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CHAPTER 1

Variation in Form and Function: The Helix-Turn-Helix Regulators of the GntR Superfamily

Paul A. Hoskisson* and **Sébastien Rigali†**

Contents

Abstract

One of the most abundant and widely distributed groups of Helix-turn-helix (HTH) transcription factors is the metabolite-responsive GntR family of regulators (>8500 members in the Pfam database; Jan 2009). These proteins contain a DNA-binding HTH domain at the N terminus of the protein and an effector-binding and/or

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oligomerisation domain at the C terminus, where upon on binding an effector molecule, a conformational change occurs in the protein which influences the DNA-binding properties of the regulator resulting in repression or activation of transcription. This review summarises what we know about the distribution, structure, function and classification of these regulators and suggests that they may have a future role in biotechnology.

> ...endless forms most beautiful and most wonderful have been and are being, evolved.
>
> Charles Darwin, 1859

I. INTRODUCTION

As bacteria sense different micro-environments, they modify their gene expression appropriately to enable them to respond to the prevailing conditions. Often the signal sensed within the cell is a metabolic intermediate, and these are sensed by many classes of helix-turn-helix (HTH) transcription factor, through which they modulate gene expression. The effector molecules bound by these proteins are often related catabolic substrates, substrates and/or intermediates of the pathway controlled by the transcription factor.

One of the most abundant groups of HTH bacterial metabolite-responsive transcription factors is the GntR family of regulators (>8500 members in the Pfam database; Jan 2009). These multi-domain transcription factors are widely distributed throughout the bacterial world where they play a fundamental role in modulation of gene expression to respond appropriately to the environment context.

This review aims to bring together and summarise our current thinking on GntR regulators, their structure, function, evolution, and how they may be exploited in biotechnology.

II. HELIX-TURN-HELIX DNA-BINDING PROTEINS

The identification of a tri-helical domain and its critical role in DNA binding within the bacteriophage Lambda proteins, cI and cro and the *lac* operon repressor, LacI, were early advances in the pioneering work of Matthews and co-workers (Ohlendorf *et al.*, 1982, 1983) and Sauer *et al.* (1982). The importance of helix two and helix three of the domain led to the identification of what became known as the HTH motif. The third α-helix is often referred to as the 'recognition' helix, which fits within

the major groove of the DNA mediating the protein–DNA interaction (Aravind *et al.*, 2005). Ohlendorf *et al.* (1983) and Sauer *et al.* (1982) suggested, through extensive sequence analysis and secondary structure analysis, that this domain was present in several DNA-binding bacterial activators and repressors, and they hypothesised that these domains descended from a common ancestor.

Throughout the 1980s and 1990s, extensive sequencing, the emergence of whole genome sequencing and experimental work confirmed the ubiquity and central role this domain played in gene regulation in both prokaryotes and eukaryotes and led to the identification of the HTH motif in all domains of life, suggesting that the HTH domain is one of the most ancient protein folds, although it appears to be most prevalent in prokaryotes (Aravind and Koonin, 1999). The development of specific algorithms for recognition of the HTH motif has become indispensible in genome annotation such as that of Dodd and Egan (1990), enabling rapid identification of HTH-containing proteins.

III. GntR REGULATORS

The HTH-containing GntR family is widely distributed throughout the bacteria where they regulate many diverse biological processes. It was named GntR after the first member identified, the *Bacillus subtilis* repressor of the gluconate operon (Haydon and Guest, 1991; Prosite Family PS50949; Pfam family: PF00392). GntR regulators are often located on the chromosome adjacent to the genes that they control, which in many cases allows insight into the metabolites that they may bind. There are however many examples where this is not the case, and identifying their cognate ligands remains a significant barrier to understanding their function.

In general, these proteins contain a DNA-binding HTH domain at the N terminus of the protein and an effector-binding and/or oligomerisation domain at the C terminus (Fig. 1.1). Upon binding an effector molecule at the C-terminal domain, a conformational change occurs in the protein which influences the DNA-binding properties of the regulator resulting in repression or activation of transcription. The DNA-binding domain is conserved throughout the GntR family yet the regions outside the DNA-binding domain are more variable; however, this is not surprising given the diversity of molecules that they bind, and this feature is used to define the GntR-like sub-families (Rigali *et al.*, 2002). Despite the large number of GntR-like regulators identified there are few examples where their effector molecules are known and the complete regulatory circuitry elucidated. Knowledge of this is of particular importance where GntR-like regulators control genes of unknown biochemical function and can

FIGURE 1.1 Schematic representation of a GntR protein. Indicates the N-terminal helix-turn-helix DNA-binding domain and the longer C-terminal effector-binding/oligomerisation domain (E-b/O).

provide information of their cellular function and will enable these processes to be built into modelling frameworks in terms of using systems biology approaches. GntR-like regulators are known to control many fundamental cellular processes such as motility (Jaques and McCarter, 2006), development (Hoskisson *et al.*, 2006), antibiotic production (Hillerich and Westpheling, 2006), antibiotic resistance (Truong-Bolduc and Hooper, 2007), Plasmid transfer (Reuther *et al.*, 2006) and virulence (Casali *et al.*, 2006; Haine *et al.*, 2005). In all these cases the exact ligand regulating gene expression through these proteins is unknown.

There are many cases where GntR-like regulators are not located next to genes that they control (orphan regulators), or without their effectors they are activators of gene expression elsewhere in the genome. One well-studied example is FadR, the fatty acid metabolism regulator in *Escherichia coli*, where it is known to negatively control 12 genes or operons and activate transcription of at least three genes when a fatty acid precursor is bound (DiRusso *et al.*, 1993; See section VIII).

The identification of the small molecules that bind to these regulators has traditionally been difficult and has mainly relied on gene context and bioinformatics to identify possible effector molecules. This area remains a significant challenge to researchers in this field and urgently requires novel methods to aid identification of effector molecules.

IV. DISTRIBUTION OF GntR REGULATORS

Examination and analysis of GntR regulator distribution throughout completely sequenced genomes demonstrate some interesting trends in terms of their abundance and may give clues to how an organism is distributed in a particular ecological niche or the kind of plasticity it experiences within its natural environment.

There are 8561 GntR regulators in the Pfam database (PfAM family GntR: PF00392: Finn *et al.*, 2008). The bulk of these (8561 sequences) are found in 764 bacterial taxa indicating that this protein fold has been widely adopted as a regulatory mechanism. Examination of taxonomic distribution of these regulators throughout the bacteria demonstrates a wide distribution; however, the predominant phyla (from current

sequences available in Pfam) are the Proteobacteria, Firmicutes and the Actinobacteria (Fig. 1.2). Detailed examination of the distribution within well-characterised species (Fig. 1.3) shows an interesting trend, not only with increasing genome size, but also with ecological niche. The trend suggests that organisms that live in complex, highly variable environments such as soil (e.g. *Streptomyces*, *Burkholderia*, *Rhizobium*) have a larger complement of the metabolite-responsive GntR regulators than obligate intracellular parasites and endosymbionts (e.g. *Chlamydia* and *Buchnera*). This trend is reinforced even within genera with *Mycobacterium smegmatis* having a complement of about 60 GntR regulators (Vindal *et al.*, 2007), where all these have been lost in the obligate intracellular pathogen *Mycobacterium leprae* during the extensive gene decay observed in this species (Cole *et al.*, 2001). These data indicate that, whilst there is a trend to increase metabolite-responsive regulators in the genome to enable rapid response to changing conditions in complex environments, this is lost when a stable niche is occupied and this requirement ameliorated.

There are twelve GntR regulators known in the Archaea. Two known from Eukaryotes, one from the sea anemone (*Nematostella vectensis*) and one from *Trichomonas vaginalis*; however, the exact functions of these are unknown. The two known GntRs in viruses are both in bacteriophages,

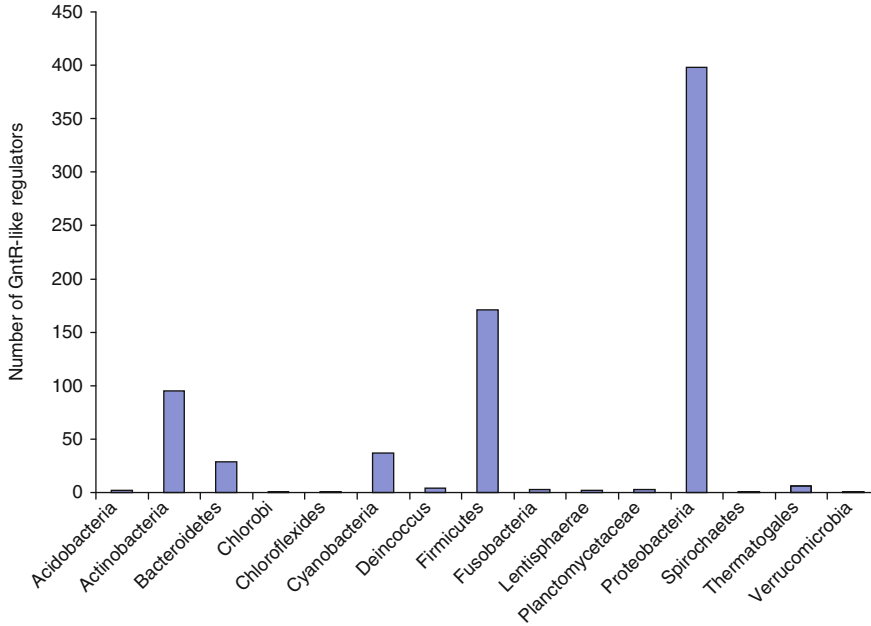

FIGURE 1.2 Distribution of GntR proteins throughout the bacterial Phyla. Please see text in Section IV. Data were taken from the sequences deposited in the Pfam database.

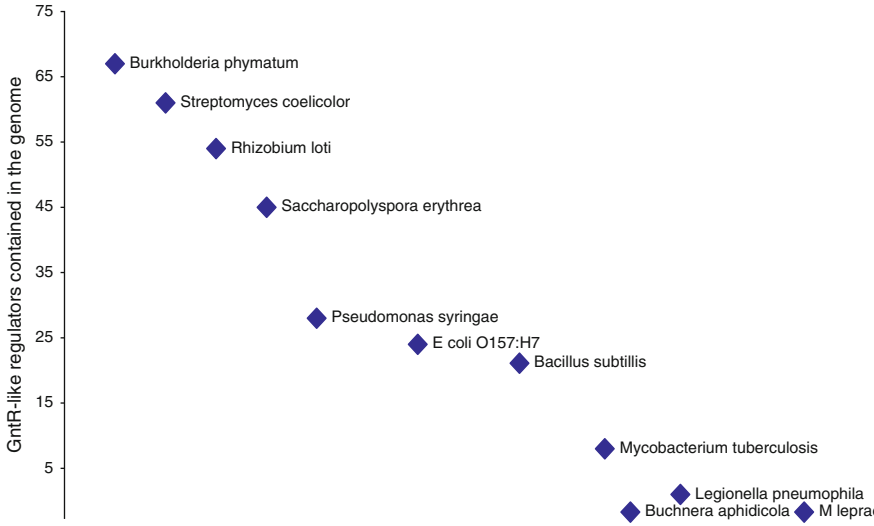

FIGURE 1.3 Distribution of GntR regulators in selected bacterial whole genomes. See text in Section V.

one in the *Streptomyces* phage ϕC31 and one in an enterophage ϕp27. Whilst the function of these within the bacteriophages is unknown, it is likely that they have been acquired from host strains.

V. STRUCTURE AND CLASSIFICATION OF GntR REGULATORS

Haydon and Guest (1991) first described the GntR family based on a common sequence at the N terminus of the proteins. They showed that a highly conserved 69-amino acid N-terminal region, containing a predicted HTH motif was conserved (Fig. 1.4). Further analysis of the domain using Pfam has indicated that the HTH domain can be refined to an average of 62.2 amino acids within the GntR domain (Finn *et al.*, 2008). Whilst overall sequence identity in the N-terminal HTH domain is low, the prediction of secondary structure is highly conserved with the three α-helices, characteristic of the HTH domain being apparent (Fig. 1.4). Despite the abundance of GntR sequences in the databases there are few crystal structures available to fully examine structure/function relationships at a detailed level.

Haydon and Guest (1991) also noted that there was extensive variation in the C-terminal domain suggesting heterogeneity in the effector molecules that they bind. Interestingly analysis of all full-length GntR-like sequences in the Pfam database indicates that on average the N-terminal

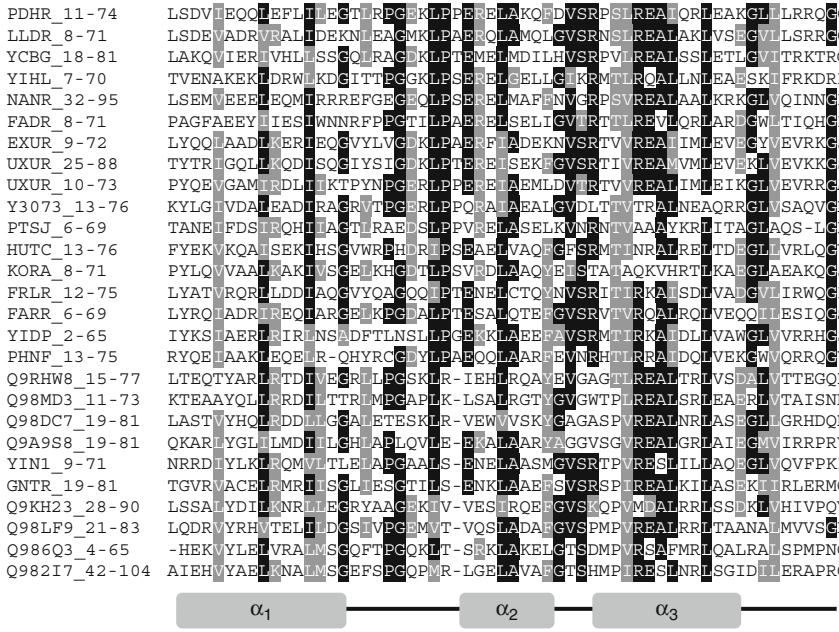

FIGURE 1.4 Alignment of the HTH domain of GntR regulators. This alignment demonstrates conservation of sequence structure within the HTH domain. Alignment was performed using the Seed alignment from Pfam (Finn *et al.*, 2008) used to generate the GntR family hidden Markov Model; the alignment was performed in ClustalW (Larkin *et al.*, 2007) with the residues coloured using Boxshade (http://www.ch.embnet.org/software/BOX_form.html). Secondary structural predictions were performed using the ProteinPredict webserver (Rost *et al.*, 2004) and were checked against the 3D structure of FadR.

domain accounts for 21.8% of the amino acid sequence, yet homology across the whole protein is around 30% reinforcing the concept of extensive C-terminal heterogeneity.

Despite only limited knowledge of two operator sequences at the time, Haydon and Guest (1991) noted that the recognition sequence for the GntR and HutC regulators was identical at seven of the eleven residues. These observations indicated that there are three interacting components of the GntR regulator: the DNA-binding domain, the so-called effector-binding and/or oligomerisation domain (E-b/O) and the *cis*-acting operator sequence. Rigali *et al*. (2002) exploited this idea through extensive analysis of the C-terminal domain of 270 GntR sequences, the N-terminal DNA-binding domain and the operator site. This led to the first extensive work on the family after its initial designation by Haydon and Guest (1991) and the formation of four sub-families within the GntR regulators,

and subsequent work led to the designation of a further three: the AraR, DevA and PlmA sub-families (Franco *et al.*, 2006; Hoskisson *et al.*, 2006; Lee *et al.*, 2003).

Due to the abundance of sequences in the databases, the sub-division of HTH DNA-binding proteins has become an issue in bioinformatics with regard to informing genome annotators. There are relatively few studies where extensive analysis of a protein and its operators has been attempted to inform on protein function (Brown *et al.*, 2003; Busenlehner *et al.*, 2003; Korner *et al.*, 2003; Maddocks and Oyston, 2008; Molina-Henares *et al.*, 2006; Weickert and Adhya, 1992).

The classification of the GntR sub-families was based on alignment of the C terminus and secondary structural predictions to reveal distinct structural topologies within each sub-family (Rigali *et al.*, 2002). Extensive analysis was verified by comparison of the predicted topology with that of the known crystal structure of FadR (van Aalten *et al.*, 2000a) which added confidence to the findings. Comparison of the predicted secondary structures is shown in Fig. 1.5 and illustrates the diversity and length differences in each sub-family described so far.

The most abundant GntR sub-family is FadR, named after the fatty acid biosynthesis and degradation regulator of the same name. This sub-family accounts for approximately 40% of GntR regulators, with the C terminus averaging 160 amino acids and consisting of six or seven α-helices. The crystal structure of the C-terminal of FadR of *Escherichia coli* is known (van Aalten *et al.*, 2000a,b, 2001) and served as a validation of the secondary structural predictions of Rigali *et al.* (2002). FadR is an acyl-CoA-responsive member of the GntR family (van Aalten *et al.*, 2001). The regulator exhibits an unusual protein fold overall. The winged helix-turn-helix (wHTH) is fused to a seven α-helix bundle which has crossover topology, containing a large internal cavity required for binding acyl-CoA (Raman and DiRusso, 1995; van Aalten *et al.*, 2000a,b). One unclear aspect of this study was how an effector-binding domain, located 30 Å from the DNA-binding domain, can affect transcription. van Aalten *et al.* (2001) elucidated this through showing, that upon binding acyl-CoA, the protein backbone undergoes dramatic conformational shifts, which results in a 7.2-Å movement of the DNA recognition helix preventing DNA binding and subsequent transcriptional repression.

Recently a cluster within the FadR sub-family was identified that appears to have evolved in Gram-positive organisms for citrate utilisation, with the authors inferring that this lineage arose through E-b/O domain replacement in an ancestral protein (Blancato *et al.*, 2008).

The second sub-family is the HutC grouping, which represents a highly diverse family in terms of effector molecules and processes regulated and accounts for around 30% of GntR regulators. The HutC sub-family C terminus consists of α-helices and β-sheets and averages 170

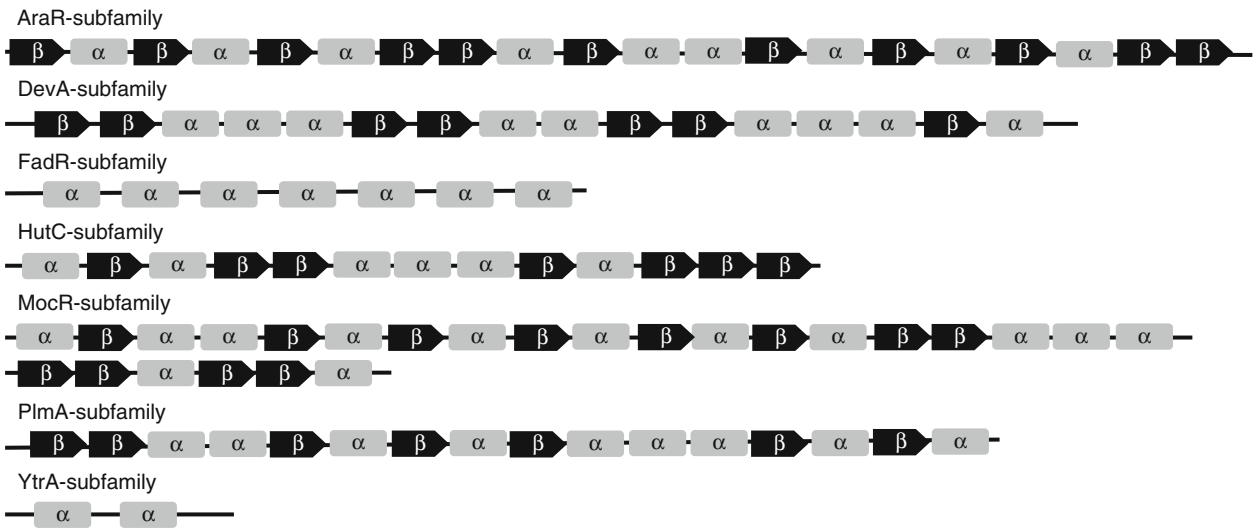

FIGURE 1.5 Structural representation of the C-terminal domains of the GntR sub-families. Secondary structural predictions were performed using the ProteinPredict webserver (Rost *et al.*, 2004) and were checked against the 3D structure of FadR. The α-helices and β-sheets are not to scale and just represent the structural arrangement of these domains.

amino acids in length. Named after the Histidine utilisation operon regulator, members of this family have been shown to regulate processes as diverse as amino acid uptake and plasmid transfer (Allison and Phillips, 1990; Kendell and Cohen, 1988). Three-dimensional crystal structural and analysis of the HutC family member, PhnF from *E. coli* (the regulator of transport and biodegradation of phosphonates) (Gorelik *et al.*, 2006), confirmed earlier bioinformatic work of Aravind and Anantharaman (2003) that the C-terminal domain had homology to the chorismate lyase fold, comprising a six-stranded antiparallel β-sheet, a two-stranded parallel β-sheet and four short α-helices. There is a large cavity present on the surface of the PhnF C terminus which may represent a putative effector molecule-binding pocket which shows conservation within other HutC family members (Gorelik *et al.*, 2006).

The MocR sub-family is remarkable for the average length of the C terminus being 350 amino acids and the presence of good homology to the class I aminotransferase enzymes. These enzymes act as dimeric proteins to catalyse the transfer of an amino group to an acceptor molecule such as a keto acid (Ko *et al.*, 1999). Interestingly this raises the possibility of the C terminus of MocR-like regulators being catalytic or that homology is linked to the dimerisation of these proteins and may suggest the recruitment of such a domain to facilitate protein–protein interaction (Rigali *et al.*, 2002).

The YtrA sub-family contains the shortest E-b/O domain, which consists of only two α-helices and often appears to be associated with ATP-binding cassette (ABC) transporters. The homology observed between this group is low (Rigali *et al.*, 2002), which may indicate further sub-division of this family may occur. Interestingly the shortened E-b/O in this sub-family, averaging only 50 amino acids, may not be efficient for binding effector molecules, but may still be effective for dimerisation (Rigali *et al.*, 2002; Yoshida *et al.*, 2000). It is interesting to hypothesise that given the shortened C terminus of the YtrA sub-family, the binding of small molecules may impair dimerisation resulting in loss of DNA binding.

The DevA sub-family of GntR regulators has only been found in streptomycetes (Hoskisson *et al.*, 2006). DevA-like proteins are most similar in the N-terminal domain to the HutC sub-family; however, the C-terminal domain has a novel topology (Fig. 1.5). Phylogenetic analysis and BLAST results confirmed that DevA and its relatives (orthologues in *Streptomyces avermitilis* and *S. scabies*) form a novel GntR sub-family due to the distinct C-terminal topology. The exact role of these proteins has yet to be elucidated; however, DevA is required for correct development in *S. coelicolor* (Hoskisson *et al.*, 2006).

The PlmA sub-family of GntR regulators represent a minor grouping that were first identified in playing a role in plasmid maintenance in the filamentous Cyanobacterium *Anabaena* sp. Strain PCC7120 (Lee *et al.*, 2003).

This sub-family has a novel domain topology when compared to other GntR sub-families (Fig. 1.5) and appears to be confined to the Cyanobacteria. Alignments of the N-terminal appear to cluster this sub-family closely with the MocR and YtrA sub-families suggesting that despite their similarity in the DNA-binding domain, the E-b/O domain may have been replaced during evolution.

The AraR sub-family is another minor grouping (Franco *et al.*, 2006; Lee *et al.*, 2003). The GntR regulator AraR from *B. subtilis* is responsible for the control of a range of carbon catabolic genes. The N-terminal domain is classically GntR-like; however, the C-terminal domain has extensive homology to the LacI/GalR regulator family, reinforcing the paradigm of modular repressors in a one-component regulatory system. This protein has reasonably well-characterised binding sites (Mota *et al.*, 1999, 2001) which results in a high-level repression by co-ordinately binding two in-phase operators causing DNA looping. The second results from single operator binding which is autoregulatory and results in low levels of transcription.

The emergence of further GntR sub-families is highly likely and preliminary screening of bacterial genomes for novel C-terminal domains fused to a GntR-like DNA-binding domain has already revealed three new effector-binding domain topologies (Rigali, Unpublished). The structure/function relationship of these new GntR domains is currently under investigation in order to highlight whether these chimeric anomalies have emerged within strains in response to a specific need or a particular environmental context.

VI. DNA BINDING, OPERATOR SEQUENCES AND REGULATION

The HTH DNA-binding domain is the best characterised of all transcription factors, exhibiting significant structural and functional versatility despite its simple structural scaffold (Aravind *et al.*, 2005). Most prokaryotic HTH transcriptional regulators bind DNA as homodimeric proteins either stable in solution or dimerisation occurring on binding (Raman *et al.*, 1997). Studies on FadR (Raman *et al.*, 1997; van Aalten *et al.*, 2000a, 2001) demonstrated the protein binds DNA as a dimer through interaction of specific regions of the C-terminal domain, as does the crystal structure of PhnF (Gorelik *et al.*, 2006). Surface-enhanced laser desorption ionisation (SELDI) mass spectrometry analysis of DevA also revealed a likely dimeric arrangement of this protein (Jakimowicz and Hoskisson, Unpublished). These data suggest that this is a common arrangement of these proteins; however, this must also be considered along with the operator-binding site. Operator-binding sites can be in the form of

inverted repeats or as directed repeats (Rigali *et al.*, 2002). The directed repeat arrangement of the operator would impact on the dimerisation mode and also on the arrangement of the operator site, which would lack the classical palindromic sequence. This lack of a symmetrical operator is the likely arrangement for TraR (Rossbach *et al.*, 1994), AphS (Arai *et al.*, 1999), BphS (Watanabe *et al.*, 2000) and FucR (Hooper *et al.*, 1999).

Rigali *et al.* (2004) suggested that sterical constraints on the HTH domain from the binding of effector molecules ultimately impact on the accommodation of the HTH in *cis*-acting elements. Considering this, alignment of the known symmetrical operator sequences by Rigali *et al.* (2002) showed that the family binds a palindromic operator comprising 5′-$(N)_y$GT$(N)_x$AC$(N)_y$-3′ with the number of residues (y) and the nature of the central residues (x) varying. Interestingly the operator sites of the HutC, FadR and YtrA sub-families show degrees of family identity suggesting a common ancestry. The FadR consensus is 5′-t.GTa.tAC.a-3′ and HutC sub-family consensus is 5′-GT.ta.AC-3′, which indicates high levels of conservation in the HTH domain, which is also demonstrated in the phylogenetic tree presented in Rigali *et al.* (2002). The YtrA sub-family shows only 5′-GT.AC3′ identity over a much larger palindromic sequence; this diversity observed in the YtrA operator is also reflected in the looser phylogenetic association of the family members. The remaining sub-families do not have enough or any experimentally confirmed binding sites to draw conclusions on operator sites. However given the negative autoregulatory nature of many of these proteins, insight can be gained from analysis of the upstream region of the GntR regulator. This approach was used by Rigali *et al.* (2004) in *Streptomyces coelicolor*. Using a systematic analysis of the HutC sub-family members in the *S. coelicolor* genome a consensus sequence was identified for this grouping (5′-GT-$N_{(1)}$-TA-$N_{(1)}$-AC-3′) which was then searched against the genome. The presumed target genes are then identified through appropriate location of the *cis*-acting site and a list of candidate genes for testing experimentally is compiled. Compilation of a weight matrix refined the consensus sequence and the most likely GntR regulator could then be identified. Rigali and co-workers found this strategy worked extremely well, enabling insight into the regulon of DasR through experimental confirmation of the *in silico* predictions. This work identified genes of the phosphotransferase system specific for the uptake of *N*-acetylglucosamine, and electrophoretic mobility assays confirmed their promoters were bound by DasR. Indicating that such *in silico* approaches can identify targets for experimental work. Further development of this method led to the inception of the PREDetector tool (Hiard *et al.*, 2007) which allowed the weight matrix to be constructed for all bacterial genomes to help elucidate the regulons of a range of DNA-Binding proteins.

The identification and characterisation of GntR regulators by many authors has led to increasing numbers of binding sites being characterised and identified. Interestingly the majority of these studies have demonstrated the negative autoregulatory nature of the GntR regulator. It is not unprecedented that these proteins can also act as activator proteins. In *E. coli* the FadR regulator is known to regulate 12 genes or operons, repressing genes encoding enzymes for the catabolism of fatty acids (including *fadL*, *fadD*, *fadE*, *fadBA* and *fadH*) and activating the genes of the anabolic fatty acid pathway (DiRusso and Nyström, 1998; Henry and Cronan, 1991). Indeed it is fascinating from a physiological point of view that this metabolic-responsive regulator acts in a positive and negative way to maintain poise within lipid metabolism through binding of lipid intermediates. Another member of the FadR sub-family exhibits this dual regulation role; NorG from *Staphylococcus aureus* appears to act as a repressor of an ABC-like transporter involved in cell wall autolysis and a direct activator of drug efflux proteins (Truong-Bolduc and Hooper, 2007). Interestingly transcriptional activation by GntR regulators has been observed in the citrate regulator CitO in *enterococcus* (Blancato *et al.*, 2008), antibiotic biosynthesis in *Serratia* (Fineran *et al.*, 2005), Taurine utilisation via TauR in *Rhodobacter* (Wiethaus *et al.*, 2008). Whether activator function or activator/repressor function is widespread within this family is unknown and at this point it is impossible to elucidate with reliability the differences in positively and negatively regulated operator sites. Yet it does indicate the flexibility of this modular metabolite-responsive regulator framework and sets scene for further investigation.

VII. EVOLUTION OF GntR REGULATORS

Several families of HTH-containing transcription factors are conserved throughout the bacteria and archaea. Despite the high levels of horizontal gene transfer between these groups, the distribution the GntR regulators suggests that a pan-bacterial and pan-archaeal distribution may represent a lineage that can be traced back to the last universal common ancestor, indicating that this domain is extremely ancient (Aravind *et al.*, 2005). The recruitment of sensor domains to the HTH domain has occurred frequently throughout evolution and has resulted in the diversity of functions known for this group of proteins. The sub-family separation observed for the GntR regulators indicates that domains may have been swapped or fused, creating chimeric proteins able to respond to novel metabolites and regulate cellular processes in response to changing conditions. Although there is little direct evidence for evolution of GntR regulators in this manner we can infer several scenarios from genome comparisons and sequence analysis.

The presence of multiple E-b/O domains that have fused to a common HTH domain accounts for the observed sub-families in Rigali *et al*. (2002), with the increase in each lineage likely to be the result of horizontal transfer and gene duplication events (Hoskisson, unpublished; Rigali *et al.*, 2002). This scenario would account for the higher levels of sequence similarity amongst sub-family members.

Interestingly the fusion of the HTH domain can be explained with two examples: the presence of aminotransferase-like proteins in the MocR sub-family and the presence of enzyme-like protein folds in HutC sub-family members. Fusion of genes can occur through gene fission, horizontal gene transfer or gene decay (Suhre and Claverie, 2004), so it is clear that adjacent genes can easily become fused with HTH domains. The recruitment of protein–protein interaction domains through this route may also explain the frequency of dimeric interactions in this and other HTH-containing families. Analysis of the HutC GntR-like protein in *Pseudomonas* by Aravind and Anantharaman (2003) using iterative BLAST searching on the C-terminal E-b/O domain recovered the chorismate lyase protein (UbiC) of *E. coli*. Structural analysis *in silico* revealed that this specific protein fold is widespread and is likely to have evolved for ligand binding and evolved into the enzyme chorismate lyase, whilst another version was recruited to the HTH domain and diversified to interact with various ligands from which it can mediate gene expression through ligand binding and the HTH domain.

A second scenario is gene duplication, which is an important evolutionary force that provides an organism an opportunity to evolve new functions. One of the duplicated gene copies diverges, to acquire differential regulation, or mutations occur, followed by evolution into a gene product with a new function. In the case of oligomeric proteins, duplicate copies sometimes evolve to function as hetero-oligomers (Dickson *et al*., 2000). Duplication is also used as a mechanism to acquire a varied substrate spectrum. Thus, functional variations and differential regulation can be obtained as a result of gene duplication and provide an adaptive or fitness advantage in the natural environment. Indeed, data available for *E. coli* and *S. cerevisae* suggest that gene duplication plays a key role in the growth of gene networks (Teichmann and Babu, 2004). Classically, gene duplication is thought to enable duplicates to become specialised in different tissues or developmental stages (Ohno, 1970). Recently, we identified a novel developmental locus, *devA*, in *S. coelicolor* (Hoskisson *et al.*, 2006). It belongs to a novel sub-family of GntR regulators, of which several others have been implicated in development of *S. coelicolor* (see Section X). Interestingly, the *devA* gene is duplicated on the chromosome of all sequenced streptomycetes (with the exception of *S. griseus*; Hoskisson, Unpublished). Each paralog exhibits around 60% identity to each other, and suggests divergence following the duplication event and

represents an ideal model for studying evolution of regulatory proteins following duplication and on evolution of GntR regulators.

VIII. GntR REGULATORS IN PRIMARY METABOLISM

Given that GntR regulators respond to many and varied metabolites it is unsurprising that they have been recruited to regulate many primary metabolic processes, responding to changing metabolite concentrations to modulate genes expression. This enables the cell to respond to rapidly changing conditions, or maintain precise balance of specific metabolites. GntR regulators have been shown to play roles in maintenance of fatty acids in response to changing fatty acid concentrations (DiRusso and Nyström, 1998), amino acid catabolism (Allison and Phillips, 1990; Hänssler *et al.*, 2007; Ortuño-Olea and Durán-Vargas, 2000), organic acids (Fujita and Fujita, 1987; Lee *et al.*, 2000; Morawski *et al.*, 2000; Núñez *et al.*, 2001; Pellicer, *et al.*, 1996; Robert-Baudouy *et al.*, 1981; Shulami *et al.*, 1999), regulation of carbon catabolism (Mota *et al.*, 1999; Rigali *et al.*, 2002, 2006; Titgemeyer *et al.*, 1995) and degradation of complex organics (Arai *et al.*, 1999; Watanabe *et al.*, 2000). The range of metabolites bound by GntR regulators is reflected in the diversity of C-terminal domains, and our understanding of how these regulators accommodate and respond to different metabolites is likely to increase in the future.

IX. GntR REGULATORS IN VIRULENCE

Despite the large numbers of GntR regulators associated with metabolism it is increasingly becoming apparent that these proteins can regulate virulence in pathogenic bacteria. It is likely that these proteins regulate virulence through responding to host factors through sensing and responding to specific metabolites. Recently studies have shown that the mammalian cell entry (mce) genes of *Mycobacterium tuberculosis* are regulated by a GntR regulator, *mce1R* (Casali *et al.*, 2006). Mce1R is negatively autoregulated and a mutant is impaired in its ability to survive in murine models. Mce1R is a member of the FadR sub-family and recently this gene cluster has been implicated in cholesterol transport (Mohn *et al.*, 2008) which fits with the types of molecules bound by other members this sub-family.

A screen for mutants that were impaired in virulence in *Brucella melitensis* identified three GntR regulators (Haine *et al.*, 2005). Further work is required to elucidate the regulon of these proteins, but it opens a new avenue of research into how these proteins control virulence.

Interestingly these studies again highlight the need to develop robust methods for identifying the metabolites bound by these proteins, to understand how pathogenic organisms integrate host signals to increase survival *in vivo*.

X. GntR REGULATORS IN *STREPTOMYCES* DEVELOPMENT AND ANTIBIOTIC PRODUCTION

Several GntR-like regulators have been implicated in development of *S. coelicolor* such as WhiH (Ryding *et al.*, 1998), *agl3R* and SE69 (Hillerich and Westpheling, 2006; Sprusansky *et al.*, 2003), yet none of their effector molecules are known. However a remarkable example of a GntR superfamily member acting as a master switch of gene expression is provided by DasR (Deficient in aerial mycelium and spore formation) whose predicted regulon includes hundreds of genes in *S. coelicolor* (Rigali *et al.*, 2008). The 'core' of the DasR regulon consists of chitinase genes, and genes involved in the transport and utilisation of aminosugars such as *N*,*N*′-diacetylchitobiose by the DasABC ATP-binding cassette transporter (Colson *et al.*, 2008) and *N*-acetylglucosamine (GlcNAc) by the phosphoenolpyruvate phosphotransferase uptake system (PTS^{GlcNAc}) (Rigali *et al.*, 2006). The importance of chitin's contribution to primary metabolism is obvious from end products of its complete catabolism via enzymes encoded by *nagA* (*N*-acetylglucosamine-6-phosphate deacetylase) and *nagB* (Glucosamine-6-phosphate isomerase), both DasR-dependent genes. These end products are acetate, ammonia, and fructose-6-phosphate, which are further metabolised via the tricarboxylic acid cycle, fatty acid metabolism, nitrogen metabolism and glycolysis. By controlling the access to the metabolites at the crossroad of these major catabolic pathways, DasR imposes itself as an essential checkpoint to sense and enable the cell to respond to the environment. In addition to the obvious exploitation of this rich carbon and nitrogen source in primary metabolism, GlcNAc has been demonstrated to be a strong nutritional signal for both morphological (sporulation) and physiological (secondary metabolites production) differentiations in *S. coelicolor*. Indeed, under rich culture conditions, the addition of GlcNAc completely blocks development (Rigali *et al.*, 2006), while on minimal media the addition of the same molecule triggers *S. coelicolor* antibiotic production (Rigali *et al.*, 2008). The inactivation of *dasR*, *dasA* and central PTS^{GlcNAc} genes results in developmental arrest of *S. coelicolor*, clearly indicating that sensing and uptake of chitin and/or cell wall and their degradation products are key elements in the decision of streptomycetes to produce reproductive aerial mycelium (Colson *et al.*, 2008; Rigali *et al.*, 2006).

Recently, in *S. coelicolor* we identified another GntR-like regulator of development, *devA*, which forms a new sub-family of these regulators (Hoskisson *et al.*, 2006). Interestingly, the *devA* gene is duplicated (*devE*) on the chromosome of all sequenced streptomycetes including the adjacent divergently transcribed genes *devC* and *devD* genes. *devA* is co-transcribed with a putative phosphatase/hydrolase, *devB*, which is of unknown function, but it would appear that tight control of *devB* during growth is required for normal development, as a mutant of *devB* is conditionally bald. *devB* is also unusual in that it is one of only a handful of developmental genes known in *Streptomyces* that does not encode a regulatory protein.

The recruitment of metabolite-responsive regulators to sporulation and antibiotic production pathways demonstrates that tight control of these developmental gene cascades is required to respond to changing environmental conditions to ensure survival in stressful or unfavourable conditions, linking primary metabolites to secondary metabolism and development.

XI. BIOTECHNOLOGY IMPLICATIONS

The tight regulation and highly responsive nature of the GntR superfamily of regulators offers an interesting opportunity in the development of inducible expression systems. The modular nature of these regulators enables the creation of chimeric proteins that respond rapidly and predictably to changing conditions, whether these are intrinsic changes in the environment or through the addition of inducer molecules. Whilst a great understanding of the molecular mechanisms of regulation is required, the three interacting components of the GntR regulator system can be exploited to facilitate the construction of vector systems containing well-characterised operator, DNA-binding domain and a metabolite-responsive effector-binding domain, whether natural or synthesised by molecular biology.

The elucidation of the GlcNAc/DasR regulon has highlighted the first complete signalling cascade for antibiotic production from nutrient sensors to antibiotic pathway-specific activators leading to a new strategy for activating pathways for secondary metabolite biosynthesis in streptomycetes, thereby offering new prospects in the fight against multi-drug resistant pathogens and cancers. Activating these pathways has proved challenging and several approaches to activate these pathways have proved unsuccessful. Rigali *et al.* (2008) demonstrated that *N*-acetylglucosamine (GlcNAc) acts as a checkpoint in the onset of secondary metabolism in several streptomycetes and a simple screen, using the addition of GlcNAc to cultures, is sufficient to induce antibiotic pathways through

derepression of DasR-regulated promoters and offers the opportunity to develop novel bioactive metabolites for the clinic in a wide range of producing strains.

XII. CONCLUDING REMARKS

The one-component regulatory proteins of the GntR family represent a highly diverse group of proteins from a structural, functional and biochemical aspect. They are widespread throughout the bacterial kingdom and regulate a wide range of processes from primary metabolism to developmental processes. The fusion of a DNA-binding domain to a diverse range of effector-binding/oligomerisation domains enables flexibility and has probably contributed to the expansion of the gene family through recruitment of new effector-binding/oligomerisation domains. This flexibility makes these proteins prime candidates for use in biotechnology creating highly effective inducible systems, responding to well-characterised metabolites in a rapid and predictable way.

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CHAPTER 2

Biogenesis of the Cell Wall and Other Glycoconjugates of *Mycobacterium tuberculosis*

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Abstract The re-emergence of tuberculosis in its present-day manifestations – single, multiple and extensive drug-resistant forms and as HIV-TB coinfections – has resulted in renewed research on fundamental questions such as the nature of the organism itself, *Mycobacterium tuberculosis*, the molecular basis of its pathogenesis, definition of the immunological response in animal models and humans, and development of new intervention strategies such as vaccines and drugs. Foremost among these developments has been the precise chemical definition of the complex and distinctive cell wall of *M. tuberculosis*, elucidation of the relevant pathways and underlying genetics responsible for the synthesis of the hallmark moieties of the tubercle bacillus such as the mycolic acid–arabinogalactan–peptidoglycan complex, the phthiocerol- and trehalose-containing effector lipids, the phosphatidylinositol-containing mannosides, lipomannosides and lipoarabinomannosides, major immunomodulators, and others. In this review, the laboratory personnel who have been the focal point of some to these developments review recent progress towards a comprehensive understanding of the basic physiology and functions of the cell wall of *M. tuberculosis*.

