrease) of virulence. Consistent with this observation, *Y. pestis* makes neither Inv nor YadA (ROSQVIST et al. 1988).

2.4 Iron Acquisition

As for any invasive pathogen, the degree of pathogenicity of yersiniae depends on its capacity to acquire iron to sustain growth. It is not yet clear whether yersiniae secrete siderophores but it is well established that they possess iron-inducible receptors for several iron chelators such as hemin and various exogenous siderophores. For recent reviews, see PERRY (1993) and BÄUMLER et al. (1993).

3 The Panoply of Virulence Functions Encoded by the pYV Plasmid

3.1 Calcium Dependency

In vitro, virulent yersiniae restrict their growth after two generations when they are shifted from 28°C to 37°C in the absence of millimolar concentrations of Ca^{2+} ions. Growth can be reinitiated if the cultures are returned to 26°C or if Ca^{2+} is added to the medium. This growth restriction phenomenon, called Ca^{2+} dependency, is associated with the massive production of a set of about ten proteins called Yops. Although it is quite obvious that massive production of a given set of proteins must divert the metabolic potential from other biosynthetic pathways, we still do not know whether Ca^{2+} dependency involves a specific repression of growth.

The physiopathological significance of regulation by Ca^{2+} is far from clear. BRUBAKER (1983) suggested a correlation between Ca^{2+} dependency and the known, distinct levels of Ca^{2+} in the mammalian intracellular (micromolar range) and extracellular (about 2.5 m*M*) fluids. According to this suggestion, the Yops would essentially be produced in the intracellular environment. Although appealing, this hypothesis is contradicted by the accumulating evidence that yersiniae spread and multiply essentially outside cells (LIAN et al. 1987; HANSKI

et al. 1989, 1991; SIMONET et al. 1990). There is thus a paradox: in vivo, yersiniae proliferate in conditions which are supposed to be nonpermissive for Yops production, but, yet, they do produce Yops (see below).

3.2 The Yops

The Yops are identified by a letter which is identical for the homologous proteins in the three species. YopB, YopD, YopE, YopH, YopM, YopN, YopO and LcrV have been described in the three species. YopO is also described as YpkA (CORNELIS et al. 1987; GALYOV et al. 1993) and YopN is sometimes described as LcrE. YopP, YopQ and YopR have only been described in *Y. enterocolitica* so far while YopJ, YopK and YopL have only been described in *Y. pestis* (STRALEY 1988; STRALEY and CIBULL 1989). It is not yet known whether these two groups of designations cover the same proteins.

The Yops were initially described as outer membrane proteins (PORTNOY et al. 1981; STRALEY and BRUBAKER 1981; BÖLIN et al. 1985) but this status has been revised and they are now considered as secreted proteins (HEESEMANN et al. 1984, 1986; MICHIELS et al. 1990). Some of the Yops (LcrV, YopM, Q, R) are soluble in the culture supernatant but others (YopH, E, O, B, D, P, N) have a propensity to aggregate as visible filaments in the culture (MICHIELS et al. 1990). Their detection in the membrane fraction presumably results either from copurification of aggregated Yops with the membranes or from the adsorption of secreted Yops to the cell surface (MICHIELS et al. 1990). The name YOP, introduced by H. Wolf-Watz (BÖLIN et al. 1985) for Yersinia outer membrane protein, could thus be questioned. However, it is so popular and widely used that it was decided to keep it but to write it Yop(s) rather than YOPs to indicate that it is not a set of initials.

The Yops are highly conserved in the genus *Yersinia* but no homology exists between different Yops in a single species (FORSBERG and WOLF-WATZ 1988; BÖLIN and WOLF-WATZ 1988; MICHIELS and CORNELIS 1988; MICHIELS et al. 1990). One of the Yops, LcrV, is the V antigen (38 kDa) already described in the mid-1950s as a diffusible antigen differentiating virulent from nonvirulent strains of *Y. pestis* (BURROWS and BACON 1956).

Patients suffering an infection (MARTINEZ 1983) or mice artificially infected with *Y. enterocolitica* grown at low temperature (SORY and CORNELIS 1988) develop antibodies against the Yops, which clearly demonstrates that they are synthesized in vivo in the course of infection. The V antigen is protective in both active and passive immunization (LAWTON et al. 1963; UNE and BRUBAKER 1984). Mutants unable to express one or the other of the Yops have been constructed in the three species and most of them are less virulent than the parental strain (FORSBERG and WOLF-WATZ 1988; SORY and CORNELIS 1988; BÖLIN and WOLF-WATZ 1988; MULDER et al. 1989; STRALEY and CIBULL 1989; LEUNG et al. 1990; GALYOV 1993). Hence, the Yops constitute major antihost components of the pYV plasmid. Whether they are produced inside or outside the phagocyte remains a matter of debate. Although we tend now to consider yersiniae as extracellular

pathogens, it has been shown that the phagolysosomal environment of human macrophages allows expression of the *yop* genes (POLLACK et al. 1986).

The functions of individual Yops are now emerging. YopE is cytotoxic for cultured HeLa cells (ROSQVIST et al. 1990a). Interestingly, it is only active if it is produced by bacteria adhering at the cell surface or if it has been internalized by microinjection (ROSQVIST et al. 1990b). YopH contributes to the ability of the bacteria to resist phagocytosis by peritoneal macrophages (ROSQVIST et al. 1988). It is a protein tyrosin phosphatase (PTPase; EC 3.1.3.48) (GUAN and DIXON 1990) acting on multiple substrates in the cytoplasm of macrophages, which suggests that it interacts with macrophage regulation (BLISKA et al. 1991). Surprisingly, the COOH-terminal 262 amino acid domain of YopH is homologous to the catalytic domain of eukaryotic protein tyrosine phosphatases, which raises the appealing hypothesis that the *yopH* gene could be of eukaryotic origin. Another Yop, YopO, turned out to be a protein kinase with extensive homology to eukaryotic serine-threonine protein kinases (GALYOV et al. 1993). Because of this activity, it was renamed YpkA by Wolf-Watz and colleagues. YopM is a 41 kDa protein sharing significant similarity with the domain of the α-chain of human platelet membrane glycoprotein Ib (αGP1b) which binds thrombin and the von Willebrand factor (LEUNG and STRALEY 1989). As expected from the sequence similarity, YopM binds thrombin, inhibits platelet aggregation (REISNER and STRALEY 1992) and hence may prevent platelet-mediated host defense events such as the onset of the inflammatory response. No cytotoxic or enzymatic activity has been reported so far for YopB and YopD, two other major Yops. Interestingly, ROSQVIST et al. (1990b) observed that a mutant affected in YopD loses its cytotoxicity in spite of the fact that it still produces YopE. These authors concluded that YopD could act as an internalization factor for YopE. In accordance with the suggestion of ROSQVIST et al., YopD and YopB seem to have a transmembrane domain (HÅKANSSON et al. 1993), which is not true for YopE (MICHIELS et al. 1990), YopH (MICHIELS and CORNELIS 1988), YopM (LEUNG and STRALEY 1989) and LcrV (BERGMAN et al. 1991).

3.3 YadA and YlpA

Protein YadA, formerly called P1 or YopA (SKURNIK and WOLF-WATZ 1989), is a major outer membrane protein thought to form a fibrillar matrix on the surface of *Y. enterocolitica* and *Y. pseudotuberculosis* (KAPPERUD et al. 1987), when they are cultivated at 37°C. YadA is a polymer of 200–240 kDa formed by the association of approximately 50-kDa subunits addressed via the classical sec export pathway (SKURNIK and WOLF-WATZ 1989). The name YadA was given for Yersinia adhesin, because its presence makes the bacteria adherent to epithelial cells (HEESEMANN and GRÜTER 1987). YadA is also responsible for a marked autoagglutination (SKURNIK et al. 1984) and for binding to both collagen fibers (EMÖDY et al. 1989; SCHULZE-KOOPS et al. 1992) and fibronectin (TERTTI et al. 1992; SCHULZE-KOOPS et al. 1992). Although these properties tend to suggest that YadA could be a colonization factor, some observations indicate that, at least in *Y. enterocolitica*,

YadA plays a major role in the defense against the nonspecific immune response. Indeed, the presence of YadA reduces the deposition of C3b at the bacterial surface of *Y. enterocolitica* by binding of factor H which leads to a rapid catabolism of C3b (Tertti et al. 1987; Pilz et al. 1992; China et al. 1993). This has two consequences: first, it inhibits the formation of the complement membrane attack complex, which inhibits killing and, second, it prevents opsonization, which severely reduces phagocytosis and killing by polymorphonuclear leukocytes (B. China et al. 1994).

YlpA is a 29-kDa lipoprotein expressed by *Y. enterocolitica* and *Y. pseudotuberculosis* at 37°C, in absence of Ca^{2+} (China et al. 1990). It is related to the TraT proteins encoded by the *E. coli* sex factor F, by various resistance plasmids and by the virulence plasmid of *Salmonella typhimurium*. Although several TraT proteins have been shown to be involved in resistance to the bactericidal activity of human serum (for revue see Sukupolvi and O'Connor 1990), we could not gain any evidence for such a role in *Y. enterocolitica*. So far, the only element that pleads for a role in pathogenesis is of a genetic order: the expression of *ylpA* is regulated like that of the *yadA* and *yop* genes (see below).

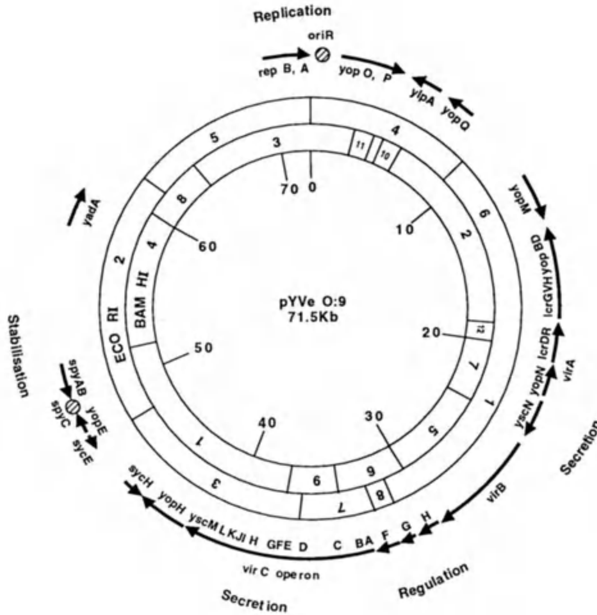
4 Organization of the pYV Plasmid

The pYV plasmid is a 70-kb plasmid maintained at about seven copies per cell by a RepFIIA replicon (Vanooteghem and Cornelis 1990) and stabilized by a partition system resembling that of bacteriophage P1 (J.-C. Vanooteghem and G.R. Cornelis, in preparation). The genetic maps of the pYV plasmids encountered in the three species are very similar except for a quadrant containing at least *yopE* and *yadA*, which has been rearranged during evolution (Biot and Cornelis 1988; Forsberg et al. 1987; Forsberg and Wolf-Watz 1988). Figure 2A gives the map of pYVe227, the archetype of pYV plasmids found in *Y. enterocolitica* strains of serotype O:9.

Genes *yadA*, *ylpA* and several *yop* genes, namely *yopE*, *yopH*, *yopQ* and *yopM* are scattered around pYV. Genes *yopO* (*ypkA*) and *yopP* form an operon located near the origin of replication. The genes encoding the V antigen, *yopB* and *yopD*, also form an operon (Mulder et al. 1989). The locus encoding the V antigen is flanked by two small genes encoding a 13 kDa protein (LcrG) (Skrzypek and Straley 1993) and a 18 kDa protein (LcrH) (Perry et al. 1986). The operon thus consists of *lcrGVHyopB,D* (Price et al. 1989; Plano et al. 1991; Bergman et al. 1991).

Mutations in any of the *yopE,H,Q,M,O,P,ylpA* and *yadA* genes simply result in the loss of the corresponding protein without alteration of the general phenotype: the mutant remains Ca^{2+}-dependent and produces all the proteins save the one(s) encoded by the mutated gene or operon. By contrast, the insertion mutants in *lcrGVHyopBD* are unable to grow at 37°C (thermosensitive phenotype) (Mulder et al. 1989). In spite of this, they nevertheless secrete the

A



B

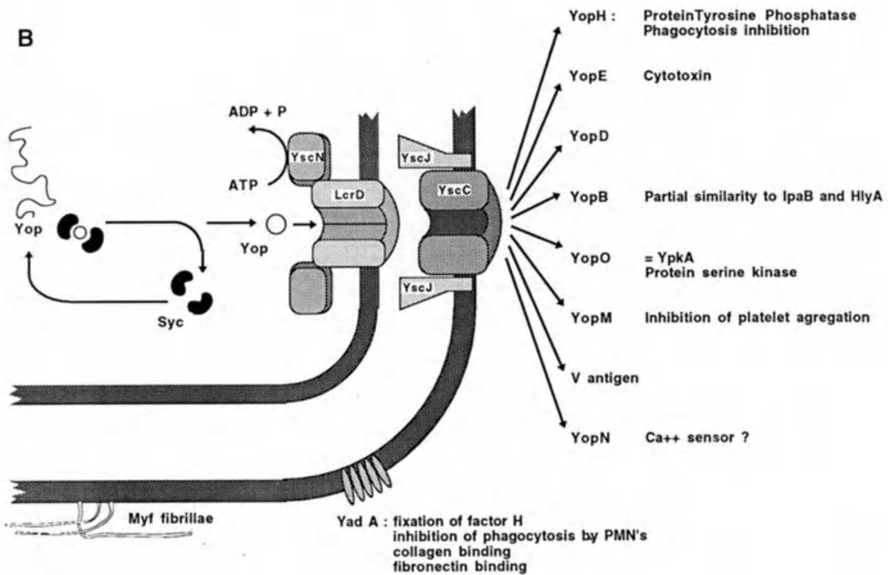


Fig. 2. A Genetic map of the pYV plasmid of serotype O:9 Y. enterocolitica. **B** The bacterial surface and Yop secretion system

other Yops at 37°C, in the absence of Ca^{2+}. The mechanism underlying this phenotype is not yet understood.

A contiguous 20-kb region of the pYV plasmid, called the Ca^{2+} dependence region, is required for the production of all the Yops. Insertion mutagenesis in this region defined a series of pleiotropic loci called *vir* (because they condition the virulence) in the *Y. enterocolitica* W22703 system and *lcr* (because they condition the low calcium response) in *Y. pseudotuberculosis* and *Y. pestis*. Some of these loci have been characterized but the information is not yet complete. These loci are *virA* (*lcrA*), *virB*, (*lcrB*), *virC* (*lcrC*), *virF* (*lcrF*), and *virG* (Goguen et al. 1984; Yother and Goguen 1985; Yother et al. 1986; CORNELIS et al. 1986, 1987b, 1989; unpublished data).

The locus *virA* contains the genes *lcrE* and the operon *lcrDR*. Mutants in *lcrE* are Ca^{2+}-blind, in the sense that they can secrete Yops even in the presence of Ca^{2+}. YOTHER and GOGUEN (1985) concluded that this gene encodes a diffusible element of the Ca^{2+} regulation pathway. Surprisingly, the product of *lcrE* turned out to be YopN (VIITANEN et al. 1990; FORSBERG et al. 1991) but YopN could never be shown to bind Ca^{2+}. *lcrD* encodes a 77-kDa inner membrane protein and the neighboring gene, *lcrR*, encodes a 16-kDa acidic protein which is thought to act as a regulator (BARVE and STRALEY 1990; PLANO et al. 1991; PLANO and STRALEY 1993). Locus *virB* has not been completely characterized yet. The first gene in this locus encodes YscN (S. WOESTYN et al. 1994). Locus *virC* contains 13 genes called *yscA-M*, arranged as a single large operon (MICHIELS et al. 1991).

Gene *virF* encodes a 30-kDa transcriptional activator which controls the expression of the *yop* genes (CORNELIS et al. 1987, 1989), of *ylpA* (CHINA et al. 1990), of *yadA* (MARTINEZ 1989; MICHIELS et al. 1991) and also of the *virC* operon (LAMBERT et al. 1992). The *yop*, *ylpA*, *yadA* and *virC* genes thus constitute what we call the *yop* regulon. The protein VirF belongs to the AraC family of regulators (CORNELIS et al. 1989). This now very large family includes regulators of degradative pathways in *E. coli* and *Pseudomonas putida* as well as regulators involved in the control of virulence of *Shigella*, *Yersinia*, enterotoxinogenic *E. coli* and the phytopathogen *Pseudomonas solanacearum* (GENIN et al. 1992). VirF acts as a DNA binding protein. DNaseI footprinting experiments on the *yopH* gene identified a protected region spanning 31 bp immediately upstream of the RNA polymerase binding site. This VirF binding sequence is located in an AT-rich region and only comprises an imperfect 8 bp inverted repeat (LAMBERT et al. 1992; P. WATTIAU et al. 1994).

5 The Yop Secretion Mechanism

5.1 The Secretion Signal

The secretion of Yops by yersiniae does not involve cleavage of a classical NH_2-terminal signal sequence (FORSBERG and WOLF-WATZ 1988; MICHIELS et al. 1990; REISNER and STRALEY 1992). The addressing signal is nevertheless localized in the NH_2-terminal domain of the Yops: the 48 NH_2-terminal residues of YopH, the 76

NH_2-terminal residues of YopQ and the 98 NH_2-terminal residues of YopE contain all the information required for export (MICHIELS and CORNELIS 1991). Quite surprisingly, there is no similarity between the exportation domains of these proteins with respect to amino acid sequence, hydrophobicity profile, distribution of charged residues or prediction of secondary structure, which suggests that the secretion signal is essentially conformational (MICHIELS and CORNELIS 1991).

5.2 Secretion of Hybrid Proteins

The Yop secretion system exports very efficiently hybrid proteins formed by fusing the NH_2-terminal domain of YopE or YopH and various prokaryotic proteins. Hybrid proteins consisting of 48 or more residues of YopH and either the β-subunit of cholera toxin (CT-B) or the α-peptide of β-galactosidase are efficiently secreted, even when they are encoded by a multicopy plasmid (SORY et al. 1990; MICHIELS and CORNELIS 1991). The system can also secrete hybrid proteins containing the entire PhoA enzyme. In this case however, the minimal NH_2-terminal domain of YopH required for export depends on the amount of protein to treat: it consists of only 48 amino acids if the hybrid is encoded by a low copy plasmid but it becomes 65 amino acids if the hybrid is encoded by a multicopy plasmid. The efficiency of the system is thus higher if the addressing domain is a little longer than the minimal 48 amino acids (MICHIELS and CORNELIS 1991). Engineered *Y. enterocolitica* strains can also secrete chimeric eukaryotic proteins: SORY et al. (1992) showed the massive secretion of a hybrid protein made of 168 amino acids of YopE and 313 amino acids of the repetitive antigen CRA from *Trypanosoma cruzi*.

5.3 The Secretion Machinery

Secretion of proteins outside the bacterial cell is rather unusual for gram-negative bacteria. There are, however, protein secretion pathways (for a recent short review, see SALMOND and REEVES 1993). Type I is exemplified by the hemolysin HlyA secreted by uropathogenic strains of *E. coli*. The proteins targeted through this pathway have no classical NH_2-terminal signal sequence, show sec-independent translocation to the external medium and require essential COOH-terminal sequences. The Type II pathway, exemplified by the pullulanase of *Klebsiella*, is considered as the general secretion pathway (GSP) found in most gram-negative bacteria except *E. coli* and some closely related species (PUGSLEY 1991). The first step in this pathway is thought to be identical to sec-dependent export to the periplasm in *E. coli*. It thus requires the cleavage of an NH_2-terminal sequence signal.

The Yop export pathway is clearly different from both type I and type II pathways. It thus appears as the archetype of a new system, which was called type III by SALMOND and REEVES (1993). The Yop transport system is encoded by the

Table 1. Some homologs in the type III secretion pathway

Yersinia	Shigella	Salmonella	Pseudomonas salanacearum	Xanthomonas campestris pv. vesicatoria	Bacillus subtilis (flagellum)
LcrD[1]	MxiA[3]	InvA[6]	HrpO[10]	HrpC2[9]	FlhA[8]
YscC[2]	MxiD[5]	InvG[14]	HrpA[10]	HrpA1[9]	
YscJ[2]	MxiJ[4]		HrpI[10]	HrpB3[9]	
YscF[2]	MxiH[4]				
YscL[2]			HrpF[12]		
YscN[13]	Spa47[11]		HrpE[12]	HrpB6[9]	FliI[7]

References: 1, PLANO et al. 1991; 2, MICHIELS et al. 1991; 3, ANDREWS and MAURELLI; 4, ALLAOUI et al. 1992; 5, ALLAOUI et al. 1993; 6, GALAN et al. 1992; 7, ALBERTINI et al. 1991; 8, CARPENTER and ORDAL 1993; 9, FENSELAU et al. 1992; 10, GOUGH et al. 1992; 11, VENKATESSAN et al. 1992; 12, Van Gijseghem et al., unpublished; 13, Woestyn et al., unpublished; 14, KANIGA and GALAN 1993.

vir (*lcr*) region of the pYV plasmid. The identification of the individual genes involved in this process turned out to be uneasy because none of the many pYV mutants that have been constructed accumulates large amounts of intracellular Yops. This probably results from a feedback inhibition of Yops synthesis when export is compromised. The distinction between regulatory genes and genes involved in secretion could be based on two criteria. First, using sensitive immunodetection methods, one could find small amounts of the Yops in some *vir* mutants but not in the typical regulatory *virF* mutants (MICHIELS et al. 1991). Second, we reasoned that mutations in the Yop secretion pathway should prevent the export of Yops but not of YadA since YadA possesses the structure of a protein exported via the classical sec pathway. The *virC* and the *virA* mutants fulfilled these two criteria: they transcribe the *yop* genes, produce small amounts of intracellular Yops, but do not secrete them and they express YadA at the bacterial surface. Hence we concluded that the *virC* and *virA* loci encode at least some of the components of the Yops secretion machinery (MICHIELS et al. 1991). The 8.5 kb *virC* locus constitutes a single large operon composed of 13 genes called *yscA* to *yscM* (for Yop secretion) (MICHIELS et al. 1991). Open reading frames (ORFs) *yscB* to *yscK* are all contiguous with four cases of overlap between a stop codon and the start of the next gene (MICHIELS et al. 1991). The putative *yscC* gene product has a signal sequence and it shares significant homology with outer membrane proteins known to be involved in the secretion of pullulanase by *Klebsiella pneumoniae* (PulD) (D'ENFERT et al. 1989) or in the release of filamentous bacteriophages (gene IV product) (BRISSETTE and RUSSEL 1990). This similarity is an indication that the *virC* operon could indeed be involved in secretion but it is also a source of confusion between the type II and type III systems. YscJ is a 27 kDa lipoprotein that we initially described as YlpB (CHINA et al. 1990). The putative products of *yscB, E, F, G* and *I* are proteins of less than 16 kDa and with no particular addressing signal. In order to prove the existence of these elements and to investigate their role in secretion, we engineered a battery of nonpolar mutants. Their analysis demonstrated unambiguously that, at least YscC, YscF,

YscI, YscJ and YscK are required for the traffic of the Yops while YscH seems to be dispensible (A. Allaoui and G.R. Cornelis, in preparation). A previous complementation analysis of polar mutants in *virC* already demonstrated that YscD and YscL are also required to secrete the Yops (MICHIELS et al. 1991). In conclusion, most of the 13 genes of the *virC* operon are indeed involved in secretion of the Yops. It is presumably not the case of *yscM*, the last gene of the operon. It encodes a 12.4 kDa protein having a significant similarity to YopH, the product of the neighboring gene. The domain of similarity spans residues 52–130 of YopH, a domain located between the secretion recognition domain (residues 1–48) and the tyrosine phosphatase domain (residues 206–468) (MICHIELS et al. 1991). From their studies in *Y. pseudotuberculosis*, RIMPILÄINEN et al. (1992) concluded that, LcrQ, the homolog of YscM, is a regulator.