ABBREVIATIONS

Ara*f*	arabino-furanose
Gal*f*	galacto-furanose
Gal*p*	galacto-pyranose
LAM	lipoarabinomannan
LM	lipomannan
Man*p*	manno-pyranose
myo-Ins	*myo*-Inositol
PG	peptidoglycan
PI	phosphatidyl-*myo*-inositol
PIMs	phosphatidyl-*myo*-inositol mannosides
PPM	polyprenol-monophospho-mannose

I. INTRODUCTION

The latest complete data as assembled by such bodies as the World Health Organization (http://www.who.int/tb/publications/global_report/en/index.html, 2008) indicate an estimated 9.2 million new cases of TB in 2006 (139 per 100,000 population), including 4.1 million new smear-positive cases (44% of the total) and 0.7 million HIV-positive cases (8% of the total). This is an increase from 9.1 million cases in 2005, due to population growth. India, China, Indonesia, South Africa and Nigeria

rank first to fifth, respectively, in terms of absolute numbers of cases. The African Region has the highest incidence rate per capita (363 per 100,000 population). There were an estimated 14.4 million prevalent cases of TB in 2006. There were an estimated 0.5 million cases of multi-drug-resistant TB (MDR-TB) in 2006 and an estimated 1.5 million deaths from TB in HIV-negative people and 0.2 million among people infected with HIV.

What is now recognized as a human tragedy of the first order, compounded by the fear of even more widespread dissemination of MDR and XDR strains of *Mycobacterium tuberculosis*, has had the benefit of evoking new interest and resulting research towards a better understanding of the organism and its effects on the host, driven by the need for new diagnostic tools and new intervention strategies, whether chemotherapeutic or immunological. The cell wall of *M. tuberculosis*, its most distinctive feature and the basis of much of the characteristics of tuberculosis pathogenesis, is a case in point benefitting from this renewed attention.

The envelope of *M. tuberculosis* consists of two distinct parts: the plasma membrane and the cell wall as such (Brennan and Draper, 1994); the question of a true capsule is controversial (see below). Clearly, *M. tuberculosis*, especially within a host, is devoid of a classical capsule adhering to the cell with sufficient tenacity to be observed by simple techniques such as negative staining and India ink. On the other hand extracellular materials do exist – glucans, arabinans and arabinomannans and some proteins, mostly reflective of internal structures – representing more of an outer layer than a true capsule (Daffé and Draper, 1998). As for Gram-positive bacteria, there probably exists an anatomically and functionally distinct periplasmic space (Matias and Beveridge, 2006). The cell wall and the membrane can readily be mechanically separated and have been studied independently (Crick and Brennan, 2008). Repeated attempts have been made to relate chemical and ultrastructural features, and these attempts have been successful as far as the major components and compartments of the cell envelope are concerned, such as those of the insoluble 'cell wall core', namely peptidoglycan (PG), arabinogalactan (AG), and mycolic acids and the plasma membrane. Thus, the images of two electron dense layers separated by a transparent zone are in accord with a typical plasma membrane bilayer. Additional triple-layered images have been attributed to the cell wall proper, and these consist of an inner layer of moderate electron density, reflective of mycobacterial peptidoglycan; a wider electron-transparent layer, attributable to the arabinogalactan–mycolic acid complex; and an outer electron opaque layer of extremely variable appearance and thickness, apparently the negatively charged so-called outer capsular layer containing mostly polysaccharides, glucans, arabinans and arabinomannans (Lemassu and Daffé, 1994; Ortalo-Magné *et al*., 1995).

However, the arrangement and the distribution of the numerous soluble cell wall entities, such as lipoarabinomannan (LAM) and lipomannan (LM), the other phosphatidylinositol-containing glycolipids, the phthiocerol- and trehalose-containing lipids, (lipo)proteins, so important to the pathogenesis of *M. tuberculosis*, are poorly understood. An exception is the elegant topological studies of the MspA porin in the cell wall of *Mycobacterium smegmatis* and its possible extrapolations to *M. tuberculosis* (Faller *et al.*, 2004) and, in that context, the clear evidence from cryo-electron tomography of an outermost morphologically symmetrical lipid bilayer, despite the presence of considerable amounts of mycolic acids with their inherently asymmetrical hydrocarbon chains (Hoffmann *et al.*, 2008; Zuber *et al.*, 2008). In this review we present the rather spectacular progress made since the re-emergence of TB as a pressing public health problem, in the chemical definition of the cell wall/envelope of *M. tuberculosis*, in its assembly and underlying genetics, and functions in the disease induction process.

II. THE MYCOBACTERIAL CELL ENVELOPE

The cell envelope distinguishes species of the *Mycobacterium* genus from other prokaryotes. It consists of three major segments: the plasma membrane, the cell wall core and the outermost layer (Fig. 2.1). The cell wall core is made up of PG in covalent attachment via phosphoryl-*N*-acetylglucosaminosylrhamnosyl linkage units with AG which is in turn esterified to α-alkyl, β-hydroxy long-chain (C_{70}–C_{90}) fatty acids known as the mycolic acids. The outermost layer consists of a variety of non-covalently attached (glyco)lipids, polysaccharides, lipoglycans and proteins, including pore-forming proteins. In *M. tuberculosis*, the surface-exposed material of this outer layer, also called capsule, is essentially composed of polysaccharides and proteins with only minor amounts of lipids (Lemassu and Daffé, 1994; Ortalo-Magné *et al.*, 1995). Thus, *M. tuberculosis* is known to expose a hydrophilic surface.

Until recently, our view of the cell envelope architecture was essentially based on the model proposed by Minnikin (1982). In this model, the mycolic acids are packed in a monolayer, parallel to each other, and oriented perpendicular to the plasma membrane. Interspersed somehow are the free lipids of the outer membrane (OM) with their fatty acyl chains intercalating into the mycolic acid layer. It was proposed that the covalently bound mycolic acids of the cell wall core and the free lipids of the outermost layer form the inner and the outer leaflets, respectively, of a highly impermeable asymmetrical bilayer that confers to mycobacteria their characteristic resistance to many therapeutic agents (Jarlier and Nikaido, 1994; Minnikin, 1982). A second model proposed by Rastogi (1991)

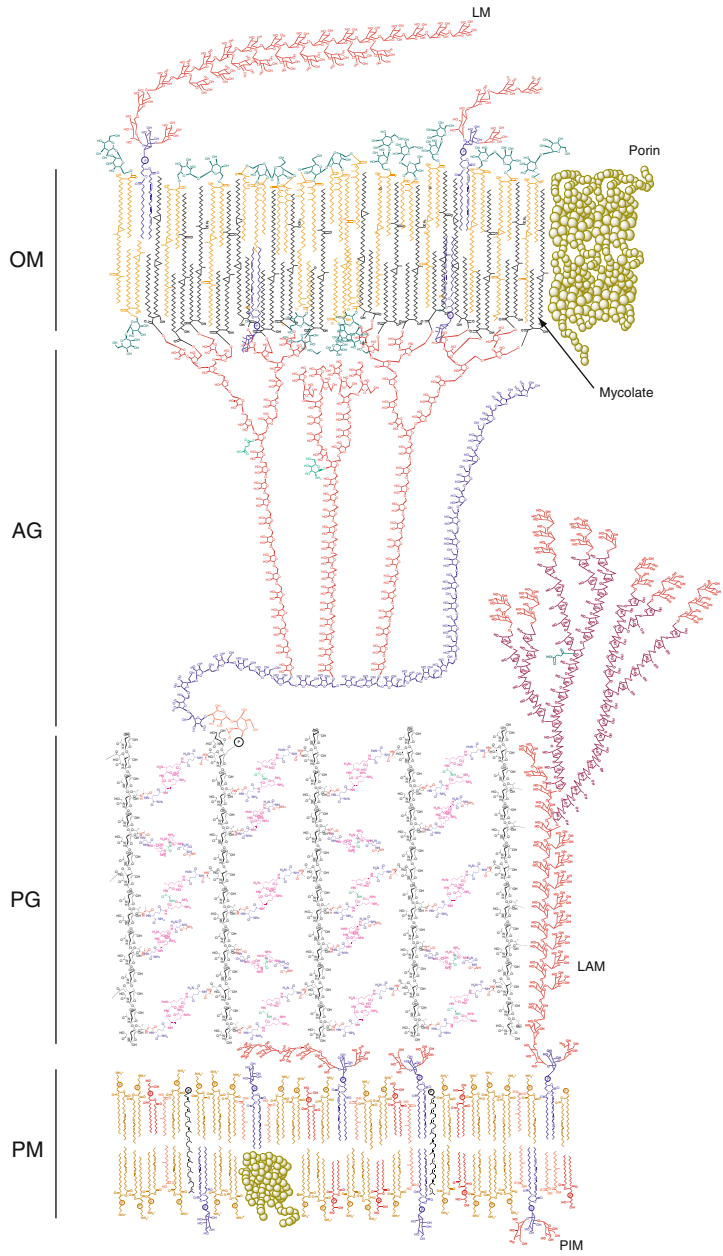

FIGURE 2.1 A current perspective of the cell envelope of *Mycobacterium tuberculosis*. Represented is a theoretical model of the outer membrane (OM) wherein similar extractable lipids are present in both leaflets and the meromycolates of bound mycolic acids span the entire hydrophobic region. Another model proposes the meromycolate

suggested a similar organization except that the non-covalently linked lipids form a monolayer that does not intercalate with the mycolic acids. Only recently have developments in microscopy techniques allowed the different layers of the mycobacterial cell envelope to be visualized in their native state (Hoffmann *et al.*, 2008; Zuber *et al.*, 2008). Cryo-electron microscopy observations of vitreous sections of *M. smegmatis*, *M. bovis* BCG and of the closely related *Corynebacterium glutamicum* provided direct evidence of the existence of an outer bilayer and periplasmic space in these species. Although these studies confirmed mycolic acids as key components of the OM, the findings that the outer bilayer is apparently symmetric and much thinner than expected from the proposed models led to a significant revision of the current view of the cell envelope's architecture. In a revised model presented in Fig. 2.1, similar extractable lipids are present in both leaflets of the OM and the meromycolate chain of bound mycolic acids span the entire hydrophobic region (Hoffman *et al.*, 2008). Another model proposes the meromycolate chains of mycolic acids to be folded upon themselves to create a more compact structure, compatible with the observed thickness of the OM (Zuber *et al.*, 2008).

III. THE CAPSULAR POLYSACCHARIDES

The outermost compartment of the cell envelope of pathogenic mycobacterial species consists of a loosely bound structure called capsule (Chapman *et al.*, 1959; Daffé and Draper, 1998; Hanks, 1961). Although mycobacteria shed some of this material in the culture medium during *in vitro* growth (Ortalo-Magné *et al.*, 1995), capsular components clearly coat *in vivo*-grown bacteria (Schwebach *et al.*, 2002), probably retained by the phagosomal membrane (Daffé and Draper, 1998). The capsular material primarily consists of proteins and polysaccharides (~97% of the total material) with only small amounts of lipids (Lemassu and Daffé, 1994; Ortalo-Magné *et al.*, 1995).

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chains to be folded upon themselves to create a more compact structure, compatible with the observed thickness of the OM (see text for details). The capsular material is not represented here. Extractable lipids represented in the outer membrane include PIMs and acyltrehaloses whereas those of the plasma membrane include phospholipids and PIMs. PM, plasma membrane; PG, peptidoglycan; AG, arabinogalactan; OM, outer membrane. The Gal*f* and Ara*f* residues of AG are represented in blue and red, respectively. The succinyl and galactosamine residues of AG are in green. Man*p* residues in PIM, LM and LAM are in red; Ara*f* residues are purple.

Three types of polysaccharides are found in the capsular material of *M. tuberculosis*: a high-molecular-weight (>100,000 Da) α-D-glucan composed of a →4-α-D-Glc-1→ core branched every five or six residues by oligoglucosides, D-arabino-D-mannan (AM), and a D-mannan composed of a →6-α-D-Man-1→ core substituted at some positions 2 with a α-D-Man residue (Dinadayala *et al.*, 2004; Lemassu and Daffé, 1994; Ortalo-Magné *et al.*, 1995). All are neutral compounds, devoid of acyl substituents (Lemassu and Daffé, 1994). The structure of AM appears to be identical to that of LAM (see Section IV.A), except for the loss of the phosphatidyl-*myo*-inositol anchor, suggesting that it may be formed from LAM by a specific hydrolytic enzyme. However, there is at present no evidence for this hypothesis.

α-D-glucan is the major carbohydrate constituent of the capsule of *M. tuberculosis*, representing up to 80% of the extracellular polysaccharides. Recent studies have begun to shed light on its biosynthesis. Based on the observation that α-D-glucan displays a glycogen-like structure, the orthologues of the *glg* genes involved in the biosynthesis of glycogen in *Escherichia coli* were identified in *M. tuberculosis* H37Rv and inactivated by allelic replacement. Biochemical analyses of the mutants indicated that the biosynthetic pathways of α-D-glucan and glycogen involve common enzymes, among which are the α-1,4-glucosyltransferases Rv3032 and GlgA (Rv1212c) (Table 2.1), the ADP-glucose pyrophosphorylase GlgC (Rv1213) and the branching enzyme GlgB (Rv1326c) (Sambou *et al.*, 2008) (see Section VI.B). The disruption of *glgC* or *glgA* reduces by half the capsular α-D-glucan content of *M. tuberculosis* H37Rv. The involvement of common enzymes in the synthesis of the intracellular glycogen and capsular α-D-glucan suggests that the latter polysaccharide is synthesized inside the cytoplasm before being translocated to the cell surface by as yet unknown transporters.

Various biological functions have been associated with the polysaccharides of the capsule suggestive of a role in immunopathogenesis. Cywes *et al.* showed that capsular polysaccharides, among which the α-D-glucan, mediated the non-opsonic binding of *M. tuberculosis* H37Rv to Complement Receptor 3 (Cywes *et al.*, 1997). Given that CR3 is one of the principal phagocytic receptors of monocytes and neutrophils and that CR3-mediated phagocytosis can result in the diminution or absence of respiratory burst and suppression of IL-12 secretion, it was proposed that this route of entry may be favourable to the intracellular survival of the tubercle bacillus (Ehlers and Daffé, 1998; Fenton *et al.*, 2005). Other studies have highlighted the anti-phagocytic and immunomodulatory activities of the capsular polysaccharides of *M. tuberculosis* (Gagliardi *et al.*, 2007; Stokes *et al.*, 2004). Owing to its glycogen-like structure, α-D-glucan was also proposed to be involved in *M. tuberculosis* evasion of the immune

TABLE 2.1 The glycosyltransferases of *M. tuberculosis*

Protein		Function	GT fold	CAZy family	PDB code	References
PIM, LM and LAM						
Rv2610c	PimA	α-D-Mannose-α-(1,2)-phosphatidyl; *myo*-inositol mannosyltransferase	GT-B	GT4	**2GEJ (79)**	Korduláková *et al.* (2002)
Rv0557	PimB	Mannosyltransferase	GT-B	GT4	2gej (23), 3c4v (20), 2jjm (19), 2iv3 (18), 2r66 (14), 2iw1 (13)	Schaeffer *et al.* (1999) and Tatituri *et al.* (2007)
Rv2188c	PimB′	α-D-Mannose-α-(1,6)-phosphatidyl; *myo*-inositol mannosyltransferase	GT-B	GT4	2gej (24), 2jjm/3c4v (23), 2iw1 (19), 2r66 (15), 2iv3 (16)	Lea-Smith *et al.* (2008)
Rv2051c	Ppm1	Polyprenol phosphomannose synthase	GT-A	GT2	1qgq (10), 3bcv (17)[a]	Gurcha *et al.* (2002)
Rv1159	PimE	Polyprenol-P-Man; α-(1,2)-mannosyltransferase	GT-C	GT87	N.D.	Morita *et al.* (2006)
Rv2174	MptA	Polyprenol-P-Man; α-(1,6)-mannosyltransferase	GT-C	NC	N.D.	Kaur *et al.* (2007) and Mishra *et al.* (2007)
Rv1459c	MptB	Polyprenol-P-Man; α-(1,6)-mannosyltransferase	GT-C	NC	N.D.	Mishra *et al.* (2008)

Rv2181		Polyprenol-P-Man lipo (arabino)mannan; α-(1,2)-mannosyltransferase	GT-C	GT87	N.D.	Kaur *et al.* (2006, 2008)
Rv3793	EmbC	Arabinosyltransferase	GT-C	GT53	N.D.	Zhang *et al.* (2003)
Rv1635c		Polyprenol-P-Man ManLAM-capping; α-(1,2)-mannosyltransferase	GT-C	GT83	N.D.	Dinadayala *et al.* (2006)
Arabinogalactan						
Rv3265c	WbbL	dTDP-Rha: α-D-GlcNAc-PP-C_{50} α-(1,3)-L-Rhamnosyltransferase	GT-A	GT2	1qgq (12), 3bcv (14)	Mills *et al.* (2004)
Rv3782	GlfT1	UDP-Gal*f* : Galactan β-(1,4)-/β-(1,5)-galactofuranosyltransferase	GT-A	GT2	1qgq (13), 3bcv (14)	Belanová *et al.* (2008) and Mikušová *et al.* (2006)
Rv3808c	GlfT2	UDP-Gal*f* : Galactan β-(1,5)-/β-(1,6)-galactofuranosyltransferase	GT-A	GT2	1qgq (9), 3bcv (11)[a]	Belanová *et al.* (2008), Kremer *et al.* (2001) and Mikušová *et al.* (2000)
Rv3792	AftA	Arabinosyltransferase	GT-C	GT85	N.D.	Alderwick *et al.* (2006)

(*continued*)

TABLE 2.1 (*continued*)

Protein		Function	GT fold	CAZy family	PDB code	References
Rv3794	EmbA	Arabinosyltransferase	GT-C	GT53	N.D.	Escuyer *et al.* (2001)
Rv3795	EmbB	Arabinosyltransferase	GT-C	GT53	N.D.	Escuyer *et al.* (2001)
Rv3805c	AftB	β-(1,2) Arabinosyltransferase	GT-C	GT89	N.D.	Seidel *et al.* (2007)
Rv2673	AftC	α-(1,3) Arabinosyltransferase	GT-C	NC	N.D.	Birch *et al.* (2008)
Glycogen, glucan and MGLPs						
Rv3032		α-(1,4)-Glucosyltransferase	GT-B	GT4	3c4v (26), 2gej (24), 2jjm/2iw1 (19), 2r66 (16), 2iv3 (17)	Sambou *et al.* (2008) and Stadthagen *et al.* (2007)
Rv1212c	GlgA	α-(1,4)-Glucosyltransferase	GT-B	GT4	3c4v (26), 2gej (21), 2jjm (20), 2iv3 (17), 2r66 (16), 2iw1 (14)	Sambou *et al.* (2008)
Rv1208		Glucosyl-3-phosphoglycerate synthase	GT-A	GT81	**3CKQ (83)**	Empadinhas *et al.* (2008)
Acyltrehaloses, *p*HBADs and phenolic glycolipids						
Rv3490	OtsA	Trehalose-6-phosphate synthase	GT-B	GT20	1uqu (32)	Pan *et al.* (2002)

Rv2958c		PGL/*p*HBAD Rhamnosyltransferase	GT-B	GT1	1rrv (20), 1iir (18), 2d0r/2iyf/2p6p/ 2vg8/ 2c9z (17), 1pnv (16), 2iya/ 2acw/2pq6 (15)	Pérez *et al.* (2004)
Rv2962c		PGL/*p*HBAD Rhamnosyltransferase	GT-B	GT1	2d0r/1rrv/1pnv/ 1iir (17), 2iyf/ 2c9z/2iya (16), 2p6p (15), 2pq6 (14), 2vg8/2acw (13),	Pérez *et al.* (2004)
Rv2957		PGL/*p*HBAD Fucosyltransferase	GT-A	GT2	1qgq (15), 3bcv (16)	Pérez *et al.* (2004)
Mycothiol						
Rv0486	MshA	UDP-GlcNAc: 1L-*myo*-inositol-1-P α-*N*-acetylglucoaminyltransferase	GT-B	GT4	**3C4V (51)**	Newton *et al.* (2003)
Glycoproteins						
Rv1002c		Protein-*O*-mannosyltransferase	GT-C	GT39	N.D.	VanderVen *et al.* (2005)
LOS						
Rv1500	LosA	?	GT-A	GT2	1qgq (11), 3bcv (16)	Burguière *et al.* (2005)

Uncharacterized GTs: GT-A fold; Rv0539 (GT2), Rv0696 (GT2), Rv1514c (GT2), Rv1516c (GT2), Rv1518 (GT2), Rv1520 (GT2), Rv1525 (GT2), Rv3631 (GT2) and Rv3786c (GT2); GT-B fold, Rv1524 (GT1), Rv1526c (GT1), Rv2739c (GT1), Rv0225 (GT4), Rv2153c (GT28) and Rv1328 (GT35); GT-C fold, Rv0051 (N.C.), Rv0236c (N.C.), Rv0541c (N.C.), Rv1508c (N.C.) and Rv3779 (N.C.).N.C., Non-classified in CAZy (http://www.cazy.org); N.D., no structures determined.

PDB codes indicate representative protein structures for each GT family (see http://www.pdb.org). The percentage identity to the M. tuberculosis *protein is indicated between parentheses. PDB codes in bold indicate percentages of identity to the* M. tuberculosis *protein greater than 50%.*[a] Only the GT domain of the mycobacterial enzyme was used for sequence alignment.

system by molecular mimicry (Lemassu and Daffé, 1994). Independent from its role in tuberculosis, α-D-glucan was reported to exhibit anti-tumour activity (Wang *et al.*, 1995; Zlotta *et al.*, 2000).

IV. THE NON-COVALENTLY BOUND GLYCOCONJUGATES OF THE OUTER MEMBRANE

A. Phosphatidylinositol mannosides, lipomannan and lipoarabinomannan

1. Localization in the cell envelope and biological activities

One of the unique features of the mycobacterial cell envelope is its high content of mannosylated molecules, including (lipo)glycans and (lipo)glycoproteins. The mannosyl-phosphatidyl-*myo*-inositol-based glycolipids and metabolically related lipoglycans comprising phosphatidyl-*myo*-inositol mannosides (PIMs[1]), lipomannan (LM) and LAM in particular, are found in abundant quantities in the cell envelope of all mycobacterial species. These molecules are non-covalently anchored through their phosphatidyl-*myo*-inositol (PI) moiety to the inner and the outer membranes of the cell envelope (Pitarque *et al.*, 2008). The importance of these molecules in promoting the entry of *M. tuberculosis* inside phagocytic cells through mannose-specific C-type lectins, regulating phagosome maturation and modulating the host immune response *in vitro* has been well documented (for recent reviews, Briken *et al.*, 2004; Fenton *et al.*, 2005; Gilleron *et al.*, 2008). However, care should be taken in the interpretation of these studies since the majority of them have focused on the interactions of purified PIM, LM and LAM with cellular models and, thus, may not accurately reflect the relevance and individual contribution of these glycoconjugates in mycobacterial infections. In support of this assumption, a recent study on isogenic knock-out mutants of *Mycobacterium bovis* BCG and *Mycobacterium marinum* deficient in the synthesis of mannose-capped LAM (ManLAM) (see Section IV.A.2) revealed only marginal differences between wild-type and mutant strains in terms of uptake by phagocytic cells, phagosome/lysosome fusion, replication *in vivo* and induction of immune responses in infected animals (Appelmelk *et al.*, 2008). On the other hand, clinical isolates of *M. tuberculosis* defective in some aspects of the synthesis and exposure of ManLAM at the cell surface were found to display important defects in phagocytosis by human primary macrophages (Torrelles *et al.*, 2008). Further studies are thus clearly required to determine the individual contribution of ManLAM

[1] PIM is used to describe the global family of PIM that carries one to four fatty acids and one to six Man*p* residues. In Ac_xPIM_y, *x* refers to the number of acyl groups esterified to available hydroxyls on the Man*p* or *myo*-inositol residues, *y* refers to the number of Man*p* residues; for example Ac_1PIM_6 corresponds to the phosphatidylinositol hexa-mannoside PIM_6 carrying two acyl groups attached to the glycerol (the diacylglycerol substituent) and one acyl group esterified to the Man*p* residue.

to the pathogenicity of virulent species of the *Mycobacterium tuberculosis* complex. A better defined role for the most polar forms of PIMs (containing up to six Man*p* residues) in virulence has recently emerged from the study of an *M. tuberculosis pimE* mutant deficient in the production of these lipids. The mutant was found to be greatly impaired for replication and persistence in mice (Larrouy-Maumus *et al*., unpublished results). The impact that polar PIM production has on the permeability of the mycobacterial cell envelope is likely to account, at least in part, for the role of these lipids in intracellular survival (Larrouy-Maumus *et al*., unpublished results).

Despite nearly two decades of studies, the role of PIM, LM and LAM in the physiology of mycobacteria is less clear. Emerging data suggest that PIMs play important roles in the permeability barrier of the cell envelope, cell membrane integrity and regulation of cell septation and division (Korduláková *et al*., 2002; Larrouy-Maumus *et al*., unpublished results; Morita *et al*., 2005, 2006; Parish *et al*., 1997; Patterson *et al*., 2003). The physiological functions of LM and LAM are unknown. While the non-pathogenic species *Mycobacterium smegmatis* appears to be permissive to a variety of mutations affecting PIM, LM and LAM synthesis, the inability to generate mutants of *M. tuberculosis* deficient in the acylation of PIMs or LAM synthesis (Goude *et al*., 2008; Jackson *et al*., unpublished results) indicates that these molecules probably play a more crucial role in the physiology of the tubercle bacillus than in that of fast-growing *Mycobacterium* species.

2. Structure of PIM, LM and LAM

PIMs, LM and LAM all share a conserved PI anchor with mannosylation extension at the C-6 position of the *myo*-inositol (*myo*-Ins) indicative of a metabolic relationship (for a recent review on the structure of PIM, LM and LAM, see Gilleron *et al*., 2008) (Figs. 2.1 and 2.2A). The anchor structure is heterogeneous, with variations occurring with respect to the number, location and nature of the fatty acids. The major PIM species are PI-dimannosides (Ac_1PIM_2 and Ac_2PIM_2) and PI-hexamannosides (Ac_1PIM_6 and Ac_2PIM_6). Ac_1PIM_2 and Ac_1PIM_6 contain one fatty acid linked to the Man*p* residue attached to position 2 of *myo*-Ins in addition to a diacylglycerol moiety, whereas Ac_2PIM_2 and Ac_2PIM_6 contain a fourth fatty acyl chain linked to position 3 of *myo*-Ins (Figs. 2.1 and 2.2A). LM and LAM possess a mannan core composed on average of 20–25 $\alpha(1\rightarrow6)$-linked Man*p* residues occasionally substituted at C-2 by single Man*p* units in all mycobacterial species investigated with the exception of *M. chelonae* which was reported to have branching occurring at C-3 (Guérardel *et al*., 2002). The length of the mannan core of LM and degree of branching varies depending on the mycobacterial species. Recently, the use of MALDI-TOF mass spectrometry has allowed the precise length

of the mannan domains of LM in *M. bovis* BCG, *M. smegmatis* and *M. tuberculosis* H37Rv to be defined (Gilleron *et al.*, 2006; Kaur *et al.*, 2007). A few unanswered questions remain, however, concerning the structure of this molecule. Firstly, it is at present unclear whether the linear and branched portions of the mannan core form distinct domains or whether 2,6-Man*p* and 6-Man*p* units intercalate at frequent regular intervals within the chain. Secondly, little is known about the position and

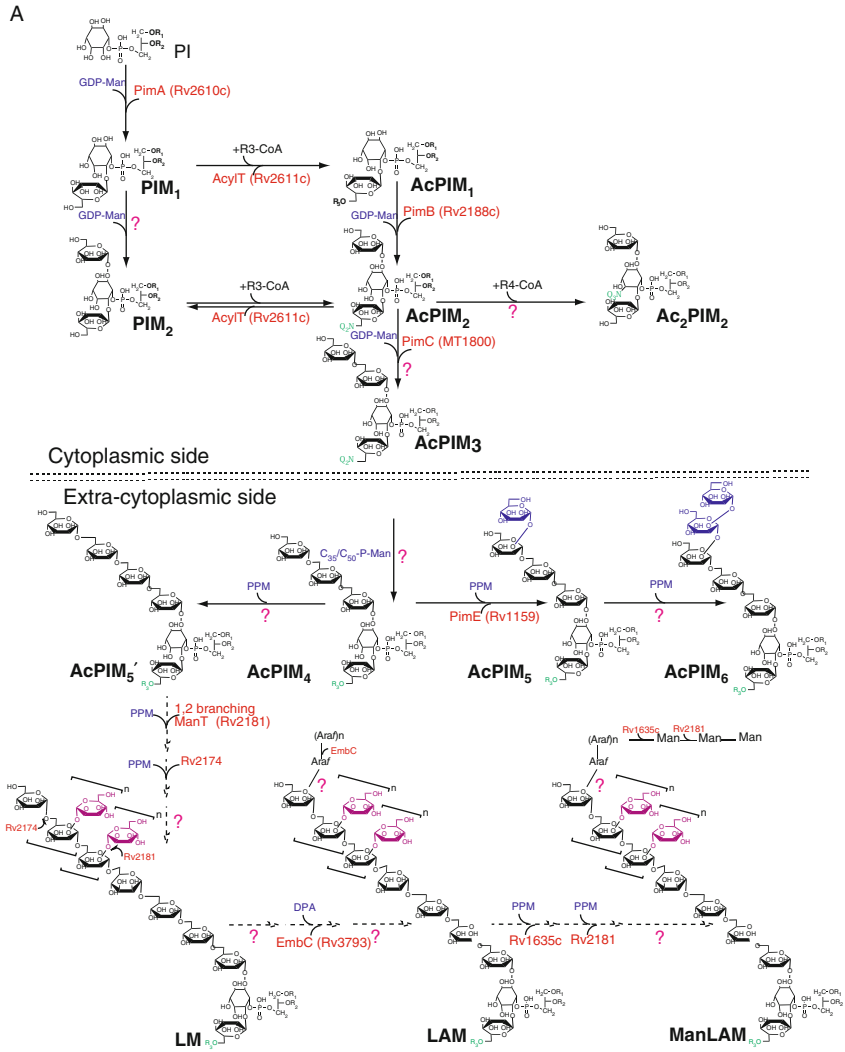

FIGURE 2.2 (Continued)

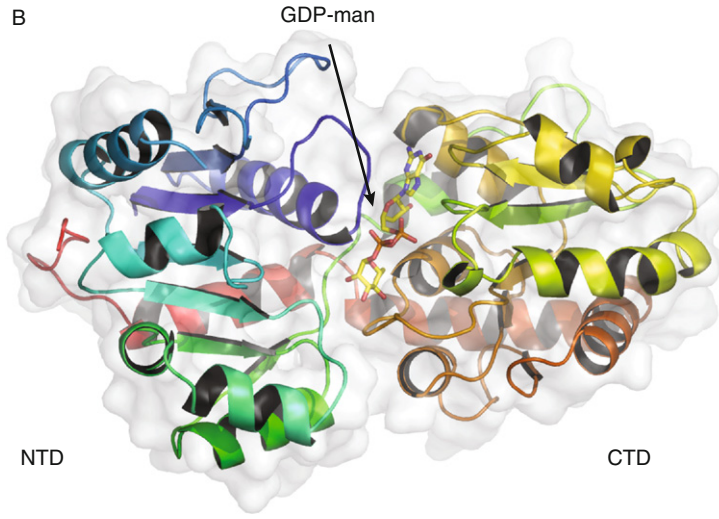

FIGURE 2.2 Biogenesis of PIM, LM and LAM. (A) Schematic representation of the current understanding of the PIM, LM and LAM biosynthetic pathways in *M. tuberculosis*. (B) Three-dimensional structure of a PimA–GDP-Man complex. Representation of the monomeric form of PimA from *M. smegmatis*. The structure is "colour ramped" from the N terminus (NTD) (blue) to the C terminus (CTD) (red).

number of arabinofuranose (Ara*f*) residues attached to the LM backbone. In the case of LAM, a branched arabinan polymer is further attached to the mannan core and cap motifs decorate the non-reducing termini of the arabinosyl side chains. The arabinan polymer contains about 60 Ara*f* units and consists of a linear α(1→5)-linked Ara*f* backbone punctuated with branched hexarabinofuranosides (Ara_6) and linear tetraarabinofuranosides (Ara_4). A recent study of the LAM-arabinan of *M. smegmatis* suggested the occurrence of an Ara_{18} motif resembling the internal structure of AG arabinan (Shi *et al.*, 2006) (see Section V.A.1). However, the length of the terminal extensions linked at the non-reducing ends of this motif seems to vary in LAM-arabinan (Ara_{18}–Ara_{22}) (Shi *et al.*, 2006). The nature of the motifs capping the non-reducing termini of the arabinan domain of LAM differs among mycobacterial species. To date, three types of LAM have been described: mannose-capped LAM (ManLAM) found in *M. tuberculosis*, *M. bovis*, *M. bovis* BCG, *M. leprae*, *M. avium*, *M. xenopi*, *M. marinum* and *M. kansasii*; phospho-*myo*-inositol-capped LAM (PILAM) found in *M. smegmatis* and *M. fortuitum*; and non-capped LAM (AraLAM) found in *M. chelonae*. LAM also differs between species in terms of the presence of recently discovered substitutions such as succinyl group and 5-deoxy-5-methylthio-*xylo*-furanose (MTX) (Delmas *et al.*, 1997; Guérardel *et al.*, 2003; Joe *et al.*, 2006; Treumann *et al.*, 2002; Turnbull *et al.*, 2004).

The fundamentals of how LM and LAM are built up are unknown, whether on a lipid carrier, growing from the reducing or non-reducing end, being built in blocks that are then put together, assembled on an enzyme complex much like polyketides or growing one glycosyl residue at a time analogous to an *N*-linked glycan.

3. Biosynthesis of PIMs

a. The early steps of PIM synthesis The first step in PIM synthesis involves the transfer of a Man*p* residue from GDP-Man*p* to the 2-position of the *myo*-Ins ring of PI to form phosphatidyl-*myo*-inositol monomannoside, PIM_1 (Fig. 2.2A). We have identified PimA (Rv2610c) as the α-mannosyltransferase (ManT) responsible for this catalytic step (Korduláková *et al.*, 2002). The characterization of PimA and the acyltransferase Rv2611c, which substitutes the 6-OH group of the Man*p* residue transferred by PimA with a fatty acyl chain, provided evidence that two distinct pathways then lead to the formation of Ac_1PIM_2 from PIM_1 (Fig. 2.2A) (Korduláková *et al.*, 2003). PimA is essential for the growth of *M. smegmatis* and *M. tuberculosis* (Korduláková *et al.*, 2002; Jackson *et al.*, unpublished results). Recently, the crystal structure of PimA from *M. smegmatis* has been solved (Guerin *et al.*, 2007) (see later) (Table 2.1). PimB (Rv0557) was originally characterized as an α-ManT responsible for the synthesis of Ac_1PIM_2 from Ac_1PIM_1 and GDP-Man in *M. smegmatis* (Schaeffer *et al.*, 1999). However, the fact that the disruption of *pimB* did not affect the biosynthesis of PIMs in *M. tuberculosis* suggested that either compensatory activities existed in the cells or that PimB performed another function in *M. tuberculosis* (Kremer *et al.*, 2002; Tatituri *et al.*, 2007). Recently, the second mannosylation step in the biosynthesis of PIM in *C. glutamicum* has been shown to be catalysed by PimB′ (NCgl2106, orthologous to Rv2188c), whereas PimB, now renamed MgtA, was implicated in the synthesis of a novel mannosylated glycolipid (1,2-di-*O*-$C_{16}/C_{18:1}$-(α-D-mannosyl)-(1→4)-(α-D-gluco-pyranosyluronic acid)-(1→3)-glycerol) and hypermannosylated 'LM-like' variant produced by *C. glutamicum* (Lea-Smith *et al.*, 2008; Tatituri *et al.*, 2007). Although *Rv2188c* was able to restore Ac_1PIM_2 synthesis in the *NCgl2106* mutant of *C. glutamicum*, clear definition of the substrate specificities of Rv2188c in mycobacteria will require its purification to homogeneity and direct demonstration of *in vitro* ManT activity using purified substrates. Ac_1PIM_2 can accumulate as an end product or be further modified with an acyl chain and/or additional Man*p* residues to form higher PIM species, such as Ac_1PIM_3–Ac_1PIM_6/Ac_2PIM_3–Ac_2PIM_6 or LM and LAM. The ManT PimC from *M. tuberculosis* strain CDC1551 was shown to catalyse the formation of Ac_1PIM_3 from GDP-Man and Ac_1PIM_2 in cell-free extracts (Kremer *et al.*, 2002). However, this enzyme is absent from most *M. tuberculosis* isolates as well as *M. smegmatis* suggesting that another as yet

unidentified enzyme is involved. Likewise, the ManT that catalyses the transfer of a Man*p* residue onto PIM_3 to produce PIM_4 remains to be identified. A summary of the characterized GTs of *M. tuberculosis* is provided in Table 2.1.

b. The biosynthesis of polar PIMs PimE (Rv1159) was recently identified as a ManT involved in the synthesis of the most polar forms of PIM bearing α(1→2)-linked terminal Man*p* residues. PimE belongs to the GT-C superfamily of integral membrane polyprenol-monophospho-sugar-utilizing GTs, suggesting that, in contrast to the ManTs, PimA, PimB, PimB′ and PimC, it probably utilizes polyprenol-phosphomannose (PPM) as a sugar donor and catalyses Man*p* transfer on the periplasmic side of the plasma membrane (Berg *et al.*, 2007). The disruption of *pimE* in *M. tuberculosis* H37Rv and *M. smegmatis* resulted in mutants which failed to produce PIM_6 (Morita *et al.*, 2006; Larrouy-Maumus *et al.*, unpublished results). Using *M. smegmatis* cell lysates as an enzyme source, evidence was provided that cell-free extracts from the *M. smegmatis pimE*-deficient mutant were able to produce PIM intermediates up to PIM_4 but not PIM_5 and PIM_6 (Morita *et al.*, 2006). While this result implicated PimE in at least one α(1→2)-Man*p* transfer onto PIM_4 to form PIM_5, the use of *M. smegmatis* extracts in the assay made it impossible to distinguish whether the transfer of the sixth Man*p* residue was also the result of PimE or that of another endogenous enzyme. Using a recombinant form of *M. tuberculosis* PimE produced in *E. coli* and purified $AcPIM_4$ and PPM as acceptor and donors substrates respectively, we recently showed that PimE catalyses the formation of one single PIM_5 product and not that of PIM_6, thereby clearly implicating this enzyme in the transfer of only one Man*p* residue onto PIM_4 to form PIM_5 (Larrouy-Maumus *et al.*, unpublished results). The α(1→2) ManT responsible for the transfer of the sixth and last Man*p* residue in the formation of PIM_6 thus remains to be identified. Like PimE, this enzyme is expected to belong to the GT-C superfamily of integral membrane PPM-utilizing GTs.

It is proposed that PIM_6 is a terminal product, as it contains two α(1→2)-linked Man*p*, a structure which is not found in LM and the mannan core of LAM (Khoo *et al.*, 1995). Thus, PIM_4 appears as the last common intermediate in the biosynthesis of polar PIMs and LM/LAM. LpqW (Rv1166), a putative lipoprotein, was shown to play a role in regulating polar PIM and LAM biosynthesis in *M. smegmatis*. Disruption of the *lpqW* gene of *M. smegmatis* yielded a mutant with wild-type apolar and polar PIM profiles but unable to make LAM, although it apparently retained the ability to produce LM (Kovacevic *et al.*, 2006). The mutant displayed, however, an unstable phenotype and rapidly recovered the ability to synthesize LAM at the expense of apolar PIM, a property accounted for by the accumulation of secondary mutations in the *pimE*

gene (Crellin *et al.*, 2008). Altogether, results thus suggested that LpqW might function to channel the PIM intermediate, $AcPIM_4$, into LAM synthesis. The crystal structure of the LpqW protein of *M. smegmatis* has been reported (Marland *et al.*, 2006).

c. Topology of PIM synthesis As evidenced by the nature of the GTs involved, the biosynthesis of PIM is topologically complex. Whereas the first three mannosylation steps of the biosynthetic pathway involve GDP-Man-dependent GT-B enzymes and occur on the cytoplasmic face of the plasma membrane (Morita *et al.*, 2004, 2005), further steps in the pathway of polar PIMs (PIM_4–PIM_6), LM and LAM most likely rely upon integral membrane GT-C-type GTs and take place on the extra-cytoplasmic side of the plasma membrane (Berg *et al.*, 2007; Besra *et al.*, 1997; Morita *et al.*, 2005) (Fig. 2.2A). Such a compartmentalization implies that as yet unidentified transporters/flippases translocate PIM intermediates from the cytoplasmic to the periplasmic side of the plasma membrane. It is at present not clear whether a form of PIM_2, PIM_3 or PIM_4 is translocated (for simplicity, only Ac_1PIM_3 is shown in Fig. 2.2A).

Little is known of the mechanisms that govern the interaction of GTs with membranes and/or lipid substrates. Members of the GT-B superfamily, to which PimA, PimB, PimB′ and PimC belong, bind membranes using a variety of mechanisms including transmembrane α-helices, amphipathic α-helices and protein–protein interactions (Martinez-Fleites *et al.*, 2006; Miley *et al.*, 2007; Wang *et al.*, 2008). The determination of the crystal structure of PimA from *M. smegmatis* in complex with the donor substrate GDP-Man and subsequent *in vitro* studies have shed some light on the molecular mechanisms involved in the very initial steps of PIM synthesis (Guerin *et al.*, 2007) (Fig. 2.2B). The notion of membrane-association through electrostatic interactions is consistent with the finding of an amphipathic α-helix and surface-exposed hydrophobic residues in the N-terminal domain of PimA. Although the sugar transfer is catalysed between the mannosyl group of GDP-Man and the *myo*-Ins ring of the PI acceptor substrate, both fatty acyl chains of PI are absolutely required for the transfer reaction to occur. Further, the fact that PimA was capable to bind mono-disperse PI but that its activity was stimulated by high concentrations of non-substrate anionic surfactants indicated that the reaction requires a lipid–water interface. Altogether, experimental data thus supported a model of interfacial catalysis in which PimA recognizes the fully acylated PI substrate with its polar head within the catalytic cleft and the fatty acid moieties only partially sequestered from the bulk solvent (Guerin *et al.*, 2007). Membrane attachment is likely to be mediated by an interfacial-binding surface located in the N-terminal domain of the protein. Structural changes or allosteric effects are expected to result from this interaction with the membrane and/or the lipid substrate and may be necessary for the formation of a competent enzyme–substrate complex.

A search for possible proteins responsible for flipping PIM intermediates (whether PIM_2, PIM_3 or PIM_4) across the plasma membrane has highlighted two possible candidates in the vicinity of the putative α(1→6) ManT Rv1459c, implicated in the initial steps of the elongation of the mannan core of LM/LAM from PIM in *C. glutamicum* (Mishra *et al.*, 2008) (see Section IV.A.4). Three genes, *Rv1458c*, *Rv1457c* and *Rv1456c*, which are conserved in all *Corynebacterinaeae*, are apparently translationally coupled with *Rv1459c* (Wang *et al.*, 2006). These genes encode two ABC-transporter integral membrane proteins, with *Rv1458c* encoding the putative ATP-dependent binding protein. Rv1458c exhibits remote structural similarities to sugar-binding proteins of ABC carriers, such as the sugar-binding protein of *Pyrococcus horikoshii* (Marabotti *et al.*, 2004) or the maltose/maltodextrin-binding protein MalK of *E. coli* (Lu *et al.*, 2005). Rv1457c encodes a permease component of an ABC-2-type transporter, typically involved in the export of drugs and carbohydrates (Reizer *et al.*, 1992). As transmembrane channels of ABC-2-type transporters are either homo- or hetero-oligomers and Rv1456c also displays features of a transporter protein, it is reasonable to assume that Rv1456c forms with Rv1457c a membrane channel coupled to the GT, Rv1459c. On the basis of the altered mycolic acid profile of a *Corynebacterium matruchotii* transposon mutant carrying an insertion in the *Rv1456c–Rv1459c* cluster, Wang *et al.* (2006) proposed one or more of these genes to be involved in mycolic acid transport. However, in light of the recent finding that Rv1459c catalyses the initial α(1→6) elongation of the mannan core of LM/LAM in *C. glutamicum*, this change in mycolylation may only have been an indirect consequence of the loss of LM/LAM in this species. Moreover, with our recent data suggesting a different function for Rv1459c in mycobacteria than in corynebacteria (see Section IV.A.4), it has become clear that the further examination of the *Rv1456c–Rv1459c* cluster directly in mycobacterial species will be required to precisely define the role of these genes in the transport of cell envelope precursors across the plasma membrane.

4. Biosynthesis of LM and LAM

Nothing was known until recently about the enzymes, intermediates and steps involved in the biosynthesis of the mannan backbone of LM/LAM. 'Linear LM', devoid of mannan core α(1→2) branching, has been recognized as a precursor but only through metabolic labelling of cell-free extracts (Besra *et al.*, 1997). Given the complexity of the mannan chain found in LM and LAM, a number of ManTs are expected to be involved in its elongation and branching.

Through genetic and biochemical studies, the membranous GT-C superfamily members relying on polyprenyl-linked sugar donors have emerged as the GTs responsible for much of its synthesis (Dinadayala

et al., 2006; Kaur *et al.*, 2006, 2007; Mishra *et al.*, 2007, 2008) (Table 2.1). The NCgl1505 protein of *C. glutamicum* (MptB; which shares ~35 % amino acid identity with Rv1459c from *M. tuberculosis*) has been shown to be an α(1→6) ManT involved in the initiation of the mannan core of corynebacterial LM (Mishra *et al.*, 2008). Although Rv1459c and its orthologue in *M. smegmatis*, MSMEG3120, demonstrated similar α(1→6) ManT activity in cell-free extracts, the facts that these enzymes were not able to restore LM production in the *C. glutamicum* mutant and that the disruption of *MSMEG3120* in *M. smegmatis* had no effect on LM/LAM synthesis, suggest that Rv1459c and MSMEG3120 may have a distinct function in whole mycobacterial cells. Our recent work on a recombinant strain of *M. smegmatis* overexpressing *Rv1459c* tends to support this hypothesis (see Section V.A.2). Hence, the α(1→6) ManTs responsible for the initial polymerization steps leading to the mannan backbone of LM from PIM_4–PIM_5 in mycobacteria is (are) currently unknown.

We and others recently characterized Rv2174 (MptA) as the α(1→6) ManT involved in the latter stages of the elongation of mannan in mycobacteria and corynebacteria (Kaur *et al.*, 2007; Mishra *et al.*, 2007). We also identified Rv2181 as the α(1→2) ManT responsible for the synthesis of the α(1→2) Man*p*-linked branches, characteristic of the mannan backbone of LM and LAM (Kaur *et al.*, 2006). LM is further arabinosylated to produce LAM in a set of poorly defined reactions involving the GT-C protein, EmbC (Zhang *et al.*, 2003). EmbC is an essential enzyme of *M. tuberculosis* (Goude *et al.*, 2008). Site-directed mutagenesis of an aspartic acid in the GT-C motif of EmbC led to the abolition of LAM synthesis (Berg *et al.*, 2005). Furthermore, introduction of point mutations in the conserved proline motif of EmbC proximal to the C-terminal domain led to the synthesis of smaller arabinan domains largely devoid of linear Ara_4, resembling the arabinan structure of AG (Berg *et al.*, 2005). Altogether, these data suggest that EmbC is most likely a multi-functional protein possessing polymerization and chain length regulating activities in addition to arabinosyltransferase (AraT) activity. The presence of two to three Ara*f* residues attached to the mannan backbone of LM in an *M. smegmatis embC* knock-out strain (Zhang *et al.*, 2003) suggests the existence of additional AraTs required for arabinan priming/synthesis.

Based on the hypothesis that the Man-capping of ManLAM probably takes place on the periplasmic side of the plasma membrane and on the assumption that the enzymes responsible should be missing from those *Mycobacterium* spp. devoid of ManLAM (e.g., *M. smegmatis*), we previously identified the GT-C member MT1671 of *M. tuberculosis* CDC1551 (orthologous to *Rv1635c* of *M. tuberculosis* H37Rv) as responsible for the addition of the first Man*p* unit of the mannose capping motifs of ManLAM (Dinadayala *et al.*, 2006). However, the extension of this strategy to search for the subsequent α(1→2) ManTs responsible for the formation of

the immunomodulatory di- and tri-mannoside caps of ManLAM failed to identify putative candidates. The only other putative GT-C enzyme devoid of an orthologue in *M. smegmatis* is Rv3779. However, the encoding gene belongs to an AG biosynthetic gene cluster (Berg *et al.*, 2007). An alternative hypothesis was that the mannoside caps arose from the action of a promiscuous α(1→2) ManT, such as PimE (Rv1159) or Rv2181, responsible for the transfer of α-1,2-linked Man*p* units to PIM_4 and the mannan backbone of LM and LAM, respectively (Kaur *et al.*, 2006; Larrouy-Maumus *et al.*, unpublished; Morita *et al.*, 2006). Indeed, the α(1→2)-linkages of the di- and tri-Man*p* units on the non-reducing ends of ManLAM, the terminal mono- and di-Man*p* units of PIM_5 and PIM_6, the α(1→2) Man*p* branching residues of the mannan core of LM/LAM and the di- and tri-mannoside units of the 45–47 kDa glycoprotein of *M. tuberculosis*, are all identical. Moreover, the enzymes catalysing the addition of these Man*p* residues use lipid-linked sugar donors, belong to the GT-C superfamily of GTs, and catalyse the reactions on the extracytoplasmic side of the plasma membrane (Kaur *et al.*, 2006; Morita *et al.*, 2006; vanderVen *et al.*, 2005) (Table 2.1). To investigate this hypothesis, knock-out mutants of *pimE* and *Rv2181* were generated in *M. tuberculosis* H37Rv. Structural analyses of the LM and LAM variants produced by the *M. tuberculosis Rv2181* mutant revealed the presence of but a single Man*p* residue on the non-reducing arabinan termini of LAM in addition to a complete absence of α(1→2)-linked Man*p* branching on the mannan backbones of LM and LAM (Kaur *et al.*, 2008). A novel recombinant strain was constructed in ManLAM-deficient *Mycobacterium smegmatis* that co-expressed *Rv2181* and *Rv1635c* – the ManT responsible for the addition of the first Man*p* capping residue of ManLAM. Analysis revealed LAM termini fully capped with di- and tri-Man*p* motifs in addition to α(1→2) Man*p* branching on the mannan backbones of LM and LAM, confirming the involvement of the α(1→2)ManT Rv2181 in the dual role of Man-capping and mannan-core branching (Kaur *et al.*, 2008). It is intriguing then that the expression of *Rv1635c* in *M. smegmatis* yielded a LAM population that had t-Ara*f* residues capped only with a single Man*p* (Dinadayala *et al.*, 2006), despite *M. smegmatis* expressing an active orthologue of *Rv2181* (*MSMEG_4247*) (Kaur *et al.*, 2006). Indeed, while MSMEG_4247 from *M. smegmatis* clearly participated in mannan-core branching, it displayed no detectable Man*p*-capping activity on ManLAM. The catalytic activity of MSMEG_4247 may be too low to mannosylate this heterologous substrate in addition to the mannan backbone. Alternatively, if LAM-capping has evolved to play a role in host cell interactions, it is tempting to speculate that Rv2181 from *M. tuberculosis* has acquired the ability to α(1→2)-mannosylate ManLAM while MSMEG_4247 from *M. smegmatis* has not. Further delineation of the

structure-function aspects of Rv2181 is required to understand its role in the biosynthesis of LM and ManLAM.

Interestingly, disruption of *pimE* in *M. tuberculosis* H37Rv led to a significant and consistent decrease in the amounts of the dimannoside motif capping ManLAM in addition to the abolition of polar PIM synthesis (Larrouy-Maumus *et al.*, unpublished). Both phenotypes were restored in the complemented mutant strain. Whether PimE is directly or only indirectly involved in ManLAM-capping, however, is at present not clear given the exclusive role Rv2181 seems to be playing in this process (Kaur *et al.*, 2008). Rv2181 and PimE may act jointly in ManLAM capping, as proposed for other mycobacterial GT-Cs involved in the biosynthesis of AG (Khasnobis *et al.*, 2006), the first enzyme playing the predominant role. Clearly, the precise delineation of the functions of PimE will have to await the development of cell-free assays using purified enzyme and substrates.

The biosynthetic origins of the MTX motif linked to t-Man*p* residues of ManLAM and succinyl residues linked to the arabinan domain of LAM have not yet been determined.

B. Acyltrehaloses

The search for virulence factors and immunodominant species-specific antigens in the envelopes of mycobacteria has led to the definition of a remarkable array of glycolipids, among which are several families of trehalose-containing lipids and the phenolic glycolipids (PGL) described in Section IV.C.

Trehalose is a simple non-reducing disaccharide of glucose (α-D-Glc*p*-(1 ↔ 1)-α-D-Glc*p*) found in bacteria, yeast, fungi, plants and invertebrates, but not in mammalian cells. It is found free in the cytosol of mycobacteria or esterified to a variety of fatty acyl groups in the OM of their cell envelope. Under its free form, trehalose is thought to function as a storage carbohydrate and as a stress protectant. The acyltrehaloses found in the cell envelope of *M. tuberculosis* include sulphatides (SL), diacyltrehaloses (DAT), triacyltrehaloses (TAT), polyacyltrehaloses (PATs), trehalose monomycolate (TMM) and trehalose dimycolates (TDM) (Fig. 2.3). In addition, a few strains of *M. tuberculosis*, known as Canetti strains, produce highly polar species-specific lipooligosaccharides (LOS) (Daffé *et al.*, 1991) (Fig. 2.3).

Mycobacteria are unusual amongst microorganisms in possessing three pathways for trehalose synthesis (de Smet *et al.*, 2000). One pathway involves condensation of glucose-6-phosphate with UDP-glucose to form trehalose-6-phosphate followed by dephosphorylation to release the free disaccharide. These reactions are catalysed by trehalose-6-phosphate synthase (OtsA, Rv3490) (Pan *et al.*, 2002) and trehalose-6-phosphate phosphatase (OtsB2,

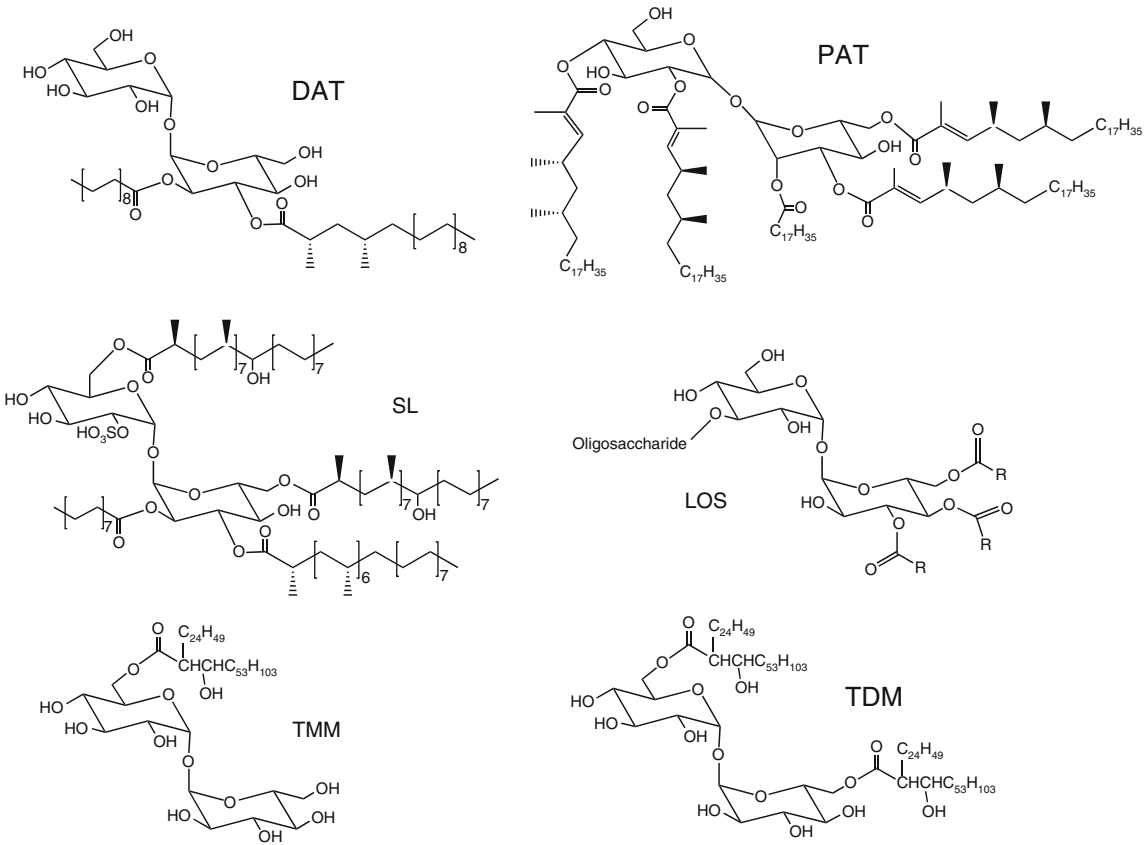

FIGURE 2.3 (Continued)

Rv3372). The second pathway generates trehalose from glycogen in a two-step mechanism involving the maltooligosyltrehalose synthase (TreY, Rv1653c) and the maltooligosyltrehalose trehalohydrolase (TreZ, Rv1562c). The third pathway consists of the conversion of maltose to trehalose by the trehalose synthase (TreS, Rv0126). While the three pathways are functionally redundant in *M. smegmatis* (Woodruff *et al.*, 2004), the OtsAB pathway was found to be predominant in *M. tuberculosis* (Murphy *et al.*, 2005). OtsB2 was demonstrated to be strictly essential for growth in *M. tuberculosis* (Murphy *et al.*, 2005). Thus, consistent with its pleiotropic role in several other functions than stress protection alone, trehalose is essential for growth of *M. tuberculosis* (Murphy *et al.*, 2005). In view of the absence of trehalose in mammalian cells and essentiality, the OtsB2 enzyme may provide a useful target for novel drugs.

In TDM, trehalose is esterified by mycolates at positions 6 and 6′. It was proposed that mycolic acids can be transferred via a mycolyl-mannosylphosphoheptaprenol donor (Besra *et al.*, 1994) to trehalose 6-phosphate, arising from the OtsAB pathway, to yield phosphorylated TMM, which then can be dephosphorylated to yield TMM (bearing only one mycolyl chain) (Takayama *et al.*, 2005). The subsequent synthesis of TDM, from two TMM molecules and the transfer of mycolates to the non-reducing ends of AG have been shown to involve antigens 85A, 85B and 85C (Belisle *et al.*, 1997; Jackson *et al.*, 1999). However, the precise functions of these enzymes remain unclear, as is the process through which mycolates are translocated from the cytoplasm where they are synthesized to the cell envelope.

The reactions leading to the acylation of trehalose with short chain fatty acyl substituents and long-chain multi-methyl-branched fatty acids to form SL, DAT, TAT and PAT have not been fully elucidated either. The only two acyltransferases characterized to date are PapA2, involved in the transfer of a palmitoyl group to the 2′-position of trehalose-2-sulphate in

FIGURE 2.3 Structures of some trehalose-derived molecules from *Mycobacterium tuberculosis*. The major sulpholipid SL-I (2,3,6,6′-tetraacyl α-α′-trehalose-2′-sulphate) is represented. In SL-I, trehalose is sulphated at the 2′ position and esterified with palmitic acid and the multi-methyl-branched phthioceranic and hydroxyphthioceranic acids. In DAT (2,3-di-*O*-acyltrehalose), trehalose is esterified with stearic acid and the multi-methyl-branched mycosanoic acid. In PAT, trehalose is esterified with stearic acid and the multi-methyl-branched mycolipenic acids. In TMM and TDM, trehalose is esterified with mycolic acids. The oligosaccharide of the LOS of *M. tuberculosis* Canetti strains consists of 2-*O*-methyl-α-L-Fuc*p*-(1→3)-β-D-Glc*p*-(1→3)-2-*O*-methyl-α-L-Rha*p*-(1→3)-2-*O*-methyl-α-L-Rha*p*-(1→3)-β-D-Glc*p*-(1→3)-4-*O*-methyl-α-L-Rha*p*-(1→3)-6-*O*-methyl-α-D-Glc-(1→1)-α-D-Glc. R are 2,4-dimethylhexadecanoic acid and 2,4,6,8-tetramethyloctadecanoic acid residues.

SL biosynthesis, and PapA1, apparently responsible for the transfer of the first (hydroxy)phthioceranoyl group onto the product of PapA2 (Kumar *et al.*, 2007). Both acyltransferases are essential for the synthesis of SL-1 (Bhatt *et al.*, 2007; Kumar *et al.*, 2007). The identity and the localization of the enzymes responsible for the final acylation steps have yet to be determined. Based on what is known of the biosynthesis of related polyketide-derived lipids known as the phthiocerol dimycocerosates, it is tempting to speculate that the biosynthesis of the entire acyltrehalose molecules takes place on the cytoplasmic side of the membrane and that biosynthesis and translocation are coupled (Jain and Cox, 2005). Alternatively, the accumulation within interior layers of the cell envelope of partially acylated SL intermediates by *M. tuberculosis* mutants deficient in the membrane transporter MmpL8 could be interpreted as the initial acylation reactions taking place in the cytosol and the remaining in the cell envelope (Converse *et al.*, 2003; Domenech *et al.*, 2004). At present, there is no clear experimental evidence supporting any of these two models.

Sft0 (Rv0295c) was characterized as the sulphotransferase responsible for the formation of the trehalose-2-sulphate moiety of SL (Mougous *et al.*, 2004). The topics of the synthesis of the polyketide or mycolic acid moieties of these molecules are beyond the scope of this review but have been extensively reviewed recently (Guilhot and Daffé, 2008; Jackson *et al.*, 2007; Marrakchi *et al.*, 2008; Natarajan *et al.*, 2008). Likewise, the translocation and biological activities of trehalose esters have been reviewed recently and will thus not be further detailed here (Bertozzi and Schelle, 2008; Glickman, 2008; Guilhot and Daffé, 2008; Jackson *et al.*, 2007).

Although the genetics of the species-specific LOS of *M. marinum* (Burguière *et al.*, 2005; Ren *et al.*, 2007) has begun to be explored, nothing is known of LOS biosynthesis in the Canetti strains of *M. tuberculosis*.

C. *p*-Hydroxybenzoic acid derivatives and phenolic glycolipids

Recent interest has focused on the PGL produced by some strains of *M. tuberculosis*. Their structure is presented in Fig. 2.4. Although their role in the pathogenesis of some W-Beijing isolates has been documented (Reed *et al.*, 2004; Tsenova *et al.*, 2005), most strains of *M. tuberculosis* do not produce PGL due to a frameshift mutation in the polyketide synthase gene *pks15/1*, which is required for the assembly of the lipid moiety of the molecule (Constant *et al.*, 2002). However, all *M. tuberculosis* isolates analysed to date have retained the ability to produce and secrete *p*-hydroxybenzoic acid derivatives (*p*-HBADs)-glycoconjugates which share with PGL the same glycosylated aromatic nucleus (Fig. 2.4) (Constant *et al.*, 2002). The biosynthesis and biological activities of PGLs

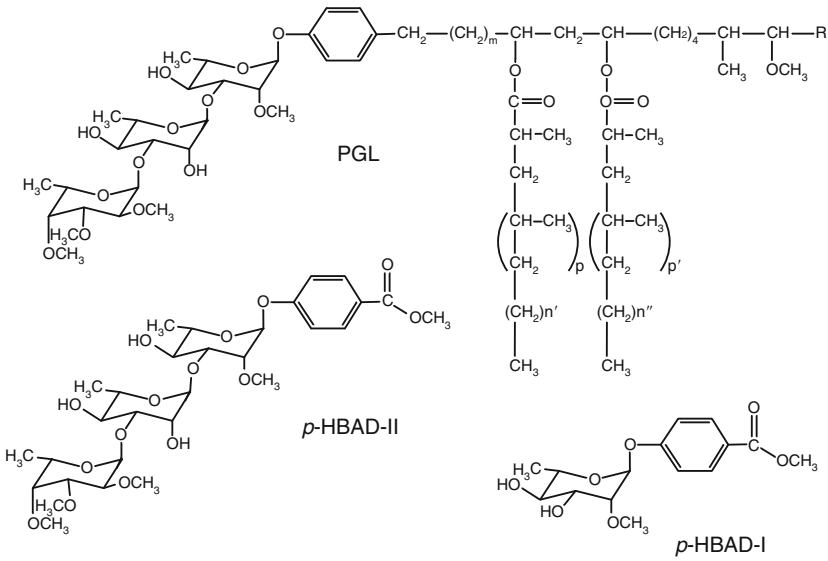

FIGURE 2.4 Structures of phenolic glycolipids and *p*-hydroxybenzoic acid derivatives from *M. tuberculosis*. The lipid core of PGL from *M. tuberculosis* is composed of phenolphthiocerol esterified by mycocerosic acids ($m = 15–17$; $n = 20–22$, $n', n'' = 16, 18$; $p, p' = 2\text{-}5$; R = $-CH_2-CH_3$ or $-CH_3$). The trisaccharide substituent of PGL and *p*-HBAD-II consists of 2,3,4-tri-*O*-methyl-α-L-Fuc*p*-(1→3)-α-L-Rha*p*-(1→3)-2-*O*-methyl-α-L-Rha*p*. The monosaccharide substituent found in *p*-HBAD-I consists of 2-*O*-methyl-α-L-Rha*p*.

and related *p*-HBADs have been reviewed recently and will thus not be described here (Berg *et al.*, 2007; Guilhot *et al.*, 2008; Jackson *et al.*, 2007; Malaga *et al.*, 2008).

D. Mannosyl-β-1-phosphomycoketides

Mannosyl-β-1-phosphomycoketides consist of a mannosyl-β-1-phosphate moiety identical to that found in mannosyl-β-1-phosphodolichols from mammalian cells and a C30–C34 fully saturated 4, 8, 12, 16, 20-pentamethylpentacosyl unit (Fig. 2.5) (Matsunaga *et al.*, 2004). They have been found in *M. tuberculosis*, *M. avium* and *M. bovis* BCG but not in the rapidly growing saprophytes, *M. phlei*, *M. fallax* and *M. smegmatis*. Suggestive of a role in pathogenesis, this family of lipids activates human CD1c-restricted T-cells (Matsunaga *et al.*, 2004). With the exception of the polyketide synthase Pks12 which has been found to be required for the production of these lipids (Chopra *et al.*, 2008; Matsunaga *et al.*, 2004) the biosynthetic pathway of mycoketides has not yet been elucidated.

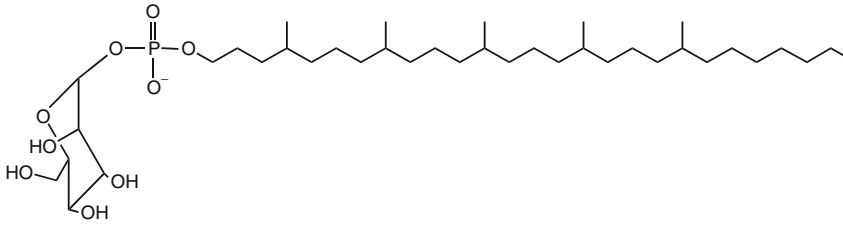

FIGURE 2.5 Structure of the predominant mannosyl-β-1-phosphomycoketides from *M. tuberculosis* H37Rv.

E. Glycoproteins

The glycosylation patterns of the only two mycobacterial glycoproteins that have been biochemically characterized to date – the MBP83 antigen of *M. bovis* and the 45–47 kDa (Apa) antigen of *M. tuberculosis* – indicate that they are modified at threonine residues with one to three α(1→3)-(MBP83) or α(1→2) (Apa)-linked Man*p*, a glycosylation pattern reminiscent of eukaryotic short-chain mannoproteins (Dobos *et al.*, 1996; Michell *et al.*, 2003). The decorating mannose motifs of Apa have been implicated in various biological activities, including the ability of this protein to induce a delayed-type hypersensitivity response in guinea pigs, stimulate primed T-cells *in vitro*, and bind C-type lectins such as the surfactant protein A and, potentially, DC-SIGN (Horn *et al.*, 1999; Pitarque *et al.*, 2005; Ragas *et al.*, 2007; Romain *et al.*, 1999). Several other proteins of *M. tuberculosis* are believed to be glycosylated even though their glycosyl appendages have not been characterized (Espitia and Mancilla, 1989; Garbe *et al.*, 1993).

The glycosyltransferases involved in protein glycosylation in *M. tuberculosis* remain largely unknown. Bioinformatic approaches identified a single *M. tuberculosis* protein, Rv1002c, sharing amino acid identity with the *O*-mannosyltransferases of *Saccharomyces cerevisiae* and a similar hydropathy profile (VanderVen *et al.*, 2005). This integral membrane protein, which belongs to the GT-C superfamily of glycosyltransferases, was shown to catalyse the initial step of the mannosylation of Apa. Moreover, evidence was provided that, analogous to eukaryotic systems, Sec-translocation is required for protein mannosylation in *M. tuberculosis*. The glycosyltransferases involved in the further elongation of the α(1→2) or α(1→3)-linked oligomannoside motifs have not yet been identified. Our recent work on a knock-out mutant of *M. tuberculosis* H37Rv deficient in the α(1→2)-mannosyltransferase Rv1159 (PimE) suggests that this enzyme is not involved in the glycosylation of Apa (Larrouy-Maumus *et al.*, unpublished).

V. THE GLYCOCONJUGATE POLYMERS OF THE CELL WALL CORE

A. Arabinogalactan

1. Structure of AG

AG was recognized as the major cell wall polysaccharide of mycobacteria as early as the 1950s. Its apparent function as a whole is the tethering of the mycolic acid layer to the PG. The function of the galactan region beyond where arabinan attaches is unknown, but it was proposed to produce a viscous hydrophilic region between the PG and mycolic acid layers. The most recent model of AG indicates that it contains 125 glycosyl residues in total distributed between a galactan domain made of 30 Gal*f* residues, three arabinan domains each containing 31 Ara*f* residues, and a specific linker unit ensuring its covalent attachment to PG made of a rhamnosyl residue attached to a *N*-acetylglucosaminosyl-1-phosphate residue (Bhamidi *et al.*, 2008). The galactan of AG is made of a disaccharide repeating unit, $[\rightarrow 6\text{-D-Gal}f\beta 1\rightarrow 5\text{-D-Gal}f\beta]_{15}$. Arabinan chains are attached to *O*-5 of Gal*f* residues 8, 10 and 12 of galactan. The characteristic non-reducing termini of the arabinan domain of AG consist of an Ara_6 motif, $Araf\beta 1\rightarrow 2Araf\alpha 1\rightarrow 5(Araf\beta 1\rightarrow 2Araf\alpha 1\rightarrow 3)$-$Araf\alpha 1\rightarrow 5Araf\alpha 1\rightarrow$, where position 5 of both the terminal β-Ara*f* and the penultimate 2-α-Ara*f* serve as the anchoring points for the mycolic acids. Under physiological conditions, approximately two-thirds of the Ara_6 motifs are mycolylated (McNeil *et al.*, 1991). The inner core of the arabinan domain is essentially made of stretches of α-1,5-linked Ara*f* residues with a critically positioned α-3,5-branch site (Fig. 2.1). Some of the interior α-3,5-Ara*f* residues are substituted at position 2 with either a non-*N*-acetylated galactosamine residue or a succinyl residue (Bhamidi *et al.*, 2008; Lee *et al.*, 2006). The inner core of the D-arabinan portion of AG is very similar to that of LAM in that the same linkages of Ara*f* units are found and both structures share an internal Ara_{18} motif extending from the α-3,5-Ara*f* interior residues (Fig. 2.1). The interior regions of LAM arabinan, however, have been shown to be more variable in terms of the length of this Ara*f* motif (Ara_{18}–Ara_{22}) (Bhamidi *et al.*, 2008; Shi *et al.*, 2006).

2. AG biosynthesis

The galactan and arabinan domains of AG are synthesized as a unit on a decaprenyl phosphate (Dec-P) carrier lipid before being transferred onto PG by an as yet unidentified ligase (Yagi *et al.*, 2003). The synthesis of AG is thus initiated on a Dec-P molecule with formation of the linker unit, followed by what appears to be the simultaneous addition of Gal*f* and Ara*f* residues (Mikušová *et al.*, 1996, 2000; Yagi *et al.*, 2003) (Fig. 2.6). Many of the enzymes involved in this complex process have been identified.

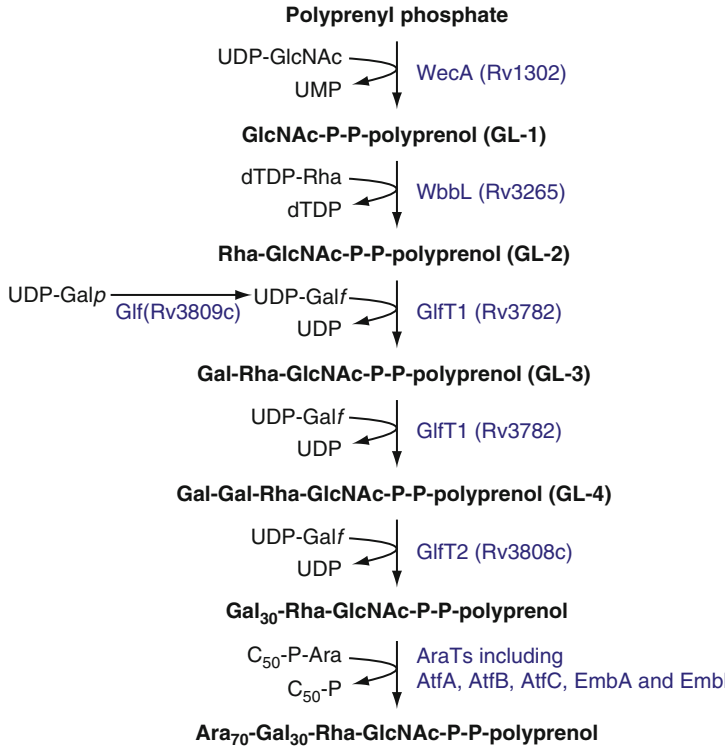

FIGURE 2.6 Proposed pathway for the biosynthesis of mycobacterial arabinogalactan. AG synthesis is initiated by a transfer of GlcNAc-1-phosphate onto a polyprenyl-phosphate and continues with the sequential addition of glycosyl residues to this lipid carrier. The enzymes that have been proposed to be involved in this process are indicated on the figure.

Detailed reviews of the biosynthesis of polyprenyl phosphate have been published (Barry *et al.*, 2007; Crick and Brennan, 2008; Crick *et al.*, 2001; Wolucka *et al.*, 2008). It is predicted that a transferase encoded by *Rv1302* (a putative orthologue of WecA) in the genome of *M. tuberculosis* H37Rv transfers GlcNAc-1-phosphate to Dec-P to form Dec-P-P-GlcNAc (GL-1) (Dal Nogare *et al.*, 1998; McNeil, 1999; Mikušová *et al.*, 1996). This step is followed by the attachment of a rhamnosyl residue to the 3 position of GlcNAc in a reaction catalysed by WbbL (Rv3265c) to form GL-2, 'the linker unit' (Mills *et al.*, 2004). dTDP-Rha, which serves as the Rha donor in this reaction, is synthesized from glucose-1-phosphate through a four-step reaction catalysed by the α-D-glucose-1-phosphate thymidylyl transferase RmlA (Rv0334) (Ma *et al.*, 1997), the dTDP-D-glucose

4,6-dehydratase RmlB (Rv3464) (Ma *et al.*, 2001), the dTDP-4-keto-6-deoxy-D-glucose 3,5 epimerase RmlC (Rv3465) (Stern *et al.*, 1999) and the dTDP-rhamnose synthetase RmlD (Rv3266c) (Hoang *et al.*, 1999).

Gal*f* residues are then added to the linker unit from UDP-Gal*f* which originates in UDP-Gal*p* in a reaction catalysed by UDP-Gal*p* mutase (Glf; Rv3809c) (Mikušová *et al.*, 2000; Weston *et al.*, 1997). UDP-Gal*p* is synthesized from UDP-glucose by UDP-Gal*p* epimerase, likely encoded by *Rv3634*. The galactosyltransferase Rv3782 (GlfT1) catalyses the first stages of galactan synthesis transferring the two first Gal*f* residues onto the linker unit (Belanová *et al.*, 2008; Mikušová *et al.*, 2006), while Rv3808c (GlfT2) continues with the subsequent polymerization events (Belanová *et al.*, 2008; Kremer *et al.*, 2001; Mikušová *et al.*, 2000; Rose *et al.*, 2006). GlfT2 is a bifunctional galactosyltransferase that catalyses the synthesis of the alternating 5- and 6-linked, linear, galactofuran in a processive manner. It is thought to be responsible for the polymerization of the bulk of the galactofuran.

As in the case of LAM, knowledge of the enzymes involved in the elongation of the D-arabinan structures of AG is more limited. Polyprenol-monophosphoryl-β-D-arabinose (DPA) is the only known Ara*f* donor in mycobacteria (Wolucka *et al.*, 1994). It is synthesized from 5-phosphoribose-pyrophosphate (Scherman *et al.*, 1995, 1996) following four catalytic steps which have been characterized (Huang *et al.*, 2005, 2008; Mikušová *et al.*, 2005). Since DPA is the only known Ara*f* donor, it is expected that the arabinosylation of AG and LAM is catalysed by membrane-associated polyprenyl-dependent glycosyltransferases (GTs) on the periplasmic side of the plasma membrane (Berg *et al.*, 2007). We and others have recently reported of the potential existence of seventeen such enzymes in *M. tuberculosis* H37Rv, the implication of twelve of which in the glycosylation of various proteins, glycolipids or polysaccharides has now been established (for a review, Berg *et al.*, 2007; Birch *et al.*, 2008; Kaur *et al.*, 2007; Mishra *et al.*, 2008; Seidel *et al.*, 2007). Arabinofuranosyltransferases characterized to date include AftA (Rv3792), involved in the transfer of the very first Ara*f* residues to the galactan domain of AG (Alderwick *et al.*, 2006; Shi *et al.*, 2008), the terminal β1,2-capping arabinosyltransferase AftB (Rv3805c) (Seidel *et al.*, 2007), Rv2673 (AftC) involved in the α-1,3-branching of the arabinan domain of AG (Birch *et al.*, 2008) and the EmbA and EmbB proteins involved in the formation of the Ara_6 motif (Escuyer *et al.*, 2001; Khasnobis *et al.*, 2006). Although EmbA and EmbB, acting alone or as heterodimers, have also been proposed to participate in the α-1,5-elongation of the linear portion of arabinan, experimental evidence for this assumption is still lacking (Bhamidi *et al.*, 2008). Our most recent results suggest that Rv0236c, the largest putative GT-C identified in the genome of *M. tuberculosis* displays, like Rv2673, α-1,3-branching arabinofuranosyltransferase activity, catalysing *in vitro* the transfer of an Ara*f* residue from DPA to a synthetic linear α-1,5-linked Ara_5 acceptor.

We found the orthologue of *Rv0236c* in *M. smegmatis* mc^2155 to be an essential enzyme and its activity to be rate limiting for the synthesis of AG as a whole (Skovierová *et al*., unpublished results). As proposed earlier for the Emb proteins (Berg *et al*., 2005, 2007), the large size of Rv0236c (1400 amino acids) suggests that it might have additional functions – for example controlling the length of the various interior or exterior segments of the arabinan polymer, acting a scaffold for a multi-enzyme machinery involved in arabinosylation – in addition to the direct transfer of Ara*f* units from a Dec-P-Ara*f* donor. Interestingly, our recent results also indicate that Rv1459c, another GT-C enzyme whose distantly related orthologue in *C. glutamicum* was shown to be an α-1,6-mannosyltransferase involved in the elongation of PIM_2 (Mishra *et al*., 2008), displays *in vitro* the same α-1,3-branching arabinosyltransferase activity as Rv2673 and Rv0236c on a synthetic linear α-1,5-linked Ara_5 acceptor. In contrast to what has been reported (Mishra *et al*., 2008), we found the orthologue of *Rv1459c* in *M. smegmatis* mc^2155 to be essential for growth (Korduláková, Škovierová *et al*., unpublished). The reason for this discrepancy between the results of these two studies is unknown. The reason for the apparent existence of three non-redundant α-1,3-arabinosyltransferases, Rv2673, Rv1459c and Rv0236c, is at present unclear but could be related to the existence of a multi-protein complex participating in the formation of the arabinan domain or to their different involvement in the branching of the interior or outermost domains of arabinan in AG and/or LAM (Fig. 2.1). Alternatively, one cannot exclude that under physiological conditions, these enzymes participate in the α-1,5 elongation rather than in the branching of the arabinan domains of AG and LAM. Indeed, although elongating α-1,5 arabinofuranosyltransferase activities – some of which apparently unrelated to the Emb proteins – have been detected in cell-free assays using mycobacterial cell wall preparations and synthetic linear or branched arabinan acceptors (Lee *et al*., 1997, 1998; Zhang *et al*., 2007), the identity of the responsible enzymes remains to be determined. Likewise, the enzymes responsible for the transfer of the galactosamine and succinyl residues onto some of the interior α-3,5-Ara*f* residues of arabinan are not yet known.

The fact that the addition of Gal*f* and Ara*f* residues appears to occur simultaneously makes it unclear whether polymerization events leading to the formation of AG take place inside or outside the plasma membrane. While the involvement of GT-C enzymes in the polymerization of the arabinan domain clearly points to the periplasmic localization of arabinan formation, the donor of Gal*f* residues in galactan synthesis is a nucleotide sugar expected to be cytoplasmic. The fact that the data leading to the conclusion of simultaneous galactosylation and arabinosylation events were generated using cell-free assays in which the natural topology of the membrane was disrupted may be the basis of this apparent

discrepancy. In that case, the transporters involved in the translocation of the galactan polymer synthesized inside the cytosol to the periplasm remain to be identified.

Interestingly, many of the genes involved in the biogenesis of the galactan and arabinan domains of AG and LAM, the formation of mycolic acids and the transfer of mycolates onto AG or trehalose are clustered in a region of the *M. tuberculosis* chromosome often referred to as the 'cell wall biosynthetic cluster' (Fig. 2.7). This cluster also carries a number of as yet uncharacterized sugar-modifying enzymes and transporters.