The group of H. Wolf-Watz called *lcrK* the region corresponding to the distal part of the *virC* operon (ROSQVIST et al. 1990a; RIMPILAÏNEN et al. 1992). It consists of *lcrP, O, Ka, Kb, Kc* and *Q*, corresponding the *yscH, I, J, K, L* and *M*, respectively. The *yscBCDEF* genes of *Y. pestis* have been analyzed by HADDIX and STRALEY (1992).

Interestingly, homologs of the *yscJ*, *yscF* and *yscC* genes have been discovered in other animal pathogens such as *Shigella flexneri* (ANDREWS and MAURELLI 1992; ALLAOUI et al. 1992, 1993; VENKATESSAN et al. 1992) and *S. typhimurium* (GALAN et al. 1992) but also in plant pathogens such as *Xanthomonas campestris pv. vesicatoria* (FENSELAU et al. 1992), *P. solanacearum* (GOUGH et al. 1992) and *Pseudomonas syringae pv. syringae* (XIAO et al. 1992). According to sequence analysis, none of the 13 gene products of the *virC* operon encodes an ATP-binding protein which could act as the energizer of the system.

The *virB* locus is also devoted to Yop secretion. It consists of eight genes which will be called *yscN* to *yscU*, in the three species, according to a proposal made by H. Wolf-Watz (University of Umeå, Sweden). Interestingly, YscN, the product of the first gene could act as an energizer of the system (WOESTYN et al. 1994). It is a 48-kDa protein containing a putative ATPase domain consisting of the two nucleotide binding motifs A and B identified by WALKER et al. (1982). YscN shares significant homology to the β subunit of the F_0F_1 proton translocase of *E. coli* and to FliI of *S. typhimurium* (VOGLER et al. 1991) and *Bacillus subtilis* (ALBERTINI et al. 1991), an ATP-binding protein presumably involved in the flagellum specific export pathway. The deletion of the Walker box A by site-directed mutagenesis completely abolishes Yop secretion, which indicates first that this protein is indeed involved in the secretion pathway and, second, that the integrity of the ATPase domain is required for this action (WOESTYN et al. 1994). The second gene of locus *virB*, *yscO*, encodes a basic 19-kDa protein (S. Woestyn et al., unpublished observations). Genes *yscO*, *yscR* and *yscS*, only described in *Y. pestis* so far, are also involved in Yop secretion. YscR is a 24-kDa inner membrane protein containing four transmembrane domains (FIELDS et al. 1994). The last gene of the locus, *yscU*, characterized in *Y. enterocolitica* encodes a 40-kDa protein containing four transmembrane segments anchoring a large cytoplasmic carboxyl-terminal domain to the inner membrane (ALLAOUI et al. 1994).

The entire *lcrB* (*virB*) region has also been sequenced in *Y. pseudotuberculosis* (H. Wolf-Watz, personal communication). Taking into account these elements, it appears that the *virB* (*lcrB*) locus of *Y. enterocolitica*, *Y. pestis* and *Y. pseudotuberculosis* is the counterpart of the *spa* locus from *S. flexneri* and *S. typhimurium*: genes *yscN, O, P, Q, R, S T, U* are the counterparts of *spa47, 13, 32, 33, 24, 9, 29, 40* from *S. flexneri* (VENKATESAN et al. 1991; SASAKAWA et al. 1993) and *spa L, M, N, O, P, Q, R, S* from *S. typhimurium* (GROISMAN and OCHMAN 1993), respectively. The degree of conservation varies between 20% and 50% for each individual gene but every gene is positioned in all three genera at the same relative position. In addition, YscU and YscN are the homologs of HrpN and HrpE from *P. solanacearum* (GOUGH et al. 1993; Van Gyseghem, personal communication).

Apart from the *ysc* genes, the Yop secretion pathway also involves *lcrD*, a gene of the *virA* locus which encodes another inner membrane protein (PLANO and STRALEY, 1993). As it is the case of some Ysc homologs, homologs of LcrD are involved in flagellar biogenesis in *Caulobacter crescentus* (SANDERS et al. 1992), in *Campylobacter jejuni* (MILLER et al. 1993), in *S. typhimurium* (VOGLER et al. 1991) and in *B. subtilis* (CARPENTER and ORDAL 1993).

If one accepts that *virC* probably contains ten genes involved in Yop secretion, this pathway thus requires at least 19 genes excluding the *syc* genes encoding the individual chaperones (see below). Many of these 19 genes have counterparts in export systems from other bacterial pathogens and in flagellum assembly systems. These homologies suggest that the Yersinia Yop secretion pathway is a representative of a new secretion pathway, sometimes referred to as type III, and essentially devoted to pathogenesis in plants and in animals. It probably derives from the secretion system involved in the export of the flagellum components. This new pathway, which is the subject of a microreview by VAN GIJSEGHEM et al. (1993), will be treated in several other chapters of this volume.

5.4 The Yop-Specific Chaperones

One of the peculiarities of the Yop secretion system is that it makes use of cytoplasmic chaperones that are specific for individual Yops (WATTIAU and CORNELIS 1993). We called these chaperones "Syc," for specific Yop chaperone, followed by the code letter of the corresponding Yop. If a *syc* gene is mutated, the corresponding Yop is no longer exported but the secretion of the other Yops is not affected. The genes encoding chaperones *sycE* and *sycH* are localized next to the corresponding *yop* genes, namely, *yopE* and *yopH*. We hypothesize that the role of these chaperones is to lead the nascent Yop proteins to the translocon. The Syc proteins would thus be the counterparts of SecB in the sec-dependent pathway. The major difference is that the Syc proteins are specific for some Yops (WATTIAU and CORNELIS 1993) while SecB is multivalent. SycE, the chaperone of YopE, is a 14.7 kDa protein with a very acidic (4.4) isoelectric point. It probably exists as a dimer in the cytoplasm (WATTIAU and CORNELIS 1993). SycH is 16 kDa protein that

also has an acidic isoelectric point (P. Wattiau et al. 1994). The chaperone serving YopB and YopD is LcrH, an acidic 18-kDa protein previously described as a regulator. We propose to rename it SyeD (P. Wattiau et al. 1994).

6 Genetic Regulation

6.1 Two Regulatory Circuits

We have seen in the previous sections that growth restriction and Yops secretion only occur at 37°C and in the absence of Ca^{2+} ions. By contrast, the production of YadA is thermodependent but independent of the Ca^{2+} concentration. Hence, temperature and Ca^{2+} influence two different regulatory networks. The first one, responding to temperature, regulates all the pYV encoded virulence functions while the second one, responding to Ca^{2+}, only regulates the production of the Yops and YlpA. None of these two regulatory networks is perfectly understood so far but it is quite clear that they are independent from each other.

Transcription of the *yop* genes and the *vir* (*lcr*) genes, including *virF*, is strongly thermodependent (Cornelis et al. 1986, 1987b, 1989; Forsberg and Wolf-Watz 1988; Goguen et al. 1984; Mulder et al. 1989; Hoe et al. 1992; Straley and Bowmer 1986; Yother et al. 1986). Transcription of a cloned *virF* is thermodependent in a *Y. enterocolitica* strain cured of pYV (Cornelis et al. 1989), which indicates that *virF* is itself thermoregulated by a chromosomal gene.

6.2 The Modulator YmoA

In order to identify the chromosomal regulator, we mutagenized a *Y. enterocolitica* strain which carries *lacZ* fused to *yopH*. Two chromosomal mutants transcribed *yopH*, *yopE* and *yadA* strongly at 28°C but nevertheless did not secrete the Yops at low temperature (Cornelis et al. 1991). Transcription of the regulatory gene *virF* itself was increased at 28°C, which may account for the increased transcription of the genes of the regulon. Although the elements of the *yop* regulon were overexpressed at low temperature in the mutants, there was still an increase of transcription upon transfer to 37°C. Hence, the thermal response was not abolished in these two mutants but rather "modulated." The phenotype was thus not that of a classical repressor minus mutant. In both mutants, the transposon was inserted in the same gene that we called *ymoA*, for *Yersinia* modulator. The *ymoA* gene encodes a 8064-dalton protein extremely rich in positively and negatively charged residues. Although there is no sequence similarity between YmoA and HU, IHF or H-NS (H1), it is very likely that YmoA is a histone-like protein (Cornelis et al. 1991).

All the properties of the *ymoA* mutants are strikingly reminiscent of the *osmZ* mutants of *E. coli* and the *virR* mutants of *Shigella flexneri*, both lacking H-NS (Higgins et al. 1988; Dorman et al. 1990; Göransson et al. 1990). YmoA is, however, not the *Yersinia* counterpart of H-NS from *E. coli* or *Shigella*. Nevertheless, the *hns* mutants, but not the wild-type of *E. coli*, express *virF* in a thermodependent manner (G.R. Cornelis, unpublished observation). Thus, H-NS and YmoA may recognize the same structures or sequences.

An homolog of YmoA was recently discovered in *E. coli*, as regulator of hemolysin production (Nieto et al. 1991). The gene encoding this regulator, called *hha*, can complement the *ymoA* mutations of *Y. enterocolitica* provided it is expressed at an adequate level (A. Mikulskis and G.R. Cornelis 1994). YmoA and Hha are thus the first representatives of a new class of histone-like proteins regulating the expression of topologically sensitive promoters. Hha can therefore be listed along with H-NS, IHF, FIS, HU and LRP. It is striking that the search for a thermoregulator in *Yersinia*, like in *Shigella* and in uropathogenic *E. coli*, converged on histone-like proteins.

6.3 Control by Temperature

The fact that *virF* is thermoregulated can explain why the Yops are only produced at 37°C. However, it does not demonstrate that temperature is only involved in the regulation of *virF*. Indeed, when *virF* is transcribed at low temperature from a tac promoter, the *yop* and *yadA* genes are only poorly transcribed and no Yops are produced (Lambert et al. 1992). By contrast, at 37°C, the response to IPTG mimics the normal response to thermal induction (Lambert et al. 1992). VirF is thus not the only key to the thermal control of Yops production.

This poor transcription of the *yop* regulon at 25°C in the presence of VirF could be due to an inadequate conformation of the promoter (Lambert et al. 1992). Indeed, in *ymoA* mutants, the *yopH* promoter is active at 37°C in the absence of VirF and it is extremely active at 25°C in the presence of VirF (Lambert et al. 1992). This indicates that chromatin structure can be involved in transcription activation of the *yop* genes. Hence, we suggest that, in vivo, the promoters of the *yop* regulon are more susceptible to VirF activation at 37°C. Thus, chromatin structure can influence transcription from *yop* promoters in addition to the effect on transcription of *virF* itself.

We hypothesize that, somehow, temperature modifies the structure of chromatin, making the promoters more accessible to VirF. We have, however, no information on the nature of the DNA structure change occurring during the temperature shift. One can only speculate that some histone-like protein, possibly YmoA, is involved in this change. This observation is just another one pointing to the role of chromatin structure in transcription regulation. This phenomenon, which seems now to be classical in the regulation of bacterial virulence functions, is reviewed and discussed in detail by Higgins et al. (1990) and by Dorman and Ni Bhriain (1993).

6.4 Control by Calcium

While temperature controls nearly all the virulence functions, the presence of Ca^{2+} regulates the production of the Yops. This second form of regulation appears to be very complex and, in spite of many efforts, it is far from being understood, yet. Transcription of the *yop* and *vir* genes is reduced in the presence of Ca^{2+} ions (Cornelis et al. 1987b; Leung et al. 1990; Forsberg and Wolf-Watz 1988; Bölin et al. 1988; Price et al. 1989; Mulder et al. 1989). The *lcrGVHyopBD* operon is involved in this regulation but the mechanism is not known yet (Price and Straley 1989; Bergman et al. 1991). Some models confer a negative regulatory role to LcrH in response to the presence of Ca^{2+} (Price and Straley 1989; Bergman et al. 1991) but we rather view LcrH as the YopB and YopD chaperone (see here before). According to Forsberg et al. (1991), YopN could be the Ca^{2+} sensor.

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Surface-Associated and Soluble Components of *Vibrio cholerae* Involved in Bacteria-Host Interactions

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1 Introduction

Cholera is a disease which has been known for many centuries and is readily identified by description in even in the most ancient of writings. Typically, it has been associated with the Indian subcontinent, but has been able to cross the globe. Since the beginning of the nineteenth century, seven pandemics have spread in waves, facilitated by increased mobility of the human population. The etiological agent *Vibrio cholerae* was first described by Pacini in 1854 and subsequently rediscovered in 1883 by Koch, who demonstrated the causal relationship with the disease. (An excellent treatise on the history of cholera has recently been published; Barua 1992.)

Up until very recently (Cholera Working Group 1993) cholera has been associated with *Vibrio cholerae* of the O1 serotype, which exists as two separate biotypes, E1 Tor and classical, that can be differentiated on the basis of a number of phenotypic and biochemical characteristics. However, the newly described O139 strains also have most of the properties associated with seventh pandemic

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El Tor strains, except for lipopolysaccharide structure (U.H. Stroeher and P.A. Manning, manuscript in preparation), and thus will not be considered separately in this article.

1.1 The *Vibrio cholerae* Life Cycle

V. cholerae is a water-borne pathogen, propagated via the fecal-oral route, that appears to have developed a complex set of traits which act at different phases of a life cycle to ensure its continuance in the natural habitat, transmission to and multiplication in the human host and release into the aquatic environment where it can persist for long period before reinfecting. Although vibrios can be usually isolated from rivers and water supplies in endemic areas, the actual numbers detected are often a significant underestimate of the level of contamination because of their ability to enter the viable but nonculturable state. This form resembles a state of suspended animation. The bacteria cannot be reactivated by simple addition of laboratory nutrient media, but require in vivo environmental signals in order to begin replicating. Presumably this reflects the complex set of stimuli which are associated with activating the expression of genes contained within the various virulence regulons (see below), as well as those required for basic cellular syntheses. The various apparent phases of the *V. cholerae* life cycle can be summarized as follows:

1. Organisms enter host via ingestion of contaminated food or water.
2. The cholera bacteria sense the host environment and modify their gene expression accordingly.
3. Various colonization enhancers including adhesins and hydrolytic enzymes are produced.
4. Further modification of gene expression leads to colonization of the intestinal epithelium and release of toxins.
5. The bacteria sense the changing environment mediated by the effects of the toxins and bacterial multiplication.
6. The organisms sense that it is an appropriate time to detach and leave the host, and/or there is selection of variants due to the development of an immune response.
7. The bacteria further modify their gene expression to facilitate persistence in the natural environment.

Although this model is somewhat speculative there is reasonable circumstantial evidence for most of the stages. Indeed it is clear that *V. cholerae* has multiple genetic factors which can operate and that many are coordinately regulated either at the level of gene expression or secretion.

Since the numerous gene products that play important roles in the *V. cholerae* life cycle are not constitutively expressed, the organism must be able to

sense the changes in the environment. Thus, upon entering the host, the bacterium must be able to detect the alterations, for example in oxygen tension, pH, osmolarity, viscosity, temperature and nutrient status, so that those factors appropriate to this stage and location are elucidated. The factors which would appear to be most relevant to the disease process are extracellular/secreted proteins and those associated with the bacterial cell surface (MANNING 1987, 1992).

2 Extracellular/Secreted Proteins

V. cholerae produces a plethora of secreted proteins some of which are best classified as toxins. Others are primarily hydrolytic enzymes which can influence the colonization process and which also have the potential to provide a source of basic nutrients.

2.1 Colonization Modifiers

The hydrolytic enzymes secreted by *V. cholerae* include DNases, proteases, chitinase and neuraminidase.

Two DNases have been identified (FOCARETA and MANNING 1987, 1991) and although mutants lacking both do not appear to be significantly attenuated in the infant mouse cholera model, one can still imagine a role for them. The mucus coat on the epithelium is rich in DNA presumably due to the high turnover of epithelial cells (FERENCZ et al. 1980). This would contribute to the viscosity and reduce the ability of bacteria to penetrate this natural defense system. However, the production of the DNases could provide a ready means of not only facilitating passage, but in doing so, also provide additional nutrients in the form of the products of DNA hydrolysis.

Multiple proteases have been inferred from mutant isolation studies (SCHNEIDER and PARKER 1978). One of these, the soluble hemagglutinin protease (Hap), is a particularly interesting molecule. It has been shown to have mucinase activity, to be able to cleave fibronectin, and it has been suggested to be involved in the proteolytic activation of cholera toxin (FINKELSTEIN et al. 1983; BOOTH et al. 1984). It is closely related to the elastase of *Pseudomonas aeruginosa* and has the enzymatic activities mentioned above (HÄSE and FINKELSTEIN 1991). Studies of a *hap* mutant in animal models have suggested it has a further function (FINKELSTEIN et al. 1992). Pretreatment of cultured epithelial cells with Hap prevents adherence, and *hap* mutants, although unaffected in virulence, show reduced shedding. Thus, besides facilitating mucus breakdown Hap also acts as a detachase, breaking down receptors for a number of adhesins and so aiding release of bacteria into the environment.

The potent neuraminidase (NanH) would seem to have a role during the main part of the infection. Cholera toxin recognizes ganglioside GM1 as its receptor;

however, GM1 is not the most abundant glycolipid in the membrane. Instead other polysialogangliosides predominate. NanH converts these molecules to GM1 by removing the additional sialic acid residues, resulting in an increased receptor density for cholera toxin (GALEN et al. 1992). This could be readily shown to lead to increased cholera toxin binding in vitro but a similar effect in vivo would be difficult to demonstrate.

Chitinase production presumably has relevance to the aquatic environmental phase. *V. cholerae* is commonly found in association with chitinous particles (TAMPLIN et al. 1990) and the secretion of a chitinase may facilitate attachment and nutrient acquisition.

2.2 Toxins

Cholera toxin (CT) is one of the best studied bacterial toxins, genetically, structurally and in its mode of action. CT is composed of two subunits, A and B, encoded by separate genes encoded within the *ctxA,B* operon (see below; MEKALANOS et al. 1983). There are five B subunits per A and they form a pentameric ring into which the A subunit is inserted (HOLMGREN et al. 1991). In the mature toxin the A subunit is proteolytically nicked to A_1 and A_2, which are held together via a single disulfide bond. The B subunit provides the receptor recognition and the A subunit has the toxic activity; both are required for CT function. Little is known regarding internalization of the A subunit; however, it must pass through the cytosol of the enterocyte to the basement membrane, where its target, the adenylate cyclase, is located. The A_1 fragment ADP-ribo-sylates the N_S (G_S) component of the adenylate cyclase leading to an increase in formation of cyclic AMP. This is associated with Cl^- and HCO^-_3 secretion and inhibition of NaCl absorption, and accompanied by changes in water movement resulting in fluid secretion in diarrhea.

The CT genes, *ctxA,B*, are part of a virulence gene cluster flanked by repeated DNA sequences designated RS1 (Fig. 1; PEARSON et al. 1993). There are four other genes encoded within this cluster: *zot*, *ace*, *cep* and *orfU* (see below; FASANO et al. 1991; PEARSON et al. 1993; TRUCKSIS et al. 1993). This whole unit is able to undergo amplification by means of a site-specific recombination system encoded within

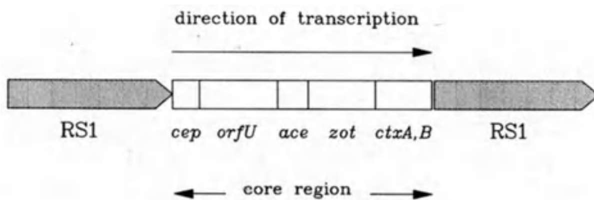

Fig. 1. Genetic organization of the virulence cassette flanked by RS1 elements. The genes contained within the core region are: *cep*, core encoded pilin; *orfU*, unknown open reading frame; *ace*, accessory cholera enterotoxin; *zot*, zonula occludens toxin; and *ctxA,B*, cholera toxin A and B subunits

RS1, and as a consequence several tandemly duplicated copies of this cluster can be generated. This has been observed after in vivo passage in animal models and the organization of this region in different isolates is also suggestive of such an amplification (MEKALANOS 1983). This provides a potent means of amplifying the products of these virulence determinants and especially in enhancing toxigenicity.

Zot (zonula occludens toxin) was identified as the result of a search for a toxin responsible for the residual diarrhea found with CT-deleted strains (FASANO et al. 1991). It appears to alter the structure of the epithelial tight junctions (zonula occludens) which leads to an increase in conductance in Ussing chamber experiments. This would result in an increase in intestinal permeability. Ace (accessory cholera enterotoxin) is the third toxin within the RS1-linked virulence gene cluster (TRUCKSIS et al. 1993). It has been shown to be enterotoxic in ligated rabbit ileal loops and would consequently be expected to be diarrheagenic.

Production of a soluble hemolysin, designated HlyA, for sheep red blood cells is a characteristic used to differentiate E1 Tor from classical strains. However, the hemolytic status of E1 Tor strains now seems to be quite variable making it less reliable as a biotyping character. HlyA has been shown to have both cytotoxic/enterotoxic and cytolytic activities (HONDA and FINKELSTEIN 1979; ALM et al. 1988, 1991). The structural gene has been cloned and the protein has been shown to undergo several cleavages suggestive of a pre-pro-protein (ALM et al. 1988; YAMAMOTO et al. 1990). Interestingly, classical strains appear to have a common defect in *hlyA*, an 11 bp deletion, which results in production of a truncated nonhemolytic form (ALM et al. 1988; RADER and MURPHY 1988; ALM and MANNING 1990a) predicted to contain enterotoxic activity (ALM et al. 1991).

HlyA induces pathological changes in the infant mouse gut and would appear to be expressed at a different stage of the infection than CT (ALM et al. 1991). Its function during an infection may be to provide iron since it is in part under control of Fur (see below); however, it is also directly controlled by the HlyU protein (WILLIAMS and MANNING 1991; WILLIAMS et al. 1993). Lysis of enterocytes also releases other nutrients which can be readily accessed due to the variety of secreted enzymes which *V. cholerae* possesses.

2.3 Protein Secretion/Export

Considerable interest has emerged in protein secretion in recent years and there appear to be a series of common underlying mechanisms (see PUGSLEY 1993 for review). Some years ago a mutant, *V. cholerae* strain M14, defective in CT secretion was isolated (HOLMES et al. 1975). Recently, it has been possible to identify the affected gene and to demonstrate that there are further linked genes also associated with protein secretion (SANDKVIST et al. 1993; OVERBYE et al. 1993). These genes, designated *eps* genes, are homologous to those associated with protein secretion found in a variety of gram-negative bacteria including *Klebsiella oxytoca* (PUGSLEY and REYSS 1990), *Erwinia chrysanthemi* (LINDBERG and COLLMER

1992) and *Pseudomonas aeruginosa* (BALLY et al. 1992). These genes are not only required for secretion of CT, but also for protease and chitinase (OVERBYE et al. 1993), and one wonders whether they are also required for all of the other secreted proteins as well, given the pleiotropic effects of some classes of protease-defective mutants (SCHNEIDER and PARKER 1978).

Homology has been found between genes for protein secretion and type 4 fimbrial assembly with a number of organisms (see PUGSLEY 1993). This also holds true for *V. cholerae*. Several of the genes necessary for biosynthesis of toxin coregulated pilus show homology (see below; Fig. 4; OGIERMAN et al. 1993a; KAUFMAN et al. 1993). Thus, the presumed ATP-binding component of these systems is present in both the *tcp* gene cluster (*tcpT*; OGIERMAN et al. 1993a) and in the *eps* gene cluster (*epsE*; SANDKVIST et al. 1993). It will be interesting to see whether a third homologue exists for the more typical type 4 fimbrial system that is also present in *V. cholerae* (see below; EHARA et al. 1991; A. Fallarino and P.A. Manning, unpublished data).

3 Surface Components

Due to the critical nature of the colonization process in the establishment of a cholera infection, the components of the cell envelope have been suggested as prime targets for vaccine development (MANNING 1987, 1992). Besides containing a variety of specific proteins, the outer membrane also contains lipopolysaccharide (LPS) and is associated with anchoring a number of surface structures.

3.1 Outer Membrane Proteins

The outer membrane of the cell envelope of *V. cholerae* is similar to that of other gram-negative enteric pathogens in terms of the types of proteins that are present. This includes several porin-like proteins and an OmpA-like protein (MANNING et al. 1982; ALM et al. 1986). Although many of the outer membrane proteins of *V. cholerae* are clearly immunogenic in both humans and animals (MANNING and HAYNES 1984; SEARS et al. 1984; KABIR 1986), their significance in pathogenesis is ill-defined.

OmpV is a major immunogenic protein (MANNING and HAYNES 1984), and antibodies have been readily detected in many convalescent sera by western blot analysis (P.A. Manning, unpublished data). However, a mutant with a Tn*phoA* insertion in the structural gene could compete equally well with the wild type in vivo, indicating that OmpV does not have a role in colonization (TAYLOR et al. 1987). The structural gene, *ompV*, has been cloned, the nucleotide sequence determined, and the location of the antigenic epitopes with the protein defined (STEVENSON et al. 1985; POHLNER et al. 1986a,b). *ompV* is very poorly expressed in *E. coli*, apparently due to a novel translational control mechanism (POHLNER et al. 1988).

The gene *ompW*, encoding a 22 kDa outer membrane protein, has also been cloned and sequenced, and its expression analyzed in a number of hosts (MANNING et al. 1985; JALAJAKUMARI and MANNING 1990). Although a minor protein in *V. cholerae*, it is very immunogenic and expressed at much higher levels in heterologous hosts. No evidence exists for a role in pathogenesis.

Two proteins, OmpU and OmpT, have been also identified that are inversely regulated by the ToxR,S,T regulon (see below); however, no role in pathogenesis has been established (Taylor et al. 1987).

3.2 Flagellum and Chemotaxis

Motility, and the associated phenomenon of chemotaxis, is an important virulence property for *V. cholerae* (GUENTZEL and BERRY 1975; FRETER et al. 1981). The bacterium has a single polar flagellum which is sheathed in what appears to be an extension of the outer membrane lipid bilayer, in that it contains LPS (FUERST and PERRY 1988) and specific proteins (HRANITSKY et al. 1980). Nonmotile mutants are less virulent than their corresponding motile parents, although both are still capable of inducing diarrhea and can adhere to intestinal epithelium (GUENTZEL and BERRY 1975; ATTRIDGE and ROWLEY 1983). It has also been suggested that a component of the flagellum is important for virulence.

Studies on the regulation of virulence determinants (see below) indicate that the cell is able to sense various molecules which are critical for expressing these factors. Thus, it is highly likely that there will be a close relationship between the chemotactic response to some of these molecules and gene regulation. The presence of a flagellum is of course essential for this response.

It is interesting to speculate about the role of the *hlyB* gene product, which was originally thought to be involved in secretion of the E1 Tor hemolysin HlyA (MANNING et al. 1984; ALM and MANNING 1990b). It is now clear that HlyB is highly homologous to the family of chemotactic transducers which are involved in signaling the flagellar motor in response to particular environmental stimuli. HlyB is up-regulated, along with HlyA, by HlyU (WILLIAMS and MANNING 1991), suggesting that an understanding of the associated chemotactic stimuli may help define the requirements for HlyA expression. The high degree of conservation of motifs within the chemotactic transducers of *E. coli*, *Salmonella* and *hlyB* suggests that other transducers in *V. cholerae* could be readily identified and facilitate elucidation of the stimuli that can moderate chemotaxis in conjunction with both pathogenesis and envioronmental interactions.

3.3 Lipopolysaccharide and Serotype Switching—A Host Avoidance Mechanism?

Lipopolysaccharide is the most abundant molecule on the cell surface of gram-negative bacteria and functions as a protective barrier against both hydrophobic molecules and detergents such as bile salts. It is the O antigen of the LPS which

provides the basis for serotypic differences. LPS of *V. cholerae* O1 is highly immunogenic and is clearly a protective antigen (NEOH and ROWLEY 1972; MANNING et al. 1986). Strains of both biotypes can be further subdivided into three serotypes, depending on the structure of the O-antigens of the LPS, designated Inaba, Ogawa and Hikojima. The three serotypes share a common antigenic determinant referred to as the A antigen. In addition, there are two specific antigens, B and C, which are expressed to varying degrees on the different serotypes: Inaba strains express only C, while Ogawa strains express both B and C, although C is present in a much reduced amount compared to Inaba (see MANNING et al. 1994 for discussion). The serotype of *V. cholerae* O1 strains is not fixed, but can undergo a conversion or switching between the Inaba and Ogawa serotypes. This conversion is nonreciprocal and occurs at a frequency of approximately 10^{-5} for the Ogawa to Inaba conversion but significantly lower for the converse. Serotype conversion has been demonstrated in vitro as a result of exposure of the organism to antisera. The isolation of Inaba strains from Ogawa cells grown in the presence of anti-Ogawa serum has been reported but the converse experiment of growing Inaba cells in anti-Inaba serum only gave rise to rough strains. Thus, it appeared that only the serotype conversion from Ogawa to Inaba could be detected. Initially, in vivo reports of serotype conversions were dismissed as multiple infections of patients or patients being reinfected with another serotype but in 1967 GANGAROSA et al. reported Ogawa to Inaba switching. Isolation of Inaba organisms was followed by a relapse, suggesting that the organism had multiplied in the intestine and that a change in serotype may have enabled the host immune response to be evaded. In 1966 (SHEEHY et al. 1966), in a laboratory which used exclusively Inaba organisms, a worker acquired an infection and after 3 days began to secrete Ogawa organisms. Thus, it may be easier to detect Inaba to Ogawa serotype convertants in humans in whom the *V. cholerae* multiply to large numbers and presumably a selection against the original serotype can occur via the immune response.

Studies using germ-free mice have confirmed the ability of *V. cholerae* to undergo serotype conversion (SACK and MILLER 1969) and demonstrate the change not only from Inaba to Ogawa and vice versa but also from smooth to rough strains lacking O antigen and then back to smooth strains usually of the same original serotype. Thus, it appears as though *V. cholerae* can change serotype and also the presence or absence of O antigen. In addition, mouse experiments and use of immunosuppressive drugs indicate that serotype convertants are selected for by the specific antibodies.

Data to support the notion that serotype conversion is important to the persistence of the disease come from observations of recent epidemics. In Latin America in 1991 a cholera epidemic began for the first time this century. Extensive biochemical analyses and rRNA RFLP analysis has shown that the epidemic strain, an E1 Tor Inaba is unique to Latin America (D.N. Cameron, T. Popovic, I.K. Wachsmuth, P.I. Fields, personal communication; SALAZAR-LINDO et al. 1991). However, Ogawa isolates that were identical to the epidemic strain in all other respects began to appear in about the seventh month of the epidemic suggesting that the epidemic strain had undergone a serotype conversion.

Sequence analysis and the construction of defined mutants have shown that Ogawa strains can be converted to Inaba by any change which leads to a defective RfbT protein, whereas an Inaba can only switch to Ogawa if the specific *rfbT* mutation in that strain is precisely reverted (Fig. 2). This has occurred in the Latin American Inaba strains that gave rise to the Ogawa form during the cholera epidemic in 1991 (Cameron et al., cited in MANNING et al. 1994).

More recently, a previously unidentified serotype, O139, also referred to as *V. cholerae* synonym Bengal, has arisen and is causing a dramatic epidemic in India and Bangladesh (CHOLERA WORKING GROUP 1993). A variety of analyses suggests that these strains are indistinguishable from seventh pandemic E1 Tor isolates except for their serotype (Cameron et al., personal communication). Indeed it would appear that the strains have a deletion within the *rfb* region and have acquired an enzyme which permits the LPS core to be modified so that it is immunologically novel (U.H. STROEHER and P.A. MANNING, manuscript in preparation). Part of the genetic changes which have occurred appear to be associated with *rfbQ,R,S* which shows a high degree of homology to Rhs elements associated with chromosomal rearrangements in *E. coli* (Fig. 2; ZHAO et al. 1993).

Interestingly, the ability to construct isogenic strains differing only in serotype has enabled the demonstration that, at least in animal models, there is no difference in naive animals in their virulence (STROEHER et al. 1992). Thus, the facility to change serotype as a consequence of immune pressure by the host has a decided advantage to the bacterium without compromising its pathogenic potential.

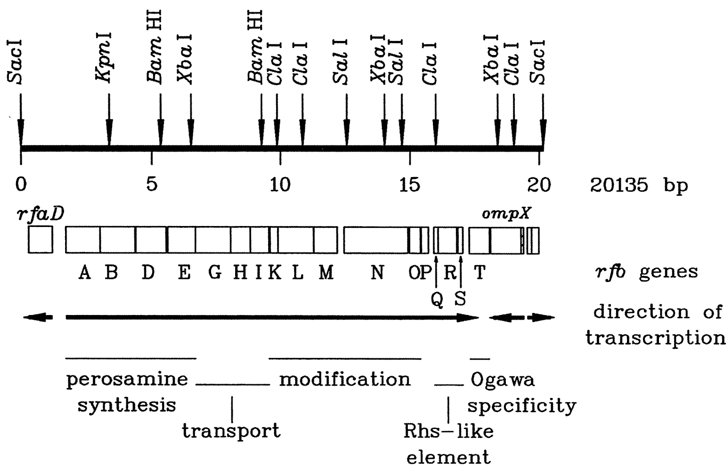

Fig. 2. Physical map of the *rfb* region encoding biosynthesis of the O-antigen of the lipopolysaccharide. The functionally defined regions are indicated. *rfaD* probably is associated with LPS-core oligosaccharide synthesis and *ompX* is a putative porin

3.4 Hemagglutinins, Grappling Hooks and Anchors

Several different hemagglutinins (HAs) have been shown to be expressed by *V. cholerae* and, by analogy with other systems (e.g., enterotoxigenic *E. coli*), these factors may also be the adhesins involved in colonization. These molecules differ in the specificity of erythrocytes which are agglutinated, the phase of growth and temperature at which they are expressed, their sensitivity to sugars and the requirement for divalent cations, and expression, at least in vitro, may be biotype-dependent (HANNE and FINKELSTEIN 1982). Thus, the major cell-associated HA of E1 Tor strains is the chicken erythrocyte HA which is sensitive to D-mannose and D-fructose and is also used in biotyping. The classical HA is L-fucose sensitive. These and other HAs have been shown to correspond to fimbriae/pili and will be discussed below. However, other HAs are poorly defined.

The gene for a mannose-fucose resistant cell-associated HA (MFRHA) has been cloned, the nucleotide sequence determined and a specific mutant constructed (FRANZON and MANNING 1986; FRANZON et al. 1993). There are conflicting data as to which of two ORFs corresponds to the MFRHA (FRANZON and MANNING 1986; VAN DONGEN and DEGRAAF 1986). This is complicated by the fact that neither of the two open reading frames (ORF's) encoded within the *mrhA,B* operon look like typical surface-associated proteins (A. Barker and P.A. Manning, unpublished data). Mutants defective in this locus show an at least 100-fold higher LD_{50} in the infant mouse model than in the corresponding parent strain (FRANZON et al. 1993). Such mutants also show a marked defect in their ability to compete in vivo with the parent strains.

The region of the bacterial chromosome where *mrhA,B* is located is unusual (Fig. 3). Part of this region has been sequenced and pulsed field electrophoresis and Southern hybridization have provided further details (FRANZON et al. 1993; BARKER et al. 1994). Within about 100–200 kb there are more than 40 copies of a 124 bp repeated sequence which flanks the individual genes in a direct orientation within the sequenced region. The function of the repeat is unknown but TAKEDA et al. (1991) identified an isolate in which it was found flanking the ST-toxin gene not normally present in *V. cholerae* O1. Perhaps this region of the chromosome represents a pathogenicity island?

FINN et al. (1987) isolated a mutant, SB001, derived from JBK70, a CT deleted derivative of the E1 Tor strain N16961; the mutant was shown to be deficient in a cell-associated HA of *V. cholerae*. This mutant was dramatically reduced in its colonization ability, but could still induce an immune response. The introduction of this mutation also abolished the fluid accumulation and deaths seen in rabbits with JBK70, making it potentially more useful as an attenuated cholera vaccine candidate. In contrast to these data, TEPPEMA et al. (1987) demonstrated that a strain lacking mannose-resistant hemagglutinating activity in vitro could still associate with the small intestinal surface.

The exact nature of the above HAs is unknown, however, several fimbriae/pili types, perhaps best pictured as specific grappling hooks or anchors, have been identified. The analogy to a grappling hook or anchor seems most apt in that these

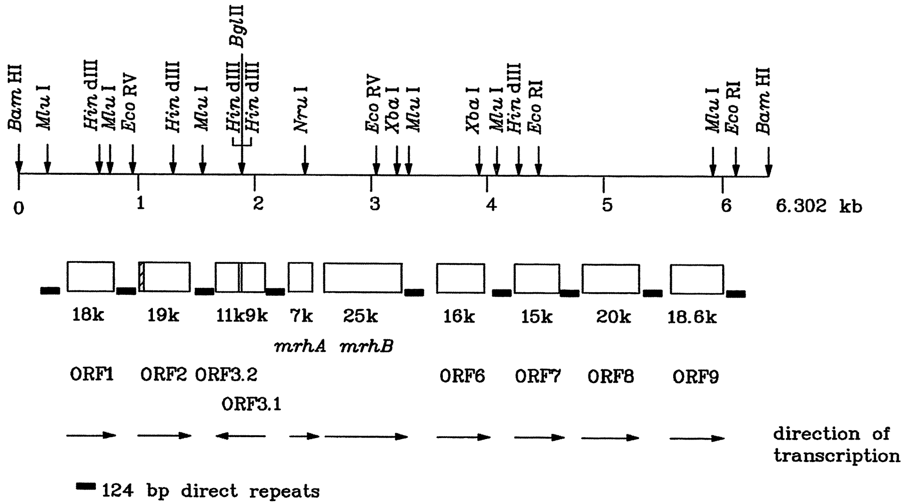

Fig. 3. Genetic organization of the region of DNA in the vicinity of the *mrfA, B* locus encoding a mannose-fucose resistant hemagglutinin. The position of the various open reading frames and the sizes of their products are indicated. The positions of the 124 bp VCR direct repeat sequences flanking the various genes are shown as *solid boxes*

structures are effectively dangling from the cell to grasp the appropriate surface in order to hold the bacterium in place. Thus, they reduce the likelihood that the bacteria will be swept away from the surfaces to which they are seeking to adhere and colonize.

Early studies disagreed as to the existence of pili/fimbriae on *V. cholerae* strains but HALL and colleagues (1988) have clearly identified the existence of several types. TAYLOR et al. (1987) first provided convincing data on a colonization pilus which they designated TCP (toxin-coregulated pilus) because it was under the same genetic control as CT (see below). Studies in the infant mouse cholera model have shown that TCP is a critical virulence determinant in classical strains and that passive antibodies are protective (SHARMA et al. 1989; SUN et al. 1990). TCP could also be shown to be essential for colonization of the human gut (HERRINGTON et al. 1988), although its status as a protective antigen is questionable given the poor immune responses compared to other antigens (HALL et al. 1991). The significance of TCP to the E1 Tor biotype is even more obscure. E1 Tor strains have been demonstrated to produce TcpA (JONSON et al. 1991a; VOSS and ATTRIDGE 1993), the major structural subunit, but assembled TCP have only recently been identified (ATTRIDGE et al. 1993). Mutants in *tcpA* are dramatically attenuated for both biotypes.

The entire gene cluster encoding TCP biosynthesis has been cloned and sequenced and the genes and their products are highly conserved between the biotypes with a few notable exceptions (Fig. 4; FAAST et al. 1989; OGIERMAN and MANNING 1992a,b; OGIERMAN et al. 1993, 1994). The *tcpA* gene and the regulatory regions between *tcpI* and *tcpP*, and *tcpH* and *tcpA* show marked differences

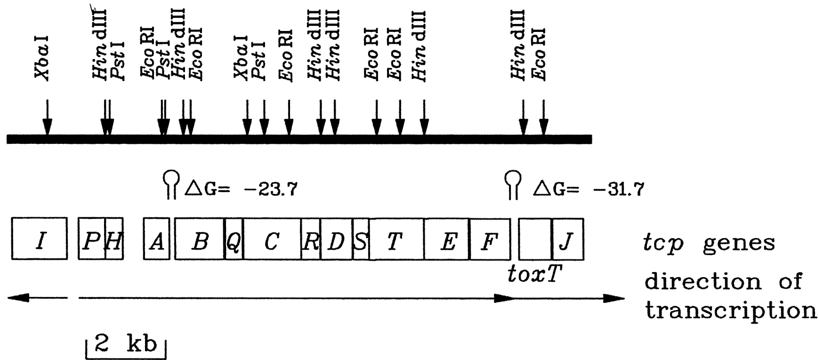

Fig. 4. Genetic map of the *tcp* region encoding biosynthesis of the toxin coregulated pilus. *tcpA* encodes the major structural subunit or pilin. The various functions of the products of the other genes have not been well defined except for *tcpJ*, which encodes a specific signal peptidase, and *toxT*, which encodes a transcriptional activator and is part of the ToxR,S,T regulon

between the biotypes but rigid conservation within the biotypes (OGIERMAN et al. 1994; J.R. IREDELL and P.A. MANNING, manuscript in preparation). These differences result in variation in expression and epitope differences in TcpA (JONSON et al. 1991a; OGIERMAN et al. 1994; VOSS and ATTRIDGE 1994; ATTRIDGE et al. 1993).

As noted above some of the Tcp proteins show homology to proteins associated with type 4 pilus biogenesis and protein secretion; however, based on databse searches, most of the proteins appear to be unique, suggesting a novel assembly mechanism. Although TCP has a number of features in common with type 4 fimbriae, another fimbrial subunit has been described which has an NH_2-terminal almost identical to that of typical type 4 pilins (EHARA et al. 1991). This structure probably corresponds to the MSHA characterized by JONSON and colleagues (1991b). Mutants in this structure are not yet available to assess the role of the mannose sensitive haemagglutinin (MSHA) in colonization. Mutants affecting an accessory colonization factor (ACF) encoded by a series of *acf* genes under the control of the ToxR, S, T regulon show decreased colonization in animal models (PETERSON and MEKALANOS 1988). ACF is thought to represent a minor pilus type.

4 Coordinate Regulation of Virulence Determinants

Studies emanating from the laboratory of John Mekalanos have been instrumental in demonstrating the presence in *V. cholerae* of a regulon of virulence determinants under the control of the ToxR,S,T system (see DI RITA 1992 for review). This regulon controls production of CT, TCP, ACF, OmpT, OmpU and possibly other molecules. It is clearly the most significant of virulence gene regulatory systems in *V. cholerae* and can, both positively and negatively, affect

gene expression. This system is referred to as a cascade because of the complexity of regulatory events which lead to gene expression by ToxR activation (or repression) or subsequent ToxT activation. ToxR is a cytoplasmic membrane-anchored regulatory protein which is activated as a result of environmental stimulation through ToxS (MILLER et al. 1987; DI RITA and MEKALANOS 1991). It also appears to activate ToxT which is a member of the AraC family of transcriptional regulators (OGIERMAN and MANNING 1992a; HIGGINS et al. 1992). Only *ctxA,B* appears to be direcly activated by ToxR whereas the other genes of the ToxR,S,T regulon require ToxT. Although a binding site for ToxR has been identified based on studies with the *ctxA,B* operon (MEKALANOS et al. 1983), the heptameric binding site TTTTGAT has not been identified in association with other ToxR regulated genes.