3. AG biosynthesis and drug discovery

The inhibitory activity of ethambutol, a first-line anti-TB drug shown to target the Emb proteins, first illustrated the essentiality of AG in *M. tuberculosis* and other mycobacteria (Belanger *et al.*, 1996; Lee *et al.*, 1995). Other studies have since provided genetic evidence of the essentiality of multiple steps of the biogenesis of AG (Barry *et al.*, 2007). Genes found to be essential in *M. tuberculosis* or *M. smegmatis* include those involved in the biogenesis of Dec-P (Eoh *et al.*, 2007), the formation of dTDP-Rha (Li *et al.*, 2006; Ma *et al.*, 2002), the linker unit (Mills *et al.*, 2004), the UDP-Gal*f* mutase gene *Rv3809c* (Pan *et al.*, 2001), the galactosyltransferase genes *Rv3782* and *Rv3808c* (Pan *et al.*, 2001) and the arabinosyltransferase or putative arabinosyltransferase genes *embA*, *embC*, *Rv3792* (*AftA*), *Rv1459c* and *Rv0236c* (Amin *et al.*, 2008; Shi *et al.*, 2008; our unpublished results).

In view of the absence of most of the biosynthetic pathway of AG in mammalian cells and essentiality, there is considerable interest in exploiting several of the enzymes of this pathway as therapeutic targets. The few drug discovery enzyme assays that have been designed and the target enzymes whose crystal structures have been determined have recently been reviewed (Barry *et al.*, 2007; Brennan and Crick, 2007; Wolucka, 2008).

B. Peptidoglycan

Two opposing models of the secondary structure of the mycolyl–AG–peptidoglycan complex (mAGP) have been proposed and reviewed (Crick and Brennan, 2008). One predicts that the PG and galactan are parallel to the plasma membrane (McNeil and Brennan, 1991). This orientation is consistent with traditional models of the PG structure; however, other modelling studies suggest that the PG and AG strands may be coiled and perpendicular to the plane of the plasma membrane (Dmitriev *et al.*, 1999, 2000). Recently, the relevant studies on the secondary structure of PG in Gram-negative bacteria were reviewed (Vollmer and Holtje, 2004), and the preponderance of data appears to favour the parallel model in these organisms, but the perpendicular model recently

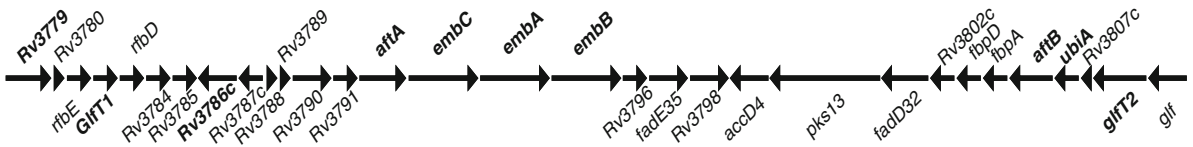

FIGURE 2.7 A schematic representation of the *M. tuberculosis* H37Rv cell wall biosynthetic gene cluster (*Rv3779-Rv3809c*) encompassing genes involved (or likely to be) in the biosynthesis of mycolic acids (*accD4*, *pks13*, *fadD32*, *fbpD*, *fbpA*), arabinogalactan (*glfT1*, *Rv3790*, *Rv3791*, *aftA*, *embA*, *embB*, *aftB*, *ubiA*, *glfT2*, *glf*), and LAM (*embA*, *embB*, *embC*, *aftB*, *ubiA*). Genes annotated or suggested as glycosyltransferases are marked in bold.

gained support from the three-dimensional solution structure of a synthetic fragment of the cell-wall as determined by NMR. These results indicate that the glycan backbone of the synthetic PG fragment forms a right-handed helix with a periodicity of six sugar residues, leading the authors to conclude that the glycan strand of PG is orthogonal to the plane of the membrane *in vivo* as opposed to the parallel hypothesis (Meroueh *et al.*, 2006). Thus, the overall structure and topology of the mAGP complex in *Mycobacterium* spp. remains open to debate as does the three-dimensional structure of PG in other eubacteria.

The detailed primary structure of the PG of *Mycobacterium* spp. has been reviewed many times, as recently as 2008 (see Crick and Brennan, 2008) (Fig. 2.8). PG is a complex polymer forming a rigid layer outside the plasma membrane providing cellular shape and the strength to withstand osmotic pressure, as well as a scaffold for the structures described (Fig. 2.1). PG from *M. tuberculosis* has been classified as A1γ as has that of *Escherichia coli* and *Bacillus* spp. according to the classification system of Schleifer and Kandler (1972) and is composed of linear chains of *N*-acetyl-α-D-glucosamine (GlcNAc) and modified muramic acid (Mur) substituted with peptide side chains that are heavily cross-linked providing added structural integrity to the bacterium; the overall degree of cross-linking is 70–80% in *Mycobacterium* spp. (Matsuhashi, 1966) compared to 50% in *E. coli* (Vollmer and Holtje, 2004). The tetrapeptide side chains of PG consist of L-alanyl-D-isoglutaminyl-meso-diaminopimelyl-D-alanine (L-Ala-D-Glu-A_2pm-D-Ala) with the Glu and A_2pm being further amidated (Adam *et al.*, 1969; Kotani *et al.*, 1970; Lederer *et al.*, 1975; Petit *et al.*, 1969; Wietzerbin-Falszpan *et al.*, 1970). About two-thirds of the peptide cross-links found in *M. tuberculosis* PG are between the carboxyl group of a terminal D-Ala and the amino group at the D-centre of A_2pm resulting a D, D bond (Wietzerbin *et al.*, 1974), and approximately one-third of the peptide cross-links occur between the carboxyl group of the L-center of one A_2pm residue and the amino group of the D-center of another A_2pm residue forming a L,D-cross-link (Wietzerbin *et al.*, 1974). L,D-cross-links are relatively rare, but recently have also been reported in the PG of *Streptomyces* spp., *Clostridium perfringens* (Leyh-Bouille *et al.*, 1970) and stationary phase *E. coli* (Goffin and Ghuysen, 2002; Templin *et al.*, 1999). In addition, in mycobacterial PG some of the Mur residues are *N*-acylated with glycolic acid (MurNGlyc) rather than *N*-acetylated (MurNAc) (Mahapatra *et al.*, 2000, 2005a; Raymond *et al.*, 2005), and the hydroxyl moiety of C-6 of some of the Mur residues forms phosphodiester bonds to C-1 of MurN-Glyc which in turn is (1→3) linked to a α-L-rhamnose (Rha) residue providing the 'linker unit' between the galactan of AG and PG (McNeil *et al.*, 1990).

In terms of PG biosynthesis, the arrangement of genes responsible for PG synthesis in *M. tuberculosis* is similar to that in other bacteria

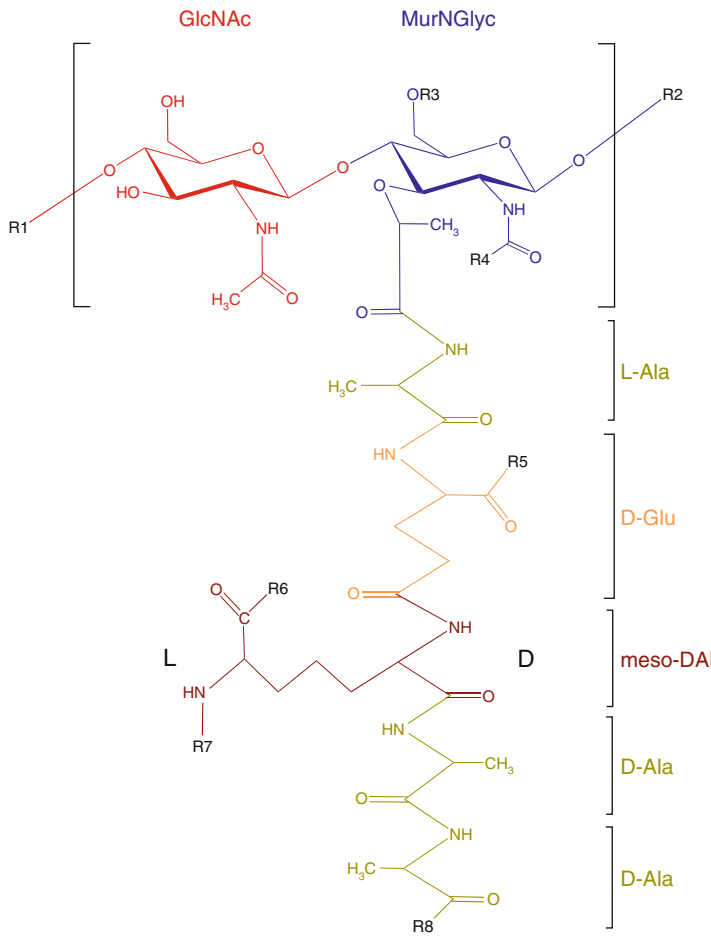

FIGURE 2.8 Structure of a representative monomer of mycobacterial PG prior to peptide trimming. R_1, *N*-glycolylmuramic acid residue of another monomer; R_2, *N*-acetylglucosamine residue of another monomer; R_3, H or the linker unit of AG; R_4, H, $COCH_3$ (*N*-acetyl) or $COCH_2OH$ (*N*-glycolyl); R_5, R_6, R_8, OH, NH_2 or OCH_3; R_7, H, or cross-linked to penultimate D-Ala or to the D-centre of another *meso*-DAP residue.

(Mahapatra *et al.*, 2000), and in all probability, so is the biochemistry (Mahapatra *et al.*, 2005b). Hence, the excellent reviews on bacterial PG synthesis, in general (van Heijenoort, 1994, 1996, 1998, 2001a,b), are applicable. However, there are some unusual aspects related to PG synthesis in mycobacteria. As noted earlier, the glycan chains are composed of alternating units of β 1→4 linked GlcNAc, MurNAc and MurNGlyc, whereas, most other bacteria contain *N*-acetylmuramic acid. The oxidation of the *N*-acetyl group to *N*-glycolyl is catalysed by a recently identified enzyme

designated NamH (Raymond *et al.*, 2005). Although there is no indication that this modification is essential for *M. tuberculosis* survival, *M. smegmatis* mutants devoid of *namH* show increased sensitivity to β-lactams and lysozyme (Raymond *et al.*, 2005). In addition, the free carboxylic acid groups of the A_2pm or D-isoglutamic acid residues of mycobacterial PG may be amidated, and some of the free carboxylic groups of the D-isoglutamic acid residues may also be modified by the addition of a glycine residue in peptide linkage (Adam *et al.*, 1969). The importance of these modifications to the survival of the bacillus awaits identification of the enzymes responsible.

VI. CYTOSOLIC GLYCOCONJUGATES

A. Polymethylated polysaccharides

Polymethylated polysaccharides (PMPS) are unusual carbohydrates unique to the order *Actinomycetales*. Although all mycobacteria were originally thought to produce two classes of them, the 3-*O*-methylmannose polysaccharides (MMPs) and the 6-*O*-methylglucose lipopolysaccharides (MGLPs), our preliminary evidence suggests that MMPs may be restricted to fast-growing mycobacterial *spp*. and thus, absent from *M. tuberculosis* (Stadthagen *et al.*, 2007). MGLPs and MMPs were first isolated from *M. phlei*, *M. smegmatis* and *M. tuberculosis* in Dr. Clinton Ballou's laboratory (Gray and Ballou, 1971; Lee, 1966; Lee and Ballou, 1964), and much of the information we have about these molecules comes from early work from this group. A remarkable property associated with PMPS is their ability to form stable 1:1 complexes with long-chain fatty acids and acyl-coenzyme A derivatives and to regulate the activity of Fatty Acid Synthase I *in vitro* (reviewed in Bloch, 1977). These findings have led to the suggestion that they may be important regulators of lipid metabolism in mycobacteria. Physiological evidence for this assumption is however lacking.

The MGLPs of *M. tuberculosis* are cytoplasmic lipopolysaccharides of intermediate size containing up to 20 sugar units, many of which are partially *O*-methylated. The structure of MGLPs from the closely related species *M. bovis* BCG has been characterized in detail (Tuffal *et al.*, 1998a) (Fig. 2.9A). The fact that both MMPs and MGLPs are composed of hexose units predominantly or exclusively in α-(1→4)-linkage confers on these molecules a proclivity to assume the helical conformation characteristic of amylose. The fact that many of these hexoses are partially *O*-methylated further confers on the molecules a slight hydrophobicity. These structural features in turn confer on PMPS the ability to form complexes with fatty acyl chains (Hindsgaul and Ballou, 1984; Machida and Bloch, 1973;

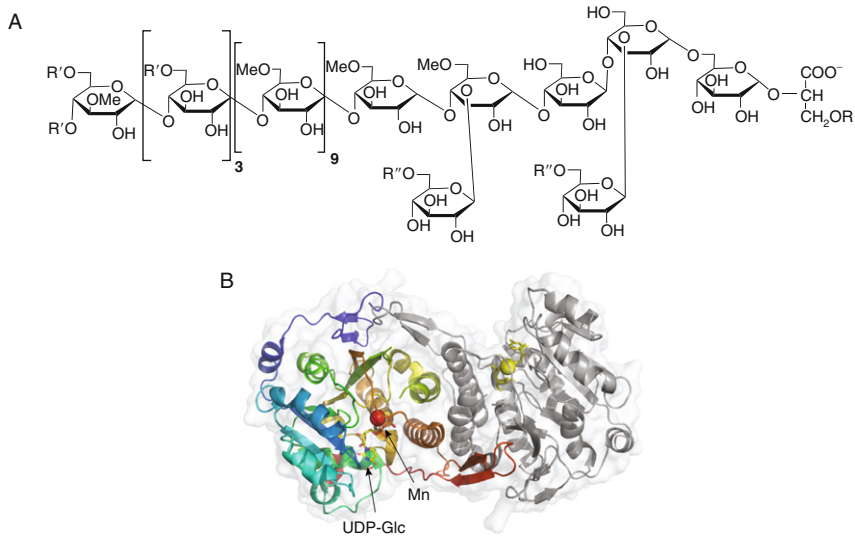

FIGURE 2.9 Methylglucose lipopolysaccharides. (A) Structure of the MGLPs from *M. bovis* BCG. The non-reducing end of the polymer is acylated by a combination of acetate, propionate and isobutyrate (R′), whereas octanoate (R) esterifies position 1 of glyceric acid, and zero to three succinate groups (R″) esterify the Glc residues of the reducing end. MGLPs occur as a mixture of four main components that differ in their content of esterified succinic acid. (B) Three-dimensional structure of the glucosyl-3-phosphoglycerate synthase from *M. avium* subsp. *paratuberculosis* (MAP2569c) in complex with UDP-Glc. Representation of the dimeric form of the MAP2569c. One monomer is 'colour ramped' from the N terminus (blue) to the C terminus (red). The second monomer is in grey and UDP-Glc is shown in yellow.

Maggio, 1980; Tuffal *et al.*, 1998b; Yabusaki and Ballou, 1978; Yabusaki *et al.*, 1979), accounting for their unique regulatory roles on fatty acid metabolism *in vitro* (Bloch, 1977; Forsberg *et al.*, 1982; Yabusaki and Ballou, 1979).

Only in recent years has the genetics of the biosynthesis of MGLPs in *M. tuberculosis* begun to be explored. Two gene clusters have been described which seem to carry much of the genes required for the glucosylation, acylation and methylation steps of these molecules (Kaur *et al.*, unpublished results; Sambou *et al.*, 2008; Stadthagen *et al.*, 2007). The glucosyl-3-phosphoglycerate synthase from *M. tuberculosis* (Rv1208) and orthologue in *M. avium* subsp. *paratuberculosis* (MAP2569c), which likely catalyse the first glucosyl transfer in the pathway (Empadinhas *et al.*, 2008), have been crystallized and the structure of the latter enzyme solved without substrate and in complex with the donor substrate, UDP-Glc (Fulton *et al.*, 2008a,b; Gest *et al.*, 2008). The structure of the protein displays the typical organization of GT-A fold enzymes with a conserved

DxD motif that coordinates a Mg^{2+} ion (Fig. 2.9B). Current knowledge of the biosynthesis and genetics of MGLPs in *M. tuberculosis* has recently been reviewed, and we would like to refer the reader to this publication (Jackson and Brennan, 2009).

B. Glycogen

Glycogen is an intracellular polysaccharide which serves as a major carbohydrate reserve in most bacteria. Glycogen content in mycobacteria, including *M. tuberculosis*, was shown to vary with growth phase and nitrogen concentration in the medium (Antoine and Tepper, 1969a,b; Elbein and Mitchell, 1973). Furthermore, the constant recycling of this molecule during exponential phase seems to be essential for growth of *M. smegmatis* (Belanger and Hatfull, 1999).

The structure of glycogen is almost undistinguishable from that of the capsular α-D-glucan described earlier (see Section III). Recently, the application of analytical centrifugation and dynamic light scattering to α-D-glucan and glycogen purified from *M. bovis* BCG indicated, however, that the α-D-glucan possessed a slightly higher molecular mass (13×10^6 versus 7.5×10^6 Da) and was more compact than glycogen (Dinadayala *et al.*, 2008).

Not surprisingly, the biosynthetic pathways of glycogen and α-D-glucan share common enzymes (see Section III) (Sambou *et al.*, 2008). Two partially redundant α-1,4-glucosyltransferases, GlgA (Rv1212c) and Rv3032, apparently participate in the elongation of glycogen in *M. tuberculosis*, although in whole cells Rv3032 seems to be the main enzyme involved in this task (Sambou *et al.*, 2008). The molecular mechanisms underlying the preferred involvement of GlgA and Rv3032 in one or the other pathway are at present not known. Rv3032 is also involved in the elongation of the MGLPs described earlier (Stadthagen *et al.*, 2007). GlgB (Rv1326c) was identified as the branching enzyme and reported to be essential for growth (Garg *et al.*, 2007; Sambou *et al.*, 2008). To our knowledge, this is the first glycogen-branching enzyme reported to be essential in a prokaryotic organism. The reason of its essentiality in the tubercle bacillus is unknown. As GlgB seems to be the only branching enzyme committed in the synthesis of α-D-glucan and glycogen, its essentiality may be related to the physiological requirement of *M. tuberculosis* to produce at least one of these two polysaccharides. This assumption is also supported by the inability to generate a double *glgA/Rv3032* knockout of *M. tuberculosis* (Sambou *et al.*, 2008). By analogy with *glgB* mutants of *E. coli* (Lares *et al.*, 1974), one may also speculate that a deficiency in GlgB could lead to the accumulation of poorly water-soluble linear polymers of 4-linked α-glucosyl residues, either in the cytosol or in the periplasmic space of the cell envelope, causing the death of the bacterium.

The fact that significant amounts of α-D-glucan and glycogen were still produced by an ADP-Glc pyrophosphorylase *glgC* mutant of *M. tuberculosis* H37Rv indicates that the GlgC-dependent ADP-Glc pathway is not the only route to glycogen/glucan synthesis in *M. tuberculosis* H37Rv. Other as yet unidentified enzymes with low sequence similarity to the usual prokaryotic ADP-Glc pyrophosphorylases may exist in *M. tuberculosis*. It is also possible that one of the two α-1,4-glucosyltransferases, Rv3032 or GlgA, utilizes UDP-Glc instead of ADP-Glc as the sugar donor in the elongation reactions. A recent study has implicated the trehalose synthase, TreS, of *M. smegmatis* in the (reversible) utilization of trehalose for the production of glycogen, in addition to its role in the interconversion of trehalose and maltose (Pan *et al.*, 2008). The TreS enzyme of *M. tuberculosis* is likely to function similarly. Finally, the presence of a putative amylomaltase gene (*Rv1781c*, *malQ*) in the *M. tuberculosis* genome suggests that this bacterium might have the ability to synthesize α-1,4-glucans when grown on maltose or maltodextrin (Preiss and Romeo, 1989).

C. Mycothiol

Mycothiol is a low-molecular-weight thiol produced by Actinobacteria whose primary role is to maintain a reducing environment in the cells which is necessary for standard metabolic activities to occur. It also represents a stabilized form of cysteine which is required for protein and CoA synthesis in the cells. Mycothiol has been shown to protect mycobacteria against oxidative stress, alkylating stress, acid stress and a broad range of antibiotics (Fig. 2.10A) (for a review, Buchmeier *et al.*, 2003, 2006; Miller *et al.*, 2007; Newton *et al.*, 2008; Rawat *et al.*, 2002, 2007). Like glutathione in Gram-negative bacteria and eukaryotes, mycothiol also serves as an enzyme cofactor (Newton *et al.*, 2008; Rawat and Av-Gay, 2007), and it is thus thought that either mycothiol or mycothiol-dependent proteins account for the protection of mycobacteria against oxidants and other toxic agents (Rawat and Av-Gay, 2007).

Mycothiol biosynthesis proceeds through a five-step pathway. *N*-acetylglucosaminylinositol phosphate is the product of the UDP-GlcNAc:1L-*myo*-Ins-1-P α-N-acetylglucoaminyltransferase MshA (Rv0486), a nucleotide-sugar utilizing GT-B glycosyltransferase (Newton *et al.*, 2003, 2006a). An unidentified phosphatase dephosphorylates this molecule to yield *N*-acetylglucosaminylinositol (GlcNAc-Ins) (Newton *et al.*, 2006a). Deacetylation of GlcNAc-Ins by the deacetylase MshB (Rv1170) yields 1-D-*myo*-inosityl-2-deoxy-α-D-glucopyranoside (GlcN-Ins) (Newton *et al.*, 2000, 2006b) which is then linked to cysteine by the ATP-dependent ligase MshC (Rv2130c) to yield Cys-GlcN-Ins (Sareen *et al.*, 2002). The final step

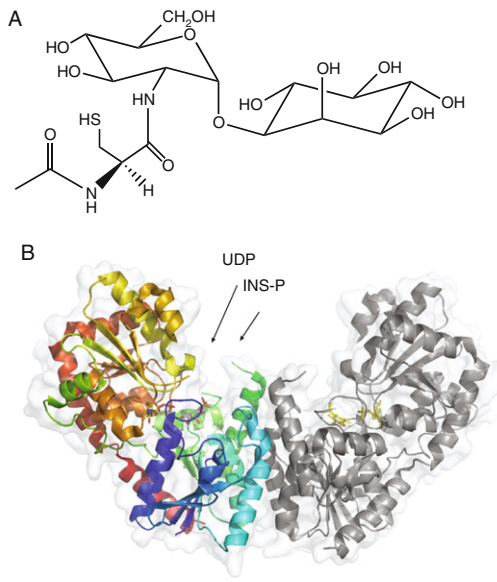

FIGURE 2.10 Mycothiol. (A) Structure of mycothiol; (B) Three-dimensional structure of a *Cg*MshA–UDP–Ins-P complex. Representation of the dimeric form of *Cg*MshA. One monomer is 'colour ramped' from the N terminus (blue) to the C terminus (red). The second monomer is in grey and the UDP and Ins-P substrates are shown in yellow. The dimer interface is entirely composed of residues from the N-terminal domain.

of the pathway is the conversion of Cys-GlcN-Ins to acetyl Cys-GlcN-Ins (mycothiol) by the acetyltransferase MshD (Rv0819) (Koledin *et al.*, 2002).

Gene disruption studies initially showed that *mshA* and *mshC* are essential for the growth of *M. tuberculosis*, thereby implicating mycothiol as vital components of the tubercle bacillus (Buchmeier and Fahey, 2006; Sareen *et al.*, 2003). This assumption was recently questioned when spontaneous isoniazid- and ethionamide-resistant mutants of *M. tuberculosis* carrying mutations in *mshA* and deficient in mycothiol production were found to display a wild-type growth phenotype *in vitro* and in mice (Vilchèze *et al.*, 2008). The reason for this discrepancy is unknown but could be due to the different *M. tuberculosis* strains used in the two studies or to the ability of the spontaneous mutants to produce undetectable but sufficient amounts of mycothiol to sustain growth. *mshB* and *mshD* are clearly not essential in *M. tuberculosis* as knock-out mutants still produced significant amounts of mycothiol and biosynthetically related thiol compounds (Buchmeier *et al.*, 2003, 2006). In view of the absence of mycothiol in mammalian cells and potential essentiality, the MshA and MshC proteins may represent attractive targets for novel anti-TB drugs. Consistently, the crystal structures of MshB and MshD from *M. tuberculosis*

and MshA from the closely related *C. glutamicum* have been determined, and inhibitors of MshB and MshC have been reported (Metaferia *et al.*, 2007; Newton *et al.*, 2006c).

The catalytic mechanism of 'retaining' GTs that leads to retention of the anomeric sugar binding is still a matter of debate and controversy. A double-displacement mechanism via the formation of a covalent glycosyl-enzyme intermediate was first proposed. However, in the absence of direct evidence of a viable covalent intermediate, an alternative mechanism was suggested, the S_Ni 'internal return' (Lairson *et al.*, 2008). The crystal structure of MshA (*Cg*MshA, pdb code 3C4V), solved in the absence of substrates and in complex with UDP and Ins-P, has shed some light into the catalytic mechanism of 'retaining' GTs (Fig. 2.10B) (Vetting *et al.*, 2008). Vetting *et al.* (2008) proposed a substrate-assisted mechanism, in which the β-PO_4 of UDP-*N*-acetylglucosamine promotes the nucleophilic attack of the 3-hydroxyl group of Ins-P and assists in the cleavage of the sugar-nucleotide bond.

VII. CONCLUSIONS AND FUTURE PROSPECTS

From this comprehensive review one can see that the primary biosynthetic pathways responsible for the synthesis of the individual entities of the cell wall of *M. tuberculosis* have been defined or are nearing so, and the roles of the individual enzymes and genes in the processes are known. Certainly this general statement applies to the synthesis of peptidoglycan, much of the arabinogalactan, the mycolic acids, LAM, the phthiocerol- and trehalose-containing lipids and glycolipids. In these respects the next great challenges are the definition of the mechanisms of assembly of the final mAGP complex, precisely the enzymology and genetics of the ligation of peptidoglycan to the arabinogalactan and recognition of the juncture at which mycolic acids are attached to the complex. Beyond these challenges are the issues of the assembly of the whole, a topic still to be faced in the case of cell wall assembly of the better studied Gram-positive and Gram-negative bacteria. Nevertheless, the elegant studies on the assembly of Gram negative LPS provide a paradigm for such future studies (Raetz and Whitfield, 2002). For instance completion of the Smooth-LPS molecule involves the ligation of the *O*-polysaccharide to the nascent Lipid A-Core and these events are located on the periplasmic face of the inner membrane, and the *waaL* gene product may be solely responsible for this ligation. Moreover the central role of MsbA in the delivery of the Lipid A-Core to the periplasmic face is now well recognized. Already searches in the *M. tuberculosis* genomes for homologues of such as the *msbA* gene, an ABC transporter, are under way. Likewise from the literature on LPS deposition (Raetz and Whitfield, 2002), we know of

the roles of, for instance, WecA, WbbE and F, in the assembly of O polysaccharides in a synthase-dependent pathway, and the necessity that the *O*-polysaccharide be still in its polyprenyl-linked format. Thus, we speculate that the AG complex of *M. tuberculosis* is exported to the cell wall in an equivalent form perhaps mediated by WecA, WbbE and F homologues. Thus, despite impressive progress over the past 20 years in our understanding of the biogenesis of the cell wall of *M. tuberculosis*, the challenges remaining are substantial. Yet, this avenue of research is particularly pressing in light of the present-day reality of drug-resistant TB and the need for new antidotes. Enzymes located beyond the cytoplasmic membrane involved in unique assembly mechanisms offer great promise as new therapeutic target with novel mechanisms of action.

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CHAPTER 3

Antimicrobial Properties of Hydroxyxanthenes

Joy G. Waite and **Ahmed E. Yousef**

Contents

Abstract

Hydroxyxanthenes are commonly used as dyestuffs in the food, cosmetics, and textile industries. These compounds also have medical applications due to their unique staining and fluorescent properties. The halogenated hydroxyxanthenes exhibit antimicrobial properties that may be useful for reducing or eliminating bacterial pathogens from a variety of environments, including drinking water and food products. Antimicrobial characteristics of Eosin, Erythrosine, Phloxine, and Rose Bengal have been known for many years, but their application as antimicrobial agents has been

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limited primarily to selective agents in microbiological growth media. The primary mechanism of bacterial inactivation by hydroxyxanthenes is photooxidation. When halogenated hydroxyxanthenes are photooxidized, a variety of compounds are produced (e.g., singlet oxygen, superoxide anion and other radicals); these exhibit toxicity to the microbial cell. Gram-positive bacteria are particularly sensitive to inactivation by photooxidation of halogenated hydroxyxanthenes; however, different species vary in their sensitivity. Gram-negative bacteria are inherently resistant to inactivation by these compounds due to the barrier properties of the outer membrane that prevent the necessary localization. Treatments such as chelation and ultrahigh pressure may be used to destabilize the outer membrane leading to sensitivity of Gram-negative bacteria to halogenated hydroxyxanthenes.

Several xanthenes derivatives are approved for food, drug, and cosmetic use, in the United States and elsewhere, as coloring agents. The microbicidal properties of xanthenes have been reported since the early 1900s; however, the full antimicrobial potential of this class of compounds needs to be explored. Incidence and severity of food-transmitted diseases are increasing and these emerging hazards have not been matched with discoveries of new and effective food-grade antimicrobial agents. This is an overview of current literature on hydroxyxanthenes, an important subgroup of xanthenes, with emphasis on the potential applications for these compounds as antimicrobial agents. This review begins with definition and classification of xanthenes, and a brief account of their synthesis and industrial applications. Subsequently, details are provided about the antimicrobial potential of these compounds including mechanisms of microbial inactivation. The chapter is concluded with a discussion of the prospects of maximizing the efficacy of xanthene derivatives, particularly against Gram-negative bacteria.

I. DEFINITIONS AND CHEMICAL STRUCTURES

Chemically, xanthene is the heterocyclic organic compound, dibenzo[a,e] pyran (Fig. 3.1). Derivatives of xanthene, which are simply referred to as xanthenes, are divided into three subgroups: fluorenes, fluorones, and rhodols (Horobin and Kiernan, 2002). Fluorenes are amine-containing xanthene derivatives (aminoxanthenes), fluorones are the hydroxyl derivatives (hydroxyxanthenes), and rhodols are aminohydroxyxantnenes. Emphasis will be on the hydroxyxanthenes, which include fluorescein, Eosin, Erythrosin, Phloxine, and Rose Bengal (Fig. 3.2).

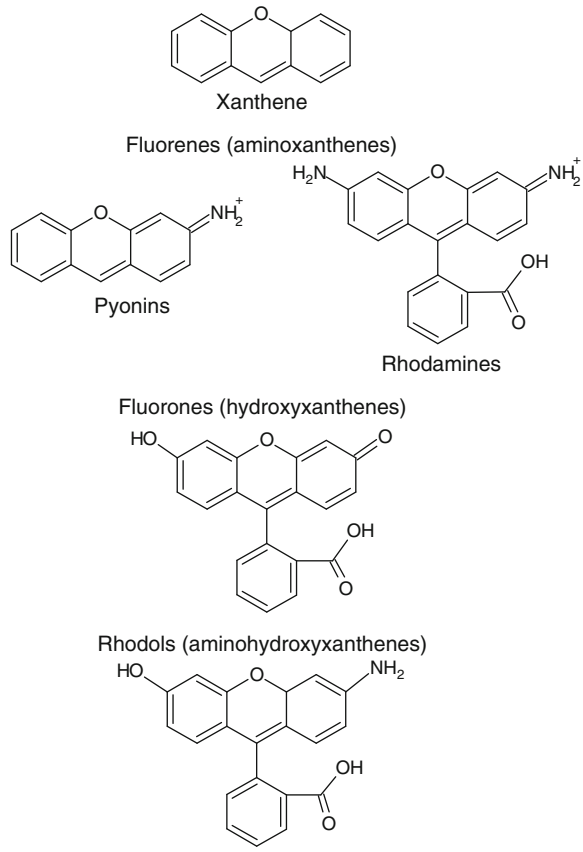

FIGURE 3.1 Basic structures of xanthene derivatives.

II. SYNTHESIS

Fluorescein was discovered by von Baeyer in 1871. Modification of this compound by halogenation to produce Eosine (1874), Erythrosine B (1872), Rose Bengal (1880–1884), and Phloxine (1887) was subsequently accomplished by other researchers (Hewitt, 1922; Neckers, 1987). Fluorescein is commercially synthesized by condensation of resorcinol and phthalic anhydride using heat or a condensing agent (Fig. 3.3). This condensation may be catalyzed by zinc chloride (Baeyer synthesis) or sulfuric acid (Ambler *et al.*, 1927; Hewitt, 1922; Jacobs, 1947; Neckers and Valdes-Aguilera, 1993).

Treatment of fluorescein with elemental bromine and iodine produces the halogenated hydroxyxanthenes, Eosin and Erythrosin, respectively.

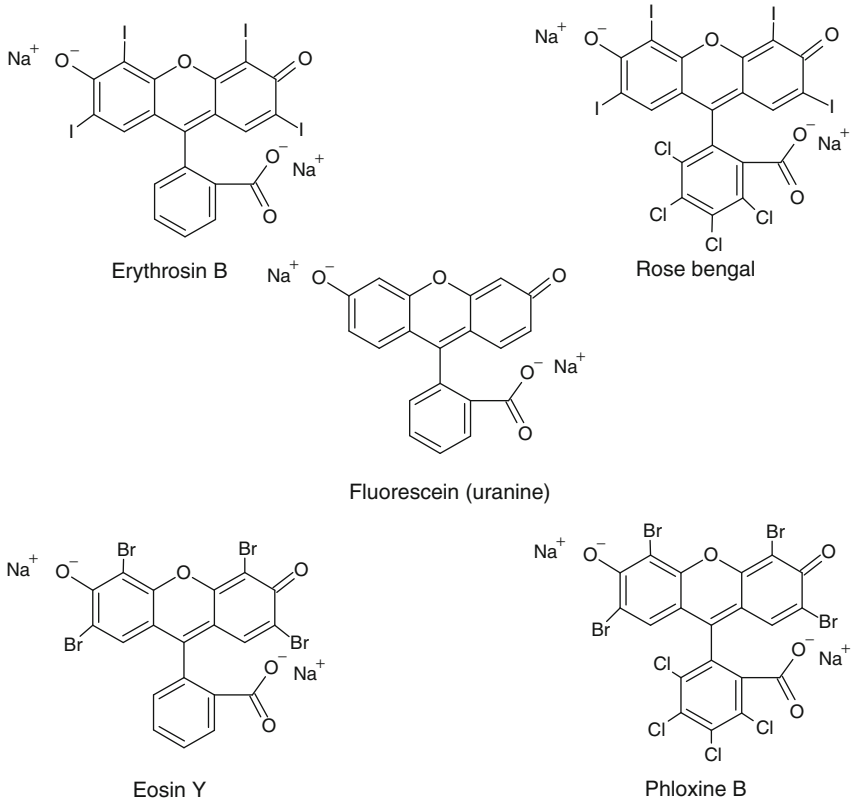

FIGURE 3.2 Chemical structures of major hydroxyxanthenes.

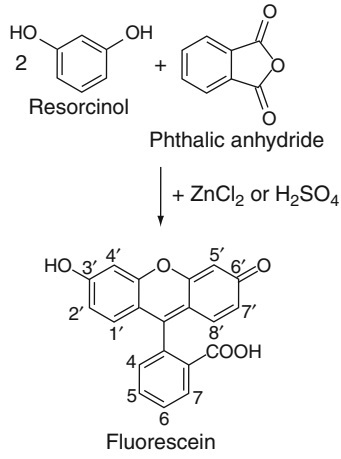

FIGURE 3.3 Chemical synthesis of fluorescein and numbering of fluorescein molecule.

The substituting agents (i.e., bromine or iodine) will first attack positions 4′ and 5′, followed by positions 2′ and 7′ (See Fig. 3.3 for position numbers). Synthesis of Phloxine and Rose Bengal (each contains four chlorine atoms) requires production of the tetrachlorinated fluorescein prior to bromination or iodination. Production of 4,5,6,7-tetrachloro-fluorescein is accomplished by the acid condensation of resorcinol and tetrachlorophthalic acid (or anhydride). Alkaline hydrolysis is used to produce the disodium salts following halogenation (Hewitt, 1922).

III. APPLICATIONS

A. Dyestuffs

Xanthene derivatives are among the oldest and most commonly used of all synthetic dyestuffs. In 1938, 32 xanthene dyes were approved for certification by the United States Food and Drug Administration (US FDA) in the three categories: food, drug, or cosmetic. Approval of some xanthene dyes was later reversed due to safety concerns, but most were delisted due to lack of commercial impact (Lipman, 1995). Currently, 10 xanthenes derivatives remain certifiable by FDA. The US Code of Federal Regulations (2003) lists the current regulatory status, characteristics, and purity of the certifiable xanthene derivatives. Hydroxyxanthenes are difficult to purify and a number of contaminants from the fluorescein-producing reaction, along with byproducts resulting from incomplete halogenations, are commonly present in commercial products, including the US FDA certified colorants (Marshall, 1976). FD&C Red No. 3 (Erythrosine B) was one of the first seven color additives allowed by the U.S. Food and Drug Act of 1906 and is currently the only xanthene derivative to be certified for food use in the United States (Parkinson and Brown, 1981; United States Government, 2003). The dye form of FD&C Red No. 3 received permanent listing status for ingested drugs and foods in June of 1969 (Blumenthal, 1990). FD&C Red No. 3 closely matches primary red and is often used for blending; however, this compound is sensitive to light. Thus the predominant applications are confections, powders, and baked products (Henkel, 1993; Parkinson and Brown, 1981). The provisional listing for the lake (the water insoluble form) of FD&C Red No. 3 was terminated in 1990 due to potential safety concerns.

B. Pesticides

Xanthene derivatives have been known, since the late 1920s, to have light-dependent phototoxic insecticidal properties (Heitz, 1997). Crounse and Heitz (1984) listed a number of xanthene derivatives as effective

insecticides against a large number of insect species. These compounds produce toxicity in insects following ingestion, and therefore a bait is included in the formulation to attract insects and encourage their consumption of the insecticide (Crounse and Heitz, 1984; Heitz, 1997). Appropriate bait selection provides the specificity of the insecticidal effects without damaging beneficial insect populations. Lethality of xanthene derivatives can also be improved by the inclusion of adjuvants, such as Tween 80 and Triton X-100, in the product formulation (Heitz *et al.*, 1997).

C. Medical applications

Hydroxyxanthenes are used in a number of medically related procedures. Fluorescein is commonly used for ophthalmic examinations and applied topically to determine the extent of corneal damage. The compound may also be injected intravenously for a procedure termed retinal angiography (Kim, 2000). Erythrosin has been used for many years as a plaque-disclosing agent in dental evaluations, and its antimicrobial activity toward dental microbiota has been noted (Caldwell and Hunt, 1969; Wood *et al.*, 2006). Application of Eosin as a histological stain has been known since the 1800s. Leukocytes are observed microscopically after staining with Eosin; hence, these are described as eosinophils (Neckers and Valdes-Aguilera, 1993). Rose Bengal has been used to test liver function in humans (Neckers, 1987). Emergence of antibiotic-resistant strains of human pathogens constitutes a serious medical challenge. According to Rasooly and Weisz (2002), Phloxine B is potent antimicrobial agent against methicillin-resistant *Staphylocccus aureus*.

D. Antimicrobials

Application of hydroxyxanthenes as antimicrobial agents is currently limited. Some of these compounds are used as selective ingredients in microbiological media. Eosin and Rose Bengal are well-known selective inhibitors that favor the growth of Gram-negative bacteria (i.e., Eosin Methylene Blue Agar) and fungi (i.e., Rose Bengal Chloramphenicol Agar), respectively (Begue *et al.*, 1965). Rose Bengal has been used to preferentially isolate fungi from soil samples for at least 60 years (Ottow, 1972).

Recent findings suggest that hydroxyxanthenes are potentially useful antimicrobial food additives (Waite, 2007; Waite and Yousef, 2008). Additional antimicrobial applications have been proposed, including wastewater treatment, seawater treatment, and as *Salmonella*-control agents for *in vivo* applications in chickens (Adams *et al.*, 1982; Bezman *et al.*, 1978; El-Adly, 2008; Jemli *et al.*, 2002; Martin and Perez-Cruet, 1987;

Rengifo-Herrera *et al.*, 2007). Rasooly (2005) demonstrated the antimicrobial efficacy Phloxine B against several *Bacillus* spp. The author also showed that Gram-negative bacteria became sensitive to the xanthene compound when their cells were pretreated with ethylenediaminetetraacetic acid (EDTA). Phloxine B is potentially useful as a bactericidal agent against the plant pathogen, *Agrobacterium tumefaciens* (Willeford *et al.*, 1998). Rose Bengal has also been found to reduce the infectivity of the parasite *Trypanosoma cruzi* (Cruz *et al.*, 1984).

IV. ANTIMICROBIAL MECHANISM

Use of hydroxyxanthenes as antimicrobial agents is currently limited, but these compounds are potentially valuable for emerging applications in food, medicine and the environment. To explore their applicability in these fields, researchers should be aware of the mechanism of action of these compounds against various microorganisms. Reviewing the current understanding of the mechanism of antimicrobial action may help researchers develop suitable strategies for new applications.