Iron, in conjuction with the Fur protein, controls the expression of a set of genes which include potential virulence deteminants (GOLDBERG et al. 1990, 1991). Growth under iron-limiting conditions leads to the increased expression of several outer membrane proteins, hemolysin and the iron siderophore vibriobactin (STOEBNER and PAYNE 1988; SIGEL and PAYNE 1982; SCIORTINO and FINKELSTEIN 1983). However, in the case of at least some of these genes, iron availability is not the sole determining factor. IrgB has been identified as a transcriptional activator of IrgA and functions in conjuction with Fur (GOLDBERG et al. 1991). Hemolysin production is also activated by a small regulatory protein, HlyU, which is related to a family of helix-turn-helix DNA binding proteins often associated with heavy metal resistance (WILLIAMS and MANNING 1991; WILLIAMS et al. 1993). Mutants in *hlyU* are attenuated to a greater degree than *hlyA* mutants and also show a small but reproducible defect in their ability to compete in vivo with isogenic *hlyU* strains, suggesting an additional defect in a colonization factor. A 28 kDa protein found in culture supernatants is up-regulated by HlyU (WILLIAMS et al. 1993), and, interestingly, there are two copies of this gene present in *V. cholerae* (S.G. WILLIAMS, L. VARCOE, P.A. MANNING, manuscript in preparation).

Both *toxR* and *hlyU* mutants are attenuated, showing a marked increase in LD_{50} in animal models (TAYLOR et al. 1987; WILLIAMS et al. 1993) and suggesting that both sets of determinants are required for full virulence potential. This reinforces the notion of a defind sequence of events during pathogenesis which are activated as a result of those preceding and that a block in this sequence will lead to attenuation. Furthermore, these regulons may be subject to an even more global control system such as DNA supercoiling (see chapter by HIGGINS).

5 Conclusions

Vibrio cholerae O1 represents a challenging adversary for those interested in studying the molecuar requiremens for pathogenesis because of the highly diverse array of factors and networks that it possesss. This is a dramatic contrast to enterotoxigenic *Escherichia coli* (ETEC), one of the most ubiquitous diarrheal

pathogens, which expresses basically an adhesin, usually referred to as a colonization factor antigen (CFA) and one or both of two toxins, the CT related LT (heat labile toxin) and ST (heat stable toxin). By comparison, ETEC are very simple pathogens lacking the barrage of colonization facilitators and are not even capable of efficiently secreting their toxins.

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Biology of the Pathogenic *Neisseriae*

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1 Introduction

N. gonorrhoeae and *N. meningitidis*, the causative agents of gonorrhoea and meningitis, were discovered in 1879 and 1887 by Albert Neisser (gonococci) and Anton Weichselbaum (meningococci), respectively, although the disease

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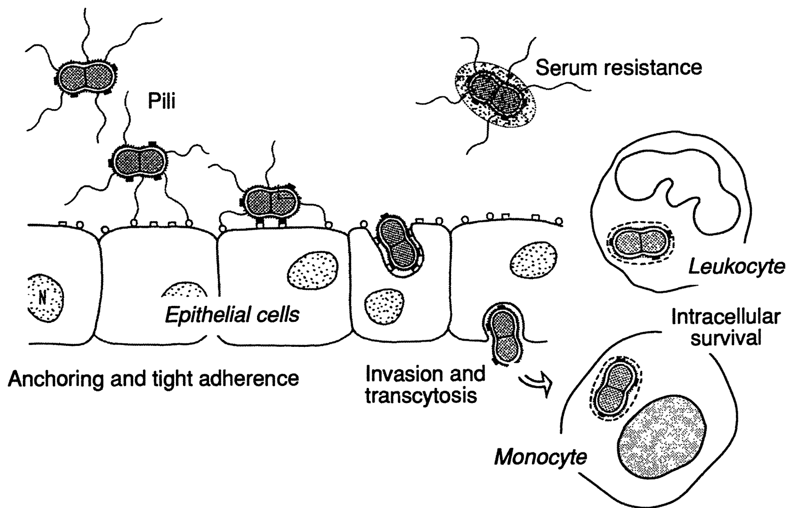


Fig. 1. Critical events of a localized neisserial infection. The bacteria first attach to epithelial cells via their pili and may then transcytotically pass to subepithelial tissues. Professional phagocytes represent important targets for the intracellular accomodation of the bacteria within phagosomal vacuoles. Extracellular bacteria use sialic acid-containing capsules to resist humoral host defenses. Only rarely the bacteria disseminate into the blood stream and other host organs. For further details, see text

gonorrhoea had been recognized since antiquity. The *Neisseriae* are gram-negative bacteria usually diplococcal in shape. They include a wide variety of commensal species and two pathogenic species. Meningococci and gonococci, as well as some of their commensal relatives, only infect humans as their natural host. They represent typical mucosal colonizers. While localized infections with *N. meningitidis* (e.g., of the nasopharynx) of normal human individuals occur frequently and are usually asymptomatic, reminiscent of the mucosal colonization by commensal *Neisseria* species, under rare, as yet undefined, conditions pathogenic *Neisseria* disseminate to cause life-threatening or other severe diseases including meningitis, bacteremia, pelvic inflammatory disease (PID) and septic arthritis.

Localized neisserial infections involve a series of receptor-mediated interactions between the bacteria and the primary target cells (Fig. 1). The formation of pili is a prerequisite for attachment to the epithelial cell surface. The surface-attached bacteria often penetrate the epithelial cells and—whilst contained in a vacuole—may transcytose towards subepithelial tissues. The pathogenic *Neisseriae* strongly interact with phagocytic cells, such as neutrophils and macrophages, which seem to provide them with an intracellular habitat.

Neisserial pathogenesis is characterized by a strong inflammatory response to the infection rather than by the action of distinct bacterial toxins. Clearly, the neisserial infection is a multifactorial process. With regard to their putative virulence attributes meningococci and gonococci are similarly equipped, with

perhaps one major difference, namely, the lack of a polysaccharide capsule in gonococci. Therefore, most of the topics discussed in this review are relevant to both species, despite the different disease spectra caused by the two pathogens.

2 Evolutionary Context and Horizontal Exchange Between *Neisseria* spp.

2.1 Evidence of Horizontal Exchange

A series of recent studies emphasizes the significance of horizontal genetic exchange in the evolution and epidemiology of *Neisseria* species. It appears that a continuous horizontal flow of genetic material affects the chromosomal composition not only of the pathogenic *Neisseria* species, but also of many commensal species. In fact, there exists ample circumstantial evidence for horizontal exchange of genes between commensal and pathogenic *Neisseria* species, between meningococci and gonococci, and within a distinct *Neisseria* species, generating mosaic genes (Halter et al. 1989; Manning et al. 1991; Feavers et al. 1992; Spratt et al. 1992; Zhou and Spratt 1992). It is difficult to give precise estimates of the frequencies with which such horizontal exchanges occur in nature, however, in a few cases it is plausible that such events may have happened in recent years (Achtman 1994). Conceivably, horizontal genetic exchange can be envisioned as a longterm adaptive mechanism suitable for responding to gross environmental changes and for securing the genetic flexibility of *Neisseria* species as a collective group of seemingly independent but truly interconnected traits. Consistent with the fact that the *Neisseria* species evolved from, and still rely on, a common pool of genes, we propose regarding these organisms as a collective, rather than entirely distinct, species.

2.2 Clonal Networks

How does this concept of a species collective fit with the clonality of *Neisseria* species as is particularly evident in the case of *N. meningitidis* (Wang et al. 1992; Achtman 1994)? This issue has recently been addressed by Maynard Smith and colleagues (1993), who proposed, based on gene linkage disequilibrium measurements, a significant difference in the clonal structures of *N. meningitidis* vs other clonal species, such as *Salmonella* and *Escherichia coli*. The authors define several types of population structures, an entirely clonal structure, the epidemic structure and, at the other extreme, the panmictic structure with no evidence of linkage disequilibrium. These population structures are the result of two competing genetic processes, i.e., the genetic drift (mutagenesis) occurring within the scope of a species, and horizontal exchange (recombination) affecting this species extrinsically. *Salmonella* is little influenced by horizontal

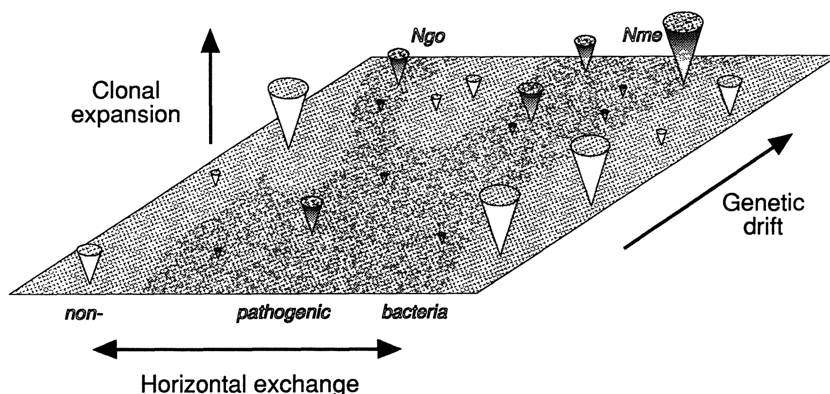


Fig. 2. Simplified representation of the clonal network structure formed by the *Neisseria* species. *Cross-points* in the plain indicate recombination events involving two bacteria of the same strain, of the same species, or of the whole population. Bacteria well adapted owing to horizontal exchange (recombination) and/or genetic drift (mutation) lead to clonal expansion (*cones*). Both pathogenic (*light cones*) and nonpathogenic clones (*shaded cones*) may occur. *Shaded areas* of the network plain represent pathogens

exchange and thus appears as a clonal population. Contrastingly, *N. gonorrhoeae* constitutes a panmictic population in which horizontal exchange is overwhelming (O'ROURKE and STEVENS 1994). *N. meningitidis* occupies an intermediate position in which occasional clonal outgrowths occur in an otherwise panmictic context.

The latter population structure could be termed a "clonal network" (Fig. 2) comprising both sexual and clonal elements. Interestingly, such a clonal network structure is evident at several levels of the genus *Neisseria* as a whole. Likewise the species *N. gonorrhoeae* can be regarded as a "large epidemic clone" of the genus *Neisseria*, still connected with the remainder of the network by horizontal exchange (HOBBS et al. 1994). Conversely, on a short scale, following up the infection route of individual *N. gonorrhoeae* strains among sexual contacts, a similar clonal network structure is generated under the influence of genetic variation (a rapid and specialized form of mutagenesis discussed below) and horizontal exchange, for example, in individuals infected with multiple strains. Thus, a clonal network is evident at several levels, i.e., strains, epidemic clones, and species. The fine-structure of a clonal network is influenced by several parameters; (1) the sexual activity between species, epidemic clones and strains, (2) their spatial relationship and (3) the selective advantage of novel recombinants. According to this theory, *N. gonorrhoeae*, despite being part of the *Neisseria* clonal network, developed and functioned as an "independent" *Neisseria* species because it was unaffected by dramatic sexual exchange with the rest of the genus, owing to its ecological isolation (VÁZQUEZ et al. 1993). The theory also explains why, despite active horizontal exchange among the *Neisseria* species, some geno-types, including that for the polysaccharide capsule and IgA protease, are restricted to distinct *Neisseria* species (FROSCH et al. 1989; FACIUS

and MEYER 1993) while the distribution of many other putative virulence determinants is not species-restricted, not even to the pathogenic *Neisseriae* (AHO et al. 1987).

2.3 Genome Plasticity

The striking contrast between the strict clonality of *Salmonella* and *E. coli* and the clonal network structure of *Neisseria* appears to be reflected by distinct differences in the chromosomal organization of the genes of these species. The *E. coli* chromosome exhibits a considerable degree of organization concerning, for example, the linkage of related genes, the presence of operons and the transcriptional orientation of genes. Such a high degree of genomic organization is not seen in the gonococcus. By contrast, the recent construction of physical maps of the gonococcus (BIHLMAIER et al. 1991; DEMPSEY et al. 1991; DEMPSEY and CANNON 1994) revealed that many functionally related genes are distributed over the genome. Examples are the structural and accessory *pil* genes (e.g., *pilS*, *pilE*, *pilC1,2*, *pilD*, *pilT*) (BIHLMAIER et al. 1991; DEMPSEY and CANNON 1994), which are not genetically linked, quite unlike other type 4 pilus systems (OGIERMAN et al. 1993). Similarly, the *opa* genes, 11 copies of which exist in the *N. gonorrhoeae* genome, are spread over the whole genome. The same is true for other gene families such as *pilC*, *pilE/S* and *laa*. Also, transcriptional coupling of genes (i.e., operons) is rarely found in *Neisseria*, although a few exceptions exist, including the tightly organized *cps* capsular gene cluster of the meningococcus (FROSCH et al. 1989). Very often genes normally organized in operons in other species are found at separate loci in the gonococcus, e.g., *carA/carB* (Rudel and Meyer, unpublised results) and the *gal* loci (ROBERTSON et al. 1993; JENNINGS et al. 1993; HAMMERSCHMIDT et al. 1994). Furthermore, many neighboring genes in *N. gonorrhoeae* are transcribed in opposite directions; thus, a general transcriptional orientation with regard to the location of the chromosomal origin of replication is not evident. Another remarkable phenomenon of *Neisseria* is the occurrence of multiple gene copies (gene families) which usually contain significantly diverse sequences. Whether the distinct chromosomal organization of *Neisseria* species is causally related to other unique features of these micro-organisms, including the natural transformation competence, intrastrain variability (discussed below) and the above-mentioned population structures, is not well understood. Above all, it is unknown whether the genetic plasticity found in *Neisseria* species is related to the life style of these organisms, characterized by an extremely narrow host range and the ability to cause persistent infections, or whether it simply reflects the fact that these bacteria are young in evolutionary terms and still at the beginning of a long-term adaptative process with regard to their host.

3 Natural Competence for Transformation

The ability of *Neisseria* spp to undergo DNA transformation under natural conditions was recognized some 30 years ago (CATLIN and CUNNINGHAM 1961; SPARLING 1966). Until today no natural process other than transformation has been reported that could account for the horizontal exchange of chromosomal genes in *Neisseria*. Bacteriophages, and in particular transducing phages, have not been identified and, although many conjugative plasmids exist, conjugative (Hfr-like) mobilization of chromosomal determinants is not known to occur under natural conditions. Thus, the only known mechanism that could account for the observed horizontal exchange among *Neisseria* is transformation.

Horizontal exchange of chromosomal markers via transformation is readily observed by cocultivation of different *Neisseria* strains in vitro (FROSCH and MEYER 1992; ZHANG et al. 1992). In a typical experiment the efficiency of transfer between two gonococcal strains after 1 h of cocultivation was in the order of 10^{-5} per cell and genetic locus. The process is completely inhibited by the presence of DNase in the culture medium indicating that, despite the apparent viability of the cultured cells, there is a substantial release of DNase-accessible DNA into the medium. How the DNA is released into the medium has not been studied in detail; however, the spontaneous autolysis observed for the gonococcus may be one explanation (HEBELER and YOUNG 1975).

Natural transformation competence differs markedly from the artificial transformation used for the cloning of genes, e.g., in *E. coli*. In *Neisseria* the transforming DNA is taken up as a linear molecule and requires the RecA function and homologous sequences in the resident DNA of the recipient cell in order to allow recircularization of a plasmid or incorporation of the DNA into the chromosome (BISWAS et al. 1986; KOOMEY et al. 1987). The DNA homology requirement is probably the most effective mechanism of protection against bacterial transformation with unrelated DNA. Whether there is any role for the abundant DNA restriction and methylation systems of *Neisseria* species (SULLIVAN and SAUNDERS 1989; GUNN et al. 1992; GUNN and STEIN 1993) on transformation is currently not well understood. In addition to these restrictions, the *Neisseria* species recognize a specific DNA sequence, 8 bp in length, that serves as a signal for the efficient uptake of DNA (GOODMAN and SCOCCA 1988). Usually, the uptake signal is part of a transcriptional terminator where it constitutes the palindromic stem region; thus, a typical neisserial terminator consists of two inverted uptake signals. Whether the uptake signal only serves for DNA recognition or whether it also represents a site for DNA linearization and/or a signal for the direction of DNA transport is currently not known.

A panel of chemical mutants have been generated which led to the dissection of the gonococcal transformation into: (1) uptake and conversion of the transforming DNA into a DNase-resistant state and (2) the subsequent processes (BISWAS et al. 1989). One such DNA uptake deficient mutant (*dud1*) has been further characterized biochemically (DORWARD and GARON 1989). Early studies

suggested a role for gonococcal pili (which belong to the type IV or *N*-methyl-Phe class of pili) in transformation competence (GIBBS et al. 1989; Facius and Meyer, unpublished results). This interesting observation has recently been specified in that pilin (PilE), the major pilus subunit, rather than intact pilus, is required for DNA uptake (GIBBS et al. 1989; Facius and Meyer, unpublished results). One intriguing aspect is that gonococci can spontaneously shut down PilE synthesis in vitro by irreversible deletion of the expressed gene copy (*pilE*). This not only results in an absolute transformation defect but also implies a role of these terminally differentiated gonnococcal variants in the infection process (see below).

In addition to PilE, the minor pilus-associated protein, PilC (JONSSON et al. 1991), is required for DNA uptake (Facius and Meyer, unpublished results). The involvement of pilus-associated factors in neisserial transformation competence is not unexpected since DNA import (for example in *Bacillus*) and type IV pilus assembly obviously share common structural elements (HOBBS and MATTICK 1993). That competence gene products are often involved in other essential cellular processes may explain why respective defects are often pleiotropic. An example of a nonessential competence determinant is *comA*, identified in *N. gonorrhoeae* and other competent *Neisseria* species. ComA appears to be a typical inner membrane protein and is involved in a transformation step subsequent to the initial DNA uptake (Facius and Meyer 1993). Its distribution among neisserial species suggests that the transformation mechanism is similar in different *Neisseria* species. Future studies on the natural transformation will hopefully shed more light on the significance of this interesting process for the evolution and the pathogenic properties of the *Neisseria* species.

4 Strategies for Rapid Microenvironmental Adaptation

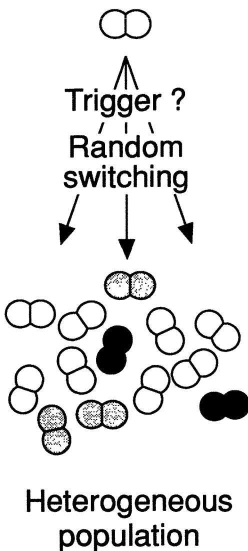
4.1 Genetic Variation vs Gene Regulation

Microbial populations not only need to adapt to the gross long-term environmental changes encountered during co-evolution with the host but, in addition, encounter frequent usually recurrent microenvironmental changes, for example, during the course of an infection. In order to respond to such recurrent changes, microorganisms maintain retrievable genetic programs. There are two principle types of adaptive programs used by microorganisms (Fig. 3): (1) Genetic variation concerns spontaneous changes in the DNA which are inherited to the progeny and are often reversible. These changes, although temporally random, take place at spatially distinct loci and ultimately lead to the synthesis of altered gene products. As a consequence, genetic variation generates heterogeneous populations of a distinct microbial strain, such that a fraction of this population is likely to show an improved microenvironmental adaptation. (2) By contrast, the second microbial adaptation strategy, gene regulation, influences the bacterial

population as a whole. In response to a certain environmental stimulus, such as temperature, osmolarity, or specific substances, the bacteria alter the expression of responsive genes in a coordinated fashion.

Obviously both strategies have specific advantages for microorganisms: While genetic variation better protects minor parts of the population against a large variety of unpredictable changes, gene regulation effects a well-determined adaptive process for the benefit of the whole population. However, genetic variation and gene regulation are not exclusive and are often interconnected. Likewise, the frequency and the direction of a switch may be influenced by environmental effectors, and conversely, a phase-variable regulator protein may control the expression of genes (ROBERTSON and MEYER 1992).

Although *Neisseria* represents a paradigm of genetic variability, gene regulation processes seem to play equally important roles in these bacteria. Likewise, stress responsive systems sensitive to heat shock and/or other stress conditions, such as nutrient starvation and iron limitation, have been identified (see Sect. 6). The genetics of some of these systems, including the regulation of pilin synthesis, is currently being studied (TAHA et al. 1988, 1992; FYFE et al. 1993). Furthermore, recent investigations suggest the possibility of global regulatory changes, such as DNA superhelicity, in the regulation of certain gonococcal genes and the modulation of their virulence properties (R.F. Rest, personal communication; Heuer, Kahrs and Meyer, unpublished results).



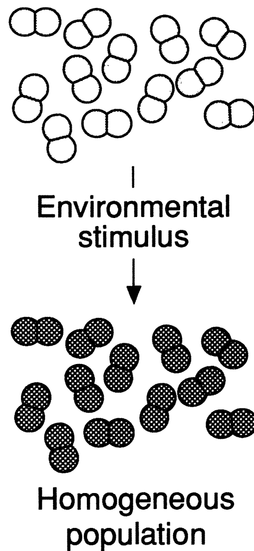


Fig. 3. Two principle genetic mechanisms, genetic variation and gene regulation, used for the environmental adaptation by microorganisms. See text for details

4.2 Gene Families and Mechanisms of Genetic Variation in *Neisseria*

An interesting feature of some (but not all) variable surface proteins in *Neisseria* is that they are represented in the genome by gene families rather than individual genes. This applies to at least three variable surface proteins which have essential functions in the infection process, i.e., the PilE, PilC and Opa proteins (ROBERTSON and MEYER 1992), while other variable factors, such as the meningococcal Opc (OLYHOEK et al. 1991) and class I proteins (BARLOW et al. 1989), are encoded by single copy genes. Two principle mechanisms by which these proteins vary their structures can be distinguished based on whether or not the variation process requires a functional RecA protein (KOOMEY et al. 1987; ROBERTSON and MEYER 1992).