A. Photooxidation and antimicrobial properties

Hydroxyxanthenes are potent photosensitizers with photooxidation believed to be the primary mechanism for their antimicrobial efficacy. The antibacterial activity of hydroxyxanthenes via photooxidation was first reported in 1904 by Jadlbauer and von Tappeiner and in 1908 by Reitz (Harrison, 1967; Karrer *et al.*, 1999). Photooxidation is a light-dependent process in which the photosensitizer reacts with other compounds in the system to produce oxidized end products (Foote, 1991). The photosensitizer undergoes photoexcitation and transfers its energy to other molecules. Photooxidation is also referred to as photosensitized oxidation or the photodynamic effect. This process is shown schematically in Fig. 3.4. The singlet (ground state) photosensitizer contains two electrons with opposite spins in a low-energy orbital. Absorption of a photon of light results in the movement of one of the electrons to a high-energy orbital while maintaining its spin direction; this is referred to as first excited singlet state. Compounds at this state can lose energy by emitting light (fluorescence) or by giving off heat (internal conversion) to return to the ground state. Alternatively, the spin direction of the excited electron may invert, that is, become parallel with its partner electron, to form an excited triplet state of the photosensitizer. This triplet state is relatively long lived (microseconds). Molecules at the excited triplet state may lose energy in three different pathways: (i) reaction with cellular or soluble components (type I reaction); (ii) reaction with triplet oxygen, that is, oxygen in its

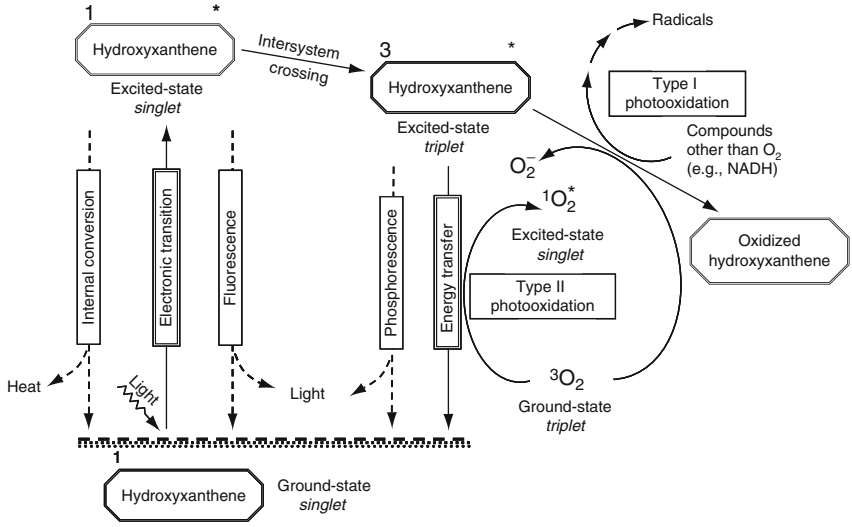

FIGURE 3.4 Illustrated interpretation of photooxidation of a generic hydroxyxanthene as a photosensitizer. Adapted from Castano *et al.* (2004).

ground state, to form the excited-state singlet oxygen (type II reaction); or (iii) light emission (phosphorescence). These reactions are not mutually exclusive for a particular photosensitizer and the ratio of reaction outcomes is dependent on the photosensitizer and concentrations of reactants (Castano *et al.*, 2004). Hydroxyxanthene triplets are presumed to favor type I reactions in biological systems due to the abundance of reductants, including NADH, resulting in reduced dye species (Martin and Logsdon, 1987a).

According to earlier studies, fluorescein had no antimicrobial potency against various Gram-positive and Gram-negative bacteria, and yeast at concentrations up to 500 μM (Martin and Logsdon, 1987b; Wang *et al.*, 2006). In a recent study (Waite and Yousef, 2008), antimicrobial activity of hydroxyxanthenes against *Listeria monocytogenes* has been correlated with increasing substitutions of halogen on the fluorescein base (Fig. 3.5). The nonhalogenataed hydroxyxanthene (fluorescein) and its brominated derivative (Eosin), at 10 ppm level, were ineffective at decreasing the population of *L. monocytogenes*. Erythrosin B, Phloxine, and Rose Bengal produced considerable inactivation of *L. monocytogenes* under the conditions tested. Iodinated hydroxyxanthenes were significantly more effective than their brominated counterparts at inactivating *L. monocytogenes*. Likewise, tetra-chlorinated derivatives were more effective than non-chlorinated species at reducing the target population. This general trend of antimicrobial efficacy has been reported previously against a variety of

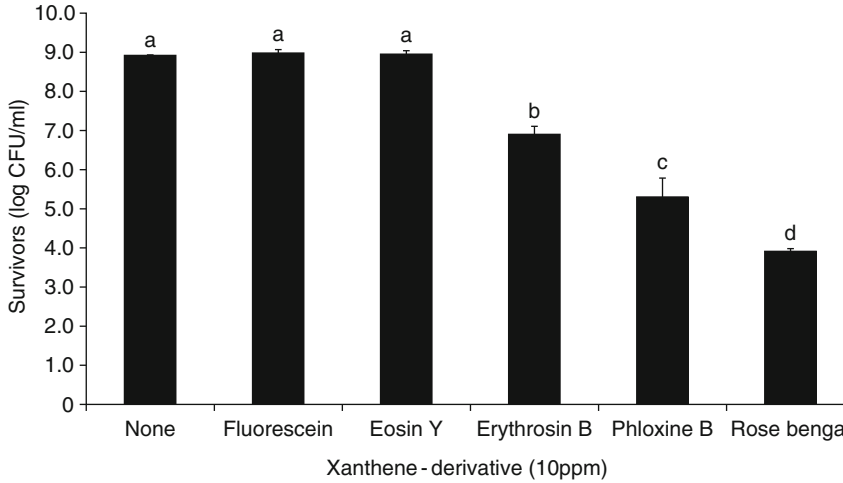

FIGURE 3.5 Surviving *Listeria monocytogenes* OSY-328 following treatment with various xanthene derivatives (10 ppm) and light exposure of 45 min in citrate–phosphate buffer, pH 7.0 (Adapted from Waite and Yousef, 2008). Error bars indicate standard error, $n = 2$. Different letter designations indicate significant differences (p-value <0.05) in survival of *L. monocytogenes* OSY-328 following treatment.

target microorganisms (Harrison, 1967; Iwamoto *et al.*, 1989; Karrer *et al.*, 1999).

Heavy halogen atoms, such as bromine and iodine, likely enhance the intersystem crossing of the excited singlet state of the photosensitizer molecule by spin perturbation to yield high levels of the reactive triplet molecule and also high phosphorescence quantum yields (Krasnoff *et al.*, 1999; Rosenthal *et al.*, 1988; Wang *et al.*, 2006). The quantum yields of fluorescence, triplet formation, and singlet oxygen generation by selected hydroxyxanthenes are shown in Table 3.1. Increasing halogen substitution results in an increase in intersystem crossing from singlet to triplet state, thus, increasing the quantum yield of phosphorescence at the expense of that for fluorescence. Higher quantum yields of triplet formation for hydroxyxanthenes correlate with increased singlet oxygen yield and enhanced antimicrobial activity, particularly for Rose Bengal (Garland and Moore, 1979; Wang *et al.*, 2006). Caution must be exercised in making generalizations about photooxidative pathways of different hydroxyxanthenes. Assumptions about Rose Bengal may not be valid for Erythrosin and vice versa. For example, Rose Bengal was found to be a potent photooxidizer of NADH, whereas Erythrosin was virtually incapable of causing NADH oxidation, indicating that the lethal mechanism may vary with chemical structure (Martin and Burch, 1988).

TABLE 3.1 Quantum yields of fluorescence, triplet formation, and singlet oxygen for selected hydroxyxanthenes upon photoexcitation (Gandin *et al.*, 1983; Inbaraj *et al.*, 2005; Jemli *et al.*, 2002; Neckers and Valdes-Aguilera, 1993; Otterstatter, 1999)

	Quantum yield		
Hydroxyxanthene	Fluorescence (Φf)	Triplet formation (Φt)	Singlet oxygen generation (Φ_{O2})
Fluorescein	0.92–0.93	0.03	0.03–0.09
Eosin	0.20–0.63	0.28–0.32	0.39–0.57
Phloxine B		0.40	0.59–0.65
Erythrosin	0.02–0.08	0.62–0.69	0.62–0.63
Rose Bengal	0.018–0.08	0.76–0.86	0.75–0.79

Excessive light exposure of hydroxyxanthenes leads to photobleaching and formation of stable photolysis products (Schafer *et al.*, 2000). Halogenated hydroxyxanthenes bleach when irradiated in the presence of oxygen. The bleaching of the dye occurs due to disruption of delocalized electrons, most likely via peroxide formation across the quinoid; however, this bleaching may be reversible if reducing agents are added (Neckers, 1987). The antimicrobial activity of these photolysis products has only been minimally studied and photolysis products of Erythrosin were found to be nonmutagenic in the *Salmonella*-based Ames assay (Ozaki *et al.*, 1998; Schafer *et al.*, 2000).

Irradiation of halogenated hydroxyxanthenes may also lead to dehalogenation via type I photooxidation. Debromination of Eosin can occur during photooxidation if the triplet reacts with amines and phenols (Neckers and Valdes-Aguilera, 1993). Irradiation with sunlight results in the debromination of Phloxine B, likely resulting in the formation of free radicals and yielding 2′,4′,5′-tribromo-4,5,6,7-tetrachloro-fluorescein and 4′,5′-dibromo-4,5,6,7-tetrachloro-fluorescein as photolysis products (Wang *et al.*, 1998). It is not known whether the release of halogens from the hydroxyxanthene contributes to antimicrobial efficacy of these compounds.

B. Localization of hydroxyxanthenes in microbial cell

Localization of the hydroxyxanthenes to a sensitive compartment or region within the cell may be crucial for the mechanism of microbial lethality by these compounds. Hydroxyxanthenes are amphiphilic molecules and therefore should have variable affinities to different cell components. Several authors presented partition coefficients of various hydroxyxanthenes using water and octanol as solvents to estimate the

TABLE 3.2 Partition coefficients of hydroxyxanthenes

Hydroxyxanthene	Partition coefficient in different studies (log *P*)			
	Wang *et al.* (2006)[a]	Levitan (1977)[a]	Oros *et al.* (2003)[b]	Waite (2007)[a]
Fluorescein	− 0.28	− 4.77		− 0.49
Eosin Y	− 0.25	− 1.27	6.49	− 0.41
Erythrosin B	− 0.24	− 0.15		− 0.38
Phloxine			8.98	− 0.20
Rose Bengal	− 0.21	3.14	9.91	0.35

[a] Values based on partition coefficients between octanol and water.
[b] Reported as calculated hydrophobicity using computer software by Advanced Chemistry Development, Inc.

degree of hydrophobicity of these compounds (Table 3.2). Oros *et al.* (2003) found hydrophobicity to correlate with antimicrobial activity of a wide variety of colorants, including hydroxyxanthenes. Lethal mechanisms have been suggested for hydroxyxanthene localization in membranes, critical proteins or enzymes, nucleic acids, and other targets. Localization to target cell components as prerequisites for antimicrobial efficacy of hydroxyxanthenes remains an unproven hypothesis. Some researchers, for example, treated contaminated water with immobilized Eosin and Rose Bengal and reported the effectiveness of these stationary molecules at reducing microbial populations (Bezman *et al.*, 1978; Neckers and Valdes-Aguilera, 1993).

1. Localization in membranes

Wang *et al.* (2006) indicated that xanthene derivatives typically localize to the cellular membrane and subsequent photooxidation leads to destructive damage of the lipid and protein components of this structure. The lipophilic tendency of hydroxyxanthenes suggests some partition of these compounds into the cell membrane; however, the transfer from aqueous solution to the cellular membrane may be impeded by the negative charges of both cell membrane and hydroxyxanthenes (Dahl *et al.*, 1989). The cytoplasmic membrane contains unsaturated fatty acids that are susceptible to lipid peroxidation via photooxidation processes (Girotti, 2001; Neckers and Valdes-Aguilera, 1993). Excessive oxidation of the phospholipid bilayer can lead to loss of membrane integrity or to localized environmental changes that alter membrane protein functionality (Harrison, 1967; Phoenix *et al.*, 2003; Stark, 2005). Oxidation of the cytoplasmic membrane also leads to changes in functionality of ion transport channels and depolarization of the membrane (Pooler and Valenzeno, 1979; Schafer *et al.*, 2000; Stark, 2005). Oxidation of membrane components

by singlet oxygen may lead to downstream oxidative DNA damage via intermediate reactive oxygen species (Ouedraogo and Redmond, 2003).

2. Protein targets

Conway and Adams (1989) grew *Lactobacillus fermentum* in the presence of 0.1% Erythrosin, stained cells with cotton blue, and analyzed staining patterns using conventional and fluorescence microscopy. Cells associated with Erythrosin were not associated with cotton blue and vice versa. Cells were disrupted, and fractions were collected after ethanol and protease treatments. Ethanol precipitates without protease treatment contained Erythrosin, whereas ethanol precipitates with protease treatment did not contain the dye. These findings indicate that Erythrosin is likely associated with protein components of the cell wall (Conway and Adams, 1989).

Proteins and enzymes may be inactivated by direct binding of the photosensitizer and subsequent oxidation of critical amino acid residues within the protein. Protein binding is of particular importance when the photosensitizer is anionic in nature. Eosin and Erythrosin adsorbed to bovine serum albumin at approximately a 1:1 ratio (Garland and Moore, 1979). *In vitro*, Rose Bengal binds to DNA polymerase with enzyme inhibition being irreversible following light exposure but reversible without light exposure (Neckers and Valdes-Aguilera, 1993). Rose Bengal also inhibited RNA polymerase and several NAD^+ and $NADP^+$-dependent dehydrogenases, *in vitro* (Stern *et al.*, 1980). Likewise, lysozyme activity is sensitive to photooxidation by Eosin (Bezman *et al.*, 1978; Kepka and Grossweiner, 1973). Tryptophan, cysteine, methionine, tyrosine, and histidine are known to be important targets of photooxidation within protein molecules (Castano *et al.*, 2004; Neckers and Valdes-Aguilera, 1993). Within a protein, histidine and tryptophan residues are particularly susceptible to oxidation via singlet oxygen (Castano *et al.*, 2004; Estevam *et al.*, 2004).

3. Nucleic acids

DNA can be oxidatively damaged in the nucleic acid or sugar portion of the molecule. Additionally, DNA could be oxidatively crosslinked to proteins, a damage that is particularly difficult for cells to repair (Castano *et al.*, 2004). Several photosensitizers are known to cause DNA damage, usually with evidence of mutation; therefore, it has been proposed that photooxidation could lead to mutagenesis (Harrison, 1967; Phoenix *et al.*, 2003).

When investigated *in vitro*, Rose Bengal caused double-strand breaks in DNA as a result of photooxidation. These reactions are 20 times more efficient in the absence of oxygen, indicating the likelihood of a type I mechanism (Ciulla *et al.*, 1989; Neckers and Valdes-Aguilera, 1993).

However, others reported lower efficiency under anaerobic conditions, thus implicating singlet oxygen as a reaction intermediate (Nieuwint *et al.*, 1985).

In vivo, hydroxyxanthenes are not likely to interact directly with DNA due to size, charge restrictions, and accessibility. Lakdawalla and Netrawali (1988) suggested that due to the potential planarity of the hydroxyxanthene molecules, there is a likelihood for intercalation in the DNA which would result in frameshift mutations. Halogenated hydroxyxanthenes have been shown to induce DNA damage in *B. subtilis* in the recombination repair assay (Yoshikawa *et al.*, 1978). Increased mutation frequencies were reported in *Neurospora crassa*, *Serratia marcensens*, *Escherichia coli*, *B. subtilis*, *Haemophilus influenzae*, and *Penicillium notatum*, following photooxidation with Eosin or Erythrosin (Harrison, 1967; Jemli *et al.*, 2002; Lakdawalla and Netrawali, 1988). However, FD&C Red No. 3 (Erythrosin) has repeatedly been negative for causing mutagenicity in *Salmonella*/Ames reversion assays (Jemli *et al.*, 2002; Lakdawalla and Netrawali, 1988).

4. Other targets

Critical redox molecules within the cell may be affected by photooxidation; these molecules include NADH, glutathione, and cytochrome c (Martin and Burch, 1988). Biologically important quinones or hydroxyquinones may be important targets of photooxidation, leading to cell death. Gutierrez and Garcia (1998) investigated interactions and complex formation between hydroxyxanthenes (Erythrosin and Rose Bengal) and quinones which led to a red shift in the absorbance spectra. *In vitro* studies by Katsuki *et al.* (1994) detected electron transfer reactions between xanthene dyes and *p*-quinones, with light exposure, as measured by electron paramagnetic resonance spectroscopy. Quinone anion radicals were the product of photooxidation using Eosin Y, dibromo-fluorescein, and Erythrosin B, but not fluorescein (Katsuki *et al.*, 1994). *In vivo* studies have not confirmed the interaction between xanthene derivatives and cellular redox components.

C. Relative resistance of microorganisms to hydroxyxanthenes

Microorganisms vary in their sensitivity to hydroxyxanthenes. Wang *et al.* (2006) found *Saccharomyces cerevisiae* to be more sensitive to photooxidation by hydroxyxanthenes than was the Gram-positive *Staphylococcus aureus*, with the latter being more sensitive than the Gram-negative *E. coli*. Most studies have reported minimal, if any, effect of hydroxyxanthenes on Gram-negative bacteria. Erythrosin, at 0.5% and 1.0%, effectively inhibits the growth of numerous organisms found in dental plaque, including *Streptococcus* spp. and some yeast, but ineffective against

Gram-negative bacteria (Caldwell and Hunt, 1969). Inefficacy of these compounds against Gram-negative bacteria has been attributed to the barrier properties of the outer membrane (Dahl *et al.*, 1989). Efficacy of hydroxyxanthenes toward Gram-negative bacteria may be improved with increasing dye concentrations (10 μM) and excessive light exposure (240 min) (Jemli *et al.*, 2002). Hydroxyxanthenes are also more effective against Gram-negative organisms in simple media, for example, water (Bezman *et al.*, 1978; Neckers and Valdes-Aguilera, 1993). Dahl *et al*. (1988) suggested that Rose Bengal penetrates the outer membrane of Gram-negative bacteria, but the process occurs slowly.

Gram-negative bacteria are substantially more resistant to inactivation by photooxidation than are the Gram-positives, indicating the importance of the protective effect of the outer membrane (Begue *et al.*, 1965; Jemli *et al.*, 2002; Karrer *et al.*, 1999; Kreitner *et al.*, 2003; Schafer *et al.*, 2000). The barrier properties of the outer membrane may prevent localization of the photosensitizer or block the penetration of photooxidation products, for example, singlet oxygen or hydroxyl radical (Jemli *et al.*, 2002; Karrer *et al.*, 1999). Deep rough mutants of *Salmonella* and *E. coli* are sensitive to photodynamic inactivation, compared to wild-type strains; this indicates a protective barrier effect of the lipopolysaccharide of the outer membrane (Dahl *et al.*, 1987, 1989).

Disruption of the outer membrane barrier properties of Gram-negative bacteria may lead to sensitization of these organisms to the photooxidative mechanisms of hydroxyxanthenes. Recently, two approaches have been used to sensitize bacteria to xanthene derivatives: chelation and treatment with ultrahigh pressure.

1. Chelation

The hydrophobic barrier of the outer membrane of Gram-negative bacteria is primarily achieved by the lipopolysaccharide components. The inner core of these molecules is anionic and the electrostatic repulsion of neighboring molecules is neutralized by divalent cations (Mg^{2+} and Ca^{2+}) which help to stabilize the outer leaflet structure of the outer membrane (Nikaido, 1996). Rasooly (2005) investigated the sensitization of *E. coli* to Phloxine B by treating the cells with the divalent chelator, EDTA. Cells treated with EDTA and Phloxine B for extended periods of time (>5 h) showed some sensitivity to the photooxidation treatment (0.6 log reduction). These findings provide some evidence that destabilization of the outer membrane via divalent cation chelation may sensitize Gram-negative bacteria to xanthene derivatives. In our laboratory, EDTA treatments (≤25 mM) in combination with Erythrosin B (10 ppm) were ineffective at reducing *E. coli* O157:H7 populations (Waite, 2007). Additionally, fluorescent microscopy confirmed that EDTA treatment did not cause accumulation of the xanthene derivative within *E. coli* cells.

It should be noted that EDTA and Erythrosin B treatment times were significantly less than those reported by Rasooly (2005). It should also be noted that this author used an *E. coli* K-12 descendent strain which, by definition as a K-12 strain, may or may not contain a complete core structure, but lacks the O antigen (Liu and Reeves, 1996). Therefore, K-12 strains would be expected to display different barrier properties than the *E. coli* O157:H7 strain used in our laboratory (Nikaido, 1996).

2. Ultrahigh pressure

Application of ultrahigh pressure, above 200 MPa, is lethal to microorganisms suspended in different media, including food (Lado and Yousef, 2002). Pressure treatments used in food processing range from 300 to 1000 MPa with holding times commonly less than 10 min. Pressure treatments may change the outer membrane of Gram-negative bacteria and alter its barrier properties. The nature of membrane changes has not been fully elucidated; however, depending on the dose of the pressure treatment, these changes may be described as reversible or irreversible.

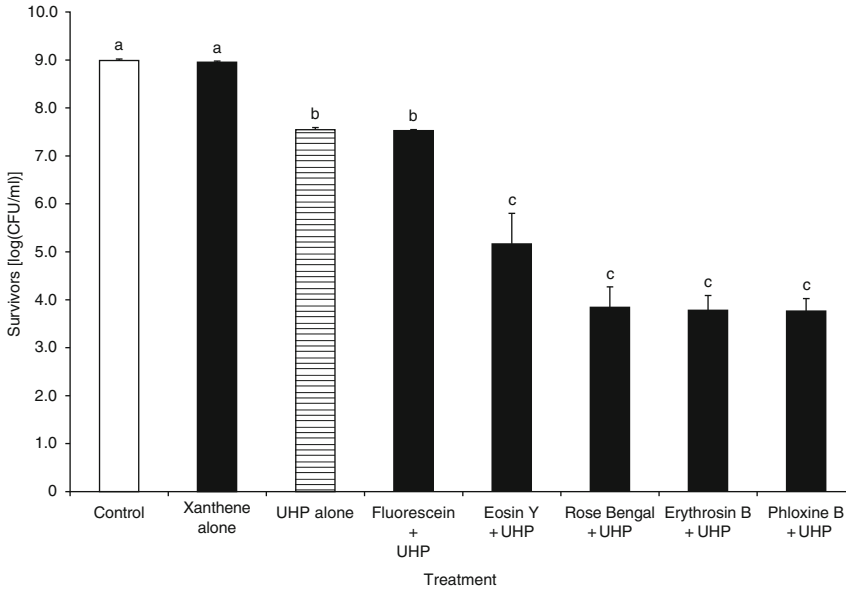

FIGURE 3.6 Surviving *Escherichia coli* O157:H7 EDL 933 populations following treatment with combinations of various xanthene derivatives (10 ppm), ultrahigh pressure (400 MPa, 1 min, 25 °C), and light exposure of 45 min in citrate–phosphate buffer (pH 7.0) (Adapted from Waite and Yousef, 2008). Error bars indicate standard error, $n \geq 3$. Different letter designations indicate significant differences (p-value <0.05) in efficacy of treatments including pressure.

Pressure treatments above 250 MPa and below 300 MPa tend to display reversible changes in the outer membrane (Hauben *et al.*, 1996; Waite and Yousef, 2009). With pressure treatments that succeed in changing the barrier properties of the outer membrane, Gram-negative bacteria became sensitive to hydroxyxanthene treatment (Waite and Yousef, 2008; Waite and Yousef, 2009). Inactivation of *E. coli* O157:H7 by combinations of xanthene-derivatives, light exposure, and ultrahigh pressure is illustrated in Fig. 3.6. The halogenated xanthene derivatives act synergistically with ultrahigh pressure against *E. coli*. These results suggest the feasibility of applying hydroxyxanthene treatments to enhance the safety of pressure-processed food products.

V. CONCLUSIONS

Halogenated hydroxyxanthenes are capable of inducing lethality toward a variety of microorganisms. The mechanism of microbial inactivation by these compounds is primarily via photooxidative pathways. Gram-negative bacteria are exceptionally more resistant than other microorganisms to these treatments. Enhancing the efficacy of hydroxyxanthenes against Gram-negative bacteria is feasible when the barrier properties of the outer membrane are disrupted by chemical or physical means. These combinations treatments may be used to enhance the safety of processed food or other products.

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CHAPTER 4

In Vitro Biofilm Models: An Overview

Andrew J. McBain

Contents

Abstract

Observing naturally occurring biofilms *in situ* or *ex situ* has revealed the wide distribution of sessile microbial communities. The ubiquity, variety and complexity of biofilms is now widely accepted by microbiologists. While they are associated with many beneficial functions such as nutrient cycling, bioremediation and colonization resistance, adverse effects including recalcitrance, their involvement in industrial fouling, contamination and infection have made

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biofilms a priority research topic. We know that most biofilms, other than within certain infections and laboratory flasks, are composed of multiple species and that there is arguably no unifying biofilm architecture. Biofilms do however share certain properties including the presence of gradients of nutrients, gasses and metabolic products, relatively increased cell density, deposition of extracellular polymeric substances and marked recalcitrance towards antimicrobial treatments. Much of our understanding of biofilm physiology and micro-ecology originates from experiments using *in vitro* biofilm models. Broadly speaking, such models may be used to replicate environmental conditions within the laboratory or to focus on selected variables such a growth rate or fluid flow, etc. This chapter provides an overview of some commonly used biofilm models including microtitre plate systems, flow cells, the constant depth film fermenter, annular reactors and the perfused biofilm fermenter. While perfused biofilm fermenters, in particular, enable growth rate to be controlled within thin, relatively homogenous, quasi steady-state biofilms through modulation of flow rate nutrient availability, other models provide representative modelling of *in situ* conditions where steady states may be uncommon.

I. INTRODUCTION

Biofilms have been described as "functional consortia of microbial cells within extracellular polymer matrices and associated with surfaces" (Brown and Gilbert, 1995). These "surfaces" may be more holistically considered as interfaces, particularly solid:liquid and solid:gas, although biofilms do form at liquid:gas interfaces and in the absence of substrata during flocculation. Since detailed consideration of biofilm physiology is outside the scope of the current chapter, the reader is directed to review articles such as the following which cover biofilm communities (Stoodley *et al.*, 2002), general "biofilmology" (Costerton, 1995), biofilm resistance (Gilbert *et al.*, 2001), dental biofilms (Marsh, 2009), intestinal biofilms (Macfarlane, 2008) and the role of biofilms in infections (Donlan, 2001; Wilson, 2001).

Dental microbiologists have studied the biofilms that are referred to as dental plaque for many decades, but the systematic study of biofilms as a coherent microbiological discipline can be traced back to around 1978 when the term "biofilm" was coined by Bill Costerton. It seems most likely that biofilms and the associated physiological specialization of sessile microbial growth have evolved as a microbial survival mechanism. Accordingly, the formation of biofilms is known to confer protection against desiccation (McAuliffe *et al.*, 2006), chemical inactivation (Gilbert

et al., 2002), physical disruption (Kumar and Anand, 1998), protozoan predation (Matz *et al.*, 2008), phagocytosis (Cerca *et al.*, 2006) and other inimical treatments and enables cell–cell communication and increased genetic exchange. While understanding and combating biofilm recalcitrance understandably remains a major focus of research attention, biofilms are ubiquitous and their presence is associated with benefits to human kind as well as problems.

In order to study biofilms in anything other than the natural environment (*in situ*) or by removing intact biofilm material and transporting it to the lab for analyses (*ex situ*), biofilm investigations require various *in vitro* and *in vivo* model systems. The simple definition of biofilms given earlier fails to portray the considerable diversity in composition, metabolism and structure manifested by biofilms growing within different environments or under varying physicochemical conditions. The development and validation of practical, reproducible and representative laboratory models for the study of biofilms has therefore been a challenge which those with a penchant for invention have eagerly accepted. A panoply of *in vitro* biofilm model systems have been developed, which range in complexity from a bacterial colony, growing directly upon solid nutrient media (agar), to sophisticated continuous culture fermentation systems. Variety is only an advantage where an informed decision about the best model for a given application can be made. There is, however, no single ideal model system and few standardized protocols. Selection of model systems therefore often reflects the preferences of the investigator and other pragmatic questions, together with more objective criteria. Inevitably, different models have particular strengths and weaknesses, being well suited to one application but less so for others. Systems developed for the maintenance of biofilms in the laboratory can be considered in terms of the degree of control they provide over various aspects of physiology, the realism with which they replicate *in situ* conditions and the ease with which they can be established, maintained and replicated.

The aim of this chapter is to provide an overview of methods commonly used to model biofilm growth in the laboratory and to consider common applications by providing examples of their successful deployment. Biofilm models have previously been rationally differentiated according to whether they are "closed" or "open" with respect to nutritional availability: "closed" systems being analogous to batch culture and "open" analogous to continuous culture (Brown and Gilbert, 1995). For the sake of consistency, the current chapter will adopt the same system of classification. The variety of models currently in use means that this cannot be all-inclusive. Rather, the intention is to cover a selection of models that have proved to be consistently useful.

II. CHOOSING THE EXPERIMENTAL SYSTEM

Before any decisions can be made regarding the most suitable model for a biofilm investigation, broader considerations of experimental approach must be considered, such as whether to adopt a reductionist approach with pure cultures or defined communities or alternatively to select complex communities or microcosms. A choice between closed (batch) systems or open (continuous culture) must also be made.

A. Pure culture, defined consortium or microcosm?

Just as is the case with planktonic-phase microbial investigations, when modelling biofilms, a key decision relates to selection of test microorganism(s) and whether to use axenic (pure) culture, a defined consortium (two or more organisms in combination) or a microcosm. Physiological studies are normally done in pure culture, where the response of the test organism through manipulation of a single variable can be monitored. Defined consortia are often used to investigate ecological phenomena that would otherwise be difficult to measure for reasons of complexity and culturability and make use of simplified combinations of organisms that are amenable to growth on artificial media and are readily identifiable when they have done so. An additional advantage of defined communities it is that it theoretically possible to achieve a high degree of reproducibility between experimental runs which cannot be relied upon when using complex inocula. An example of a defined consortium which has been successfully deployed in a large number of studies is often referred to as the "Marsh Consortium" (after Philip Marsh) and is composed of approximately 10 bacteria that represent important physiological and ecological groups within the mouth. McKee *et al.* (1985) developed a nine-member consortium comprising *Streptococcus mutans* ATCC 2-27351, *Strep. sanguis* NCTC 7865, *Streptococcus mitior* EF 186, *Actinomyces viscosus* WVU 627, *Lactobacillus casei* AC 413, *Neisseria* sp. A1078, *Veillonella alkalescens* ATCC 17745, *Bacteroides intermedius* T 588 and *Fusobacterium nucleatum* NCTC 10593. When provided with a complex, mucin-containing medium, the consortium can be maintained stably and, given experience, can be selectively counted using several types of selective agar. Similar approaches have also been developed by colonic bacteriologists (Newton *et al.*, 1998) and for the investigation of aquatic microbial ecosystems (Buswell *et al.*, 1997).

Julian Wimpenny defined microcosms succinctly as "laboratory models of the natural system from which they originate, but also from which they evolve" (Wimpenny, 1988). Microcosms are therefore laboratory models set up with the aim of closely mimicking the physicochemical, microbiological and nutrient conditions present *in situ* and are normally

inoculated using material removed from the environment of interest. Microcosms have the advantage of maintaining much of the complexity and heterogeneity of the original sample, enabling *in situ* bacterial community dynamics to be replicated within the laboratory environment and the manipulation of variables of interest. Microcosms of dental plaque have, for example, provided further insight into the microbial ecology and physiology of dental (McBain *et al.*, 2003a; Pratten *et al.*, 1998a), colonic (Hopkins *et al.*, 2003; McBain and MacFarlane, 2001) and many other microbial ecosystems.

B. Continuous, semi-continuous or batch culture?

The batch culture paradigm, where microorganisms are provided with finite nutrients within an enclosed vessel and growth rates are rapid is comparatively rare in nature. The majority of natural ecosystems probably function in a manner that is analogous to fed-batch or continuous culture, whereby fluid flow serves to provide substrates constantly or discontinuously. *In situ* growth rates may be slow, extremely so in oligotrophic or otherwise unfavourable environments. While batch culture models have the advantage of simplicity, continuous culture systems enable better control of growth rates and other variables. It is important to note however that when chemostats are adopted for biofilm studies by suspending or immersing substrata, the system will cease to operate as a chemostat in the strict sense, losing homogeneity and the steady-state conditions originally described by Herbert (Herbert *et al.*, 1956). Instead, mixing will be compromised and within certain limits, the nascent, sessile population will increase over time (Brown and Gilbert, 1995).

III. CLOSED SYSTEM BIOFILM MODELS

A. The agar plate: A simple biofilm model?

Bacterial colonies formed upon solid growth media reproduce many of the properties of a biofilm, particularly high cell density and gradients (gas, nutrient and metabolites). Brown and Gilbert (1995) suggested that the highest reproducibility in such models would be obtained by inoculating them as confluent bacterial lawns rather than separated colonies since colonies vary in size, and thus nutrient availability and differential gaseous gradients within each colony will increase variance (Shapiro, 1987).

The finite nutrient resources within an agar plate mean that biofilms can only be supported until nutrients become depleted and the system is inherently variable as a function of time. Nevertheless, while agar plate methods are considered simplistic, their utility should not be overlooked.

A reproducible and efficient adaptation of this approach has been described by Charaf and colleagues who used it to evaluate susceptibility towards various antimicrobials (Charaf *et al.*, 1999). Their system utilizes trypticase soy agar (40 ml) which is poured into 10×10 cm Petri dishes onto which a filter paper (Whatman quantitative No. 2, Maidstone, UK) is overlaid. Overnight cultures of the test bacterium (1 ml) are then diluted 1/10 or 1/100 and pipetted onto the filter paper so that the paper is evenly moistened. Sterile, flat coupons of test substrata are then placed on the inoculated filter paper. Depending on the test bacterium, coupons can be removed after ca. 48 h and either used immediately for biocide efficacy testing or dried at 35 °C for 40 min in order to conduct the AOAC germicidal spray test (AOAC, 2000). Data so derived appears to be reasonably reproducible and differentiates between different antimicrobials (Charaf *et al.*, 1999).

More recently, agar colonies have been used in elegant studies into the molecular biology of bacillus biofilm formation where complex colonial morphologies have been used as indicators of variations in biofilm phenotype (Kearns *et al.*, 2005; Murray *et al.*, 2009; Verhamme *et al.*, 2009).

Bacterial growth on the surface of an agar plate can be distinguished from growth upon hard surfaces since the biofilm is fed from the substratum. A similar situation may occur in biofilms associated with soft tissue infections (Wolcott *et al.*, 2009), those associated with cystic fibrosis (Costerton *et al.*, 1983) and biofilms causing food spoilage. Care is required when interpreting data derived from this type of model since the availability of cationic nutrients such as iron and magnesium may be reduced through interaction with the anionic agar polysaccharide gel and the effectiveness of cationic antimicrobials may be similarly affected if these are incorporated into the agar. However, many of the polymers within the biofilm matrix are also anionic in nature. Therefore, whilst the and binding of cations within the biofilm matrix might similarly limit their diffusion it could also result in their sequestration and subsequent utilization (Sutherland, 2001).

An issue worth considering is that if agar plate colonies are to be regarded as a form of biofilm, why is the agar disc-diffusion method a poor predictor of therapeutic failure in infections associated with indwelling devices? (Bayston *et al.*, 2007). This is probably because in disc-diffusion assays, considerable concentrations of antimicrobial will be present very shortly after inoculation, many hours before cell density has increased sufficiency to create significant gradients. Growth is therefore suppressed when the physiology of target cells is analogous to that of a planktonic culture. Once colonies have formed however, there is evidence that some of the recalcitrance properties of biofilms are manifested. Cells sampled from agar plate surfaces have exhibited markedly lower susceptibility when compared to the same cells grown planktonically in

broth culture in a range of studies including those of Al-Hiti and Gilbert (1983) and DeMatteo *et al.* (1981).

B. Biofilm models based on multi-well plates: Potential for high-throughput analyses

Microtitre (i.e., 96-well) plates can be used to good effect where a simple, quantitative assay for bacterial adherence and/or biofilm formation is required. To conduct such assays, wells containing sterile growth medium are inoculated and allowed to grow with or without mixing under aerobic or anaerobic conditions, depending on the species or consortium under investigation. Similarly, fresh human saliva can be used with or without supplemental growth medium. The basic assay relies on the time-dependent adherence to the wells, which are generally composed of polystyrene, polypropylene or polycarbonate. The biofilm prevention or removal potential of antimicrobials can be measured by adding various concentrations of test compounds to nascent or mature biofilms. Quantification of adherent biomass is achieved following removal of spent culture fluid from the wells and an optional wash in buffer to remove "loosely adherent cells" by staining for which crystal violet is commonly used as a non-specific stain. Protocols may also be modified to incorporate differential stains. The presence of biofilms is apparent as a ring at the air–medium interface from aerobic and facultatively anaerobic bacteria. The stain is then dissolved by the addition of a standard volume of ethanol and measured colorimetrically using a microtitre plate reader. This type of biofilm assay has been particularly useful in molecular genetic studies in order to screen large numbers of mutants for their ability to form biofilms. For example, Friedman and Kolter (2004) used such a system to confirm the involvement of the *pel* gene cluster in the production of a glucose-rich matrix material in a strain of *Pseudomonas aeruginosa* and to demonstrate that a second genetic locus, termed *psl* is involved in the production of a mannose-rich matrix material. The system has also been variously adapted to measure other parameters of biofilm growth including the time taken for re-growth following the application of biocides, obviating the need for staining following biocide exposure and to monitor biofilm formation as a function of opacity by applying algebra. This reportedly enabled the non-destructive generation of real-time biofilm growth curves in order to simultaneously determine MICs for planktonic and adherent populations against a range of antimicrobials (Das *et al.*, 1998).

A versatile proprietary system has been developed and patented (Ceri *et al.*, 1999) and is marketed as the Calgary (Biofilm) Device or the MBEC Device. This is essentially a 96-well plate with a lid that incorporates 96 removable pegs which can be used a biofilm substrata. Biofilms formed

upon the pegs can be exposed to antimicrobials and re-growth in fresh medium is used as an endpoint in order to determine the minimum biofilm eradication concentration (MBEC). Others have adapted a microtitre plate lid marketed as a transferable solid phase screening system (Nalge Nunc International, Rochester, NY, USA) to support biofilm growth (Sandoe *et al.*, 2006).

For the investigation of population dynamics and antimicrobial efficacy in defined consortia (*Actinomyces naeslundii*, *Veillonella dispar*, *F. nucleatum*, *Streptococcus sobrinus and Streptococcus oralis*) of dental plaque bacteria, a simple and useful model utilizing a 24-well cell culture plate has been developed into which hydroxyapatite (the dominant tooth enamel mineral) discs are placed (Guggenheim *et al.*, 2001a,b). Similar systems have also been developed which utilize whole saliva, enabling control strategies upon replicated plaque microcosms to be determined. The latter system has been used, for example, to evaluate hydrolytic enzymes as potential control dental plaque agents (Ledder *et al.*, 2009). Similar models can be used to investigate biofilm formation by a range of different bacteria upon various substrata. For example, hydroxyapatite discs can be substituted with glass beads of ca. 3 mm diameter; several of which can be added to each well, providing replicate biofilms for time-course experiments. Alternatively, other solid material of interest may cut to size and used.

IV. OPEN SYSTEM BIOFILM MODELS

A. Suspended substratum reactors

The simplest biofilm model in this class can be set up simply by adding substrata to a planktonic batch culture, which could comprise any material of interest such as glass beads, dental enamel, portions of biomaterial or glass wool. Since continuous culture has many practical advantages however, "chemostats" have often been favoured for use in biofilm experiments. Experimental validity in chemostat experiments depends on homogeneity and the establishment of steady states with respect to bacterial biomass (Herbert, 1961; Herbert *et al.*, 1956; Monod, 1950; Novick and Szilard, 1950). "Wall growth" which is biofilm growth on the glass surface of the fermentation vessel was a problem to be avoided, necessitating experimental runs to be abandoned and reducing the quality of generated data. Suspended substratum biofilm reactors (SSRs) however take advantage of the propensity for planktonic bacteria to form biofilms at solid–liquid interfaces by including removable, colonizable material within "chemostat" and other reactor vessels. Construction is relatively simple and can be achieved simply by hanging or placing substratum coupons within the vessels such that planktonic-phase cultures develop

within the fluid phase and form biofilms upon the suspended substrata. The coupons can then be removed from the vessels at various times during the experimental run and analysed.

1. SSRs in dental microbiology

As has often been the case in the biofilm field, dental microbiologists were pioneers in model development. In one widely quoted publication from the early 1980s, oral bacteria were grown planktonically in continuous culture and during the chemostat runs, the authors apparently realized that the wall growth could be sampled to provide a simulation of dental plaque (Marsh *et al.*, 1983). The same research group went on to develop an early version of an SSR where removable hydroxyapatite substrata were suspended within the chemostat vessels following inoculation with a defined consortium comprising 10 oral bacteria (Bradshaw *et al.*, 1996). The addition of sucrose to the system, without pH control, selected for cariogenic species. Importantly, the researchers were also able to ascertain that bacteria become deposited upon the hydroxyapatite in less than 1 h and that colonization density increased over 21 days thus illustrating that the sessile population developed in a time-dependent manner. The latter observation also indicates that biofilms within the model did not achieve a steady state (Bradshaw *et al.*, 1996).

2. SSRs in colonic microbiology

A similar system has been developed by microbiologists who were conducting some of the first studies into biofilm formation within the human colon. Here complex gut microcosms, routinely used to study intestinal microbial ecology and metabolism, were adapted to study colonization and sessile (biofilm) growth. Much of the surface-associated bacteria growth in the colon occurs on particulate food matter (Macfarlane and Macfarlane, 2006, 2007) and within mucous layers (Ahmed *et al.*, 2007). Instead of hanging inert solid substrata within their fermenter vessels, therefore, solidified polysaccharide and mucin "baits" were cast upon glass coupons within specially designed holder pans, which were then suspended within fermenters (see Fig. 4.1) enabling realistic modelling of the colonization of food particles. This approach is epitomized by Macfarlane *et al.* (1997) in an investigation that identified the presence of metabolically and or genotypically distinct assemblages of gut bacteria upon insoluble food particles. In a later study, the same research group observed the exclusive, apical attachment of a single species of *Clostridium* to retrograde starch granules within faecal microcosms (Sharp and Macfarlane, 2000) providing further evidence that in the intestine, chemically distinct solid-state growth substrates select for specific microbial populations.

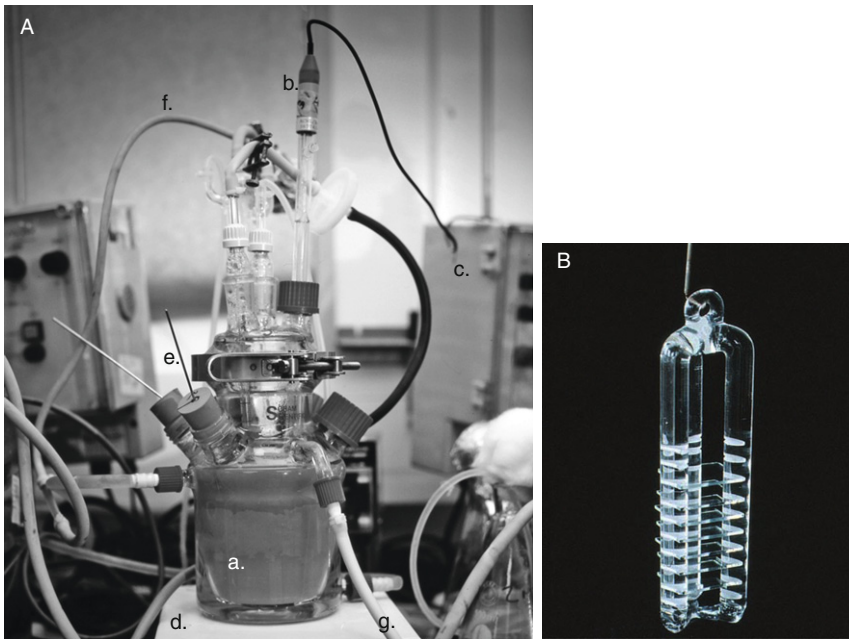


FIGURE 4.1 (A) A pH-controlled fermentation system for the growth of planktonic and sessile intestinal bacterial ecosystems (Macfarlane and Macfarlane, 2007) comprising a ca. 200-ml working volume water-jacketed vessel (a). pH is continuously measured by electrode (b) and controlled by addition of 1 M NaOH via a pH control module (c). Mixing is done by magnetic stirrer (d). The fermenter is inoculated with freshly voided human faeces and biofilms develop upon "baits" cast on glass coupons held in a removable holder (B) which are suspended from wire bait hangers (e). The system is fed continuously with a complex, mucin-containing medium via the medium inlet tube (f). The medium outlet (g) functions as per a conventional chemostat. In this image, biofilm (i.e. wall growth) can be seen on the inner glass surfaces of the fermenter.

SSRs have utilized other forms of substrata such as glass beads that can simply be placed inside fermenter vessels before autoclaving. In mixed reactors, reasonable homogeneity can be achieved and replicate biofilms are easy to obtain simply by removing beads from within the fermenter during experimental runs. Park *et al.* (2005) used such a system to establish a denitrifying microbial community that was subsequently analysed using PCR denaturing gradient gel electrophoresis.

3. SSRs and gene expression in biofilms

This type of approach has also been adopted by investigators aiming to understand differential gene expression in biofilms versus planktonic growth modes. Whiteley *et al.* (2001), for example, used DNA microarrays

to investigate differences between free-living *P. aeruginosa* cells and those growing in biofilms established upon glass beads within a "chemostat" vessel. Surprisingly few genes (ca. 1%) showed differential expression between the two growth modes. In the same investigation, the authors sought to elucidate biofilm-specific mechanisms of resistance to the antibiotic tobramycin. Exposure of biofilms to the antibiotic caused differential expression of only 20 genes. Pysz *et al.* (2004) used a range of suspended substrata to support biofilms of *Thermotoga maritima*, an anaerobic, thermophilic bacterium within a chemostat vessel. Microarray analyses revealed differential expression of 114 open reading frames in sessile cells.

These are among a number of transcriptomic and investigations conducted with the aim of identifying biofilm-specific genes. As mentioned earlier, however, biofilm physiology within SSRs is dynamic and transcriptomic studies based on this approach, although demonstrably useful may be complicated by the action of multiple variables. Alternative biofilm models which allow growth rate to be controlled more accurately and heterogeneity to be minimized, such as the perfused biofilm fermenter, are therefore arguably more suited to studies aimed at characterizing differential gene expression within biofilms.

4. The CDC biofilm reactor

This commercially available, suspended substratum reactor was developed by Donlan *et al.* (2004) of the Centers for Disease Control and Prevention (CDC) (USA) and is marketed by BioSurface Technologies Corp. (Bozeman, MT, USA). It is often used to provide reproducible biofilm samples under consistent growth conditions for the evaluation of antimicrobial agents and surface materials. The system comprises a vessel with a port mid-way up the vessel. Biofilms develop upon coupons held within eight polypropylene holders (each housing three coupons of 12.7-mm diameter) suspended from the lid and immersed in growth medium. These are arranged such that they surround a centrally located stirring vane, which is operated magnetically. Test agents can be added to the bulk fluid phase, simultaneously exposing all coupons. Sampling is achieved by removing coupon holders from the lid that can then be replaced or substituted with a stopper. Coupons made from various materials are commercially available, or specific test material could be specially cut to fit. The CDC biofilm reactor is often used in industry to compare anti-biofilm strategies and is favoured by some because a standard protocol is available (ASTM 2562-07: "Standard Test Method for Quantification of *P. aeruginosa* Grown with High Shear and Continuous Flow using a CDC Biofilm Reactor") which has been systematically evaluated. When run according to standard procedures the CDC Biofilm Reactor has been shown to be reliable and is relatively insensitive to

minor perturbations in the time allowed for initial surface colonization, culture temperature, nutrient concentration and fluid shear stress (Goeres *et al.*, 2005).

B. Rotating reactors for control of shear stress

Biofilms forming at solid–liquid interfaces may be exposed to varying fluid dynamic forces and these may have a significant effect upon composition (Dunsmore *et al.*, 2002; Purevdorj *et al.*, 2002; Stoodley *et al.*, 1999b) and recalcitrance of biofilms (Crabbe *et al.*, 2008). Efforts to maximize realism within modelled biofilms or experiments designed to investigate fluid flow as a key variable have therefore sought models which allow its control. While this can be achieved by altering flow rate and bore width in flow cells and within Robbins devices, submerged substratum reactors, incorporated rotating cylinders, have been developed and validated for a variety of applications aimed at better understanding the effects of fluid dynamic forces upon nascent and mature biofilms (see Flemming *et al.*, 2000). This type of reactor which can be operated as a batch or continuous culture system apparently originated in studies conducted by Kornegay and Andrews (1967) who were at the time interested in what were termed "biological films". "Annular reactors", named after *annulus*, the Latin word for ring were later adapted to study biofilms (Characklis *et al.*, 1982). They are available from BioSurface Technologies Corp. and comprise two concentric cylinders. The outer, static one is the outer wall of the reactor vessel, while the inner one is rotated by a variable-speed motor and holds flush-mounted, removable test coupons. Shear forces are controlled by varying rotational speed. An extension of this concept was custom manufactured from food-grade stainless steel at the University of Manchester in order to study a variety of fluid dynamic forces in real time within the same milieu and comprises four concentric cylinders. This model has been used to determine the effects of varied shear force upon the development of potable water biofilms (Rickard *et al.*, 2004).

C. The Robbins device and flow cells

Similar principles underlie the operation of these two commonly used biofilm models, whereby culture fluid is passed through a tube or cell and biofilms may be monitored microscopically (in flow cells) or formed on coupons (in some flat plate flow cells) or pegs (Robbins devices).

1. Flow cells

Flow cells are often used when microscopic examination of biofilm formation is required under fluid flow conditions that may be laminar or turbulent depending on the dimension of the cell and the rate of

fluid flow. Their use for microscopic examination of biofilms has been reviewed (Palmer, 1999). Flow cells are available commercially or bespoke units may be constructed, but they commonly involve the flow of culture fluid through a flat-walled transparent chamber such that biofilm formation can be visualized in real-time or using time-lapse photography. Access ports for the introduction of bacteria, antimicrobials or dyes may be located upstream of the cell, together with bubble traps which are often used to avoid biofilm disruption. Alternatively, flat plate flow cells can be machined from blocks of polycarbonate or other composites, with glass lids to enable visualization. Instructions for the construction of a three-cell flow chamber are available from the website of the Department of Systems Biology, Technical University of Denmark. BioSurface Technologies Corp. market several versions; some for example, can hold multiple, removable substratum coupons which can be subjected to a variety of analytical techniques and Stovall Life Science Inc. (Greensboro, NC, USA) market gamma irradiated, single-use flow cells incorporating bubble traps and feed tubing. Capillary flow cells can be constructed from square section capillary tubes so that biofilm formation can be directly visualized on the inner surfaces of the chamber and the use of small-bore capillaries enables turbulent flow and high shear forces to be developed when required. The apparatus is located onto a support block that is then sited upon the microscope stage. A good example of the deployment of a capillary flow is a study by Paul Stoodley (Stoodley *et al.*, 1999a) which used square section glass flow cells to grow defined species biofilms under laminar and turbulent flow to challenge the view that biofilms are static entities. Marked differences were observed in biofilm structure under laminar vs. turbulent flow conditions. Time-lapse microscopic imaging revealed migrated ripples within biofilms with a maximum migration velocity of 2.2×10^{-7} m s^{-1} when the liquid flow velocity was 0.5 m s^{-1}, corresponding to a Reynolds number of 1800). Flow cells have also been used by oral microbiologists in order to study coadhesion (Aspiras *et al.*, 2000) and other ecological phenomena (Rogers *et al.*, 2001).

2. The (modified) Robbins device

This commonly used model comprises a plastic or metal tube into which pegs can be inserted so that when in place, the end of the peg forms part of the wall of the tube. A version, modified by McCoy *et al.* (1981) and termed the Modified Robbins Device (MRD), comprises a rectangular-section cell into which retractable plugs are inserted and is marketed by Tyler Research Corporation (Edmonton, Canada). To use the MRD, batch or continuous culture fluid (or, e.g. tap water) is fed through the device, submerging the substrata (see Kharazmi *et al.*, 1999; Millar *et al.*, 2001 for overviews). Biofilms are sampled by removing plugs, which are then

replaced to maintain a closed system (Fig. 4.2). For obvious reasons, MRDs have been useful for studying biofilms formed within wastewater and other industrial pipe systems (Araujo *et al.*, 2004) and in medical lines (Dasgupta, 1994; Dijk *et al.*, 2000; Nickel *et al.*, 1985). MRDs are most commonly used in the laboratory but can also be connected to pipelines, etc. to study *in situ* biofilm formation.

D. Drip-fed biofilms

1. The constant depth film fermenter (CDFF)

The CDFF was originally developed by Atkinson and Fowler (1974) for growing "microbial films" and later by Peters and Wimpenny (1988). The CDFF allows the generation of large numbers of replicate biofilms. The basis of the model is that biofilms form on the top of plugs that are recessed to a measured depth within sample pans that are located within a turntable. Biofilms are fed by the drip-wise addition of growth medium onto the turntable and excess and/or spent medium flows downwards through a waste outlet (Fig. 4.3). Importantly, biofilms can be maintained at a constant depth by static scraper blades, which remove excess medium and biofilm as the turntable rotates. Various substrata, for example polytetrafluoroethylene (the pegs are manufactured from this material), hydroxyapatite, tooth enamel, dentin or other substrata may be utilized where discs of the test material are placed above the pegs before the depth is set. The system allows replication (5 plugs per pan and a total of 15 pans per CDFF) and standardization of physicochemical parameters and is particularly amenable to the study of mature biofilms, the effects of antimicrobials and different substrates on growth.

The CDFF reviewed by Wilson (1999) is favoured by many oral microbiologists because it allows the continuous culture of dental plaque while controlling biomass accumulation (i.e., depth) thus establishing what is arguably a dynamic steady state. CDFF pegs are normally set at a depths of between 50 and 500 μm but most commonly for maintenance of dental plaques, 200 μm (McBain *et al.*, 2003a,b; Metcalf *et al.*, 2006) or 300 μm (Pratten and Wilson, 1999). The model has been used to successfully monitor the effect of biocide exposure of pure (Pratten *et al.*, 1998b), defined (Pratten *et al.*, 1998a) and complex (McBain *et al.*, 2003b) cultures of oral bacteria. Vroom *et al.* (1999) monitored pH *in situ* and in real time within oral biofilms that had been grown in a CDFF using fluorescence lifetime imaging and reported localized sharp pH gradients within dental biofilms. CDFFs have also been used to evaluate novel surface disinfection technologies (Wood *et al.*, 1998), population dynamics of bacteria derived from cystic fibrosis patients (Al-Bakri *et al.*, 2004) and to model domestic drain biofilms where biofilms of 0.5 cm were stably maintained (McBain *et al.*, 2003c, 2004). Inocula can be added directly onto the

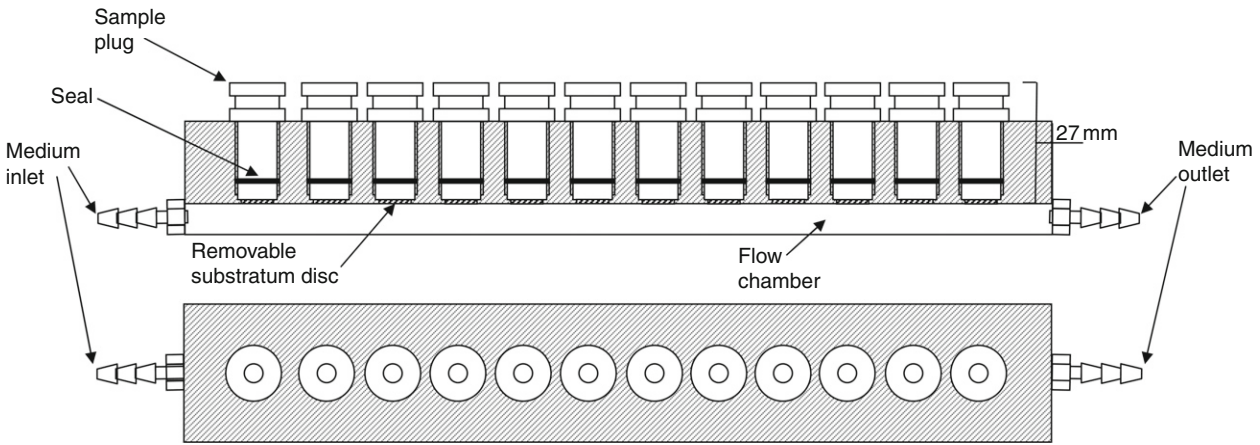
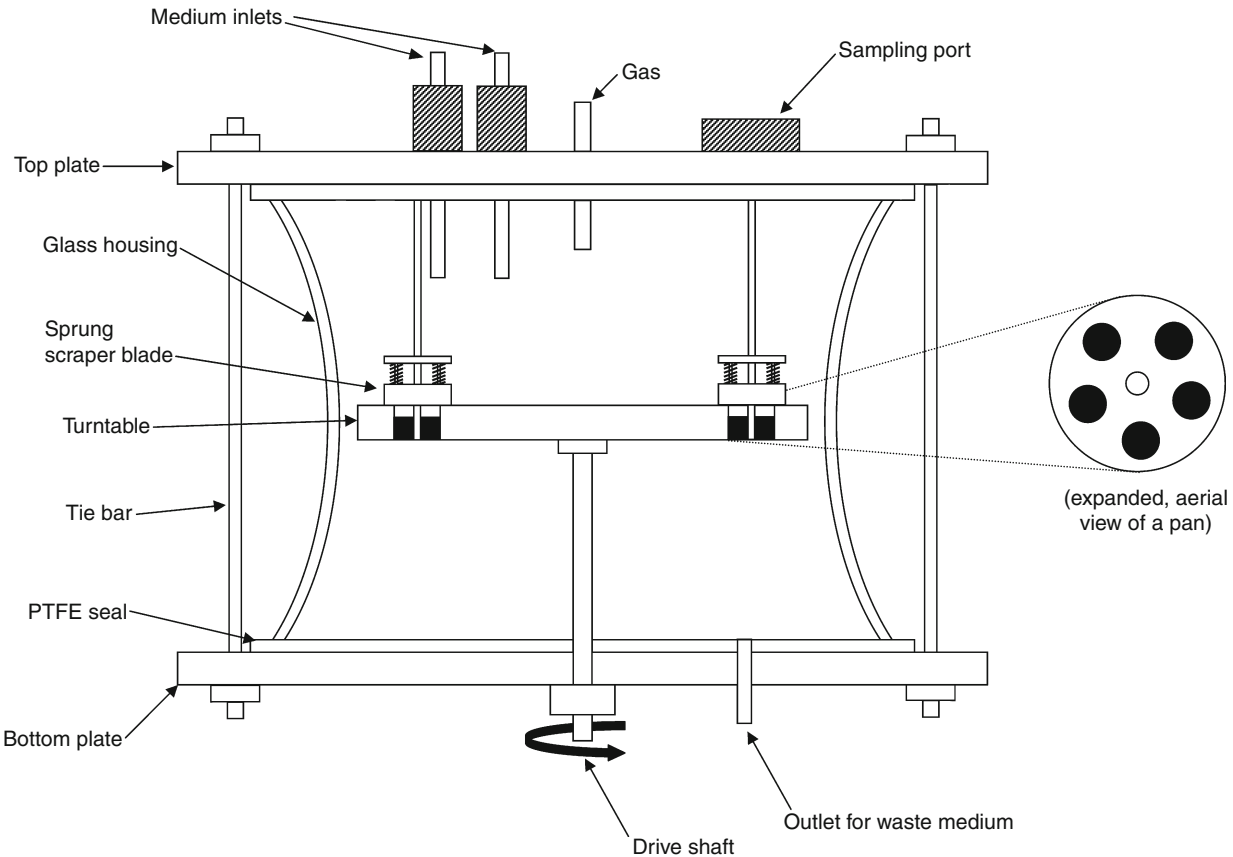


FIGURE 4.2 The modified Robbins device showing plan and elevation. These are commercially available with 12 or 25 individual ports. Biofilms form upon coupons at the end of the pegs that are flush with the upper surface of the flow chamber. These can be removed individually for sampling. (Tyler Research Corp.).


Medium inlets
Gas
Sampling port
Top plate
Glass housing
Sprung
scraper blade
Turntable
Tie bar
PTFE seal
Bottom plate
Drive shaft
Outlet for waste medium
(expanded, aerial
view of a pan)

turntable through ports machined into the top plate. When running oral microcosm experiments, several separate inoculations with fresh saliva maximize the microbial diversity that develops within the modelled biofilms. Likewise, establishing defined communities is often best achieved by inoculation of individual species in a set order (aerobes and facultative species first followed by anaerobes) with multiple inoculations over several hours, or from an established chemostat consortium.

2. The drip flow biofilm reactor

Variations of this system have been used by oral microbiologists for many years and the basic principle involves the growth of biofilms upon slides which are fed by dripping growth medium onto the surface. The Drip Flow Biofilm Reactor is available from BioSurface Technologies Corp. and comprises four individual, parallel test channels, machined into polycarbonate blocks within which microscope slides may be placed (Fig. 4.4).

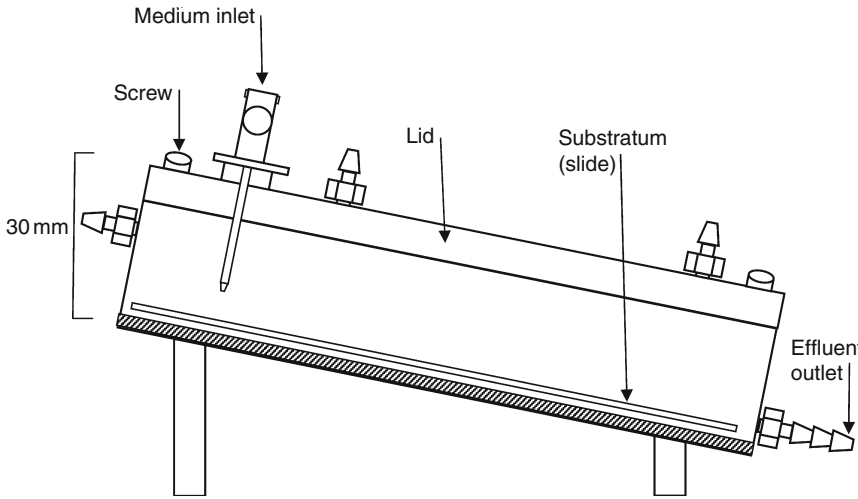


FIGURE 4.4 The drip flow biofilm fermenter. This model supports biofilms on slides that are maintained with growth medium drip-wise (BioSurface Technologies Corp.).

FIGURE 4.3 The constant depth film fermenter. This fermentation system facilitates the long-term, continuous culture of solid:liquid and/or solid/air biofilms (depending on the rate of medium addition). Biofilm depth can be approximately determined. The model is inoculated through the sampling port. For the establishment of dental and other complex communities several individual inoculations may be required. Distance between top and bottom plates, ca. 175 mm; diameter of sample plugs, 5 mm. (see Wilson, 1999).

Each chamber is fitted with a removable sampling lid and during operation; the module is placed at an incline to allow the flow of medium over the surface area of the slide. Medium inlets in the lid enable the continuous drop-wise addition of medium onto the slide that can be variously coated for specific applications. For example, hydroxyapatite-coated slides are available from Clarkson Chromatography Products Inc. (South Williamsport, PA, USA), providing an ideal substratum for the establishment of *in vitro* dental plaques.

Drip flow biofilm reactors have previously been used to study disinfection efficacy (Buckingham-Meyer *et al.*, 2007), to determine distributions of zinc and active biomass in within biofilms (Hu *et al.*, 2005) and to test the effectiveness of bacteriophage anti-biofilm strategies (Curtin and Donlan, 2006). The model can be adapted to hold other substrata. For example, sliced pork, human tissue, or portions of wound dressing may be used in order to simulate chronic wound biofilms. Whilst the Drip Flow Biofilm Reactor is a useful and robust system for the establishment of solid:liquid/solid:air interface biofilms, care is required in experimental design and in sampling because medium flow over the surface of the slide may not be uniform and hence there may be significant aerial heterogeneity over the surface of the substratum.

E. Perfused biofilm fermenters

Perfused biofilm fermenters (PBF) are constructed such that nutrients are supplied by continuous perfusion of growth medium, which is pumped through the substratum (a permeable membrane) and hence through the biofilm. Spent culture medium removes dispersed cells and metabolic products. The fact that media flow can be accurately controlled, daughter cells removed and that biofilm exposure to nutrients is proportional to flow rate means that quasi or dynamic steady states with respect to growth rate and cell turnover can be achieved, and therefore bacterial growth rate can be modulated by altering the rate of medium perfusion. The mean specific growth rates of biofilms maintained within these systems can be calculated as a function of the rate of elution of cells and the total biofilm population (Hodgson *et al.*, 1995; Spencer *et al.*, 2007). Models of this type have been particularly useful as tools to study biofilm recalcitrance associated with low growth rate which is believed to be an important determinant of biofilm recalcitrance (Gilbert *et al.*, 2002). PBFs have also been used in studies designed to elucidate differential gene expression in biofilms, compared to planktonic cultures. In this type of investigation, growth rate is a significant and often overlooked variable and is probably not well controlled in SSRs. The two most commonly used perfused models are the perfused biofilm fermenter and the sorbarod biofilm fermenter. The former utilizes a 0.2- or 0.45-μm pore size cellulose

acetate or nylon membrane as the substratum, the latter uses sorbarod filters which are cylinders of compacted cellulose fibres, similar to cigarette filters in size and composition that are marketed for plant tissue culture.

1. Perfused membrane fermenters

a. The baby machine. These were originally developed by Helmstetter and Cummings (1963). The name comes from the fact that when bacterial cells attach to the membrane in a monolayer, newly formed daughter (baby cells) detach spontaneously during division, leaving the parent cells attached. This model provides an elegant means of studying the bacterial cell cycle since, if carefully maintained, bacteria released within spent culture medium exhibit synchronous growth (Omar and Gilbert, 2005). An additional application is that bacterial senescence may be investigated since the immobilized membrane-bound cells age relative to daughter cells through each successive division cycle.

b. The perfused (membrane) biofilm fermenter (PBF). Forty years after the model was first developed, Gilbert *et al.* (1989) demonstrated that this model could serve as an elegant model for the control of bacterial growth rate within the thin biofilms that formed upon the membranes. They conducted a number of studies to validate this, while making a significant contribution to the understanding of biofilm recalcitrance, which they indicated was partially attributable to growth-rate gradients. The original apparatus for the membrane biofilm reactor used custom-made water-jacketed fermentation fermenters (Brown and Gilbert, 1995). Exponential phase cultures (ca. 50 ml) were pressure filtered through a sterile, pre-washed 47-mm diameter cellulose acetate membrane, and the filters were then aseptically inverted using sterile forceps and placed onto a fret within the fermenter (Allison *et al.*, 1999). Fresh medium was passed into a chamber above the filter by peristaltic pump and the hydrostatic head of sterile medium which developed above the filter forced medium through the filter from the aseptic side to the inoculated side and hence through the nascent biofilm at essentially the same rate at which medium was pumped into the fermenter. Daughter cells within spent culture medium (perfusate) could be counted or biochemically analysed. Bacteria within the membrane-associated biofilm were harvested destructively. During validation experiments, it was observed that following inoculation, perfusate counts decreased rapidly within the first 2 h during which time loosely attached cells were displaced from the filter by fluid dynamic forces and thereafter with care and where fermenters did not become contaminated or membranes burst, a quasi or dynamic steady state could be attained. Importantly, bacterial counts of sacrificed filters indicated that only relatively minor (ca. two-fold) increases in membrane biofilm cell densities occurred during the course of an experimental run, even if

the models were maintained continuously for 2 weeks (Gilbert *et al.*, 1989). Light microscopy revealed that the membrane associated, approximated to a monolayer of cells. The method can be used alongside chemostats so that the biofilms can be compared to planktonic cultures growing at the same rate thus allowing differences in physiology to be attributed to the biofilm phenotype without the confounding variable of growth rate. A series of studies conducted using PDFs indicated that slow growth rate was a significant mediator of biofilm recalcitrance (Evans *et al.*, 1990a,b, 1991) and that detached daughter cells were phenotypically distinct in terms of susceptibility, hydrophobicity and charge (Allison *et al.*, 1990a,b).

c. The swinnex biofilm fermenter. While the adapted Baby Machine is an excellent model for studying the effects of growth rate on biofilm physiology, multiple replicated biofilms are often required, for example when determining the effects of varying concentrations of antibiotics. In such experiments, test antibiotics could be added to the growth medium and pharmacokinetic profiles of antibiotic concentration could be generated to mimic exposure to in the body or within growth-rate-stratified biofilms. Additionally, since it is not uncommon to lose models due to leaks, contamination or burst filters, it is prudent to set up multiple systems. This was impractical with the perfused biofilm fermenter due to the expense and scale of the model and, therefore, a more practical alternative was sought. Swinnex Filter Units (Millipore, Watford, UK) which are essentially reusable, autoclavable syringe filter housings into which various filters can be inserted proved to be ideal for adaptation as low-cost, small-scale perfused biofilm fermenters. Their use as biofilm models requires little more than adding an air inlet to minimize pooling of medium on the underside of the filters (Gander and Gilbert, 1997). They have been used successfully to study the effect of growth rate on biofilm susceptibility and remain an excellent and highly cost-effective choice for any biofilm study where growth rate control is required or where dispersion is of interest. The Swinnex fermenter does not, however, generate sufficient biomass for microarray analysis. While the perfused biofilm fermenter is of a larger scale, issues of cost and practicality led to the development of a further interpretation of the theme utilizing a larger proprietary filter housing manufactured by Sartorius (Goettingen, Germany).

d. The ''Sartorious'' filter apparatus biofilm fermenter. This was developed by Omar and Gilbert (2005) as a perfused biofilm fermenter capable of generating sufficient biomass for microarray experiments with *Escherichia coli*. A number of studies had previously applied such techniques to examine patterns of gene expression in biofilm bacteria that had been maintained in various types of fermenter, and patterns of gene expression had been compared between the biofilm and the planktonic phase. Omar and Gilbert (2005) observed that the biofilms used were probably subject

to physiological heterogeneity, reducing the quality of data generated and that results of microarray experiments may identify genes affected by changes in nutrient environment and growth rate as well as biofilm formation *per se*. The model comprises a Model 16510 polycarbonate filter holder (Sartorius, Gottingen, Germany) which is designed to deliver fluid from an input reservoir to receiver through a 47-mm filter. Additional gas filter units (0.2-μm porosity) are attached to the air inlets and outlets to ensure maintenance of sterility within. The unit is assembled with a 47-mm cellulose acetate membrane filter (0.45-μm porosity, Millipore, Watford, UK) prior to sterilization by autoclaving (Fig. 4.5). The model provides a cheap, robust apparatus by which perfused biofilms may be established. The filter holder can be housed within a static incubator and is re-useable. It is less prone to contamination than the original perfused biofilm fermenter since it is not necessary to remove and invert the membrane following inoculation; instead, the housing is inverted and perfused with fresh medium without the need for disassembly. An additional advantage of this system is that during perfusion, the membrane apparently becomes slightly concave, such that perfusate can be collected without pooling or otherwise interacting with the sides of the apparatus, which reportedly results in better synchrony within re-grown perfusate cells (Omar and Gilbert, 2005). When running this model, once perfusion of medium commences, loosely attached cells are removed, as

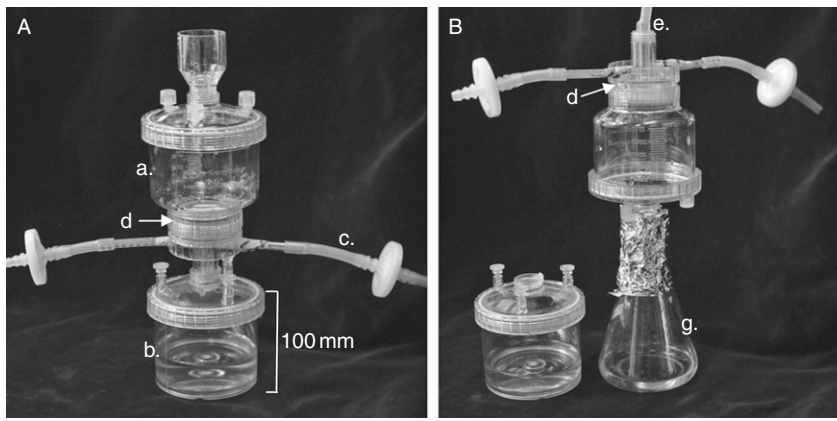


FIGURE 4.5 The "sartorious" filter unit adopted as a biofilm fermenter in assembled form (A) showing input reservoir (a), collection vessel (b) and 0.2-μm air filters (c) and location of substratum filter (d). For inoculation, the membrane is moistened by the addition of sterile growth medium (20 ml) to vessel (a) and application of a vacuum to (c) followed by addition of stationary phase culture (70 ml). The system is then inverted as shown in (B) and placed on a collection vessel (g). Growth medium is fed by peristaltic pump through the inlet (e).

indicated increases in numbers of cells within perfusate samples over the first ca. 2 h. Following this, however, the rate of "daughter cell" generation becomes relatively constant which indicates that a quasi steady state has been achieved. Net biofilm growth rates may then be calculated and the test bacteria grown in a chemostat at the same growth rate to enable the comparison of biofilm and planktonic cells at equivalent growth rates.

2. Sorbarod biofilm fermenters

a. The sorbarod biofilm fermenter (SBF). Sorbarod filters are manufactured as supports for plant tissue culture. The SBF uses single Sorbarod filters as biofilm substrates which are perfused with culture medium. The filter plug consists of a paper sleeve wrapped around a matrix of cellulose fibres. This cost-effective model generates relatively large, heterogeneous biofilms (Fig. 4.6). The model is very simple to set up using readily available components including the barrel of a 2-ml syringe which are commonly manufactured from polycarbonate and may be autoclaved several times without distortion (Fig. 4.6). Viable counts on developed biofilms can be done by sacrificing filters and if required, longitudinal sections can be archived for subsequent biochemical or molecular analysis. As with the perfused membrane biofilm reactors, conditions approaching steady states are obtainable and spent culture fluid (perfusate) can be collected to monitor dispersed cells without disrupting the steady state. SBFs have proven utility for the study of biofilm physiology. Hodgson *et al.* (1995) used an SBF to grow reproducible pseudo-steady-state biofilms over several days, where the growth rate of the biofilm was measurable and significantly slower than in broth culture. Bacteria could be visualized in dissected filters growing in microcolonies in association with cellulose fibres by light microscopy and using environmental scanning electron microscopy within the filters and the high cell densities obtained facilitated SDS–PAGE analyses, which had previously been impractical with the perfused membrane fermenter. Significant differences between the protein profiles of biofilm and eluted populations were observed.

Due to the physical scale of Sorbarod filters and the heterogeneous properties of the cellulose fibres, the sorbarod model does not lend itself to investigations where homogeneity is a key requirement. For such applications, the perfused membrane fermenter has significant advantages. However, where a large amount of biomass is required and a simple, cost-effective and reliable method is required, the SBF remains a good choice and has been adopted by several researchers for a range of applications. Parveen *et al.* (2001), for example, used SBFs to monitor the effects of ciprofloxacin on *P. aeruginosa* biofilms in real time; Budhani and Struthers (1997) tested the efficacy of β-lactam antibiotics against sessile

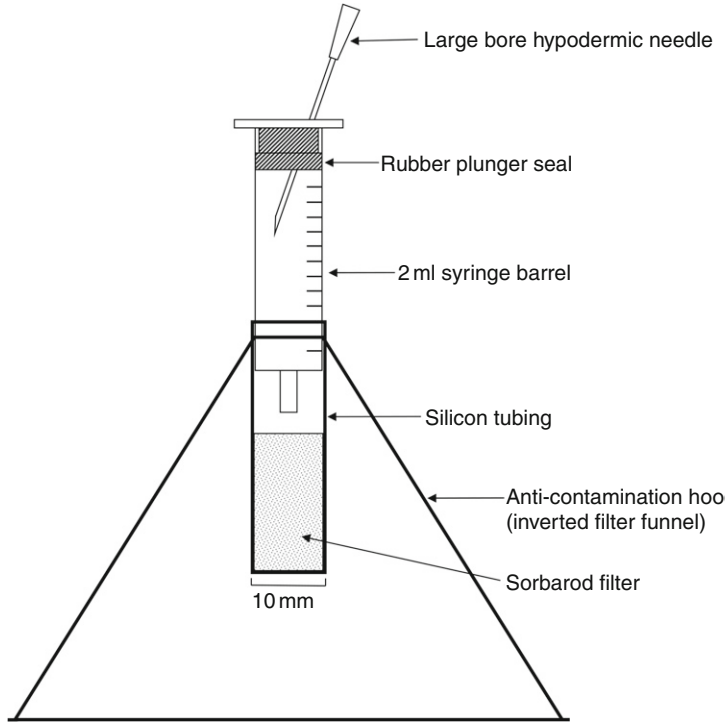


FIGURE 4.6 The Sorbarod biofilm fermenter. Construction uses readily available, low-cost materials. The medium feed line can be connected to a 2-ml syringe barrel, which is then inserted into the needle luer. The filter can be retained by stapling the end of the silicone tube. This model can be destructively sampled and/or spent culture medium (perfusate) can also be collected, enabling non-destructive, real-time sampling of dispersed biofilm cells. *Caution:* Care is necessary when manipulating needles; to reduce risk, these can be aseptically blunted using pliers following insertion through the plunger seal.

populations *Streptococcus pneumoniae* biofilms, while Al-Bakri *et al.* (2004) studied population dynamics of *P. aeruginosa* and *Burkholderia cepacia* biofilms using Sorbarod models. The model has also been used to grow a bioluminescent strain of *P. aeruginosa* for non-destructive monitoring of bacterial responses to ciprofloxacin exposure (Marques *et al.*, 2005). An adapted SBF has been used to good effect by Greenman and colleagues to investigate tongue flora and oral malodour (Spencer *et al.*, 2007).

b. The multiple sorbarod device. A significant disadvantage of the SBF is the fact that, other than relying on perfusate samples, biofilm sampling can only be achieved by dismantling the apparatus and that experiments requiring multiple biofilms necessitate establishing multiple SBFs. The Multiple Sorbarod Device (MSD) comprises a stainless steel housing

resembling a revolver barrel into which five separate Sorbarod filters can be supported (Fig. 4.7). It was originally designed as a real-time system to determine the effectiveness of biocides for controlling biofouling within paper manufacturing plants (Coulburn, 2002). The original system incorporated a pressure sensor such that biofilm accumulation increased backpressure and effective anti-biofouling agents would in theory decrease backpressure without requiring filters to be removed and without the need to perform viable counts. Subsequently however it has also proved to be an ideal system in which to grow oral biofilms and has been validated for the maintenance of complex salivary microbial ecosystems (McBain *et al.*, 2005) and for the *in vitro* reproduction of inter-individual variation within oral microbiotas (Ledder *et al.*, 2006). Furthermore, the

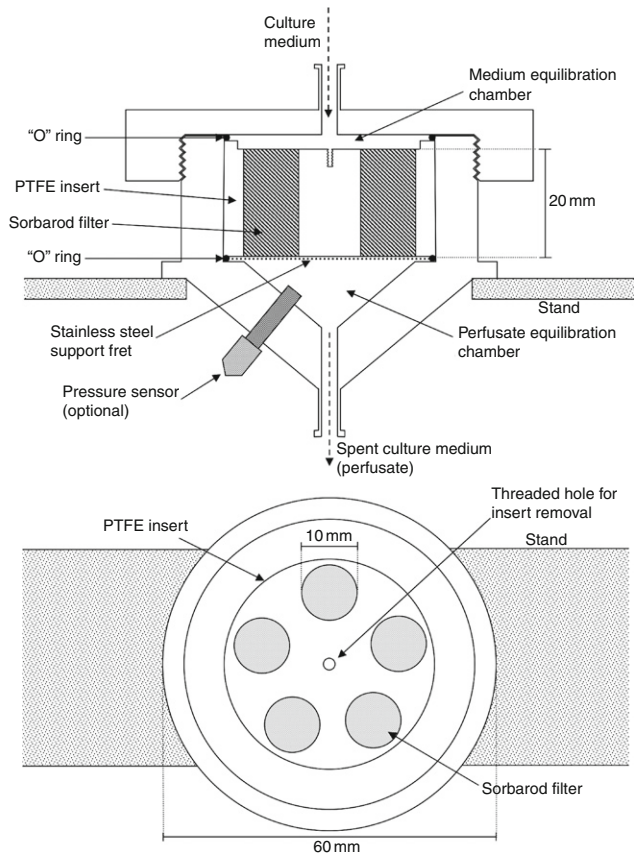


FIGURE 4.7 The multiple sorbarod device showing plan and elevation. This model allows replicate ($n = 5$) biofilms to be established on five separate Sorbarod filters within the same housing. McBain *et al.* (2005).

TABLE 4.1 An overview of some commonly used *in vitro* biofilm models

	Applications	Comments	References
Agar plate biofilm models	Basic systems for modelling biofilms, particularly those with nutritive substrata	Readily accessible and simple to run; dynamic growth (non-steady state)	Charaf *et al.* (1999)
Multi-well plate biofilms	General biofilm models for replication and quantification; commonly used in biofilm molecular genetics	Simple to run, requires readily accessible materials; high degree of replication is possible; non-steady state; limited choice of substratum	Kearns *et al.* (2005)
Submerged substratum models	Various; these are commonly used, general biofilm models	Representative of many real biofilm scenarios; large common fluid phase mean that replication of treatment requires multiple runs or several models; non-steady-state conditions	Bradshaw *et al.* (1996)
Rotating reactors	Modelling fluid flow and shear forces	Excellent for intended purpose; large common fluid phase	Characklis *et al.* (1982)
The Robbins device	Modelling biofilms in flowing systems, medical biofilms, etc. A commonly used, general biofilm model	Readily available, robust; upstream/downstream conditions may (theoretically) differ	Kharazmi *et al.* (1999)
Flow cells	Real-time biofilm visualization in flowing systems, etc	One of the only choices for the primary application	Palmer (1999)

(*continued*)

TABLE 4.1 (*continued*)

	Applications	Comments	References
The constant depth film fermenter (CDFF)	Dental biofilm and other general biofilm work	Models biofilms at the solid:air and solid:liquid interface, depending on medium flow rate; excellent for long-term continuous culture biofilm studies; biofilm depth can be set; multiple identical biofilms for time course or other replication; difficult to obtain; replication of treatments *in situ* requires multiple runs or several models	Wilson (1999)
The drip flow biofilm reactor	General biofilm work	Simple, elegant design; biofilms may be aerially non-uniform	Buckingham-Meyer *et al.* (2007)
Perfused membrane biofilms			Allison *et al.* (1999)
(i) The baby machine	Generation of thin homogenous biofilms. Synchronous growth in daughter cells possible	Good control of growth rate; technically challenging to run	Helmstetter and Cummings (1963)

(ii) Perfused biofilm fermenters	As above	As above	Gilbert *et al.* (1989)
(iii) Swinnex fermenters	Antimicrobial studies where physiological control/ homogeneity are required	Small, robust and cost-effective; low biomass, technically challenging	Gander and Gilbert (1997), p. 41)
(iv) The "Sartorious" biofilm fermenter	As above but for proteomic or transcriptomic analyses	As above; ca. 5× biomass of the Swinnex fermenter	Omar and Gilbert (2005)
The Sorbarod fermenter (SBR)	General biofilm model; antimicrobial studies, dental plaque microcosms; cystic fibrosis biofilms, etc	Robust model generating substantial amounts of biomass; growth rate control possible but considerable heterogeneity within biofilms	Hodgson *et al.* (1995)
The multiple Sorbarod device	As above but allowing replication of biofilms within a single unit	Enables the replication of Sorbarod biofilms; bespoke manufacture required (not commercially available)	McBain *et al.* (2005)

MSD is a useful model where test agents are evaluated for their ability to promote dispersion from extant biofilms since successful treatments would increase the number of viable or dead cells within perfusates which can then be monitored by direct and/or viable counting (Ledder *et al.*, 2009).