The best known example of RecA-dependent variation represents the major pilus subunit, PilE or pilin, which operates by intragenic recombination: In the bacterial genome multiple gene copies exist most of which are unexpressed, incomplete (silent/cryptic) gene copies (*pilS*), while only one or two of these represent the expressed gene copies (*pilE*). The *pilS* copies constitute the variant sequence repertoire which is used for recombination with *pilE* to generate variant pilin molecules. In *N. gonorrhoeae* MS11 this repertoire is large enough to produce potentially $\sim 10^7$ variant pilin proteins (SWANSON et al. 1987a; HAAS et al. 1992). Owing to this remarkable variability, the bacterial pili can effectively escape the human immune response, since the variability of antibodies and T cell receptors of the immune system, which uses a similar variation mechanism, is within the same order of magnitude. Recent studies have provided evidence that recombination of *pil* genes can occur by at least four pathways, i.e., reciprocal, nonreciprocal (gene conversion-like), transformation-mediated recombination and deletion of duplicated gene copies (HAAS and MEYER 1986; MANNING et al. 1991; SWANSON et al. 1986; SEIFERT et al. 1988; GIBBS et al. 1989; HILL et al. 1990; ZHANG et al. 1992; FACIUS and MEYER 1993).

In contrast to the pilE genes, the genes encoding Opa and PilC proteins represent complete, rather than incomplete, variant genes (STERN et al. 1986; JONSSON et al. 1991; Rudel and Meyer, unpublished results) which undergo frequent phase transitions (on/off switches) but rarely recombine with each other (CONNELL et al. 1988; BHAT et al. 1991). These switches occur via a RecA-independent DNA slippage mechanism involving homo- or heteropolymeric repeated nucleotide sequences within the coding sequences of the genes (CONNELL et al. 1988; ROBERTSON and MEYER 1992); variation of the number of the repeating units alters the reading frame and consequently affects the translation into functional gene products. In case of the *opa* genes, the repeating unit is a pentameric (CTCTT) sequence whereas the *pilC* genes are controlled by a run of C residues; both "coding repeats" (CR) are located in the secretory signal peptide-encoding part of the genes. Another method of controlling the expression of a gene via a repetitive sequence is realized in the meningococcal *opc* gene, where a variable homopolymer of C residues is positioned upstream of the –10 of

the *opc* promoter (SARKARI, et al. 1994) thus giving rise to altered transcriptional activities. Lipopolysaccharide (LPS) is also a variable surface structure of pathogenic *Neisseriae*. Little is known about the genetic basis of neisserial LPS variation, except that the process is RecA-independent. Whether it involves repetitive sequences as in the case of Opa proteins and the structurally related LPS of *Haemophilus influenzae* (WEISER et al. 1989) is not known.

5 Functional Relevance of Genetic Variation

5.1 The Neisserial Pili

The pili, fine, hair-like organelles protruding from the bacterial cell surface, probably represent the most variable structures produced by the pathogenic *Neisseria* spp. They are an absolute requirement for the initiation of an infection (KELLOGG et al. 1968) in that they confer the attachment of the bacteria to epithelial cells (MCGEE et al. 1981; STEPHENS and MCGEE 1981). The binding of pili appears to be specific for human cells and the pili therefore represent a major determinant of the neisserial species tropism. Owing to their exposed location they are also strong targets for an antibody response that could interfere with receptor recognition. However, efforts in generating a pilus-based vaccine have thus far failed due to the enormous variability of the pili, and in particular the pilin (JOHNSON et al. 1991). It therefore remains a crucial question of how the pili deceive the immune system while fulfilling their function as adhesins. This problem certainly has important practical and theoretical implications not necessarily restricted to the *Neisseria* model.

What are the components of pili involved in receptor recognition? Early studies suggested a direct role for pilin (PilE), the major subunit, in adherence to various cellular substrates (SCHOOLNIK et al. 1984; VIRJI and HECKELS 1984; ROTHBARD et al. 1985). While this possibility still exists, recent studies provide evidence for different adherence properties of the pili. At least three distinct adherence specificities, one for epithelial cells, one for erythrocytes and one allowing intergonococcal adherence, can be distinguished, either genetically or otherwise (RUDEL et al. 1992). This does not exclude the association of pili with yet other adherence properties, e.g., for endothelial, phagocytic and sperm cells. Furthermore, *N. meningitidis* can produce two different classes of major subunits (class I and class II pilins, VIRJI et al. 1989) which may effect the adherence properties (VIRJI et al. 1992a). Interestingly, the adherence to epithelial cells and the interbacterial interaction are influenced by the variation of pilin while pilus-dependent hemagglutination is not (LAMBDEN et al. 1980; RUDEL et al. 1992; NASSIF et al. 1993). Pilin can be modified by phosphorylation and/or glycosylation (ROBERTSON et al. 1977; SCHOOLNIK et al. 1985; VIRJI et al. 1993b), and it has recently been shown that the variant-dependent glycosylation of gonococcal pilin can

influence receptor recognition (VIRJI et al. 1993b). However, the observed effect of pilin variation on adherence may be indirect and does not preclude a role for potential minor subunits in adherence.

Recent studies in fact suggest an association of minor protein components with the gonococcal pilus (MUIR et al. 1988; PARGE et al. 1990). One of these components, the PilC protein, has been characterized genetically and implicated in the biogenesis of pili (JONSSON et al. 1991) and transformation competence (Facius and Meyer, unpublished results). Nonetheless, pili can be assembled in the absence of either of the two known PilC1 and PilC2 proteins (RUDEL et al. 1992), probably by utilizing an alternative PilC-like assembly factor (Rudel and Meyer, unpublished results). Such pili still hemagglutinate but lack the potential to adhere to epithelial cells (RUDEL et al. 1992) indicating a role for PilC in epithelial cell adherence. We recently succeeded in the purification of a gonococcal PilC protein to homogeneity from a PilE-negative strain, and in raising specific antibodies (Rudel, Scheuerpflug, Meyer, submitted). Experiments carried out with these reagents firmly indicate that PilC proteins represent epithelial cell-specific pilus-associated adhesins. Furthermore, the purified PilC protein effectively competes with the binding to epithelial cells of both gonococci and meningococci producing different PilC and PilE proteins. This led us to the intriguing conclusion that the pili of pathogenic *Neisseria* species, irrespective of their structural variability, recognize the same, or a closely related, group of receptor(s).

5.2 Cell Tropisms of *Opaque* (Opa) Proteins

The Opa proteins (previously referred to as class V proteins of the meningococcus and P.II proteins of the gonococcus) are major constituents of the outer membranes of mainly pathogenic *Neisseria* spp. Computer predictions suggest a β-pleated sheet structure for Opa proteins, typical of many outer membrane proteins, whereby three variable loop regions and a fourth conserved loop are oriented towards the bacterial surface (MEYER et al. 1986; BARRITT et al. 1987; BHAT et al. 1991). The number of variant *opa* genes present in gonococci (~11) is considerably higher than that in meningococci (3–4) and *N. lactamica* (~2). Interestingly, independent *N. gonorrhoeae* isolates rarely possess *opa* genes of identical sequence (HAAS et al. 1992), indicating that the repertoire of variant *opa* genes within the gonococcal population is substantially larger than that of a single strain.

The role of Opa proteins in various adherence functions, such as interbacterial adhesion and interaction with human epithelial and phagocytic cells (KING and SWANSON 1978; LAMBDEN et al. 1979; VIRJI and HECKELS 1986; FISHER and REST 1988; MAKINO et al. 1991; BELLAND et al. 1992; VIRJI et al. 1993a), has long been recognized. Recent work suggests that Opa proteins not only cause bacterial adherence but also trigger important cellular functions. Invasion of *N. gonorrhoeae* into human epithelial cells was shown to depend on the production of one distinct variant Opa protein produced by this strain (MAKINO et al. 1991;

WEEL et al. 1991a). Using a reverse genetics approach, the remaining Opa proteins of the same strain were subsequently shown to confer binding to human polymorphonuclear cells (PMNs) but not to epithelial cells (KUPSCH et al. 1993). Consistently, chemoluminescense of PMNs was only induced if certain Opa proteins were expressed (BELLAND et al. 1992). Yet a different set of Opa proteins, including the one that reacts with epithelial cells, triggers uptake and chemoluminescense in human peripheral blood monocytes (PBMs). Interestingly, for all Opa proteins of strain MS11 at least one binding specificity is observed, indicating that each variant gene encodes a functional protein. The specific properties of Opa proteins are maintained if the genes are cloned and expressed in a different neisserial host (KUPSCH et al. 1993). A similar pattern of functional variation is seen for other *Neisseria* strains, some of which have also been assessed with regard to the interaction with endothelial cells (Aubel and Meyer, unpublished results). In the much smaller Opa protein repertoire of meningococci Opa proteins required for epithelial cell invasion (VIRJI et al. 1993a) and PMN stimulation (Heuer and Meyer, unpublished results) have been identified. This species often carries a copy of the phase variable *opc* gene whose product, although structurally unrelated, has a function similar to the epithelial cell-specific Opa proteins (VIRJI et al. 1992b). It is thus evident that the variable Opa and Opc adhesins represent important cell tropism determinants of *N. gonorrhoeae* and *N. meningitidis* and that the variability of these proteins allows multiple cellular interactions.

5.3 Immune Escape vs Structural Adaptation

It is useful to distinguish between two principal functions of genetic variation, i.e., an escape function and an adaptive function, examples of which seem to be given by the *pil* and the *opa* systems, respectively (Fig. 4). A typical escape mechanism is antigenic variation: This term describes the competition between two variable systems operating with similar mechanisms, i.e., the host immune system and a bacterial population. The main interest on the bacterial side, in this case, is to avoid molecular interaction, e.g., with an antibody. The opposite applies for the adaptive variation model: Here any structural change must lead to a productive molecular fit; a trial-and-error mechanism would cause unnecessary bacterial extinction. We will briefly discuss to what extent the genetic systems underlying pili and Opa variation conform to this model.

The extreme variability of the major pili subunit (PilE) classifies it as an escape factor. The mechanism used for its variation, i.e., more or less random intragenic recombination, provides little chance that novel recombinants will conform to a specific molecular fit. The question therefore arises of how functional integrity is maintained? At least two conserved functions of the pili can be identified, i.e., one for pili polymerization and—as our recent results suggest—one for the interaction with a conserved receptor. The polymerization function is nonproblematic because it involves the conserved hydrophobic regions of PilE which are neither

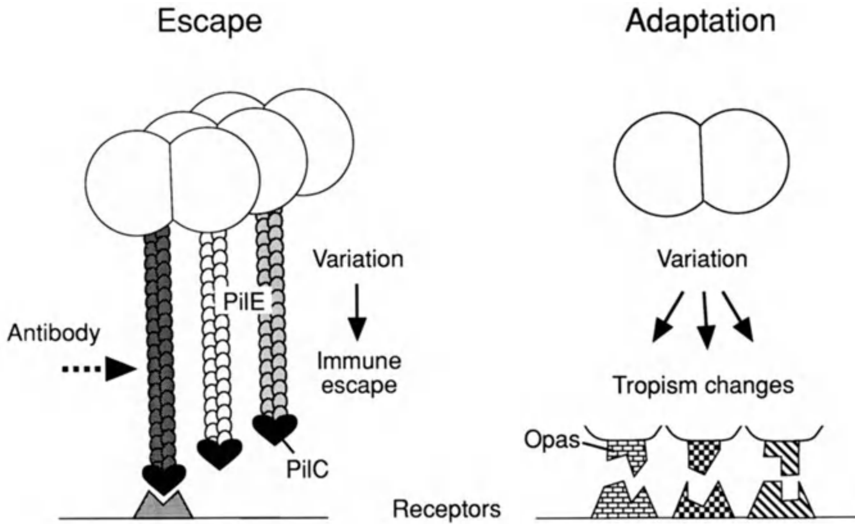


Fig. 4. Escape and adaptive function of genetic variation. The variation of the neisserial pili with their major subunit, PilE, represents an example of immune escape; despite the extreme variability of the pili the interaction with the host cell receptor via PilC is functionally conserved. Variation of the Opa outer membrane proteins primarily serves as an adaptative process for the interaction of Neisseria with a variety of different host cell receptors

surface-exposed nor immunosusceptible. The dilemma, however, is how to accomodate a conserved receptor binding function within the highly variable context of the pili? To solve the problem, the bacteria probably make use of a minor, pilus-associated adhesin, PilC, which is far less variable. In conclusion therefore, PilE variation primarily serves to protect pili from interacting with immunoglobulins but probably is inadequate to modulate the receptor specificity.

For adaptive variation, the model underlying the genetic mechanism ought to avoid mutation and recombination which could lead to nonproductive phenotypes, and rather to utilize a selected set of preexisting genes. This situation is found in the *opa* gene system which exhibits limited phenotypic variability: Recombination, bearing the risk of generating nonproductive hybrids, is a rare event among the *opa* genes (STERN et al. 1986; CONNELL et al. 1988) and all native *opa* genes encode functional Opa proteins capable of recognizing distinct cellular receptors (KUPSCH et al. 1993). In contrast, in vitro engineered hybrid *opa* genes often encode nonfunctional Opas. Hence, it is evident that the mechanism of Opa variation favors productive interaction with target cells but its overall variability is probably too small to support an efficient immune escape reaction.

If this applies, why is it so essential for the pili rather than for the Opa proteins to vary antigenically? Why is it that many other surface proteins of *Neisseria*, such as the major outer membrane protein P.I. (JUDD 1989), do not undergo intrastrain variation at all? This question leads us to yet another neisserial variation system,

i.e. the variable lipopolysacharide (LPS). In contrast to the highly exposed pili, the neisserial membrane-associated proteins seem to be efficiently protected against host immunity through a variable carbohydrate mantle produced by the bacteria.

5.4 Withstanding the Extracellular Environment: Function of Lipopolysaccharide Variation and of the Capsule

The *Neisseria* species produce a short type of LPS which lacks any repetitive O-side chains. Nonetheless LPS preparations from in vitro cultured bacteria reveal multiple size classes indicating a structural heterogeneity of the neisserial LPS (SCHNEIDER et al. 1988). Antibodies can be raised against distinct LPS species which can be used to demonstrate sectored colonies on plates, and molecular mimicry with host cell glycolipids (MANDRELL and APICELLA 1993). Several lines of evidence suggest that LPS variation also occurs in vivo. In meningococcal carriers, the majority (70%) of bacteria isolated from the nasopharynx are unencapsulated and preferentially express a short LPS species (BROOME 1986), whereas in the diseased state, 97% of the blood and CSF isolates are encapsulated and of the long LPS species (JONES et al. 1992). Likewise, experimental gonococcal infection in human volunteers indicates that bacteria isolated early in the infection have a short LPS, whereas after development of inflammatory response a different bacterial phenotype with a long LPS species predominates (SCHNEIDER et al. 1991).

A major difference among the variant LPS molecules is the presence of additional carbohydrate residues in the longer LPS forms that can be externally modified by a membrane-associated bacterial sialyltransferase using host-derived or endogenous CMP-NANA as sialyl donor (SMITH 1991; MANDRELL and APICELLA 1993; VAN PUTTEN 1993). Given the presence of CMP-NANA, LPS variation thus determines whether or not the LPS is sialylated. The functional relevance of the LPS phase transitions has recently been elucidated (VAN PUTTEN 1993) and appears to lie in the expression of variable amounts of sialic acid incorporated in the different forms of LPS. A low sialylation phenotype, as found early in the infection (SCHNEIDER et al. 1991), enables entry of the bacteria into mucosal cells, but makes them susceptible to bactericidal activity. In contrast, highly sialylated bacteria are incapable of entering epithelial cells but are resistant to phagocytosis and killing by antibodies and complement, allowing persistence of infection. Thus depending on the degree of sialylation the bacteria are either adapted to the extracellular environment and are capable of resisting humoral immune mechanisms, or they become sensitive to bactericidal activities but then readily enter the intracellular milieu via Opa-mediated cellular interactions.

The function of highly sialylated LPS has many similarities with that of polysialic capsules produced by *N. meningitidis* and many commensal *Neisseria* species (FROSCH et al. 1989), in that they protect extracellular bacteria against both

specific and nonspecific host responses. Although phase variation of capsule expression has been observed, this structure appears to be primarily regulated by environmental factors (BRENER et al. 1981). Loss of capsule expression favors an Opa and Opc-mediated interaction of *N. meningitidis* with target cells and thus seems to be a prerequisite for the cellular invasion (VIRJI et al. 1993a). Therefore, recent advances made in the analysis of capsular (cps) gene organization and function (FROSCH et al. 1989) are important for our understanding of the conditions favoring invasive meningococcal infections.

6 Examples of Regulatory Responses in *Neisseria*

6.1 Iron Acquisition and Virulence

Iron is an essential nutrient for most bacteria, and since in vivo the free iron concentration is extremely low, microorganisms have developed sophisticated iron acquisition mechanisms. In recent years considerable progress has been made in unravelling the major high affinity iron acquisition mechanism of the pathogenic *Neisseria* (for review, see VAN PUTTEN 1990). In contrast to many microorganisms, but apparently characteristic for most nonenteric mucosal pathogens, gonococci and meningococci do not produce siderophores but acquire iron directly from the iron-binding proteins transferrin (Tf), lactoferrin (Lf), and heme. At this time, several components of the Tf-iron acquisition system have been identified, and they seem to be conserved among the pathogenic *Neisseriae* (Fig. 5). In response to low intracellular iron concentrations, a specific human transferrin receptor is expressed at the cell surface. This receptor, which does not resemble its eukaryotic homologue, appears to consist of a complex of

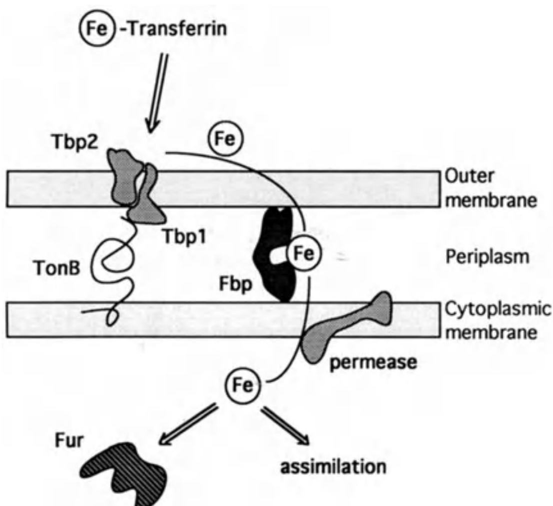


Fig. 5. Hypothetical model of the neisserial transferrin-iron acquisition system. For detailed explanation, see text

two transferrin binding proteins (Tbps), a relatively conserved Tbp1 (approximately 100 kDa) and an antigenically and size variable Tbp2 (68–86 kDa, depending on the strain). Tbp1 is homologous to the TonB-outer membrane protein receptors of *E. coli* (CORNELISSEN et al. 1992), and binds Tf when expressed in *E. coli* (CORNELISSEN et al. 1993; PALMER et al. 1993). Tbp2, which is most likely a lipoprotein (LEGRAIN et al. 1993), is also required for Tf-mediated iron uptake, but its Tf binding activity appears to depend on the presence of Tbp1 (IRWIN et al. 1993). After binding, the iron is removed from the Tf-receptor complex, via an as yet unidentified functional TonB equivalent, and transferred to a periplasmic transport protein, named Fbp, which both genetically and functionally belongs to the family of periplasmic binding proteins (BERISH et al. 1992; CHEN et al. 1993). From here the iron is transported across the cytoplasmic membrane possibly via an inner membrane permease (CHEN et al. 1993) and either directly assimilated or stored, perhaps in the form of ferritin-like molecules. In analogy to other systems (BRAUN 1985), the activity of the neisserial iron acquisition system appears to be regulated by iron, which, by acting as a corepressor, controls the binding of the neisserial Fur protein (BERISH et al. 1993) to the operator sites in the vicinity of the promoters of the iron-regulated genes, thus influencing their transcription. Putative Fur-iron complex binding domains precede the neisserial Tbp2 gene (*tbpB*), which is arranged in tandem in the genome with the Tbp1 gene (*tbpA*) (LEGRAIN et al. 1993), the Fbp gene (*fbp*) (BERISH et al. 1990), and the putative iron-regulated cytotoxin encoding genes *frpA* and *frpC* (THOMPSON et al. 1993a, b; see below).

Besides being essential for bacterial growth, iron availability also influences the expression of bacterial virulence determinants such as capsule, pili and putative cytotoxins. This apparent coupling of nutrient availability and bacterial virulence, certainly not unique to the pathogenic *Neisseria* species (LITWIN and CALDERWOOD 1993), is a clear example of the cross-talk between the bacterium and its ecosystem, which ultimately determines the bacterial phenotype. The importance of iron in this respect is particularly illustrated by the up to 1200-fold greater virulence in mice of iron-deprived, low pH grown meningococci than their non-iron-deprived counterparts (BRENER et al. 1981). One bacterial determinant that has been associated with the highly virulent phenotype is the probably reduced growth rate-related increase in capsule thickness (MASSON et al. 1982), but other factors, including the recently identified iron-regulated putative cytotoxins FrpA and FrpC (THOMPSON et al. 1993a,b), may be of relevance as well. FrpA (128 kDa) and FrpC (198 kDa), which are rarely produced by the gonococcus, are proteins released into the extracellular environment and belong to the RTX cytotoxin family (WELCH 1991).

6.2 Aerobic vs Anaerobic Growth

Another environmental factor influencing the bacterial phenotype is oxygen. *Neisseria* can grow in the absence of oxygen with nitrite as a terminal electron acceptor (KNAPP and CLARK 1984) and this results in the expression of several

additional proteins and the repression of others (KEEVIL et al. 1986; CLARK et al. 1987). The major anaerobically induced protein is a surface-exposed 54 kDa lipoprotein Pan-1 (HOEHN and CLARK 1992a). This broadly conserved but antigenically heterogeneous antigen, which is predominantly expressed in gonococci (HOEHN and CLARK 1990), is possibly glycosylated (HOEHN and CLARK 1991), which is rather unusual for a bacterial membrane protein. The corresponding gene (*aniA*) has some sequence homology to the gonococcal lipoproteins Lip (H.8) and Laz (CANNON 1989) and seems to be under the tight transcriptional control of two overlapping promoters, including a putative gearbox promoter (HOEHN and CLARK 1992b). This type of promotor has been associated with stationary phase regulation (ALDEA et al. 1989). In other systems, anaerobic growth expression requires DNA gyrase activity and has been associated with increased DNA supercoiling (YAMAMOTO and DROFFNER 1985; HIGGINS et al. 1990), but this has not been investigated for the pathogenic *Neisseriae*. The function of Pan-1 or of the other oxygen-regulated proteins in bacterial persistence is presently not known.