Disadvantages associated with the MSD are similar to those of the single Sorbarod device and relate to the development of heterogeneous biofilms. Heterogeneity, however, is a characteristic of biofilms that arguably increases the validity of the system for microcosm studies where maintenance of microbial diversity is one of the main aims.

V. OVERVIEW AND CONCLUSIONS

A sufficient variety of biofilm models are now commercially available or may be constructed in the laboratory such that the availability of an appropriate model is unlikely to limit experimentation. One of the main issues for uninitiated researchers is making a rational choice regarding the best model to use. Generally, systems that closely reproduce *in situ* conditions should be chosen when the aim is solely to reproduce natural biofilms under lab conditions. Direct, non-destructive visualization of biofilms can be achieved using flow cells and for some physiological studies, steady-state models such as the perfused membrane fermenters and to some extent the CDFF offer major advantages. Much information is now available in the published literature and a selection of useful studies has been cited in Table 4.1. There is however, no single, ideal biofilm model for all applications; the idiom ''horses for courses'' applies.

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CHAPTER 5

Zones of Inhibition? The Transfer of Information Relating to Penicillin in Europe during World War II

Gilbert Shama

Contents

Abstract

Alexander Fleming published his first description of penicillin in 1929, but the journal articles that were to propel penicillin from its relative obscurity were those of Howard Florey and his co-workers at Oxford University. These were published in *The Lancet* in the early years of World War II and although wartime conditions restricted the flow of information on penicillin throughout Europe, they never succeeded in shutting it off altogether. In Germany an information-gathering initiative was established in the early phases of the war to systematically copy and distribute British and American scientific articles. A similar, though less well-resourced, operation was permitted to function in Occupied France. Both these operations were to yield up information on penicillin to

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their respective scientists. However, workers in other countries of occupied Europe fared less well; there was a dearth of information on penicillin in Holland but despite this, activity to produce the antibiotic still took place. Central to the production of penicillin at this time was access to a strain of Fleming's strain of *Penicillium notatum*, and an attempt to explain how this particular strain found its way to various European laboratories is given here.

I. INTRODUCTION

Full appreciation of the therapeutic potential of penicillin really only came to be made in the early years of World War II, and in many ways the war was to shape penicillin's development. On the one hand there was unprecedented cooperation amongst British and American pharmaceutical companies. Whilst on the other, steps were taken to prevent the means for producing penicillin from falling into enemy hands. News about penicillin inevitably leaked into the public domain through newspaper articles and radio broadcasts (Shama, 2008), and the authorities in Britain and the United States found themselves having to explain why this miraculous new drug was being reserved exclusively to meet military needs and was unavailable to the civilian population.

This work is primarily concerned with how information about penicillin came to be diffused amongst scientists in Europe. This includes both those at academic institutions and those in industry and particular emphasis is placed on the countries of Germany, Holland and France. Interest in penicillin was based on its therapeutic potential and if this were ever to be realized it naturally implied production of the antibiotic. Therefore, in addition to tracking information transfers and exchanges via the medium of articles in learned journals, it is necessary also to consider transfers of a somewhat more tangible sort: cultures of penicillia – the *sine qua non* of penicillin production.

Despite the war researchers in Britain and the United States continued to publish their findings on penicillin almost without any form of control or censorship. The exception to this was studies relating to the determination of the chemical structure of penicillin and those intended to achieve its chemical synthesis (Anon, 1947). This was largely as a result of perceived German superiority in organic chemistry.

II. EARLY PENICILLIN RESEARCH IN GREAT BRITAIN

The precise circumstances by which Alexander Fleming came to isolate his celebrated mould are not known nor indeed is the exact date in time when this event occurred. Naturally enough this has led to all sorts of

speculation and ultimately to a forensic-like reconstruction of the most probable sequence of events that led to the isolation (Hare, 1970). Even Hare's work convincing as it is, is predicated to a very great extent on statements made when Fleming had achieved fame and was himself trying to piece together events that had occurred some 15 years or more earlier, and so should not be thought of as definitive (Root-Bernstein, 1989).

Sticking to verifiable facts, having isolated the mould, Fleming took steps to preserve it, determined the antibacterial spectrum of the active principle that he termed 'penicillin' and published an account of his findings in the *British Journal of Experimental Pathology* in 1929.

Fleming had been unable to extract and purify the penicillin from the crude broths in which the mould, a strain of *Penicillium notatum*, had been cultivated. This impasse was also encountered by what may be referred to as the 'first wave' of researchers who attempted to further investigate Fleming's observations. These were Harold Raistrick at the London School of Hygiene and Tropical Medicine (Clutterbuck *et al.*, 1932) and Roger Reid at Penn State University (Reid, 1933, 1934). Thanks to Bennet and Chung (2001) the name of another penicillin pioneer, Bornstein (1940) has recently been brought to light.

There can be little doubt that Fleming's interest in penicillin waned in the years following his initial description of the antibiotic. However, it did not dry up altogether: working from Fleming's laboratory notebooks, Wainwright (2002) has ingeniously attempted a reconstruction of a paper that Fleming might have written, and which he speculates may even have been submitted, but not accepted, for publication.

If Raistrick and Reid could indeed be considered the first wave, the second wave was to be of tsunami-like proportions. In 1938 Howard Florey and Ernst Chain at the Sir William Dunn School of Pathology at Oxford University began a study of microbial antagonism. Having conducted an extensive literature review of the phenomenon, they narrowed their field of interest to three compounds; these were a bacteriolytic agent produced by *Bacillus subtilis*, pyocyanase and penicillin (Macfarlane, 1984). Pyocyanase proved to be too toxic to animals and therefore they turned to penicillin.

Chain was a skilled biochemist and he was soon able to extract and purify penicillin from crude mould broths. This permitted the Oxford workers to undertake crucial *in vivo* experiments. The results they obtained were to prove a dramatic testament to the chemotherapeutic powers of penicillin against deadly bacterial pathogens. Florey and his co-workers published their initial findings in *The Lancet* in August 1940 (Chain *et al.*, 1940).

The potential that penicillin held in a time of war was certainly not lost on Florey, and he set about organizing the manufacture of the antibiotic

on a scale sufficient to permit clinical trials. However, if he had had no qualms about publishing the first of his first results in *The Lancet*, something happened to make him think again before making any further disclosures. Sometime in the spring of 1941 Florey was written to by the Swiss pharmaceutical firm Ciba requesting a culture of *P. notatum*. He saw in this request the possibility that the culture might be passed on to Germany, and this was something he was evidently determined not to let happen. Florey took immediate action, he knew that Fleming would have cultures, but so too would the National Collection of Type Cultures (NCTC) and he wrote to Sir Edward Mellanby of the Medical Research Council (MRC), under whose aegis the NCTC came, as well as to Fleming.

In his response, dated 23 April 1941, Mellanby admitted that Florey had raised 'a matter of some difficulty'. Mellanby could not see how he could instruct the NCTC not to send out cultures to a neutral country such as Switzerland. But Mellanby did not see this as serious matter, assuring Florey that he was 'miles ahead of any possible competition'. Fleming replied on 25 April 1941. 'I am entirely in agreement with you that we should not pass on cultures of the penicillium to the enemy, and if Ciba, or any similar firm, approach me I will refuse'. He went on to write: 'During the past ten years I have sent out a very large number of cultures of penicillium to all sorts of places, but as far as I can remember none have gone to Germany. In this country many people have (or had) cultures, including places like Burroughs Wellcome, Evans Leischer and Webb, May and Baker, Parke Davis, and Boots. It is unlikely that they would be approached, but it possible that an application might be made to Raistrick who has written on the subject. I have talked to Raistrick on the telephone and he agrees that, seeing we are at war, he will not supply cultures to Ciba'.

Fleming's response is revealing, and its wider implications have not been fully appreciated. Quite apart from the early industrial interest, it has become almost the convention to stress how little impact Fleming's (1929) paper had had. Yet Fleming states categorically that he had sent out 'a very large number of cultures' to various persons and organizations. This was evidently in response to requests from those who had read his article or otherwise heard about penicillin.

But returning to Florey, the responses he had received from Fleming and Mellanby evidently re-assured him and he went on to publish in August 1941 the second of his *Lancet* papers on penicillin (Abraham *et al.*, 1941). This paper contained all that was then known about culturing the mould and extracting and purifying the penicillin. Apart from Chain's masterful contribution, it owed much to the experimental ingenuity of Norman Heatley. Florey must have felt secure in the knowledge that without the mould no one in Germany could undertake production despite having what was in effect a blueprint for small scale manufacture.

It is interesting to reflect on Florey's attitude to Germany and the Germans. As has already been mentioned, Florey was to undertake what was to become virtually a one-man mission to recruit the combined resources of the British and American pharmaceutical industries in achieving mass production of penicillin. Another facet of this crusade of his appears to have been an equally strong determination to deny the benefits of penicillin to the enemy. Florey never quite lost his anxieties about the possibilities of Swiss-German collusion; when he was contacted by the Red Cross on 21 October 1942 for information on penicillin he declined to supply it responding curtly that work was at an experimental stage and that it was 'very improbable that [penicillin] will be available in any quantity for the general public or for such an organization as the Red Cross for a very long time to come'. Also, during the war Ian Fraser was sent to Oxford to receive instruction from Florey himself on the administration of penicillin in the forward battle area, Florey suggested to Fraser that he should not give penicillin to German wounded, something that Fraser absolutely refused to accept (Fraser, 1995).

III. GERMANY

The war had an obvious impact on the exchange of scientific information between countries by what might be called the normal channels of communication. Books and journals published by enemy states suddenly became unavailable. Germany took steps to access firstly British publications denied to them because of the war, and then with the United States' entry into the war, American publications. Similar measures were later put in place by the United States. The details of these information-gathering operations have been extensively documented by Pamela Spence Richards in a number of publications and the following two paragraphs draw heavily from Richards (1994).

Immediately after September 1939 the German government put into action a carefully prepared operation for the collection of foreign journals. German scientific attachés in neutral countries such as Sweden, Portugal, Turkey and Mexico obtained copies of journals and had them shipped to Germany in diplomatic pouches. These publications were forwarded to the Emergency Association of German Science at the Preussiche Staatsbibliothek from where they were forwarded to the Technische Hochschule in Berlin-Charlotenburg where a special photographic unit reproduced them by photostat or microfilm. Copies of articles or whole issues of journals were then distributed to the relevant research institutions and to reviewing organs such as *Chemisches Zentralblatt*, *Physikalische Berichte* and the medical reviews produced by Springer.

Well planned as this scheme was, the intensification of the war soon began to hamper the process of acquisition particularly once the United States had joined the conflict. Interruptions to the flow of foreign scientific publications into Germany were evidently seen as having a negative impact on the German war effort, and the state intervened to remedy the situation. In 1941 a new organization was formed, the German Society for Documentation, which came under the direct control of the Minister of Education. One of this organization's most useful achievements was to publish in December 1943, an inventory of all foreign journals imported into Germany since September 1939 including, most crucially, details of their precise locations in the country.

In addition to these arrangements, there were also in operation unofficial channels by which foreign articles might be acquired. German industry evidently had its own networks for getting hold of Allied scientific publications. Unsurprisingly, material that was of particular commercial interest was sometimes withheld from potential competitors. Indeed Richards documents a case of a complaint being made to Goebbel's ministry that certain companies and research institutes were keeping the best new acquisitions for themselves.

Despite these arrangements there would inevitably have been delays in the distribution of enemy material to those most qualified to assess and exploit it. Florey's *Lancet* papers did reach Germany and once they did so it is not inconceivable to imagine that a search was instigated for a culture of Fleming's mould. And Fleming was wrong: he had sent a culture of *P. notatum* to a least one person in Germany. This information first came to light in Allied intelligence reports compiled immediately after the defeat of Germany. Teams of Allied scientists followed in the wake of the advancing armies with the objective of uncovering German research and development that had taken place during the war. There were priority areas such as advances in weapons technology, and nuclear weapons in particular, but every facet of German science and technology was to come under scrutiny. The teams came under the auspices of a number of different organizations and there was much duplication of effort. The two organizations of relevance here are the Combined Intelligence Objectives Subcommittee (CIOS) and British Intelligence Objectives Subcommittee (BIOS).

The recipient of Fleming's strain was referred to in the CIOS and BIOS reports simply as 'Dr Schmidt' who was at the IG Behring works at Marburg and at least three visits were made to the IG Behring works. It seems that Schmidt had obtained the culture at an unspecified time before the war but had not attempted to culture it. When Schmidt was finally called to perform his patriotic duty he was unable to coax the culture to grow. He passed it on to microbiologists at Berlin-based pharmaceutical firm Schering, but they too failed to grow it and must have concluded that it had lost its viability in the years that Schmidt had stored it.

There was, however, an obvious source to which one might turn to for cultures of penicillia: the fall of Holland in May 1940 had delivered into German hands what was undoubtedly the greatest mould collection in the world. This was the Centraalbureau voor Schimmelcultures (CBS) located in Baarn near Utrecht.

Shama and Reinarz (2002) had previously speculated that the time when German scientists found out about penicillin was in the autumn of 1942. This date was arrived at from letters that were received at the CBS from Germany. However, a recent discovery of a letter from the CBS archives now suggests that the actual date may have been earlier. The letter, from the Dutch offices of Bayer in Amsterdam and dated 3 August 1942, refers to earlier correspondence of February and March of the same year. It is a reminder; the CBS appear to have promised to send a new strain of *P. notatum* to Prof. Dr H. Schmidt of the Marburg works, and the letter is to inform the CBS that it is now urgently required. This must be the same Dr Schmidt referred to in the CIOS and BIOS reports. The letter goes on to stress the urgency of the request and mentions Fleming and penicillin by name. If this was indeed the first request to the CBS, by working backwards it enables some estimate to be made of the interval of time that elapsed between the appearance of Florey's first article on penicillin (August 1940) and the same article landing in Schmidt's lap. If the date of Bayer's Amsterdam office first letter o the CBS is taken as February 1942, then this represents a delay of 18 months. If one allows a period of 2 months to cover first Schmidt's, then Schering's failed attempts to culture the strain, then a more realistic estimate of the delay would be 16 months.

The CBS was subsequently to receive numerous requests from Germany for *Penicillium* cultures. The first wave was received towards the end of September 1942, but a steady trickle continued more or less continuously until well into 1944. News was evidently spreading throughout the German pharmaceutical industry. Any prospective purchaser of cultures from the CBS would almost certainly have turned to their latest catalogue of cultures. This was the List of Cultures published in 1937 and the Supplementary List of Cultures dated 1938. Neither contained reference to Fleming's strain, *P. notatum*, NCTC 4222. However, listed was *P. notatum* (Westling), and it was for this stain that German organizations applied for. This is indicative that at some stage scientists in Germany reached the conclusion that the CBS did not possess Fleming's strain and that they would try the next best thing.

The requests for *P. notatum* (Westling) came from a variety of organizations and seem to have included virtually every German company, research institute and university with even the slightest connection with pharmaceuticals. Amongst the more well known names are I. G. Farben in Frankfurt-am-Main, and also at Elberfeld, The Kaiser Wilhelm Institute

for Medical Research and Chemistry, the director being the accomplished organic chemist Richard Kuhn.

The crucial question is whether *P. notatum* (Westling) produced penicillin. It may originally have done so, however, repeated subculturing since its deposition at the CBS probably just after 1911 for the purposes of maintenance would have meant that the trait would have not been preserved as it pre-dated even Fleming's work (R. H. Sansom, personal communication, 2001).

Shama and Reinarz (2002) had previously stated that the CBS did not possess the strain at the time. However, new information has come to light that suggests that this assertion may have been incorrect. The information comes from letters exchanged between the Postal and Telegraph Censorship Department and R. St. John-Brooks the Curator of the NCTC in June 1944 (reference, gratitude, etc.). A culture of *P. notatum* NCTC 4222 which St. John-Brooks had sent to the Sociedade Industrial Farmaceutica in Lisbon was intercepted by the Censor and returned to the NCTC. St. John-Brooks wrote to the Censor claiming that he 'did not know that there was any embargo on the sending of cultures of Sir Alexander Fleming's strain of *P. notatum* out of this country. This particular strain was without doubt, widely distributed on the continent before the war as it was sent to Holland in 1930 and was indeed correctly named there by Prof. Johanna Westerdijk, Director of the Centraalbureau voor Schimmelcultures at Baarn on 9 April of that year'. It is interesting that despite Mellanby's protestations to Florey that he was powerless to influence the NCTC an embargo was in fact imposed. Table 5.1 shows the organizations to whom St. John-Brooks claimed to have sent cultures of *P. notatum* NCTC 4222 to.

It now seems very likely that the CBS did after all have a culture. Moreover, the curator of the CBS, Prof. Johanna Westerdijk may have been the first to correctly identify it as *P. notatum*. In his original paper, Fleming thanks 'our mycologist, Mr la Touche, for his suggestions as to the identity of the penicillium' (Fleming, 1929). La Touche was famously incorrect in his identification; he had proposed *Penicillium rubrum*, and Macfarlane states that Raistrick was doubtful of the identification and sought the advice of the great American mycologist Charles Thom sending him a culture on 14 May 1930 (Macfarlane, 1984) *after* that received by Westerdijk.

Applying to the CBS for cultures of penicillia was one strategy, the other was to attempt isolation of strains of penicillin-producing strains of *Penicillium*. This was indeed put to effect at a number of laboratories throughout Germany. These efforts first came to light in a series of intelligence reports that were mentioned earlier and are described in greater detail in Shama and Reinarz (2002). Previous to this particular study, German activities in the field of penicillin research had been described in work published in German an excellent example being (Pieroth, 1992).

TABLE 5.1 Distribution of *Penicillium notatum* (No. 4222 – Fleming's strain) since the outbreak of war (to places abroad)

Division of Industrial Chemistry, Council for Scientific & Industrial Research, East Melbourne, Australia
Indian Institute of Science, Bangalore, India
The British Council (for export)
S.A. Brewing Co. Ltd., Adelaide, S. Australia
N.Z. Government, Strand W.C. (for export)
School of Pathology, T.C.D., Eire
Aktiebololaget Marvello, Stockholm, Sweden
Imperial Mycologist, New Delhi, India
Guinness Brewery, Dublin, Eire
Department of Pathology, University College of Dublin, Eire
Ministry of Agriculture, Belfast, N. Ireland
Laboratorio Fidelis, Lisbon, Portugal
Alexr. Lipworth Pty. Co., Johannesburg, S. Africa
Bakubhai Ambalal & Co., Bombay, India
South African Institute for Medical Research, Johannesburg, S. Africa
Clinsearch Laboratories, Johannesburg, S. Africa
Sociedade Industrial Farmaceutica, Lisbon, Portugal[a]

[a] This last item was sent but intercepted by the Postal and Telegraph Censors Department.
Copied from letter sent by R. St. John-rooks, NCTC on 9th June 1944 to Dr A. N. Drury, CBE, FRS, Lister Institute of Preventative Medicine, Chelsea Bridge Road, London, S. W. 1.

Some German workers, notably Hans Knöll at the Schott Glass works, did succeed in making penicillin. It was never to have nothing like the impact that the Anglo-American initiative was to have. This was due to a number of factors; the first was the lack of any central co-ordinating authority. This would have been essential, as different strains would have needed to be compared with one another using standardized assays for penicillin so that the most productive could be cultured industrially. As the war progressed, the frequency and destructive power of Allied air raids over Germany increased. Any novel industrial process developed on a sufficiently large scale would have therefore drawn the attention of Allied military planners who would have singled it out for destruction. Similar fears had led Florey to seek American industrial help in mass-producing penicillin simply because American plants would have been beyond the reach of the Luftwaffe. When finally the need for a coordinating committee was appreciated by German researchers and a meeting called in Berlin in October 1944, the stark realization was made that it was already too late as the necessary raw materials and plant

necessary would never be made available (Shama and Reinarz, 2002). The diverse and disparate attempts to isolate penicillin-producing moulds would almost certainly have led to the isolation of novel antibiotic compounds; an example being the work of von Kennel *et al.* (1943). Some of these compounds would undoubtedly have had therapeutic worth, whilst others would have proved themselves toxic and of no practical value.

The apparatus put in place by the German authorities to acquire Allied scientific and technical material was described earlier in some detail, and it is therefore interesting to gauge its efficiency towards the latter stages of the war. An article on penicillin appeared in a journal entitled *Chemiker-Zeitung* in October 1944 (Loewe, 1944). The journal appears to be aimed primarily at industrial chemists and engineers, and the article itself is a review of the status of work on penicillin. Despite mention in the Introduction of an embargo on publication of studies relating to the chemical structure of penicillin, some early proposals, for example Abraham *et al.* (1942) and Catch *et al.* (1942), were in fact published before the embargo was imposed. Both proved to be incorrect, but Catch *et al.*'s work is cited in Loewe's review. German workers, this article reveals, had become acquainted with the fact that penicillin was being made in the United States by submerged culture and that corn steep liquor was being used in its manufacture. The latter was apparently unknown in Germany, for it has been mis-translated as 'maisalkohol', that is grain alcohol. Information is provided to the journal's readership on the War Production Board and the names of all the American pharmaceutical companies involved in mass production. The latest American work cited dates to the latter part of 1943 and includes *Science* (July 1943), *Oil, Paint and Drug Reporter* (July 1943), *Chemical and Engineering News* (September 1943). The range of material is impressive but the elapse of time between publication in the United States and its arrival into the right hands in Germany is just over 12 months. Apart from Swiss articles dating to 1944, the article contains a citation of a piece of French work from January 1944 (Nitti, 1944) which is considered later.

Richards (1994) did not consider the Swiss contribution to German information gathering initiatives. As a neutral country, British and American publications would have been more readily available than in most of the rest of Europe. As a country having a sizeable pharmaceutical industry, publications on penicillin would have had particular interest. This led to the publications of reviews (e.g., Wettstein, 1944) that would have been readily obtainable in Germany.

Despite the attempts described and the 'near miss' with the Dr Schmidt's culture, the German authorities never quite gave up the quest for a culture of Fleming's mould. An American agent, Hamilton Southworth, visited the Pasteur Institute just after the liberation of Paris

and was told that there persisted attempts to obtain Fleming's strain until as late as June 1944.

But the story of Fleming's strain of *P. notatum* in Germany does not end there. There had been at least one another microbiologist in Germany with a culture of this particular organism – at least until the year 1933. Julius Hirsch had obtained his PhD under the pioneer biochemist Carl Neuberg (1877–1956). At some stage in his early career Hirsch worked for a period of two years in London with D. D. Woods and H. McIlwain and acquired a culture of Fleming's mould whilst in London. He returned to Germany and was appointed Professor at the University of Berlin (Bartmann and Wagner, 1962) but because he was a Jew was deprived of his post in 1933. Although she does not specifically mention Hirsch, the expulsion of Jews from academia in Nazi Germany has been described by Deichmann (1999). Hirsch managed to escape to Istanbul where he became a Professor at the Institute of Hygiene. He evidently took his strain with him because he published some accounts of his work with the mould (Hirsch, 1944). This article serves to indicate how Hirsch had access to surprisingly up-to-date information in Turkey. In addition to revealing familiarity with the names Florey and Raistrick, Hirsch cites an article published in *The Times* on 12 February 1944. Hirsch's work came to be recognized outside Turkey; his studies of a glucose-oxidizing enzyme that possessed bacteriostatic properties produced by *P. notatum* (Fleming) was mentioned in the Editorial of *The Journal of the American Medical Association* in March 1944 (Anon, 1944). This work of Hirsch's on this particular enzyme, which came to be known as notatin, was also cited by Coulthard *et al.* (1945). It is interesting that these authors betray their incredulity that Hirsch could actually be in possession of Fleming's culture, for they state 'Hirsch (1944) observed the secretion of a glucose-oxidizing enzyme by *P. notatum* Fleming (*sic*). . ..'

A most curious aspect of this particular chapter of the history of penicillin is just what Hirsch considered to be vital to him as he fled his country of birth for Turkey. Had he left behind his precious culture of *P. notatum* in Germany someone might have found it and German penicillin production would have taken a very different turn. The final irony perhaps being that Hirsch's work on notatin was cited in the article by Loewe (1944) mentioned earlier.

Work published some 25 years ago reveals an interesting example of sharing of information between wartime allies. Manfred Kiese of Berlin University wrote a review on penicillin in which he abstracted articles that had appeared in Britain and the United States, including of course *The Lancet* articles. These were published in *Klinische Wochenschrift* in the issue of 7 August 1943 (Kiese, 1943). In his review of the early history of antibiotics in Japan, Yagisawa (1980) makes reference to a certain Mrs Fusako Tsunoda who conducted research to show that this issue of

the *Klinische Wochenschrift* was actually conveyed to Japan by submarine which she managed to identify as 'I-8' which left the port of Brest 5 October 1943 and arrived in Kure near Hiroshima on 21 December of that year.

Yagisawa also (1980) mentions a Prof. Masahiko Kuroya at Tohoku Imperial University who was active in penicillin research and he coincidentally was also the subject of an intercept by the Australian Special Wireless Group made on 18 September 1944 and sent to Florey for information. The message claims that Kuroya and his assistant Shikaji Kondo had discovered 'a new type of penicillin with surprising curative effects over Fleming's penicillin'.

IV. HOLLAND

The strategy adopted by a number of German organizations in contacting the CBS for cultures of *P. notatum* and those described would of course have alerted the staff there to the interest in this particular mould. Some of the requests for cultures sent by post from Germany specifically refer to Fleming. The latter's original paper on penicillin would have been available in academic libraries in Holland even though Florey's *Lancet* papers would not have been.

The Director of the CBS was the redoubtable Johanna Westerdijk. Westerdijk had commenced her studies in botany at Amsterdam University in 1900 before going on to study in Munich and then Zurich from where she obtained her PhD. She was appointed to a chair in botany at Utrecht University in 1917 so becoming the first woman professor in Holland (Ten Houten, 1963). Under her leadership the CBS expanded greatly becoming arguably the finest in the world. In her position as Director Westerdijk would have therefore been supremely placed to inform fellow Dutch scientists of German interest in penicillin. The question of whether she did or not is considered as follows.

Reference has already been made to teams of Allied technical specialists who followed in the wake of the armies as they swept through mainland Europe. Their mission was not confined to Germany and they interviewed a number of scientists in Occupied countries as they became liberated. Between 6 June and 5 July 1945 at least three teams were charged with uncovering medical research in Occupied Holland. Each of them seems to have interviewed Prof. J. V. Konigsberger. These reports contain much common material and the following is a distillation of the most important revelations contained in them.

Konigsberger carried out the clandestine production of antibiotics in Utrecht. This work was centred on *P. expansum* that produced an antibiotic that he termed 'expansine' but was in all probability patulin.

Expansine proved to be too toxic for systemic use but had some value for treating fungal infections of the skin. Konigsberger was not under any misapprehension that he was working with penicillin. That this can be asserted is due to the following. Koningsberger admitted contact with Dr J. J. Duyvené de Wit who was in charge of research at the pharmaceutical firm of Brocades, Stheeman and Pharmacia. Stheeman was closely involved with an activity for making penicillin in Delft at a company known as the *Nederlandische Gist en Spiritusfabriek* (NG&SF) and described in recent work (Burns and van Dijck, 2000).

Workers at NG&SF managed to get hold of a German review of penicillin mentioned earlier (Kiese, 1943). However, they obtained an additional source of information by a remarkable incidence of serendipity. Andries Querido had been associated with the company, but because he was Jewish, came to be interned at Westerbork transit camp in January 1943. The Germans appear to have conferred a 'reserved occupation' status on workers at the plant, and this evidently extended to Querido. He was allowed visits to the works at Delft but was obliged to leave behind his wife and children as hostages at Westerbork. At Amsterdam station he encountered by chance S. van Creveld, a senior lecturer in child health at Amsterdam University. Van Creveld told him about a 'Swiss medical journal' (almost certainly the *Schweizer Medizinische Wochenschrift*) dealing with 'a miraculous new compound – penicillin'. Van Crefeld lent Querido the article and Querido took it to the Gist works where a programme for producing penicillin came to be initiated (Querido *et al.*, 1990).

Returning to Koningsberger, he also revealed that he had heard about penicillin from an RAF leaflet that had come into his possession. Shama and van der Els (2008) have recently speculated that the leaflet might have been one belonging to a series known as *Wervelwind*, that is Whirlwind, dropped over Holland on the night of 24/25 April 1944 (see Fig. 5.1). Finally, Koningsberger stated that he had a copy of the *Schweizer Medizinische Wochenschrift* of June 1944, and therefore would have known from this source the identity of penicillin-producing strains. In the course of his interviews Konigsberger made reference to fellow scientists with whom he was involved. Apart from the mention of Duyvené de Wit, there was also Dr B. C. P. Jansen, Director of the Laboratory of Physiological Chemistry, Netherlands Institute of Nutrition, Amsterdam, and Abraham van Luyk. The latter had been working alongside Konigsberger since 1940. Before that van Luyk had been Westerdijk's assistant from 1910 until his retirement from the CBS in December 1939 (Faasse, 2008). The interesting omission is Johanna Westerdijk. Despite this Koningsberger was aware that 'two or three dozen German institutes had obtained stains [of penicillia] from Baarn [i.e. the CBS]'.


PENICILLINE
het nieuwe
wondermiddel
door
Dr. A. M. Meerloo
Op de schimmels ziet men de druppels, waarin zich het penicilline heeft gevormd.
MOETEN we niet huiveren in de geneeskundig den naam „wondermiddel" te gebruiken? Skeuvel middelen zijn ons niet aangeprezen, die later bleken teleur te stellen? Toch hebben de laatste jaren verschillende wondermiddelen aan het licht gebracht. De strijd tegen de ziekteverwekkers en hun giften is geleidelijk gemakkelijker geworden. Salvarsan was het eerste chemische strijdmiddel tegen de microbe, die de syphilis verwekt. Het was het resultaat van jarenlang wetenschappelijk onderzoek. De laatste jaren voor den oorlog kregen de dokters de extra hulp van de zoogenaamde Sulpha-producten, die o.a. in den strijd tegen den verwekker der acute longontsteking en van de nekkramp, uitstekend voldoend. Hoe hopeloos stonden we in den strijd tegen deze laatste ziekte, doch in de laatste jaren had ik geen enkel verloren geval.
De beide ampullen bevatten een minimale hoeveelheid penicilline, verbonden aan een zout. Voor inspuitingen in den ader wordt het geneesmiddel in gesteriliseerd water opgelost.

FIGURE 5.1 Text of 'Wervelwind' Leaflet Dropped over N. Holland on the Night of 24/25 April 1944.

Konigsberger may have come across this information from van Luyk who, following his long service at the CBS, would almost certainly have maintained contacts there. Konigsberger and Westerdijk must have known each other well. Baarn is after all only a few miles from Utrecht. Faasse (2008) points out that Koningsberger and Westerdijk would have had occasion to meet formally at least once a year as both were on the board of the CBS. Faasse also states that Westerdijk corresponded with Koningsberger whilst the latter was temporarily interned. Possibly Konigsberger disapproved of her meeting requests from Germany for strains of penicillia. Possibly too he sought to prevent the knowledge of his own clandestine activities coming to the attention of the Germans, and only confided to those with whom it was strictly necessary to do so. It is however strange that with the worlds' greatest mould collection on his doorstep Koningsberger should choose to isolate his own strains of *P. expansum*.

Westerdijk was herself in a difficult position: she could hardly refuse to send cultures to Germany. Occupying a position of authority she must have been aware of the possibilities that amongst the people she came into professional contact were those who might denounce her to the Germans in order to secure preferment. According to Faasse (2008) one manifestation of such a threat at the CBS was Dr F. H. van Beyma thoe Kingma who

demonstrated collaborationist tendencies, and who was apparently able to travel freely to Germany. Huub van der Aa, a onetime employee of the CBS, has in his possession letters revealing that plans were being considered to remove the entire CBS to Germany. Van Beyma thoe Kingma was apparently party to these discussions. These plans never materialized, partly it must be assumed, because of the objections of German researchers such as Hans Knöll who argued against the practicality of such a scheme. Notwithstanding, it seems likely that Westerdijk had at least some inkling of these plans and this might have actuated her conduct towards German agencies.

Following the liberation of Holland persons in positions of authority were obliged to account for their wartime conduct. In the first instance this comprised a questionnaire containing such questions as: 'have you accepted any new position during the occupation? Have you taken over the duties of anyone who had lost his position as a result of a German measure?' Westerdijk had to submit to this process, and her answers were in the negative. When asked whether she had maintained contact with Germans for the benefit of her academic work, she replied that she had kept contact with Prof. G. Gassner of Magdeburg University (Faasse, 2008).

There is in the Florey archive one piece of evidence that suggests that Westerdijk's contacts with German scientists went deeper than she was prepared to admit. The document comprises intercepted intelligence material sent to Florey for information by a certain Dr John Barnes at Porton Down. The documents are dated July and August 1944. This material reveals that Westerdijk was in correspondence with Prof. Eugen Haagen of the University of Strasbourg and also Chief of the Luftwaffe Medical Service, over penicillin production by various strains of penicillia. Westerdijk had apparently carried out assays for Haagen. Just how Barnes came across this intelligence is unclear. He formed part of a unit known as ALSOS charged with monitoring German capability to produce biological warfare agents.[1] The unsavoury fact was that in addition to his sideline in antibiotics, Haagen was also carrying out typhus experiments on human inmates of Natzweiler-Struthof concentration camp. He was eventually indicted with war crimes and sentenced to 20 years of imprisonment, but never served more than a couple of years (Baumslag, 2005). Naturally Westerdijk would not have known any of this.

The strains mentioned in the intelligence report were *P. notatum* (Westling), *P. puberlum* (Bainier), *P. corylophilum* (Dierckx) and *P. expansum* (Link) – none of which produce penicillin. Westerdijk's responses cannot have satisfied the authorities, for she was summoned to appear before the

[1] This information was provided by G. B. Carter author of Chemical and Biological Defence at Porton Down, 1916–2000, published in 2000 at The Stationary Office, Norwich.

Committee for the Restoration and Purification of Utrecht University and suspended from giving lectures. A flavour of what course the interview took can be gauged from an unsigned and undated note that Faasse came across in Westerdijk's case file.

> After reports of the American penicillin research had reached the Netherlands and Germany, German researchers tried to produce the magic remedy. The Dutch research effort in this area went underground. The Germans contacted the CBS and demanded strains of the fungi *P. notatum* and *P. expansum*. Westerdijk had sent them these strains after first checking that they had lost the power to kill bacilli. The Germans had been unable to get anywhere with these strains.

The Committee considered the evidence and came to the conclusion that Westerdijk had not taken action that that was of significance to the German war effort. She was allowed to resume her lecturing on 20 September, the Committee writing to her that 'Albeit that we can appreciate the factors that caused you to take up the position you adopted, we nonetheless share your own feeling of regret that you did not display a spirit of resistance such as might have been possible, even in your circumstances'.

Reference to newly discovered documentary evidence discussed earlier strongly points to the CBS possessing a culture of Fleming's strain of *P. notatum*. If she did indeed possess this strain it seems that she did not hand it over. This was in itself an act of resistance that needs to be acknowledged.

The question of whether or not Westerdijk informed fellow Dutch scientists about penicillin must remain open. It seems strange that if she did possess Fleming's strain she did not pass it on to the *Nederlandische Gist en Spiritusfabriek*.

Florey seems either to have forgotten about the intelligence he had received of Westerdijk's communication with Nazi officials, or considered it to be insignificant, for after the liberation he replied to a letter of hers on 20 September 1945 stating that he was 'delighted to hear that your great fungus collection has been kept going'.

V. FRANCE

For a long time an image existed of the French population during World War II as standing united in resisting their German occupiers. Naturally this has been proved to be a myth. Studies appearing over the last 30 years or so paint a rather less heroic reality. All sorts of accommodations had to be arrived at with the Germans both at the level of the individual and of

organizations. This is not of course to diminish in any way the heroic few who refused to enter into any compromises whatsoever and chose to resist to the end.

Conflicts of this sort were being played out at the Pasteur Institute. During the occupation it was obliged to produce therapeutic sera for export to Germany whilst at the same time serving as a secret store of medical supplies that were put at the disposal of French resistance movements (Chevassus-au-Louis, 2007). Some of the workers at the Institute, such as Ernest Fourneau, came to be charged with collaboration after the war had ended (Chevassus-au-Louis, 2004), whilst others such as Federico Nitti came to be decorated for the acts of resistance they performed.

The systems in place for accessing foreign publications in France during the war years have been documented by Richards (1994) and also serve as an example of this duality.

One system that was put into operation represented what, according to Richards, was an example of successful scientific co-operation and resulted in the exchange of journals between France and Germany. The driving force behind this particular initiative was Jean Gérard, Secretary General of the Maison de la Chimie. As such he was director of one of the best scientific periodical collections in the world and, moreover, its microfilming facilities were considered amongst the best in existence. Gérard was able to reactivate the Maison's subscriptions to German journals that had been cut off by the terms of the Armistice. The scheme was fairly successful but was ultimately limited to the material that Gérard's German contacts had access to, and whilst material from certain countries was readily accessible, that from other countries including the United States, soon fell into short supply. Richard's own assessment of Gérard is that while he performed valuable services to the Germans by thoroughly indexing French scientific publishing during the occupation, and by making German research available for French scientists, she found no evidence that he provided access to Allied science that the Germans did not already have.

Richards also describes the existence of a clandestine channel that provided French scientists with access to Allied research. An illegal indexing and abstracting service was operated throughout the period of the occupation by the Centre National de la Recherche Scientifique (CNRS). In this case the leading personality was Frédéric Joliot-Curie. At the beginning of 1941 Joliot-Curie asked a young scientist, Jean Wyart, to take over the embryonic documentation service started by the CNRS in 1939. Despite the division of France into free and occupied zones, Wyart was able to travel freely between the two, and as a result, access both British and American material.

A rivalry developed between Gérard and Wyart. This had consequences for Wyart because only Gérard had the authority to distribute

paper for documentation purposes and he used that power to deny Wyart paper. However, printing – only possible with German permission – was arranged through an acquaintance of Wyart's, the head of the prestigious Hermann Scientific Editions. Thus was produced the CNRS's *Bulletin Analytique* that appeared monthly, offering three- to six-line summaries of French, British, and American articles and the possibility of ordering the original on microfilm.

Curiously the Germans made no efforts to suppress the illegal index, despite the protestations of Gérard, with whose service it was competing successfully because of its British and American content. Richards does not consider that this tolerance was in any way due to the German's need for the index as they already had an efficient system of their own as described. She considers that its very nature and low audience must have made it a low priority for the Germans.

Florey's papers may well have entered France by one of the routes described earlier. However, Quirke (2008) states that the French pharmaceutical company Rhône-Poulenc obtained 'English technical reports' at the end of 1942, and that these were probably Florey's *Lancet* papers. The approach that Ciba had made to Florey in April 1941 was described earlier; it is conceivable that news about penicillin may have circulated amongst the industrial pharmaceutical community in Switzerland. This would have included both Swiss companies and foreign companies with offices in Switzerland. Included amongst these was Rhône-Poulenc.[2] The possibility exists that Rhône-Poulenc employees may have learned about penicillin from their counterparts in Ciba and then have passed the information on to their parent company in France.

Some indication that information exchanges of this kind did occur within pharmaceutical circles is evidenced by a small wave of requests for *P. notatum* (Westling) that arrived at the CBS in Baarn starting in the summer of 1943. Indeed one can track the sequence by which news about penicillin spread. The first of these was from the Laboratoires du Dr Roussel (7 July 1943) followed by the Etablissements Byla (23 September 1943) and finally Maison L. Frere (3 February 1944). Missing from this list is Rhône-Poulenc and the reason for this must be that they already had access to Fleming's strain of *P. notatum*.

Rhône-Poulenc had a natural collaborator that they would turn to once they had copies of Florey's paper in their hands; this was the Pasteur Institute. They had previously worked closely together on research into novel of sulphonamides, and indeed, the then director Jacques Tréfouël had been instrumental along with Nitti in unravelling the mode of action of these antibacterial drugs. Most important of all for Rhône-Poulenc was

[2] Personal communication by V. Quirke, see also Quirke (2008).

the fact that the Pasteur Institute possessed a strain of Fleming's *P. notatum*. This was according to Quirke (2008) presented to the Institute by Fleming himself when he visited Paris, or alternatively, according to the testimony of one Bernard Sureau, to whom we shall return, presented to André Lwoff by Fleming when Lwoff visited London. Quirke also states that Raymond Paul, Research Director at Rhône-Poulenc, warned Nitti in late August 1943 that they should not fall behind in the penicillin race. She even mentions that the possibility that collaboration with Prof. Hörlein Director of the Bayer laboratories had been considered. The possibility cannot, therefore, be ruled out that Florey's papers may have reached Rhône-Poulenc directly from Germany. Certainly, the date that Quirke quotes 'the end of 1942' falls after the time when news about penicillin had reached German scientists.