Coisolation of gonococci and obligate anaerobic microorganisms from the site of infection (FONTAINE et al. 1982) and the generation of antibodies against the anaerobically induced proteins in the sera of patients with neisserial disease (CLARK et al. 1988) suggest that neisserial anaerobiosis does occur in vivo. Whether the anaerobic phenotype has pathogenic relevance beyond persistence in the low oxygen environment is presently unknown. However, anaerobic growth appears to influence the LPS phenotype (TSAI et al. 1983; FRANGIPANE and REST 1992) and to enhance the activity of the membrane-bound sialyltransferase, resulting in high level serum resistance (FRANGIPANE and REST 1993).

6.3 Stress-Associated Proteins

The necessity of bacteria to continuously adapt their phenotype in response to unfavorable environmental conditions encountered during infection can certainly include the response to stress. In addition to the distinct nutrient-regulated expression of antigens, the pathogenic *Neisseriae* display a uniform stress response, i.e., irrespective of the nature of the environmental stimulus (PANNEKOEK et al. 1992a). Until today, a number of stress (or heat-shock) proteins have been identified (KLIMPEL and CLARK 1989; KEEVIL et al. 1989; WOODS et al. 1990; PANNEKOEK et al. 1992a; ARAKARE et al. 1993) and particular attention has been given to a 63 kDa determinant. This most abundant neisserial stress protein is both genetically and antigenically broadly conserved among the *Neisseria* spp. and has considerable homology to the Hsp60 heat shock protein family (PANNEKOEK et al. 1992a,b; PANNEKOEK, DANKERT and VAN PUTTEN, submitted). Analyses of patients sera indicate that the antigen is expressed and immunogenic in vivo, eliciting either a broadly cross-reactive or a *Neisseria*-specific antibody response (DE HORMAECHE et al. 1990; PANNEKOEK et al. 1993). An antigenically related surface-exposed protein of similar size has been demonstrated to possess lectin binding activity (PERROLET and GUINET 1986; BENKIRANE et al. 1992). The pathogenic relevance of the neisserial

stress response has not been demonstrated in vivo. However, the possibility exists that the expression of a broadly conserved immunodominant antigen such as neisserial Hsp60 in vivo may cause an aberrant immune response. In analogy to the chlamydial Hsp60 (MORRISON et al. 1992), the gonococcal homologue has been postulated to contribute to Hsp60-related immunopathology associated with PID (Pannekoek, Dankert and van Putten, submitted).

7 IgA Protease: A Unique Extracellular Factor

A common feature of pathogenic *Neisseriae* is the production and secretion of an antigenically diverse family of endoproteinases specifically affecting immunoglobulin A1 (IgA1) of their human hosts (PLAUT et al. 1975). Like the closely related enzymes produced by *Haemophilus influenzae* these IgA proteases belong to the class of serine proteases (BACHOVCHIN et al. 1990). Although the proteases have been studied intensively during the last decade, direct evidence for an important role in bacterial pathogenesis is missing (COOPER et al. 1984). Undoubtedly, IgA1 cleavage represents one function (KILIAN et al. 1988), however, the relevance of this activity to the infection process remains obscure and additional functions should be considered.

7.1 Gene Structure and Secretion Pathway

Unlike the pilus and Opa proteins, IgA proteases are encoded by single copies of *iga* genes. Horizontal exchange of *iga* sequences resulted in numerous serologically distinct IgA proteases in *N. gonorrhoeae* and *N. meningitidis* (HALTER et al. 1989; LOMHOLT et al. 1992; MORELLI et al. 1994). Despite this sequence variability the key features of the *iga* genes and gene products are conserved (HALTER et al. 1989). The primary translation products are organized as polyprotein precursors (Iga) of approximately 170 kDa molecular mass (POHLNER et al. 1987). They consist of five distinct protein domains: an NH_2-terminal leader peptide followed by the IgA protease (IgaP), the Iga_γ, Iga_α and Iga_β domains. According to their function, the Iga domains can be grouped into regions involved in the extracellular secretion of the precursor and regions presumably active in the pathogen host interactions. Intrinsic secretion functions for bacterial inner and outer membrane transport reside in the typical NH_2-terminal leader sequence and the COOH-terminal Iga_β domain, respectively (POHLNER et al. 1987; KLAUSER et al. 1990). During transport these two Iga domains are clipped off the Iga protein and remain associated with the bacteria while the three central Iga domains are released into the supernatant as a single preprotein ($Iga_{P\gamma\alpha}$) (Fig. 6). Ultimately, the soluble $Iga_{P\gamma\alpha}$ preprotein is cleaved resulting in a stepwise maturation of three distinct products, the IgA protease, a small γ-peptide and the α-protein.

The unique secretion process of IgA protease represents one of the currently best understood examples of extracellular protein export in gram-negative bacteria (for review see KLAUSER et al. 1993b). Two interesting features of the Iga_{β} domain responsible for protease translocation across the outer membrane are its function in other gram-negative bacterial host systems, such as *E. coli*, and its suitability for export of heterologous proteins. The core region of the Igaβ translocator presumably assumes a β-barrel-type pore in the outer membrane through which the adjacent polypeptide chain is extruded (KLAUSER et al. 1993a,b). In contrast to other export systems, proteins to be translocated via Igaβ are required to be in an unfolded state, a feature which renders the Igaβ function suitable for generalized protein export (KLAUSER et al. 1990, 1992). Recent work suggests that the system can be successfully used to present functional antibody Fv fragments on the *E. coli* cell surface (KRÄMER et al., submitted).

7.2 Function of IgA Protease and Associated α-Proteins

The studies on the secretion and maturation of extracellular IgA protease have provided valuable information regarding the proteolytic specificity of this enzyme (POHLNER et al. 1987). Both uncoupling of the $Iga_{P\gamma\alpha}$ unit from the outer membrane-associated Iga_{β} domain (Fig. 6) and two subsequent processing steps in the supernatant represent autocatalytic cleavage steps. Comparison of the amino acid sequences flanking the autoproteolytic cleavage sites and the hinge region of IgA1 revealed a consensus sequence motif which is necessary and also sufficient

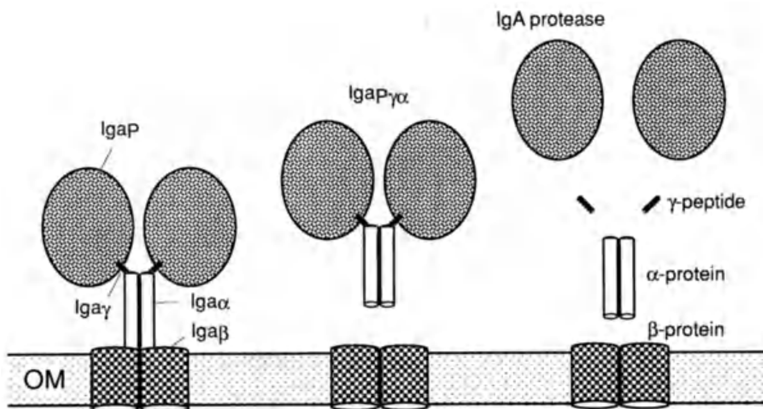


Fig. 6. Extracellular maturation of IgA protease and associated proteins. The export of the Iga polyprotein to the bacterial cell surface is known in much detail (for review see KLAUSER et al. 1993b). The exported $Iga_{P\gamma\alpha}$ polyprotein, initially anchored in the outer membrane (OM) via the Iga_{β} translocator, is released by autoproteolytic cleavage into the supernatant. Subsequent concentration-dependent autoproteolysis results in three separate proteins, IgA protease, α-protein and γ-peptide. Amphipathic α-helices within Iga_{α} cause dimerization of both $Iga_{P\gamma\alpha}$ and α-protein. The α-protein also carries determinants for cellular uptake and signals for nuclear migration

for IgA protease cleavage (POHLNER et al. 1992). Thus, IgA protease from *N. gonorrhoeae* MS11 cleaves in the central peptide bond in the sequence Yaa-Pro-Xaa-Pro, where Yaa predominantly stands for the amino acid Pro and Xaa for the amino acids Thr, Ser or Ala. Data bank searches for proteins containing such cleavage sequences identified a variety of potential targets in human proteins besides IgA1. One example is the CD8 protein of human T cells which is cleaved efficiently when a recombinant CD8 fusion protein is given as substrate (POHLNER et al. 1992). Cleavage of native, cell-associated CD8 was not observed, indicating that higher order structures and modifications may influence the accessibility of a target sequence.

The possibility that other host cell factors might be affected by IgA protease is intriguing with regard to the potential of α-proteins to enter human cells (Pohlner, Langenberg, Wölk and Meyer, submitted). Several allelic forms of α-proteins exist amongst the pathogenic *Neisseria* species. In *N. gonorrhoeae* two alleles, α_1 (12 kDa) and α_2 (24 kDa) have been described (HALTER et al. 1989) and additional alleles have been identified in *N. meningitidis.* The α-proteins have an extremely positive charge (pI > 10). Characteristic of α-proteins are predicted α-helical regions with an array of hydrophobic residues at one side of the helix. These amphipathic helices explain the observed dimerization of α-proteins and Iga precursors in solution (Fig. 6). The second important characteristic of α-proteins are sequences exhibiting structure and function of nuclear location signals (NLSs) required for the transport of proteins into the eukaryotic nuclei. Consistent with this we were recently able to demonstrate that purified α-proteins are capable of entering human epithelial cells and that they infiltrate the nucleus (Pohlner, Langenberg, Wölk and Meyer, submitted). Since α-proteins are transiently attached to IgA protease (i.e., in the form of $Iga_{P\gamma\alpha}$) this result raises the possibility that the protease may function inside human cells. *N. meningitidis*, which is endemic among humans, produces particularly large amounts of extracellular *iga* gene products. The implications this has for healthy, locally colonized individuals requires further investigation.

8 Infection from a Cell Biology Point of View

8.1 Infection Models

The fact that *Neisseriae* are exclusively human pathogens implies that most if not all studies on the bacteria-host cell interaction have to be carried out using artificial infection systems, with all of their limitations. Important aspects of pathogenesis such as the bacterial microenvironment, the generation of an inflammatory response, and the immune response cannot be properly addressed, and information on these topics has to come from specimens collected from patients.

Also the ability to test mutants for their virulence, or attenuated strains, or distinct antigens as candidate vaccines is limited due to the lack of an animal model. The most common approach used in vitro for functional analysis of neisserial virulence determinants is infection of cultured cells. In many cell lines some form of attachment of *Neisseria* takes place, and this has resulted in the dissection of adhesin functions including the PilC and Opa proteins described above. In addition, cultured cells have a variable ability to ingest attached microorganisms, an event called cellular invasion, to illustrate the active participation of the bacteria in the induction of the internalization process.

In several aspects, however, established cell lines are too degenerate to be representative of the natural infection. This seems particularly warranted for the pilus-mediated adherence process and the intracellular fate of the bacteria. In most cell lines pili are not required for adherence, which contrasts with the natural situation (KELLOGG et al. 1968) and observations with organ culture models (McGEE et al. 1981; STEPHENS and McGEE 1981; TJIA et al. 1988), and bacterial entry into transformed cells seems to be a dead end (WARD et al. 1975; WEEL et al. 1991a,b). The transformed cells probably lack the appropriate differentiation status and/or the architecture of the natural tissue to allow, for instance, transcytosis of the bacteria to deeper cell layers. In studying such a process, primary cultures or organ cultures of human cornea, nasopharyngeal tissue, fallopian tubes, or other target tissues of infection appear to be more appropriate (McGEE et al. 1981; TJIA et al. 1988; STEPHENS 1989). A recent development which combines the advantages of cell lines and organ cultures is the use of tissue spheroids, small vesicles of cultured cells which appear to possess the original tissue architecture (BOXBERGER et al. 1993).

For certain aspects of the neisserial infection, animal experiments have been useful. In mice and infant rats, virulence of meningococci and the protective effect of antibodies can be evaluated (BRENER et al. 1981; SALIT and TOMALTY 1986; SAUKKONEN 1988; NURMINEN et al. 1992). Mice seem to be susceptible to gonococcal colonization (KITA et al. 1981, 1991; TAYLOR-ROBINSON et al. 1990). Particularly valuable is the analysis of bacteria grown in subcutaneous chambers implanted in animals (VEALE et al. 1975; ARKO 1974, 1989). This approach has resulted in the identification of putative virulence determinants associated with intracellular survival of *Neisseria gonorrhoeae* in polymorphonuclear granulocytes (PARSONS et al. 1985) and in the sialylation of LPS (SMITH 1991) and has unequivocally demonstrated the importance of a functional iron acquisition system for in vivo persistence of the bacteria (GENCO et al. 1991).

The most valuable, although limited, approach to understanding the complexity of pathogenesis of mucosal infections in humans involves studies with human volunteers willing to undergo inoculation with gonococci. These experiments, which are still in progress, promise to give new insights into the biological significance of phase variation (SWANSON et al. 1987a, 1988; SCHNEIDER et al. 1991). Perhaps an equally valid but more feasible strategy for studying neisserial infections will utilize complex animal systems, for example, immunocompromised animals (e.g., SCID mice) carrying human tissue transplants or

transgenic animals that express human receptor molecules essential for an infection. This latter approach, however, first requires identification of the relevant receptors.

8.2 Mucosal Cell Receptors

Despite extensive research, the exact nature of the pilus receptor(s) on host cell surfaces has not been identified as yet. Since pilus-dependent adherence of neisserial strains seems to be restricted to human cells and some avian species, this receptor resembles a crucial species-specific determinant. It is hoped that the recent identification of PilC as a pilus adhesin (Rudel, Scheuerpflug and Meyer, unpublished results) and of other pilus-associated proteins and adherence functions (Schoolnik et al. 1984; Rothbard et al. 1985; Muir et al. 1988; Parge et al. 1990; Rudel et al. 1992; Nassif et al. 1993) will facilitate the search for the corresponding structure(s).

In many in vitro cell culture systems, pili are not required for bacterial adherence which can be conferred by members of the Opa protein family (Lambden et al. 1979; Makino et al. 1991; Virji et al. 1993a). The Opa protein-host cell interaction seems to be a two step event, a weak and reversible attachment conferred by most of the Opa proteins and a tight probably receptor-specific interaction (Bessen and Gotschlich 1986; Makino et al. 1991). This latter form, which appears to be a prerequisite for entry into host cells, is particularly evident with distinct gonococcal Opa proteins and with Opc (Weel et al. 1991a; Makino et al. 1991; Virji et al. 1992b, 1993a), both highly basic proteins (Bhat et al. 1991; Olyhoek et al. 1991). In earlier reports Opa protein was suggested to bind an oligosaccharide moiety of the LPS which mimics host cell glycolipids (Blake 1985). Further analysis of host cell determinants of Opa protein binding revealed that purified Opa protein bound to a variety of different proteins and glycoproteins, but no distinct receptor molecule(s) were demonstrated (Bessen and Gotschlich 1987). Recently, a host cell binding site for members of the Opa protein family has been identified (van Putten and Paul, unpublished results). This putative receptor, which appears to be present in all epithelial cell lines, particularly recognizes the gonococcal Opa protein associated with tight adherence and cellular entry, but hardly binds to other members of the Opa protein family. Purified receptor and receptor analogues, but not other basic proteins, totally block Opa protein mediated bacterial adherence to eukaryotic cells in vitro, and cell lines defective in receptor expression are only sparsely colonized by bacteria, suggesting that the missing structure is an essential component in the adherence process.

In a reverse approach, screening of known host cell components for their capacity to bind bacteria, several lacto- and ganglio-series glycolipids have been implicated as adhesion receptors for *N. gonorrhoeae*. High avidity binding occurred with gangliotriosylceramide (asialoG_{m2}:GalNAc(β1–4)Gal(β1–4)Glc(β1–1)Cer) and gangliotetraosylceramide (asialo-G_{m1}:Gal(β1–3)GalNAc(β1–4)Gal(β1–4)Glc.

(β1–1) Cer), and less avid binding with lactosyl-ceramide (Gal(β1–4)Glc(β1–1)Cer), lacto-*N*-triaosylceramide(GlcNAc-3Gal(β1–4)Glc(β1–1)Cer), lacto-*N*-neotetraosylceramide (paragloboside: Gal(β1–4)GlcNAc(β1–3)Gal(β1–4)Glc(β1–1)Cer), and isoglobotriaosylceramide (Gal(α1–3)Gal(β1–4)Glc(β1–1)Cer) (STROMBERG et al. 1988; DEAL and KRIVAN 1990). For one of these putative receptors (asialo-G_{m1}), the corresponding bacterial ligand has been identified as a 36 kDa lipoprotein, for which at least two gene copies are present on the bacterial genome (PURACHURI et al. 1990; Maier and Meyer, unpublished results). This protein is different from the sialic acid-specific 27 kDa sialo-adhesin, Sia-1, present in the commensal *Neisseria flava*. This putative adhesin recognizes the structure NeuAc (α2–3)Gal(β1–4)Glc on erythrocytes (NYBERG et al. 1990). The function of glycolipid binding in neisserial pathogenesis has not been investigated.

8.3 Bacteria-Directed Ingestion of *Neisseriae* by Mucosal Cells

The initial attachment of *Neisseria* to mucosal cells may be followed by ingestion of attached organisms when the appropriate phenotype is present. Morphologically, this process involves an elongation of the microvilli and, in order to encompass the bacteria, the formation of zones of intimate contact between the bacterial and the host cell membranes, along with engulfment of the bacteria via a zipper-like mechanism (WATT and WARD 1980; TJIA et al. 1988; WEEL and VAN PUTTEN 1988; WEEL et al. 1991a,b; STEPHENS 1989). As for most other pathogens, the molecular basis of these events is a current topic in *Neisseria* research. Experiments using inhibitors of different endocytic pathways and confocal microscopy of stained actin filaments and actin binding proteins suggest that the Opa/Opc protein-associated internalization event involves an actin filament process without a function for receptor-mediated endocytosis (SHAW and FALKOW 1988; MAKINO et al. 1991; Grassmé and van Putten, unpublished results). The factor initiating the bacteria-directed endocytosis is unclear. Expression of the appropriate Opa protein in *E. coli* results in a very low level of internalization compared to the parent gonococcus (maximal 2 vs 30–50 bacteria per cell, respectively) (SIMON and REST 1992; MAKINO et al. 1991; VAN PUTTEN 1993), suggesting that additional, possibly newly synthesized, factors are required to create the invasive phenotype (CHEN et al. 1991; KUPSCH et al. 1993; Kahrs and Meyer, unpublished results). It has been speculated that the insertion of the major neisserial ion channel protein I (P.I) is a critical determinant for entry (BLAKE 1985; WEEL and VAN PUTTEN 1991; WEEL et al. 1991b). Insertion of purified P.I into PMN or PBM cells interferes with cell signaling, inducing a transient membrane hyperpolarization, inhibition of phosphatidylcholine-phospholipase C activity and failure of the NADPH oxidase function (HAINES et al. 1988, 1991; Lorenzen and Meyer, unpublished results). Immunomorphological evidence supports binding to, or insertion of, the protein into the host cell membrane at the sites of bacterial

entry during infection (WEEL and VAN PUTTEN 1991; WEEL et al. 1991b). Complete internalization of the bacteria requires phosphorylation of host proteins at tyrosine residues (Grassmé and van Putten, unpublished results), but whether Opa protein, P.I., and/or other bacterial factors trigger this even is unclear. In all cases, it should be emphasized that these observations may be unique to the infection system and bacterial strain used, and may not represent uniform mechanisms or reflect the in vivo situation. Analyses of infected specimens obtained at the symptomatic stage of gonococcal disease suggest that gonococcal entry into mucosal cells may be a rare event (WARD and WATT 1972; EVANS 1977), though this may differ depending on the strain and the stage of the infection.

8.4 Intracellular Processing and Passage Across the Mucosal Barrier

During natural infection the epithelial barrier is often damaged and bacteria are found in the subepithelial tissue. How the pathogenic *Neisseria* translocate across the epithelial barrier is still unclear. In most types of cultured cells, ingested gonococci reside at first in endosomes and appear to survive for at least several hours (CHEN et al. 1991; WEEL et al. 1991a,b). Then, however, phagolysosomal fusion occurs and the bacteria are degraded (WEEL and VAN PUTTEN 1991; WEEL et al. 1991a,b). Occassionally, cytoplasmic localization of the bacteria has been reported (SHAW and FALKOW 1988), suggesting that some bacteria may have the potential to escape from the vacuole. These data suggest that either the choice of the infection system and/or the bacterial phenotype may determine the outcome of the experiment. In fallopian tube tissue, but not in nasopharyngeal tissue organ cultures, internalized *Neisseria* spp. appear to be released in the subepithelial tissue by exocytosis or by lysis of the infected cells (WARD et al. 1974; MCGEE et al. 1981; STEPHENS 1989). In unpolarized cell lines, exocytosis of ingested bacteria is difficult to measure because it is almost impossible to eliminate all extracellular bacteria from the assay system, and thus to inhibit continued bacterial ingestion (VAN PUTTEN 1991).

An obvious alternative way of gaining access to the subepithelial tissue is by disruption of the integrity of the mucosa by toxic factors, the elicited inflammatory response, and/or coinfection with other micro-organisms. Infection of human corneas in vitro results in a continuous shedding of infected epithelial cells, thus reducing the thickness of the epithelial layer (TJIA et al. 1988). In human fallopian tubes, LPS and peptidoglycan fragments released from the bacteria inhibit ciliary activity and cause a sloughing of noninfected ciliated epithelial cells, thus disrupting the architecture of the surface epithelium (for review see STEPHENS 1989). This effect, which seems to vary with the phenotype of the causative strain (DEKKER et al. 1990), is probably the result of stimulated tumor necrosis factor (TNF) production (MCGEE et al. 1992). A similar but not LPS-mediated toxic effect occurs in nasopharyngeal tissue challenged with meningococci (STEPHENS et al. 1986). In both tissue types, the mucosal damage facilitates transcellular passage of the bacteria (WARD et al. 1974; STEPHENS and FARLEY 1991).

8.5 Professional Phagocytes

Neisserial colonization of the human mucosa may give rise to an inflammatory response with recruitment and activation of professional phagocytes and activation of the host immune defense. Morphological data suggest that, in natural infection, the pathogenic *Neisseria* may reside inside phagocytes (WARD et al. 1972; DEVOE et al. 1973; SWANSON and ZELIGS 1974), but prolonged intracellular survival has been difficult to establish in vitro (SWANSON et al. 1975; for review see SHAFER and REST 1989). Dissection of the immunoresistant phenotype indicates that the bacteria possess a variety of antiphagocytic properties (see above) as well as a large repertoire of mechanisms that potentially allows them to be facultative intracellular microorganisms. An important step in *Neisseria* phagocyte interaction appears to be the distinct Opa protein-associated contact with the cells which results in induction of a respiratory burst and which may be followed by ingestion of the bacteria (KING and SWANSON 1978; REST et al. 1982; VIRJI and HECKELS 1986; FISHER and REST 1988; BELLAND et al. 1992). This nonopsonic interaction, which at least in part can be mimicked by synthetic peptides derived from the Opa protein HV2 region (NAIDS et al. 1991), is enhanced by fmlp, PMA or the calcium ionophore A23187 in combination with cytochalasin D, and inhibited by pertussis toxin, and calmodulin inhibitors (FARRELL and REST 1990), and probably involves a 19 kDa (glyco)protein that is part of the secondary granule membrane and thus up-regulated at the cell surface in activated phagocytes (FARRELL et al. 1991). The stimulation of the neutrophil oxidative metabolism does not lead to the release of detectable amounts of oxygen intermediates into the surrounding milieu which may contribute to extracellular persistence of the bacteria (NAIDS and REST 1991). Though most of these studies were performed with gonococci, it is anticipated that similar findings will be made with meningococci.

Once ingested, the bacteria probably reside in phagosomes and are exposed to the extremely hostile environment of oxidative and nonoxidative killing mechanisms, unless phagolysosomal fusion is inhibited, as may occur by the insertion of the bacterial ion channel in the vesicle membrane (WEEL et al. 1991b; WEEL and VAN PUTTEN 1991). The bacteria may counter the oxidative killing by increased oxygen consumption (KRIEGER et al. 1980; COHEN and COONEY 1984; BRITIGAN et al. 1988), the production of high levels of catalase (ARCHIBALD and DUONG 1986; JOHNSON et al. 1993), and/or by switching to anaerobic growth using nitrite as a source of energy (for review see HASSETT and COHEN 1989). In addition, expression of a long LPS type and intracellular bacteriostasis may promote resistance to nonoxidative antimicrobial agents either by preventing the binding of cathepsin or antimicrobial cationic proteins to the bacterial penicillin binding protein or inhibiting their detrimental effect, which requires active cell wall synthesis (DALY et al. 1982; CASEY et al. 1985; ROCK and REST 1988; SHAFER 1988; SHAFER et al. 1990). In this way, the nongrowing bacteria may survive for prolonged periods awaiting cytotoxic lysis or exocytotic release from the cells perhaps as clumps of multiplying bacteria enclosed in host cell remnants. These so-called infectious units have been proposed to play an important role in the persistence and transfer of neisserial infection (NOVOTNY et al. 1977). Whether the

recently identified FK506-inhibitable peptidyl-prolyl *cis-trans*-isomerase in meningococci (SAMPSON and GOTSCHLICH 1992; MCALLISTER and STEPHENS 1993), which has homology to the *Legionella* mip protein supposedly involved in initiation of intracellular infection (HACKER and FISCHER 1993), contributes to intraphagocytic survival remains to be established.

9 Conclusions and Outlook

In addition to providing the fundament for the development of vaccines and novel drugs in order to prevent disease, bacterial pathogenesis models serve as a platform for studying the complexity of interactions between microbial populations and higher organisms. The strength, and to the same extent a weakness, of the *Neisseria* model is the narrow host range of the included species. While this precludes the use of a natural animal infection model it minimizes the multitude of interactions a microbe can possibly encounter during infection and therefore may help us to specify the evolutionary forces governing microbial behavior (and that of their genes). Thus far the biological meaning of many genetic processes remains obscure, an example being the irreversible loss of essential infection determinants, such as *pilE* (SEGAL et al. 1985) and similar examples in other systems, which seems to disarm the pathogens and drive them into a dead end. Understanding the evolutionary basis of such processes is crucial if we are to explain the pathogenesis which presently is best described as an accidental case (see FALKOW 1990). In this field, the genus *Neisseria* offers a wide scenario for the comparison, on multiple levels, of pathogenic and non-pathogenic species interconnected by the flow of genetic material.

Apart from evolutionary considerations, the *Neisseria* model represents a paradigm of escape and adaptive functions and significantly contributes to the current adventure of unravelling the biochemical processes of cellular cross-talk. Despite the experimental obstacle of genetic variability and the lack of an animal model, neisserial adhesins playing critical roles in target cell interaction have been successfully defined and other signaling factors have been identified, such as P.I porin, which inserts into target cell membranes, and the Iga polyproteins, which are capable of entering the nuclei of human cells. As in other systems, cytoskeletal reorganization and phosphorylation of host cell proteins upon neisserial entry into epithelial cells can be demonstrated and the intracellular processing of bacteria contained in professional phagocytes is being studied. Research along these lines will not only extend our knowledge in terms of isolated pathogen host cell interactions but undoubtedly lead to a better understanding of the interplay between *Neisseria* and the immune system: Key questions relate to the modulation of antigen presentation and cytokine production and, finally, to the mechanism of the inflammatory response associated with neisserial infections.

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Pathogenicity Mechanisms of *Bordetella*

R. RAPPUOLI

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1 The Genus *Bordetella*

The genus *Bordetella* contains four pathogens of the upper respiratory tract. *Bordetella pertussis* and *B. parapertussis* colonize exclusively humans, causing whooping cough and a mild *pertussis*-like disease, respectively; *B. bronchiseptica* can be isolated from most mammalian species, causes kennel cough in dogs and atrophic rhinitis in pigs and is only rarely isolated from humans; *B. avium* causes turkey coryza and many other diseases in birds. The closest relatives of the genus *Bordetella* are *Alcaligenes* and *Pseudomonas* species, bacteria that are widespread in the environment (MÜLLER and HILDEBRANDT 1993; DE LEY et al. 1986) (Fig. 1). This suggests that the ancestors of *Bordetella* species were bacteria living in the external environment that evolved to infect homeothermic animals. During evolution, the first to diverge was *B. avium* which is the most distant in the evolutionary scale; then from the mainstream line of *B. bronchiseptica*, a single clone became specialized to infect exclusively humans and gave rise to *B. parapertussis* and *B. pertussis* (Fig. 1) (ARICÒ and RAPPUOLI 1987; ARICÒ et al. 1987; GROSS et al. 1989a; MUSSER et al. 1986). The latter two species are still a very homogeneous clonal population, while *B. bronchiseptica* and *B. avium* are heterogeneous populations of many subclones.

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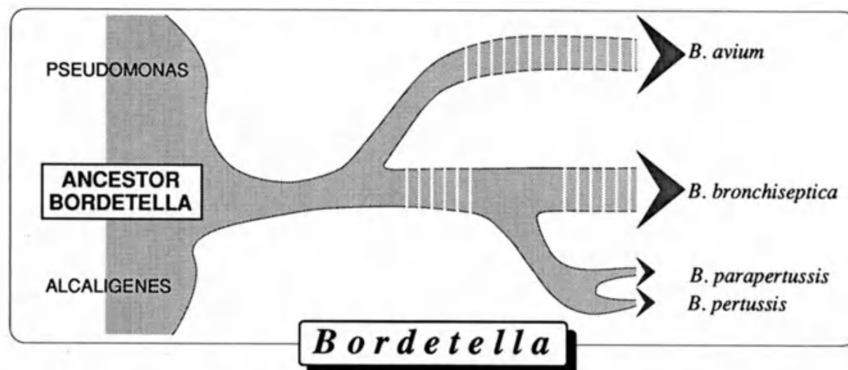


Fig. 1. The evolutionary tree of the genus *Bordetella*. The ancestor of the genus was likely to be a bacterium related to *Alcaligenes* and *Pseudomonas* species, living in the external environment, that initially acquired the ability to infect homeothermic animals. It then became so specialized for the second environment that today *Bordetella* species are isolated only from animals. During evolution, the four species further differentiated to adapt to their respective hosts: birds for *B. avium*, mammals for *B. bronchiseptica*, and humans for *B. pertussis* and *B. parapertussis*. The first species to diverge was *B. avium*. *B. parapertussis* and *B. pertussis* are homogeneous species and all isolates are of clonal origin. *B. avium* and *B. bronchiseptica* are heterogeneous species made up of different subclones

Today, the four species are so specialized that they can be isolated exclusively from their animal hosts; however, some properties still reflect their origin. For instance, the synthesis of virulence-associated factors is regulated by temperature (LACEY 1960), a system that very likely serves to distinguish the external environment, with a temperature of 20°–25°C, from the animal host at 37°C. The environmental origin of *B. bronchiseptica* is also suggested by its ability to survive for long periods in soil and in lake water (PORTER et al. 1991), and the presence of flagella (AKERLEY and MILLER 1993), a structure that is common to most pathogens that inhabit the external environment such as *Escherichia coli*, *Salmonella*, *Pseudomonas*, *Campylobacter jejuni* and *Vibrio cholerae*.

2 Virulence Factors: Structure, Export and Pathogenesis

Bordetella species colonize the upper respiratory tract of their hosts, using adhesins specific for ciliated cells of the respiratory epithelium and for alveolar macrophages. Although the four species may use mostly the same molecules for adhesion and host intoxication, most information derives from studies on *B. pertussis* and little is known about the subtle differences that may be responsible for differential pathogenesis. The following section is focused mostly on the factors that are believed to play a major role in *B. pertussis* virulence.

B. pertussis is peculiar among bacterial pathogens because it contains a redundancy of virulence factors: multiple adhesins and toxins are present and they all seem to play some role in pathogenesis. The other peculiarity of *B. pertussis* is the unusual arrangement of the virulence factors. The bacterium has been very creative and has found original ways to utilize molecules known to have a different function in other bacteria. The best examples of this are the combination of adenylate cyclase and hemolysin into a single virulence factor and the use of a peptidoglycan fragment as a toxin specific for ciliated cells. Finally, *B. pertussis* is unique because it utilizes at least three of the four different pathways used by gram-negative bacteria to export molecules to the bacterial surface or to the extracellular space. Again, even with regard to export, known systems are utilized in an unusual way: for instance, pertussis toxin is exported by a system that previously was believed to export only single-stranded DNA, and filamentous hemagglutinin is exported by a mechanism commonly used to export hemolysins. Below, the *B. pertussis* virulence factors are described: the homologies with other systems are highlighted mostly through the figures. The chromosomal location of the genes described has been mapped (Stibitz and Garletts 1992) and is reported in Fig. 2.

2.1 Filamentous Hemagglutinin

Filamentous hemagglutinin (FHA), a major adhesin of *B. pertussis*, is a protein of 220 kDa that can be purified in large amounts from the culture supernatant. The protein is encoded by the largest prokaryotic gene so far described (*fha*B, 10774 base pairs) (Relman et al. 1989; Domenighini et al. 1990), and is synthesized as a large precursor of 367 kDa (Aricò et al. 1993). The genomic location of the *fha* genes and their arrangement are reported in Figs. 2 and 3, respectively. The precursor protein is likely to be exported to the periplasm by a *sec*-dependent pathway and then further delivered to the bacterial surface with the help of a transporter protein (FhaC), encoded by a gene downstream from the *fha*B gene (Willems et al. 1994; Locht et al. 1993). The mechanism used for the surface exposure of FHA is similar to that of the Sh1A and HpmA hemolysins of *Serratia marcescens* and *Proteus mirabilis*, respectively (Uphoff and Welch 1990; Schiebel et al. 1989; Poole et al. 1988; Ondraczek et al. 1992). In this system, final secretion is mediated by Sh1B and HpmB, two proteins homologous to FhaC, which recognize and post-translationally modify an NH_2-terminal sequence on the target proteins that is present also in FHA (Delisse-Gathoye et al. 1990). Once on the cell surface, the full-length FHA molecule remains anchored to the bacterium through the COOH-terminal and binds eukaryotic cells by multiple domains located in the NH_2-terminal portion (Aricò et al. 1993). These include a domain that recognizes galactose-containing glycoconjugates on ciliated cells and macrophages (Tuomanen et al. 1988), an Arg-Gly-Asp domain that promotes adhesion to the macrophage integrin C3, and possibly other integrins on different cells (Relman et al. 1990; Aricò et al. 1993), and a heparin-binding region (Menozzi et al. 1991).

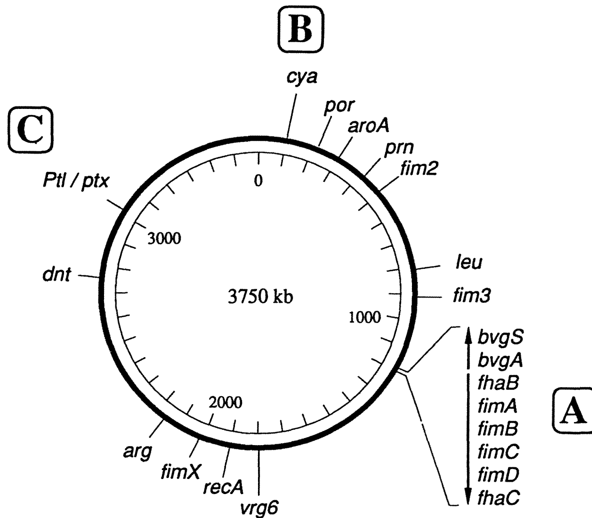


Fig. 2. Genomic map of Bordetella pertussis showing the position of the known genes. A, B and C are the loci that are shown in detail in Figs. 3–5. (Adapted from STIBITZ and GARLETTS 1992)

Fig. 3a–d. Structure of the chromosomal region A of Fig. 2. **a** This region contains the *bvg*A and *bvg*S regulatory genes; the *fha*B gene that codes for FHA and the *fha*C gene required for FHA export; and the genes *fim*A-*fim*D required for assembly and export of the fimbrial subunits. **b–d** FHA, Fim and Bvg proteins, respectively. The structural proteins are indicated as *empty arrows* or *boxes*, while the proteins involved in export and assembly of the structural proteins are shown in *black*. Below each protein the most significant protein homologies are reported to indicate how related these systems are to those of other bacterial or eukaryotic systems.

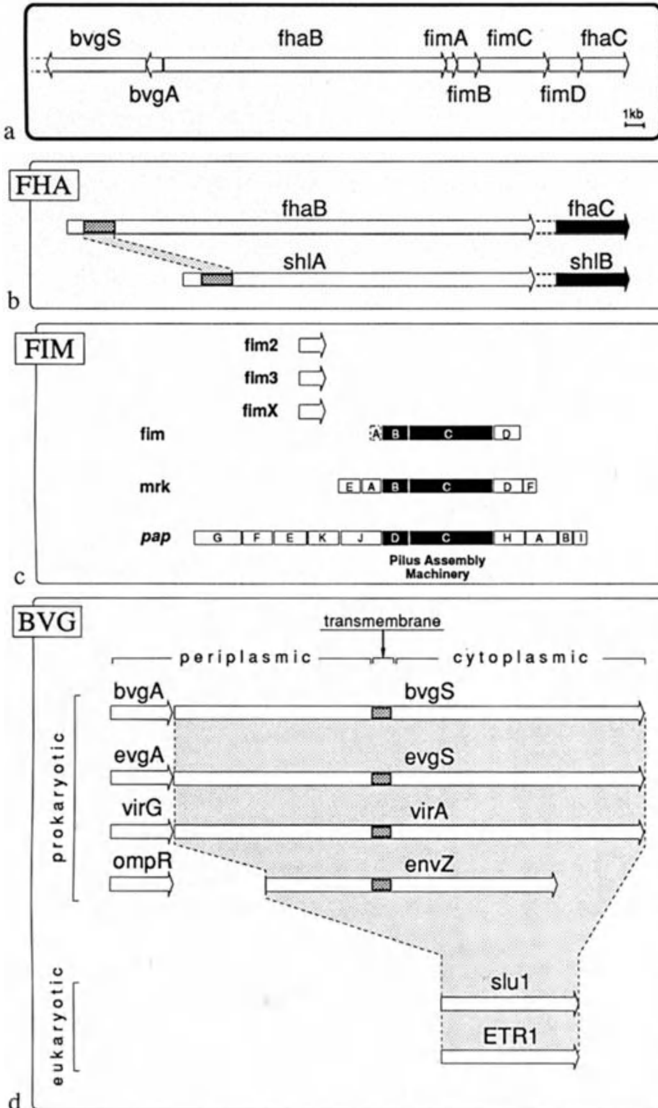
b FHA is exported by a system homologous to that of the *Serratia marcescens* hemolysin. The protein is exported to the periplasm by a signal peptide-dependent pathway and then further transported to the medium by the FhaC protein which is homologous to the *Serratia* protein ShlB. FhaC and ShlB recognize a target sequence present at the NH_2-terminal of FHA and ShlA. This is indicated by the *shaded area* (SCHIEBEL et al. 1989; SCHONHERR et al. 1993).

c The *Bordetella* fimbrial system is mostly homologous to the *mrk* fimbrial system of *Klebsiella pneumoniae* (ALLEN et al. 1991) and generally related to all other fimbrial systems, of which the prototype is the *pap* system of uropathogenic *E. coli* (JONES et al. 1992; HULTGREN et al.1993). The pilus assembly machinery is composed of two proteins FimB and FimC (indicated in *black*). By analogy with the *pap* system, it is believed that FimB binds the pilus major subunit (encoded by *fim*2, *fim*3, and *fim*X), preventing its periplasmic aggregation, and chaperones it to the FimC protein. This protein is the membrane anchor for the assembly of the pilus (JONES et al. 1992).

d BvgA and BvgS are highly homologous across the entire sequence to the *E. coli evg* system (UTSUMI et al. 1992) and contain regions of homology (indicated by the *shaded area*) to all other bacterial proteins of the two component system of which we show as examples only VirG, VirA, EnvZ and OmpR (GROSS et al. 1989b). The homologous region of the eukaryotic homologues of the two component system are also reported. (OTA and VARSHAVSKY 1993; CHANG et al. 1993)

Remarkably, most binding activities of FHA mimic eukaryotic mechanisms of cell adhesion (RELMAN et al. 1989), and in some cases it has been proposed that antibodies to FHA may interfere with leukocyte binding (TUOMANEN et al. 1993).

Finally, the NH_2-terminal 220 kDa region containing the eukaryotic cell binding domains is cleaved and released from the bacterium, possibly in order to achieve two goals: (1) to facilitate spreading, by releasing the bacterium from the bound cells and allowing it to move to other cells, tissues or organisms; (2) to coat the surrounding tissues with a sticky protein that would saturate the receptors,



compete for specific bacterial binding and facilitate subsequent nonspecific bacterial adhesion (TUOMANEN and WEISS 1985; TUOMANEN et al. 1985). The presence of FHA is also required for intracellular uptake of *B. pertussis* by HeLa cells and macrophages, a condition that has been proposed to play a role in disease (SAUKKONEN et al. 1991). The key role of FHA in bacterial adhesion has suggested that this protein should be one of the key components in new, acellular vaccines directed against whooping cough.

2.2 Pertactin

Pertactin is an outer membrane protein of *B. pertussis* that was originally named P69 or 69K protein to indicate the apparent molecular weight determined by SDS-PAGE. Cloning of the gene (CHARLES et al. 1989; MARCINAK et al. 1993) showed that this protein derives from a precursor of 93 kDa that is exported to the periplasm by a *sec*-dependent pathway and is rapidly processed to generate an NH_2-terminal protein of 60.5 kDa (pertactin), that migrates with an apparent molecular mass of 69 kDa in SDS gels and a COOH-terminal protein of 33 kDa (MAKOFF et al. 1990). *B. parapertussis* and *B. bronchiseptica* produce proteins homologous to pertactin which have a slightly different migration in SDS gels, named P70 and P68, respectively (LI et al. 1991, 1992). The role of pertactin in *B. pertussis* virulence is controversial. In vitro studies have shown that eukaryotic cells can adhere to purified pertactin and that, as in the case of FHA, an Arg-Gly-Asp-containing domain mediates this adhesion (LEININGER et al. 1991, 1992). However, in vivo the role of pertactin in bacterial adhesion has not been clearly demonstrated, and mutants lacking pertactin are not defective in adhesion (ROBERTS et al. 1991). Recently, it has been shown that pertactin mutants have a suboptimal FHA-mediated adhesion, suggesting that pertactin may help FHA in reaching the conformation that is competent for cell binding (ARICÒ et al. 1993). The ability of P68 to protect piglets from *B. bronchiseptica* infection (KOBISCH and NOVOTNY 1990), the high immunogenicity of pertactin, and its ability to induce protection in mice challenged by aerosol with virulent *B. pertussis* (SHAHIN et al. 1990) have suggested that this protein should be included in acellular vaccines against pertussis (NOVOTNY et al. 1991).

2.3 Fimbriae

Fimbriae or pili are filamentous structures of high molecular weight. They are composed of identical subunits of approximately 20 kDa, radiate from the surface of bacteria and are usually involved in bacterial adhesion. *B. pertussis* contains three fimbrial subunit genes (*fim*2, *fim3* and *fim*X) that are non contiguous in the chromosome (LIVEY et al. 1987; PEDRONI et al. 1988; CUZZONI et al. 1990; MOOI et al. 1990 and Fig. 2). *fim*2 and *fim*3 are expressed and assembled into fimbrial subunits, while *fim*X is silent in *B. pertussis* but is expressed in *B. bronchiseptica*. In *B. pertussis*, as in all gram-negative bacteria, secretion of fimbrial subunits

from the periplasm and assembly of the fimbrial structure require several accessory proteins, usually encoded by sequences that are clustered with the fimbrial structural genes (PUGSLEY 1993). In *B. pertussis* the accessory genes required for fimbrial assembly and export (*fim*B and *fim*C) are homologous to the corresponding proteins in other gram-negative bacteria. However, these genes are located far away from the structural genes and map downstream from the *fha*B gene, in a cluster containing also a gene coding for the adhesin moiety of the pilus (*fim*D) and an incomplete fimbrial structural gene (*fim*A) (WILLEMS et al. 1992, 1993; LOCHT et al. 1993 and Figs. 2, 3c). Given the redundancy of adhesins present in *B. pertussis*, the role of fimbriae in adhesion has never been clearly defined; nevertheless, it has been clearly established that serotype 2 and 3 fimbriae induce *B. pertussis* agglutinating antibodies (MING LI et al. 1988). In addition to the global *bvg* regulatory system, the fimbriae have an additional system that regulates their transcription using deletion and insertion of cytosine residues in the fim-promoter region (WILLEMS et al. 1990).

2.4 Pertussis Toxin

Pertussis toxin (PT), a protein of 105 kDa with multiple toxic properties, is produced only by *B. pertussis*. PT is a major virulence factor (WEISS et al. 1984; MONACK et al. 1989), involved both in bacterial adhesion and host intoxication (TUOMANEN and WEISS 1985), and may be responsible for the unique features of whooping cough, a disease caused only by *B. pertussis* (PITTMAN 1979, 1984). As with other bacterial toxins, PT can be divided into an enzymatically active part that is responsible for the toxicity (A protomer), and a nontoxic moiety (B oligomer) that binds to receptors on the surface of target eukaryotic cells and facilitates the translocation of the toxic A subunit across the eukaryotic cell membrane (RAPPUOLI and PIZZA 1991). The A protomer constitutes the S1 subunit, an enzyme of 26.2 kDa which is associated with the B moiety, an oligomer composed of four noncovalently-linked subunits, S2, S3, S4 and S5 of 21.9, 21.8, 12 and 11.7 kDa, respectively, that are present in a 1:1:2:1 ratio (TAMURA et al. 1982). The biological activities of PT reside both in the A and the B subunit. However, the toxicity of PT is exclusively associated with the enzymatic activity of the S1 subunit. The B oligomer binds to receptors on eukaryotic cells, using lectin domains located in the S2 and S3 subunits that share some homology with eukaryotic selections (SAUKKONEN et al. 1992). Upon binding, PT can agglutinate red blood cells, deliver signals that stimulate the proliferation of T cells, or cause calcium release. These activities of PT require very high concentration of toxin (0.5–1 μg/ml) and are unlikely to have a physiological role (SOMMERMEYER and RESCH 1990; NENCIONI et al. 1991). The toxic properties of PT are associated with the enzymatic activity of the S1 subunit which, as in the case of diphtheria, cholera and pseudomonas toxins, has an ADP-ribosylating activity and transfers ADP-ribose groups to G proteins of eukaryotic cells (KATADA and UI 1982; RAPPUOLI and PIZZA 1991). The G proteins that are modified by PT are involved in signal transduction and are responsible for the communication of cells and tissues with the external environment. Once the G

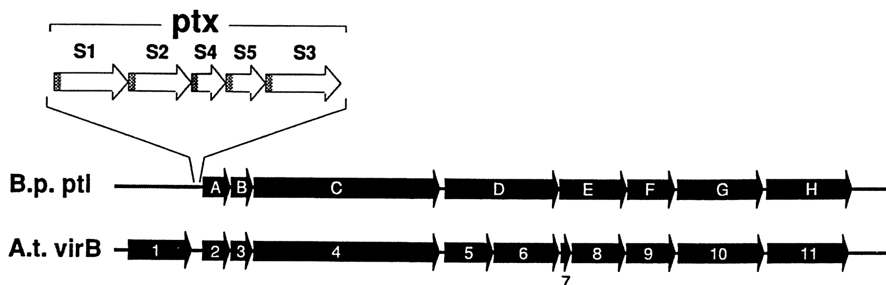


Fig. 4. Structure of the chromosomal region C of Fig. 2, encoding the pertussis toxin locus (ptx) and the locus involved in pertussis toxin secretion (*B. p. ptl*). The structural proteins are indicated as *empty arrows*, while the proteins involved in export and assembly of the structural proteins are shown in *black*. The operon required for the transport of PT from the periplasm to the medium (*ptl*) is highly homologous to the *vir*B operon of *Agrobacterium tumefaciens (A. t. vir*B), which is involved in transport of single-stranded DNA. The homology with the nopaline pTiC58 *vir*B region is shown (KULDAU et al. 1990). The figure shows that the PT operon may have evolved simply by inserting into an operon encoding the DNA transport system

proteins are modified by PT, they lose the ability to receive signals from surface receptors and this causes a generalized toxicity that has different consequences in different tissues. The amount of PT required for these activities is extremely low: the toxic activity in vitro is detected with 1 pg/ml (HEWLETT et al. 1983), while in vivo 5 ng of PT per mouse cause toxic effects that last longer than 3 months.

The genes coding for the five subunits of PT are clustered in an operon (NICOSIA et al. 1986; LOCHT and KEITH 1986) in the arrangement shown in Fig. 4. A similar arrangement is present also in *B. parapertussis* and *B. bronchiseptica*; however, in these organisms the PT operon is not transcribed (GROSS and RAPPUOLI 1988; ARICÒ and RAPPUOLI 1987). Each of the five polypeptides is independently exported by a *sec*-dependent pathway to the periplasm where the five subunits assemble into the holotoxin. Following assembly, the holotoxin is exported across the outer membrane into the external environment. Efficient assembly and secretion requires the S1 subunit, nevertheless, in its absence, the B oligomer can still be assembled and exported, although at low efficiency (PIZZA et al. 1990). Export of the assembled toxin across the outer membrane requires the products of the *ptl* operon. This is located downstream from the PT operon and contains eight genes that are homologous to the transport system used by *Agrobacterium tumefaciens* to transfer single-stranded DNA to plant cells (COVACCI and RAPPUOLI 1993; WEISS et al. 1993) (Fig. 4). The critical role of PT in virulence and its ability to induce protective immunity have made PT the major component of all acellular vaccines developed against pertussis. In order to be included in vaccines, PT is detoxified by chemical agents, a treatment which, however, reduces the immunogenicity of the protein. Recently, a strain of *B. pertussis* has been engineered by site-directed mutagenesis so that it encodes a mutant PT protein that is nontoxic but fully immunogenic (PIZZA et al. 1989). This molecule has already been extensively tested in clinical trials and is the most promising candidate for future vaccines (RAPPUOLI et al. 1992b; PODDA et al. 1992, 1993). A vaccine based on this molecule has been already approved for use in infants in Italy.

2.5 Adenylate Cyclase-Hemolysin

The adenylate cyclase-hemolysin is a bifunctional protein endowed with both adenylate cyclase and hemolytic activity, and is a major virulence factor of *B. pertussis*. Mutants lacking this protein are unable to initiate infection (KHELEF et al. 1992) and are avirulent in the mouse model (WEISS et al. 1984). The protein is synthesized as a large precursor of 1706 amino acids that can be functionally divided into the enzymatically active adenylate cyclase (residues 1–400), and the hemolysin portion (residues 401–1706) (GLASER et al. 1988a). The whole protein is post-translationally modified and secreted directly to the medium by a dedicated export apparatus that is similar in structure and function to that of the *E. coli* α-hemolysin (MACKMAN et al. 1987; PUGSLEY 1993; SHYAMALA et al. 1992). Downstream from the adenylate cyclase structural gene (*cya*A), there are three genes, *cya*B, *cya*C and *cya*E, that are involved in a *sec*-independent secretion of the protein to the medium (GLASER et al. 1988b) (Fig. 5). Upstream from the *cya*A gene, another gene, *cya*C, is necessary for activation of the hemolytic activity of the protein, possibly by a post-translational acylation mechanism (BARRY et al. 1991). This modification is necessary for interaction of hemolysin with mammalian cell membranes (ISSARTEL et al. 1991). Following binding to eukaryotic

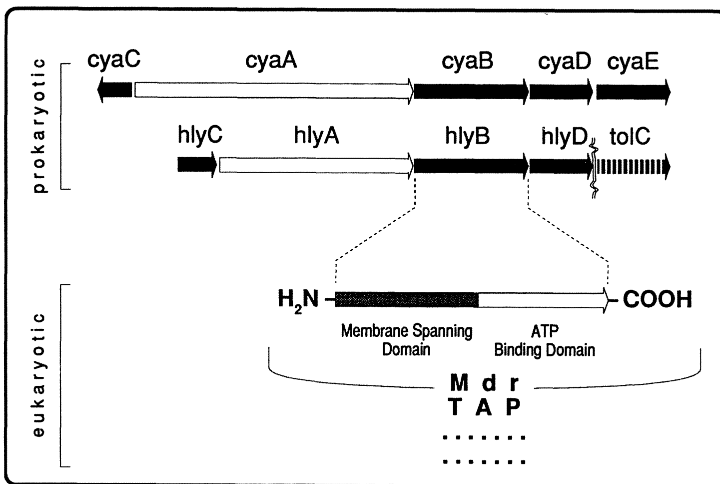


Fig. 5. Structure of the chromosomal region B of Fig. 2, encoding the adenylate cyclase-hemolysin system. The structural proteins are indicated as *empty arrows*, while the proteins involved in export and assembly of the structural proteins are shown in *black*. The structural gene *cya*A is followed by the genes *cya*B, *cya*D and *cya*E, required for export, and is preceded by *cya*C, which is involved in hemolysin activation. These genes code for proteins that are homologous to the corresponding proteins of the *E. coli* hemolysin system (*hly*) that are shown below. In *E. coli*, the *tol*C gene is not clustered with the hemolysin genes. The CyaB and HlyB proteins have a membrane spanning domain and a cytoplasmic translocation ATPase homologous to a large superfamily of membrane proteins, which are involved in the transport of a variety of substances across the membrane of bacterial and eukaryotic cells, including the Mdr multidrug resistance transporter (P-glycoprotein) and TAP proteins (BLIGHT and HOLLAND 1990; WANDERSMAN 1992)

cells, the COOH-terminal region with hemolytic activity is inserted into the membrane, triggering an increase in Ca^{2+} concentration which apparently facilitates translocation of the whole molecule across the cell membrane. Once in the cytosol, the NH_2-terminal portion of 43 kDa is proteolytically cleaved, releasing the adenylate cyclase which causes unregulated synthesis of cAMP (Rogel and Hanski 1992; Hewlett et al. 1991). This enzyme is unique because it is extremely sensitive to activation by eukaryotic calmodulin.

The double toxic activity of the adenylate cyclase-hemolysin and its absolute requirement for *B. pertussis* virulence suggests that this molecule could be a good vaccine candidate for immunization against pertussis. This has been confirmed by the observation that this is the only antigen, in addition to PT, that is able to protect mice from intracerebral challenge with virulent bacteria (Guiso et al. 1989, 1991). However, so far, the unavailability of large quantities of this protein has limited the development of such vaccines.

2.6 Tracheal Cytotoxin

Tracheal cytotoxin (TCT), a disaccharide-tetrapeptide of 921 daltons, is a fragment of the bacterial cell-wall peptidoglycan with a well defined structure: *N*-acetyglucosaminyl-1,6,-anhydro-*N*-acetylmyramylalanyl-γ glutamyldiaminopimelylalanine (Cookson et al. 1989). TCT is released into the medium during the logarithmic phase of growth by several bacteria, including *Neisseria gonorrhoeae*. It is identical in structure to the endogenous, neurologically active slow-wave sleep-promoting factor FS_{μ}, and is specifically toxic for ciliated cells (Goldman et al. 1990). During *B. pertussis* infection, TCT causes severe local damage of the ciliated cells lining the large airways, and this may be a primary cause of cough during pertussis. During *N. gonorrhoae* infection, TCT is responsible for the destruction of the ciliated cells of the human fallopian tube epithelium. The toxicity of TCT seems to be due to its ability to induce interleukin-1 (Heiss et al. 1993), which in turn induces nitric oxide, a substance known to have either cytostatic or cytotoxic effects on a variety of cells (Heiss et al. 1994). The high energy required for the continuous movement of the cilia could explain the exquisite susceptibiliy of ciliated cells to nitric oxide, a substance that targets a number of enzymes involved in ATP synthesis.

2.7 Dermonecrotic Toxin

Dermonecrotic toxin is a potent toxin that is produced by all forms of *Bordetella* species and is often called heat-labile toxin, to indicate that the toxic activity can be inactivated by heating at 56°C. The protein is a single polypeptide of 140 kDa, which when injected intradermally into suckling mice or guinea pigs induces blood vessel contraction and hemorrhagic skin necrosis. Although the molecule has been purified (Zhang and Sekura 1991), little is known about the structure and mechanism of action of this molecule.

3 Global Regulation of Virulence and Reversible Differentiation

Bordetella species sense environmental signals and modify gene expression in order to optimize their interaction with the environment. This process involves a well defined and temporally controlled program of gene expression that generates bacteria with totally different phenotypic properties. It is reminiscent of the differentiation process of higher organisms, although in this case the process is reversible (RAPPUOLI et al. 1992a). Thus, for example, bacteria grown at 25°C are non-hemolytic, avirulent and have a different colony morphology from those grown at 37°C, which are hemolytic, fully virulent and can be agglutinated by specific antisera that do not recognize the bacteria grown at 25°C. The different phenotypic properties of bacteria grown at different temperatures are schematically shown in Fig. 6, which summarizes also the molecular events that cause the phenotypic changes. *Bordetella* species grown at 25°C express a number of genes, named *vir*-repressed genes (*vrg*), which are likely to be necessary for survival in a low temperature environment outside of the animal host (BEATTIE et al. 1992; FINN el at. 1991). The flagellin gene of *B. bronchiseptica* is the best example of this class of genes (AKERLEY and MILLER 1993). Following temperature transition to 37°C, the *vrg*s are rapidly repressed and several genes

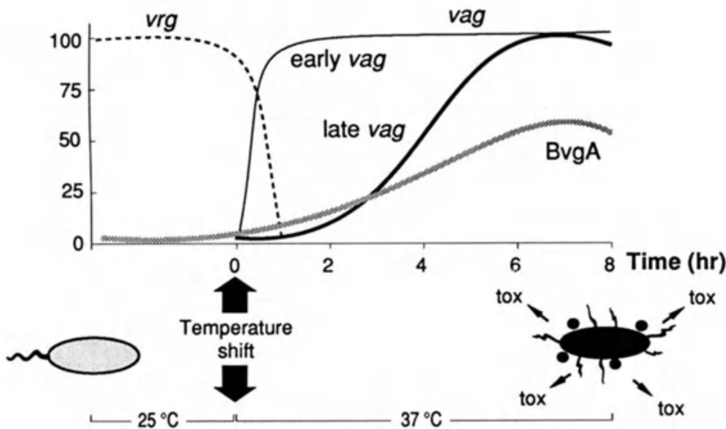


Fig. 6. Transcriptional response of *Bordetella pertussis* to a temperature variation from 25°C to 37°C and vice versa. *Top* the vir-repressed genes (*vrgs*) are expressed at 25°C. The vir-activated genes (*vags*) are expressed at 37°C. Following a temperature shift from 25°C to 37°C, the *vag*s are turned on in two steps. During the first step the genes coding for molecules involved in adhesion (FHA and fimbriae) are activated and the transcription of the *bvg* locus is also increased (*early vags*). This causes an increase of the intracellular concentration of the BvgA regulatory protein. The second step initiates when the amount of BvgA reaches a value that is approximately 20 times higher than the basal level at 25°C and consists of the activation of the genes coding for pertussis toxin, adenylate cyclase, pertactin, etc. (*late vags*). *Bottom* the bacteria grown at 25°C or 37°C are phenotypically different and well adapted to grow in different environments. This is the result of a well defined program of gene expression that resembles the differentiation of higher organisms

required for virulence in the homeothermic animal host, named vir-activated genes (*vag*), are turned on in two steps (SCARLATO et al. 1991). The first step occurs immediately after the temperature shift and involves activation of genes coding for proteins required for bacterial adhesion, such as fimbriae and FHA (early *vag*s). The second step of gene activation, which starts 2 h after temperature shift and reaches maximal levels at 6–8 h, involves the transcriptional activation of the late *vag* genes, including those coding for PT, adenylate cyclase and pertactin (Fig. 6).

The global regulation of *vrg* and *vag* is mediated by a single regulatory locus, *bvg* (WEISS et al. 1983; GROSS et al. 1989a). In addition to low temperature, *bvg* is responsible for the shut off of *vag* transcription in the presence of nicotinic acid or $MgSO_4$, although other salts may also influence expression of the *bvg* regulon (LACEY 1960; MELTON and WEISS 1993). As discussed in the introduction to the genus *Bordetella*, it is likely that the *bvg* system evolved to differentiate the external environment, with a temperature of 20°–25°C, from the animal host at 37°C. However, the nature of the physiological stimulus that regulates the synthesis of the virulence factors is still controversial; for instance, it has been proposed that the *bvg* system may shut off virulence gene expression during the intracellular stage of *B. pertussis* (MASURE 1992). The *bvg* locus maps next to the FHA gene (STIBITZ and GARLETTS 1992; Fig. 2) and encodes for BvgA and BvgS, two polypeptides of 23 and 135 kDa, respectively, that are homologous to a family of bacterial and eukaryotic proteins which regulate gene expression by using "two components." This is schematically shown in Fig. 3d (ARICÒ et al. 1989; STIBITZ and YANG 1991). The first component (see Fig. 7 for details) is a receptor (BvgS) that senses the external environment with the NH_2-terminal portion. Following a positive stimulus, the receptor dimerizes and translocates the message across the membrane, through a membrane spanning domain, to the cytoplasmic portion of the receptor which is endowed with kinase activity. This portion of the two component system is the most conserved in all bacterial homologues and has been recently shown to be conserved also in eukaryotic signal transducing systems (Fig. 3d and KOSHLAND 1993; OTA and VARSHAVSKY 1993; CHANG et al. 1993). For instance, ETR1, a system homologous to *bvg*, has been shown to be responsible for sensing ethylene in *Arabidopsis* and to regulate the expression of a number of genes involved in fruit ripening and flower senescence (CHANG et al. 1993). The second component of the system is a DNA, binding transcriptional activator (BvgA), the activity of which is regulated by BvgS through phosphorylation (MILLER et al. 1989; GROSS et al. 1989a). The intracellular amount of BvgA is quite low when bacteria are grown at 25°C, or in the presence of $MgSO_4$; however, it increases over 60-fold when the negative stimulus is removed (SCARLATO et al. 1991; MASHAKO et al. 1992). One of the questions that is not yet solved is the mechanism by which the *bvg* system differentially regulates the early and late *vag*s. BvgA has been shown to bind upstream from the FHA and *bvg* genes and activate transcription from the FHA and *bvg* promoters. Nevertheless, under the same conditions, BvgA is not able to bind the DNA regions upstream from the PT or adenylate cyclase genes (ROY et al. 1989), which are necessary for promoter activity (GROSS and RAPPUOLI 1988). Similarly, when

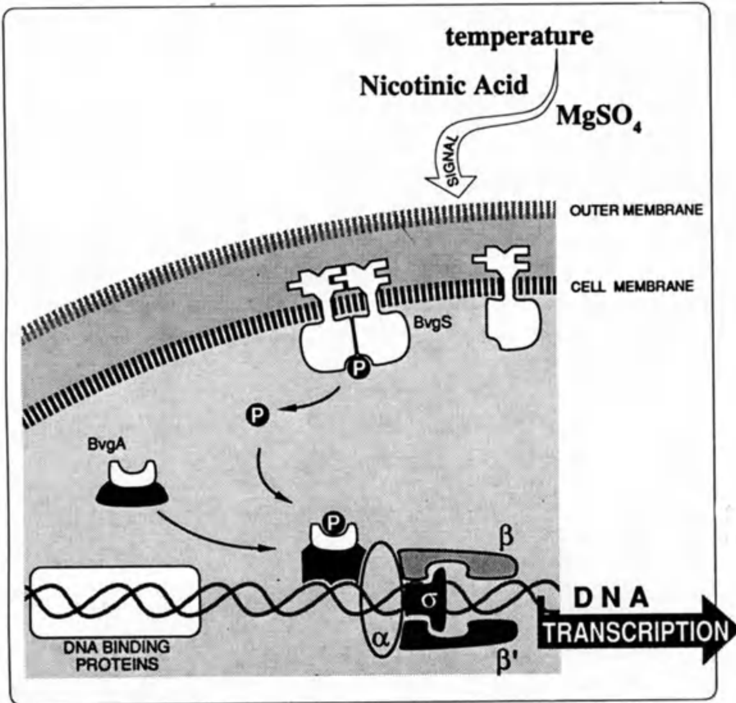


Fig. 7. The key players in regulation of virulence genes in *B. pertussis*. The external signals are sensed by *BvgS* which dimerizes and activates *BvgA*, possibly by phosphorylation, causing transcription of the *vag* genes and repression of the *vrg* genes. *BvgA* is known to bind upstream from the FHA and *bvg* promoters, but binding upstream from the pertussis toxin and adenylate cyclase promoters has not been demonstrated. The mechanism by which BvgA activates or represses transcription is not known. Some of the possible hypotheses are: (1) BvgA binds DNA and directly helps the RNA polymerase to make an open complex. (2) It interacts with other factors that in turn interact with and activate the RNA polymerase. (3) It specifically derepresses promoters that are not available for transcription because they are covered by DNA binding proteins. Other players in the transcriptional activation, shown in the figure, are DNA binding proteins and the α, β and σ subunits of the RNA polymerase

cloned in *E. coli* the *bvg* system is able to transactivate and regulate efficiently the FHA gene but not the PT gene (MILLER et al. 1989). Recently, it has been shown that the PT gene can also be transactivated in *E. coli* by the *bvg* system; however, this activation is exquisitely sensitive to DNA topology and supercoiling, and generally it does not work when the PT promoter is cloned in small plasmids (SCARLATO et al. 1993). Presently, two theories have been proposed to explain the differential regulation of early and late *vag*s by the *bvg* system: one theory suggests that, upon activation of *bvg*, transcription of an unknown transactivator specific for the late genes is initiated; the other theory does not require an additional factor but suggests that the late genes have a lower affinity for BvgA, and that they are activated only when the intracellular concentration of BvgA is 20 times or more above the basal level (SCARLATO et al. 1991; RAPPUOLI et al. 1992a).

The *bvg* system controls two well known phenomena of *Bordetella*: phase variation and antigenic modulation. Modulation is the classical name indicating that *Bordetella* "modulates" the expression of the virulence factors and consequently the immunogenicity, virulence, vaccine potency, etc., in response to temperature, $MgSO_4$ or nicotinic acid (LACEY 1960); phase change occurs when a frameshift mutation or a deletion within the *bvg*S gene cause a permanent loss of the ability to produce virulence factors (STIBITZ et al. 1989; ARICÒ et al. 1991).

From the evolutionary point of view it is interesting that a system highly homologous to *bvg*, but with an unknown function (*evg)*, has been described in *E. coli*. When this system is hyperexpressed in an EnvZ mutant, it regulates the expression of OmpC in response to temperature, $MgSO_4$ and nicotinic acid (UTSUMI et al. 1992).

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