Production started on a small scale at Rhône-Poulenc's site at Vitry, but assays were performed at the Pasteur Institute. The French researchers would almost certainly have employed the penicillin assay described in the second of Florey's Lancet papers (Abraham *et al.*, 1941). This was based on the use of small porcelain cylinders impregnated with penicillin solutions and deposited on petri dishes in which the agar had previously been seeded *Staphylococcus aureus*. However, they seemed also to have employed an assay that they had devised themselves. This was based on what they termed 'microbiophotometry' (Faguet and Nitti, 1943). The assay made use of a device that was in many ways ahead of its time and that was capable of automatically recording the growth of up to six individual cultures simultaneously. By this method the inhibitory effect of penicillin could be determined by the effect it had on the growth rate of the target organism – presumably cultures of *S. aureus*.

Bernard Sureau, who was mentioned earlier, was during the war a young intern based at the hospital connected to the Pasteur Institute. He worked with Nitti and has left an undated deposition in the archives of the Pasteur Institute that describes his involvement with penicillin. Sureau's account is a personal one but is somewhat at odds with other sources. There is no mention of Rhône-Poulenc in his account; instead Sureau attributes the initiation of penicillin research at the Pasteur Institute to Nitti having heard a BBC French service broadcast in the Autumn of 1943. Shama (2008) has established that there was in fact a French broadcast on penicillin made on 29 September 1943. In contrast to Home broadcasts, those made by the Foreign Services of the BBC were aimed at medical professionals and it is indeed possible that Nitti heard the broadcast and may have obtained useful information from it and even encouragement: it was almost certainly not what initiated penicillin research at the Pasteur Institute. Sureau gives an example of the lengths that the researchers at the Pasteur Institute were prepared to go to obtain Allied literature on penicillin. He claims that a colleague was dispatched to

Spain to acquire a copy of a special edition of *The British Medical Journal* of 15 April 1944 on penicillin.

Sureau also describes an early trial of their penicillin in January 1944 on a young infant dangerously ill with meningitis. Massive amounts of sulphonamides had been given to the infant but to no avail. An immediate improvement occurred once penicillin had been administered but their meagre stock of the antibiotic ran out and the infant succumbed once more to meningitis and finally died. This account bears a number of striking similarities to the first recorded use of penicillin in Oxford (Macfarlane, 1984). The patient was Albert Alexander who was at death's door with a relentlessly spreading infection occasioned from a scratch with a rose thorn. The precious stock of penicillin was administered with miraculous results that turned to tragedy when the penicillin was exhausted. In their desperation the Oxford medical team had even resorted to extracting the penicillin from the patient's urine and re-injecting it into him. In both cases despite the death of the patient those administering it had seen enough to become convinced of penicillin's therapeutic value.

This particular clinical case was to form the basis of a small clutch of publications in French journals. The first of these was by Nitti that appeared in print a matter of weeks after treatment of the infant ill with meningitis (Nitti, 1944). A second paper was to follow in March 1944 reporting on the same case. The authors were Martin, Sureau and Vittoz (Martin *et al.*, 1944) but interestingly not Nitti. A third paper appeared in June of the same year (Martin and Sureau, 1944).

Examining the British and American papers cited in these publications is indicative both of the efficiency of French document gathering procedures and of the time delays that researchers at the Pasteur Institute were obliged to incur. Both Nitti (1944) and Martin *et al.* (1944) cite Fleming (1929) and Raistrick's work (Clutterbuck *et al.*, 1932) both of which would have existed in Parisian libraries before the war. They also cite Florey's two *Lancet* papers (Abraham *et al.*, 1941; Chain *et al.*, 1940). The most recent British publication is a *Lancet* paper of 1943 (Clark *et al.*, 1943). They also refer to a German review on penicillin published by Kiese (1943)

Nitti's paper makes reference to Florey's *Lancet* papers and interestingly to a chemical composition for penicillin that seems to be that proposed by Abraham *et al.* (1942), but is not formally referenced. He also alludes to work of Coulthard's, possibly Coulthard *et al.* (1942). In addition, he provides his readers with some industrial intelligence – namely that a plant has been established in Canada for the production of 10 kg of penicillin per week. Martin and Sureau (1944) make reference to an article in the *Journal of Bacteriology* of August 1943 – this was almost certainly by Foster and Woodruff (1943). The latter indicates that the delay in obtaining foreign material was just less than 12 months.

The researchers at the Institute Pasteur must have published in the knowledge that their work would come to the attention of the Germans. They were possibly ignorant of the fact that no one in Germany possessed Fleming's strain. The true situation would have become plain when German officials turned up at the Institute demanding the strain. Hamilton Southworth, an American agent, who visited the Institute immediately after the Liberation and whose report on the situation prevailing is considered later, was told that about 8 months prior to his visit (i.e. January or February, 1944) – just after Nitti's publication – German officials tried to obtain the Fleming strain but were alleged to have been given a false one. The officials apparently told the researchers at the Pasteur Institute that they were organising their own programme. They tried again in June 1944, this time Schlossberger [This might have been Prof. H. Schlossberger, of the Hygiene Institute, Frankfurt] had been present. This account is verified to some extent by Bernard (1947). Nitti's paper certainly made its way to Germany, and was cited by in the review article on penicillin (Loewe, 1944) published in *Chemiker-Zeitung* in October 1944 and referred to earlier.

Hamilton Southworth was attached to the London Mission of the Office of Scientific Research and Development's Committee on Medical Research. He was the author of a weekly newsletter in which he reported on medical developments from Britain. These were not necessarily confined to British advances but also included intelligence material received in Britain from Germany and occupied Europe. In one newsletter (No. 103) dated 2 September 1944, Southworth reported on a trip to Paris he made between 28 August 28 and 2 September 1944. Charles de Gaul had marched into Paris just days before on 25 August.

Southworth went to the Pasteur Institute and met Jacques Tréfouël. He wrote: 'They were isolated, discouraged, and without equipment. Most of what they did was a continuation of their old traditions. The Germans asked for a few things like typhus vaccine and tetanus antitoxin but said very little of what they in turn were doing. When the French failed to cooperate, the breach grew even wider. Suspicion even developed between the different French workers as to which of their neighbours might be 'un peu collaborateur'. Southworth was given details of their penicillin work and of Rhône-Poulenc's involvement and that they possessed Fleming's strain. He discovered that only about 30 patients had been treated with penicillin. He commented 'most have had staphylococcal infections and the results have been good. Local use has predominated and there is particular enthusiasm for subconjunctival injection in conjunctivitis. We found no instance of the use of penicillin in syphilis, gonorrhea, or more unusual infections'. Southworth was told that Constantin Levaditi, also at the Pasteur Institute, had a strain of *P. corylophilum* DX obtained from Holland. It produced no penicillin

and its activity *in vitro* was presumed to be due to notatin. (This incidentally was one of the strains that Westerdijk had assayed for Haagen).

Southworth's analysis is contrasted with that of Pasteur Vallery-Radot (Louis Pasteur's grandson) who wrote the foreword to a compilation of articles detailing French medical research during the war (Hamburger, 1947).

> I was greatly surprised to learn that our good friends in America had never heard of our latest work in medical research. All were convinced that during those years of oppression France had been living coiled around herself and that her spirit had deserted her body... How could these men carry on with their work, under such seemingly impossible conditions? The equipment of laboratories was deficient, often the gas and electricity were cut off; to obtain the animals for necessary experiments was of the greatest difficulty.
>
> They themselves led a miserable life, underfed, shivering with cold all winter, their minds beset with anguish. They lived under the constant threat of being deprived of their freedom, worrying about the fate or of their friends who were thrown into jail, deported or shot. How could they be successful in their scientific work? And successful they were, in spite of all the torments of servitude, in spite of their physical and moral tortures, of almost overwhelming difficulties, of this wall shutting off France from the outside world, and in spite of this numbing silence about all countries not included in the German Reich.

It is the contention here that the 'wall shutting off France from the outside world' was not entirely impermeable nor that the 'numbing silence' was total.

Southworth's revelation of the total numbers of patients that had received treatment with penicillin – some 30 in all – The numbers treated were very small and the overall impact on diminishing human suffering would have been negligible and one might justifiably ask 'was it worth it?' In normal times researchers at the Pasteur Institute would have found themselves at the centre of research on penicillin. The war ultimately did not prevent their involvement in research on penicillin. Despite the privations the researchers at the Pasteur Institute were working at what today be referred to as the 'cutting edge' of antibacterial chemotherapeutics. Their efforts to make penicillin against all odds provided them with valuable experience in the preparation and extraction of penicillin and this was to become of tremendous benefit in the immediate aftermath of the Liberation and until penicillin became available for civilian use. The opportunity to apply their hard-earned skills was an unusual one. Following the liberation of Paris, that city became the centre for a number of Allied hospitals. The American army made the Pasteur Institute a

generous and valuable gift; the urine of all servicemen that were undergoing therapy with penicillin. Therèse Trefouël, also at the Pasteur Institute even published a paper on the process (Tréfouël *et al.*, 1945).

VI. CONCLUSIONS

An absolute requirement for penicillin production in the 1940s was access to the most up-to-date literature and, most crucial of all, Fleming's culture of *P. notatum*.

Written requests to Fleming for cultures have not survived,[3] but it is clear from the account given earlier that Fleming had been distributing cultures of his mould well before Florey had published his papers demonstrating the therapeutic potential of penicillin. Undoubtedly some of those who had obtained cultures in the period between Fleming and Florey – the '*inter regnum*' as Wainwright (2002) has referred to it – had not attempted to actually grow them until Florey's papers appeared. Moreover, those that did undertake work on penicillin during the *inter regnum*, for example Reid and Raistrick, cannot be said to have significantly advanced the study of the antibiotic.

It is unlikely that Fleming lived to know what the full consequences of his acts in distributing cultures were to have. Perhaps he may have divined the reason why neither Florey nor Chain had to approach him for a culture was that he had previously given one to Georges Dreyer, the former head at the Sir William Dunn School of Pathology in Oxford. Fleming would probably would not have known that when Florey and Chain were deciding which antimicrobial compound to study after pyocyanase proved to be too toxic that a certain Miss Campbell-Renton happened to be culturing *P. notatum* just along the corridor from them (Macfarlane, 1984).

Those scientists in Europe involved in research on penicillin were aware of the massive and well-resourced Anglo-American programme to mass produce penicillin through access to British and American publications that the state of war never succeeded entirely in cutting off. Their motives in pursuing their studies can only be guessed at. It would be reassuring to think that amongst them were humanitarian ones. There were undoubtedly others too including an understandable need to retain their professionalism. Perhaps just being able to produce any penicillin at all under straightened circumstances may have represented a victory of sorts. Being able to publicize the fact through journal publications, so representing a hope that just as British and American articles had reached

[3] Kevin Brown, Curator of the Alexander Fleming Museum, St Mary's Hospital, London.

them, their work might reach the Allies, may have heightened the sense of achievement. Some of those who took part in these attempts are destined to remain unnamed and unknown, but perhaps this work has succeeded in salvaging the names of a few that might otherwise have been forgotten.

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CHAPTER 6

The Genomes of Lager Yeasts

Ursula Bond

Contents

Abstract

Yeasts used in the production of lagers belong to the genus *Saccharomyces pastorianus*. Species within this genus arose from a natural hybridization event between two yeast species that appear to be closely related to *Saccharomyces cerevisiae* and *Saccharomyces bayanus*. The resultant hybrids contain complex allopolyploid genomes and retain genetic characteristics of both parental species. Recent genome analysis using both whole genome sequencing and competitive genomic hybridization techniques has revealed the underlying composition of lager yeasts genomes. There appear to be at least 36 unique chromosomes, many of which are lager specific, resulting from recombination events between the homeologous parental chromosomes. The recombination events are limited to a defined set of genetic loci, which are

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highly conserved within strains of lager yeasts. In addition to the hybrid chromosomes, several non-reciprocal chromosomal translocations and inversions are also observed. Remarkably, in response to exposure to environmental stresses such as high temperatures and high osmotic pressure, the genomes appear to be highly dynamic and undergo recombination events at defined loci and alterations in the telomeric regions. The ability of environmental stress to alter the structure and composition of the genomes of lager yeasts may point to mechanisms of adaptive evolution in these species.

I. INTRODUCTION

Yeasts have been used in the production of alcoholic beverages such as beers, wines, mead and sake for many thousands of years due to their highly developed fermentative capacity. Historically, fermentations relied on the use of endogenous yeasts, present on the substrate such as grapes or the repitching of yeast from previous fermentations, as the source of the fermenting microorganism. The yeasts used for the production of ales and lagers were originally classified based on their flocculation properties. Ale yeasts tended to float to the top of the vat at the end of fermentation and were classified of top-fermenters, while lager yeasts sedimented to the bottom of the vat and were thus named bottom-fermenters. It was not until the development of pure culture techniques by Hansen in 1893 that individual strains of yeasts were cultured and pure strain stocks used for the production of ales and lagers.

II. CLASSIFICATION

Modern genetic and taxonomical classification techniques place the lager and ale yeasts into the genus *Saccharomyces sensu stricto* (Kurtzman, 2003; Vaughan-Martini and Martin, 1995). This genus incorporates many species that are industrially important and currently includes the species *S. cerevisiae*, *S. bayanus*, *S. pastorianus*, *S. paradoxus*, *S. kudriavzevii*, *S. cariocanus* and *S. mikatae* (Naumov *et al.*, 2000b; Replansky *et al.*, 2008; Sampaio and Goncalves, 2008) and the recently described *S. arboricolus*, isolated from oak trees in China (Wang and Bai, 2008). The lager yeasts, originally referred to as *S. carlbergensis*, are now

classified as *S. pastorianus* (Rainieri *et al.*, 2006) (Fig. 6.1). Ale yeasts are predominately *S. cerevisiae*; however, *S. bayanus* strains have also been isolated from beer.

While up to eight distinctive species have thus far been identified within the *Saccharomyces stricto sensu* genus, taxonomic classification can be complicated as the species display common physiological and morphological characteristics. Additionally, recent genetic studies have identified a number of hybrid strains containing genetic information from two or more *Saccharomyces* species. The species *S. bayanus* has been sub-divided into *S. bayanus* var. *bayanus*, which contains a number of hybrid strains and *S. bayanus* var. *uvarum*, typically isolated from enological environments (Rainieri *et al.*, 2003, 2006). Hybrids between *S. cerevisiae* and either *S. kudriavzevii*, *S. bayanus* var. *uvarum* or *S. bayanus* var. *bayanus* have been isolated in wine fermentations, and a triple hybrid of *S. bayanus* var. *uvarum*, *S. cerevisiae* and *S. kudriavzevii* has been identified in vineyards in Switzerland (Masneuf *et al.*, 1998; Naumov *et al.*, 2000a, 2005).

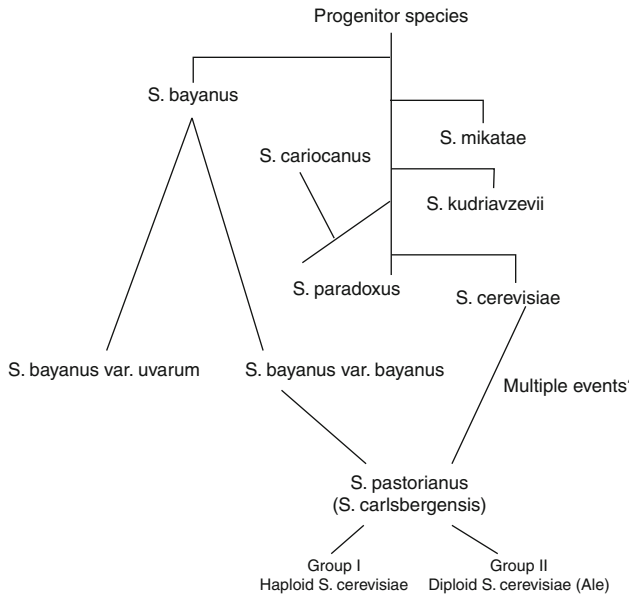

FIGURE 6.1 Origins of the species *Saccharomyces pastorianus*. *S. pastorianus* strains are interspecies hybrids of *S. cerevisiae* and *S. bayanus* var. *bayanus* and can be divided into two distinct groups. Adapted from Rainieri *et al.* (2006) and Dunn and Sherlock (2008).

III. THE LAGER YEASTS: *SACCHAROMYCES PASTORIANUS*

Sequence analysis of individual genes from lager yeast strains as well as the complete sequencing of the whole genome of one strain of *S. pastorianus* provided evidence that the lager yeasts are natural interspecies hybrids of *S. cerevisiae* and *S. bayanus* (Bond and Blomberg, 2006; Caesar *et al.*, 2007; Casaregola *et al.*, 2001; Kielland-Brandt *et al.*, 1995; Rainieri *et al.*, 2006; Smart, 2007) (Fig. 6.1). A recent study of a wide variety of lager yeast strains by competitive genomic hybridization (CGH) to *S. cerevisiae* and *S. bayanus* DNA microarrays indicates that the lager yeast strains can be further sub-divided into two distinctive groups (Groups I and II) based on the number of copies of the parental chromosomes, the location of genome rearrangements and DNA polymorphisms (Fig. 6.1) (Dunn and Sherlock, 2008). The classification of lager yeasts into two groups lends support to the notion that the lager yeast strains originated from multiple interspecies hybridization events between distinct but closely related progenitor strains. Partial sequence analysis of Group I and II sub-types suggests that Group 1 strains may have resulted from a hybridisation event between a haploid ale *S. cerevisiae* strain and a haploid *S. bayanus* strain, while Group II strains may be derived from a fusion between a diploid *S. cerevisiae* with a haploid *S. bayanus* (de Barros Lopes *et al.*, 2002; Legras *et al.*, 2007). The *S. cerevisiae* content of the Group I and II lager yeasts differs by only 0.3% indicating that the progenitor strains were highly related. The Group I strains appear to have lost large portions of the *S. cerevisiae* genome.

IV. LAGER YEAST CHROMOSOMES: TYPES

One of the earliest approaches to the characterization of the lager yeast chromosomes employed the technique of single chromosome transfer (Nilsson-Tillgren *et al.*, 1981). These classical experiments, in which single chromosomes from the lager yeasts were transferred into *kar1* mutants of *S. cerevisiae*, revealed that some chromosomes readily underwent homologous recombination with the *S. cerevisiae kar1* mutant while others displayed no ability to recombine. The former were thus identified as *S. cerevisiae*-like while the latter were referred to as lager type. The frequency of recombination differed substantially for chromosome to chromosome and, furthermore, different regions within the same chromosome more readily recombined with the *S. cerevisiae* chromosome than other regions. This led to the hypothesis that mosaic chromosomes composed of *S. cerevisiae*-like genes and lager-type genes may exist in the lager strains. The complex electrophoretic patterns of lager yeast

chromosomes provided further evidence for the hybrid nature of the lager yeast genome. Analysis of lager yeast chromosomes separated by pulse-field electrophoresis, using chromosome specific probes, identified multiple forms of certain chromosomes, for example, three forms of chromosome X in the production strain *S. carlsbergensis* were identified by this method (Casey, 1986).

More recently, the chromosomal composition of *S. pastorianus* strains has been addressed using CGH (Bond *et al.*, 2004; Dunn and Sherlock, 2008; Kodama *et al.*, 2006). CGH analysis is performed by comparing the ability of a test genome to compete with a known control genome for hybridization to *S. cerevisiae* or *S. bayanus* DNA microarrays. In the initial analysis (Bond *et al.*, 2004), DNA from two different strains of lager yeasts (6701 and CMBS) was competed with DNA from a haploid strain of *S. cerevisiae* (control genome) for hybridization to microarrays containing all known *S. cerevisiae* ORFs. Hybridization of the competing DNAs to the microarray chips depends on (i) the degree of homology between the target and control DNA to the *S. cerevisiae* DNA on the chips (generally the strain S-288C is used for the preparation of chips) and (ii) the copy number of genes in the test and control genomes. For example, hybridization of DNA from a haploid *S. cerevisiae* genome differentially labelled with Cy3- and Cy5-conjugated nucleotides should generate a ratio of hybridization (ROH) of 1.0, if there is no preferential hybridization of either labelled DNA. Using this as a control, it is possible to obtain a normalized value for the ROH to each gene on the microarray. Should the test genome contain more copies of an individual gene, the expected ROH should be greater than 1.0. Likewise if the test genome contains a gene with low levels of homology to the corresponding DNA on the chip or the gene is missing altogether from the genome, the expected ROH will be less than 1.0.

Characterization of these two lager yeast strains in this manner revealed a number of interesting features. Firstly, plotting the ROH for each gene arranged by chromosome and using the *S. cerevisiae* gene order revealed distinct changes in ROH at specific locations on eight of the sixteen chromosomes. These 'jumps' or changes in ROH reflect abrupt changes in copy number and/or homology to the *S. cerevisiae* microarrays and can be interpreted as points where recombination between the homeologous chromosomes has occurred or where *S. cerevisiae* genes have been deleted (Fig. 6.2). Such recombination events could generate mosaic chromosomes consisting of *S. cerevisiae*-like and lager-type genes as originally proposed by (Nilsson-Tillgren *et al.*, 1981). The exact location where recombination had occurred between homeologous pairs of chromosomes can be clearly defined at a single gene resolution (Table 6.1).

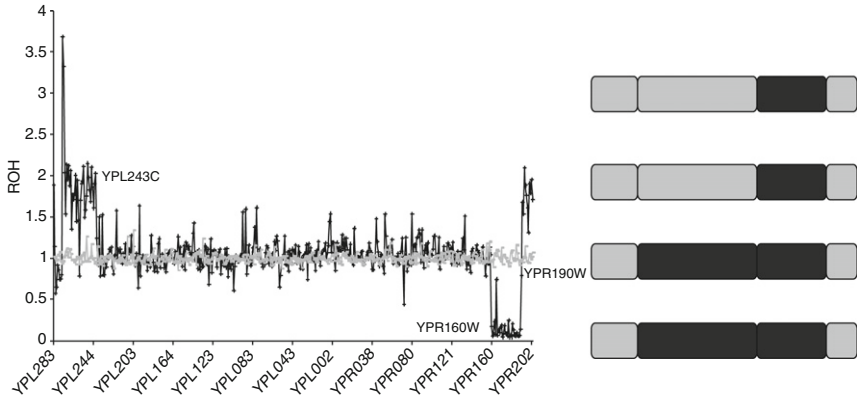

FIGURE 6.2 Ratio of hybridizations (ROH) for genes on chromosome XVI in the lager strain CMBS (black) and the haploid *S. cerevisiae* (grey). DNA isolated from these strains was competed with DNA from *S. cerevisiae* for hybridization to *S. cerevisiae* microarrays. The locations of 'jumps' in ROH are indicated by the closest ORF. Putative composition of chromosome XVI in CMBS: grey blocks represent *S. cerevisiae*-like and black blocks represent *S. bayanus*-like.

In this initial analysis, up to twenty eight locations were identified where specific changes ('jumps') in ROH occurred. Of these, nine reside in sub-telomeric X elements, which contain repetitive sequences and autonomous replication sequences (ARS), eight reside within 25 kbp of the telomere and appear to represent telomere deletions. The remaining eleven sites reside at unique sites on eight different chromosomes and all but one (YHR165C) were common to both lager yeast strains (Table 6.1). A large number of the jump sites lie next to or within a few open reading frames of a Ty element, a tRNA gene or an ARS sequences. The location of Ty and ARS elements next to some of the recombination sites points to roles for both transposon- and replication-mediated recombination in the generation of hybrid chromosomes. Surprisingly, many of the genes adjacent to the recombination loci encode proteins that play essential roles in fermentation, including AAD6, ADH2 and TDH2 (Bond *et al.*, 2004; Nakao *et al.*, 2009). Other genes at these loci include two genes encoding proteins involved in RNA metabolism (PRP8 and KEM1), and HSP82, a heat-shock protein.

Since this initial analysis, a more extensive CGH analysis of seventeen strains of *S. pastorianus* from a variety of geographic locations has been conducted using both *S. cerevisiae* and *S. bayanus* 60-mer oligo-microarrays. These experiments allowed for more fine mapping of the recombination sites between the homeologous chromosomes. In addition to verifying the

TABLE 6.1 Major chromosomal translocations in *Saccharomyces pastorianus*

	Location	ORFS	Other features	Reference
S.c/S.b				
III	YCR039C	MAT locus	MAT locus	Bond *et al.* (2004), Nakao *et al.* (2009)
VI	YFL056C/YFL052W	AAD6/Un	ARS	Bond *et al.* (2004), Dunn and Sherlock (2008)
VII	YGL173C	KEM1	tRNA, ARS	Bond *et al.* (2004), Dunn and Sherlock (2008), Nakao *et al.* (2009)
VIII	YHR165C	PRP8	None	Bond *et al.* (2004), Dunn and Sherlock (2008)
X	YJR009C	TDH2	ARS	Bond *et al.* (2004), Dunn and Sherlock (2008), Nakao *et al.* (2009)
XI	YKL044W/YKL045W	Dub/PRI2	None	Bond *et al.* (2004), Dunn and Sherlock (2008), Nakao *et al.* (2009)
XIII	YMR302C/YMR303C	YME3/ ADH2	None	Bond *et al.* (2004), Dunn and Sherlock (2008), Nakao *et al.* (2009)
XV	YOR343C	TYA	Ty2	Bond *et al.* (2004), Dunn and Sherlock (2008)
XVI	YPL240C/YPL241C	HSP82/CIN2	None	Bond *et al.* (2004), Dunn and Sherlock (2008), Nakao *et al.* (2009)
XVI	YPL160W	GPH1	tRNA, Ty1	Bond *et al.* (2004), Dunn and Sherlock (2008), Nakao *et al.* (2009)
XVI	YPL191C	QCR2	ARS, Ty4	Bond *et al.* (2004), Dunn and Sherlock (2008), Nakao *et al.* (2009)

(*continued*)

TABLE 6.1 (*continued*)

	Location	ORFS	Other features	Reference
S.c/S.c				
V/XI	Unidentified in Nakao *et al*. (2009)	Ty	Ty LTR	Nakao *et al*. (2009)
XI/XV	YKL220C/YOR381W	FRE2/FRE3	ARA, Tel X	Nakao *et al*. (2009)
S.b/S.b				
II/IV	YBRO31W/YDR012W	RPL4A/ RPL4B	None	Nakao *et al*., 2009
	YDR012W / YBRO31W	RPL4B/ RPL4A	None	Nakao *et al*. (2009)
VIII/XV	YOR18W-19W/ YHR014W-15W	ROD1-Un/ SPO13-MIP6	tRNA/Ty1	Nakao *et al*. (2009)
VIII/XV	YHR014W-15W / YOR18W-19W	SPO13-MIP6/ ROD1-Un	tRNA/Ty1	Nakao *et al*. (2009)

Un, uncharacterized; Dub, dubious; ARS, autonomous replicating sequence.

location of the recombination sites identified in the original CGH study (Bond *et al.*, 2004), up to 80 additional putative recombination sites in both sub-genomes were identified although the majority of these sites are clustered at similar genome locations on 10 chromosomes and mainly correspond to the major sites previously identified (Table 6.1).

V. GENOME SEQUENCE ANALYSIS OF A LAGER YEAST STRAIN

While CHG analysis can provide clues to the chromosomal makeup of lager yeast strains, a complete understanding of the chromosome composition of lager yeasts would not emerge until the full genome sequence analysis of *S. pastorianus* (Weihenstephan strain 34/70) was obtained (Nakao *et al.*, 2009). This whole genome sequence analysis suggests a genome size of 23.4 Mbp and a predicted number of chromosomes of 36. This is slightly more than the combined chromosome content of *S. cerevisiae* and *S. bayanus* which each contain 16 chromosomes. The sequence analysis confirmed the origin of the progenitor strains as *S. cerevisiae* and *S. bayanus*: *S. cerevisiae* orthologues (referred to as class 1 ORFs) showed on average 99.2% identity to the reference *S. cerevisiae* strain S-288C, while the corresponding paralogues (class 2 ORFS) on average were 92.7% identical to the reference *S. bayanus* strain CBS 7001 which is classified as *S. bayanus* var. *uvarum*. This lower level of identity may indicate that the progenitor strain may be more similar to *S. bayanus* var. *bayanus*, supporting prior analysis of genes from a number of lager yeasts by restriction fragment length polymorphism (RFLP) which showed the greatest similarity was to the *S. bayanus* var. *bayanus* strain NBRC 1948 (Rainieri *et al.*, 2006, 2008).

The full sequence analysis confirmed the presence of three types of chromosomes, (i) *S. cerevisiae*-like, (ii) *S. bayanus*-like and (iii) mosaic chromosomes. The location of the major recombination sites on the mosaic chromosomes previously identified by CGH analysis was also confirmed and a more detailed description of the events obtained (Table 6.1). Of the eleven major unique 'jump' sites, eight were confirmed while three of the sites (YHR165C, YOR343C and YFL056C/YFL052W) do not appear to be present in the Weihenstephen strain; seven of the conserved recombinations involved non-reciprocal translocations within homeologous alleles and one appears to be a reciprocal translocation.

The analysis also uncovered a series of chromosomal translocations which were not identified by CGH analysis (Table 6.1) and a number of gene inversions. One inversion was identified on the *S.c*-type chromosome XIV and three were identified on the *S.b*-type chromosomes XIV, V and VI, respectively. The latter two are also evident in the *S. bayanus*

progenitor strain and presumably occurred prior to the hybridization event. Of the translocation events, two reciprocal chromosomal translocations between *S.b*-type chromosomes II and IV and VIII and XV are also found in the *S. bayanus* progenitor strain and were previously identified in lager yeasts by pulse-field gel electrophoresis and Southern blotting (Ryu *et al.*, 1998; Tamai *et al.*, 1998; Yamagishi and Ogata, 1999). The two novel translocations between *S.c*-type chromosomes V and XI and between XI and XV are not present in the *S. cerevisiae* progenitor strain (Table 6.1). As predicted from the earlier CGH studies, the telomeric and sub-telomeric regions were the least conserved regions of the chromosomes and telomeric loss is evident. Surprisingly, eight novel ORFs with no corresponding orthologue in either *S. cerevisiae* or *S. bayanus* are located in the sub-telomeric regions. This finding suggests that the telomeres are highly adaptable and may be subject to adaptive evolutionary pressures.

VI. CHROMOSOME COPY NUMBER

While the presence of mosaic chromosomes within the lager yeast genome has been clearly established, it has been more difficult to definitively estimate the number and types of chromosomes present in the strain. Several lines of investigation have been used to determine the minimum number and type of chromosomes present. Firstly, gene replacement experiments, together with meiotic analysis of genes located on chromosomes VI, XI, XIII and XVI, suggest the presence of four copies of each of these chromosomes (Kielland-Brandt *et al.*, 1995). Secondly, analysis of lager yeast strains by flow cytometry (FACs) indicated a general tetraploid DNA content (Kielland-Brandt *et al.*, 1995; Usher and Bond, unpublished results). This is also consistent with DNA content estimated by DAPI-fluorometry analysis (James *et al.*, 2003). Analysis of gene copy number by real-time PCR analysis in *S. pastorianus* strains estimated copy numbers of between 1 and 6 for individual *S. cerevisiae* ORFs (Bond *et al.*, 2004). CGH analysis also provides evidence of the apparent amplification of the *S. cerevisiae* complement at multiple chromosomal sites. These regions of amplification can be interpreted in two ways. Firstly, there may be tandem amplification of *S. cerevisiae* genes at the same chromosomal location or secondly, such amplifications may represent multiple chromosome copies containing *S. cerevisiae* genes. For example, genes from the left telomere to YPL240C and from YPR192C to the right telomere of chromosome XVI show average ROH values of 1.8 (see Fig. 6.2A). Using real-time PCR to evaluate gene copy number, this value correlates with approximately four copies of *S. cerevisiae* genes. The remainder of genes on chromosome XVI show an average ROH of 1.0–1.2 with the exception of genes between YPR160W and YPR190W, which

show an ROH of 0.2. These ROH values correspond to *S. cerevisiae* gene copy numbers of 2 and 0, respectively. Since there is no supporting evidence from the whole genome sequencing for tandem gene amplification, we interpret these data as indicating the presence of at least four copies of chromosome XVI in the lager yeast strains (Fig. 6.2B). Based on the ROH values, a plausible chromosome XVI complement would be four copies in a ratio of two *S.b*-like to two *S.c*-like chromosomes with all four containing *S.b* region from YPR160W to YPR190W and *S.c*-like ORFs at the telomeres (see Fig. 6.2B).

Dunn and Sherlock (2008) have conducted FACs analysis on a variety of Group 1 and 2 *S. pastorianus* strains and estimated that Group I strains appear to have a DNA content similar to a diploid consisting of one *S. cerevisiae* genome and one *S. bayanus* genome, while Group 2 appears to be triploid with two *S. cerevisiae* genomes and one *S. bayanus* genome. This is somewhat consistent with the estimated chromosome number of 36 for the Weihenstephan strain, which appears to be of the Group 2 variety, indicative of an overall diploid with some aneuploidy (Nakao *et al.*, 2009). The discrepancies in chromosome copy number estimation based on the various approaches outlined earlier may possibly be reconciled by invoking the presence of multiple copies of identical chromosomes that are indistinguishable at a genome level, although this remains to be determined.

VII. CONSEQUENCES OF GENOME REARRANGEMENTS

The novel genomic rearrangements that have occurred in the lager yeast genome have resulted in a number of loss-of-function events due to gene deletions and/or frame shift mutations, gain-of-functions due to the emergence of novel ORFs, gene fusions resulting from chromosomal translocations or clustered regional gene amplification, as discussed earlier. The full complement of genomic rearrangements are too numerous to mention in this review, but readers are referred to the supplementary data from (Dunn and Sherlock, 2008; Nakao *et al.*, 2009) for the complete details. The most significant outcomes of these rearrangements include the apparent amplification of a number of genes that play significant roles during fermentation, such as the MAL3 cluster at the right telomere of chromosome II which is required for maltose metabolism. Likewise, the copy number of the MTT1 gene, encoding the maltotriose transporter, and MET 3 and MET 14, encoding proteins required for sulphite anabolism, have increased due to a chromosomal translocation events on chromosomes VII, X and XI respectively. Conversely, other genes encoding proteins with significant functions during fermentation such as the flocculation genes FLO 9 and 5 show reduced copy number most likely due to

telomere-associated gene loss. Likewise, there is a loss of the α-glucoside transporter MPH3 due to an apparent frameshift mutation in the ORF.

Of the eight lager specific genes identified from the whole genome sequence analysis, surprisingly, with the exception of one, all are located in the sub-telomeric regions and are between 350 and 795 amino acids in length. These novel ORFs appear to encode for proteins that may enhance the fermentative capacity of the yeasts through increased sugar and amino acid metabolism. How these novel ORFs originated is currently unknown; however, recent data suggest that the telomere regions of the lager yeasts are in a high state of flux which may be sufficient to drive such adaptive evolution (see below).

Finally, as many as 28 *S.c*-like and 33 *S.b*-like ORFS appear to encode truncated proteins as a result of frameshift or stop codon mutations in the ORFs. In general these deletions are not deleterious to the yeasts due to the presence of multiple homeologous alleles. Interestingly, these mutated genes do not coincide with any of the major genomic rearrangements listed in Table 6.1 and appear to be independent of these events. However, such genomic rearrangements, in particular, the reciprocal and non-reciprocal recombination events between homeologous chromosomes, have the potential to generate either loss- or gain-of-function phenotypes. A detailed analysis of one such recombination site on chromosome XVI at YPR160W, which encodes the enzyme glycogen phosphorylase, revealed that the recombination occurred at the 5′ end of the gene generating a hybrid gene that is *S. cerevisiae*-like at the 5′ end and *S. bayanus*-like at the 3′ end (Usher and Bond, 2009). Northern blotting and real-time reverse transcriptase-PCR analysis indicates that the hybrid gene does not produce a mature RNA transcript and is non-functional due to a frameshift mutation. There is evidence from Northern blotting for a YPR160W-like transcript. This most likely emanates from the *S.b*-like copies of chromosome XVI (Fig. 6.2A and B). This loss of function in two of the four copies of YPR160W appears to alter glycogen metabolism in the lager yeast strains, which contain glycogen levels similar to haploid strains of *S. bayanus* and *S. cerevisiae* (Usher and Bond, 2009). Currently, there are no other reported phenotypic changes associated with homeologous recombination events, although it is possible that more will be uncovered upon detailed analysis of the other recombination sites.

VIII. THE DYNAMIC GENOME OF LAGER YEASTS

The presence of stable hybrid chromosomes in all lager yeast strains that are not present in the progenitor strains indicates that recombinations between the homeologous chromosomes occurred after the species hybridization event. The recombination events appear to have occurred within

short homologous regions of homeologous alleles, which share 81–96% sequence identity. However, since the vast majority of homeologous genes in the genome would share similar sequence identity levels, it is not clear why recombination at these specific 'hot spots' is more likely than at other regions of the genome. Recombination events are rare in *Saccharomyces* species and occur at frequencies in the order of 5×10^{-8} to 2×10^{-9} in genes with this level of sequence identity (Datta *et al.*, 1997). The frequency of chromosomal recombination events can be increased by the induction of double-strand breaks (DSBs) following exposure of cells to DNA damaging agents such as UV light, gamma irradiation or DNA-modifying agents (Admire *et al.*, 2006; Haber, 2006). Polyploid and aneuploid strains of yeasts show increased sensitivity to gamma irradiation and DNA-damage-inducing agents (Mayer *et al.*, 1992) and are known to have higher rates of genome instability compared to haploid strains (Mayer and Aguilera, 1990; Storchova and Pellman, 2004). Perturbations of internal cellular processes such as DNA replication can also contribute significantly to the induction if DSBs (Aguilera and Gomez-Gonzalez, 2008). There is a growing body of evidence suggesting that natural impediments to replication fork progression can lead to gross chromosomal rearrangements (Azvolinsky *et al.*, 2006; Huang and Koshland, 2003; Lemoine *et al.*, 2005; Mirkin and Mirkin, 2007; Schmidt and Kolodner, 2006).

It is quite possible that the chromosomal rearrangements encountered in lager yeasts occurred as a consequence of exposure to the harsh environmental conditions experience during the process of fermentation to facilitate adaptation to these conditions. Lager yeasts are exposed to high osmotic and hydrostatic pressure, anaerobiosis, low pH, low temperature, high alcohol concentrations and high cell densities during fermentation (Attfield, 1997; Brosnan *et al.*, 2000; Carrasco *et al.*, 2001; Gibson *et al.*, 2008; Zuzuarregui *et al.*, 2005). The hybrid genome of lager yeasts appears to confer a very high degree of resistance to these stresses. For example, the parental species of the lager yeasts, *S. cerevisiae* and *S. bayanus*, do not survive well under industrial fermentation conditions and are much less capable of metabolising the available sugars to ethanol. Likewise, the ability of the hybrid lager yeasts to adapt to growth at low temperatures is a trait conferred from the *S. bayanus* component of the genome (Belloch *et al.*, 2008).

The hypothesis that exposure of lager yeasts to stressful environmental conditions might facilitate chromosomal rearrangements has been tested in a series of experiments in which lager yeasts were exposed to the combined stresses of high temperature and high specific gravity (James *et al.*, 2008). Colonies surviving the initial stresses were subjected to repeated rounds of selection under the same stress conditions to ensure that the stress tolerance was heritable. Three of these stress-tolerant clonal isolates

(CM5, CM6 and CM10) were compared to the parental strain using CGH analysis. These studies revealed that each had undergone gross rearrangements, small deletions, regional amplifications and changes in chromosome copy number (James *et al.*, 2008). The strains CM5 and CM6 showed a similar pattern of chromosomal rearrangements but differed from each other in chromosome copy number. The strain CM10 showed the least amount of changes but again copy number differences from the parental strain were evident. The majority of the rearrangements present in strains CM5 and CM6 mapped to the previously identified recombination sites (refer to Table 6.1); however, the study uncovered a number of novel recombination sites that had not previously been noted. These include sites close to YER139C, YGL162W, YJL050W and YKL165C.

These experiments showed for the first time that genome rearrangements can be induced in allopolyploid yeast cells by exposure to environmental stress. Furthermore, the recombination events are not random but occur at specific defined 'hot spots'. The molecular mechanisms controlling these recombination events are currently unknown, but it is possible that these 'hot spots' represent fragile chromosomal regions susceptible to DSBs induced in response to environmental stresses. The major outcome of these recombination events is a change in the ratio of *S.c*-like to *S.b*-like alleles between the stress-tolerant and parental strains. This is most evident in an analysis of chromosome III (Fig. 6.3) where the ratio of *S.c*-like to *S.b*-like genes to the left of the MAT locus has changed from 2:2 in the parent to 4:0 in the stress-tolerant CM5 and CM6 strains, a net gain of *S. cerevisiae* genes.

The stress conditions used to induce the chromosome rearrangements described earlier are severe in nature and are not likely to be encountered by lager yeasts in the environment or during fermentation. However, the cumulative effects of several stresses such as anaerobiosis (high CO_2 concentrations), high sugar concentrations (up to 22%), low temperatures (as low as 7 °C) experienced during fermentation have the potential to influence the chromosome structure and copy number. To test this, the chromosome profiles of lager yeasts were examined following fermentation in either standard fermentation conditions in medium containing 16% maltose (low-specific gravity) at 13 °C or under more severe conditions of 22% maltose (high-specific gravity) medium at room temperature, 20 °C. While gross chromosomal rearrangements were not observed in the final population of cells, the study revealed that growth of cells in the higher sugar concentrations and at room temperature resulted in greater genome instability in a number of defined regions. In general, approximately 10–15% of genes on each chromosome showed significant changes in ROH with the majority of these changes occurring at the telomeres. Secondly, there was an overall loss of the *S. cerevisiae* gene complement on

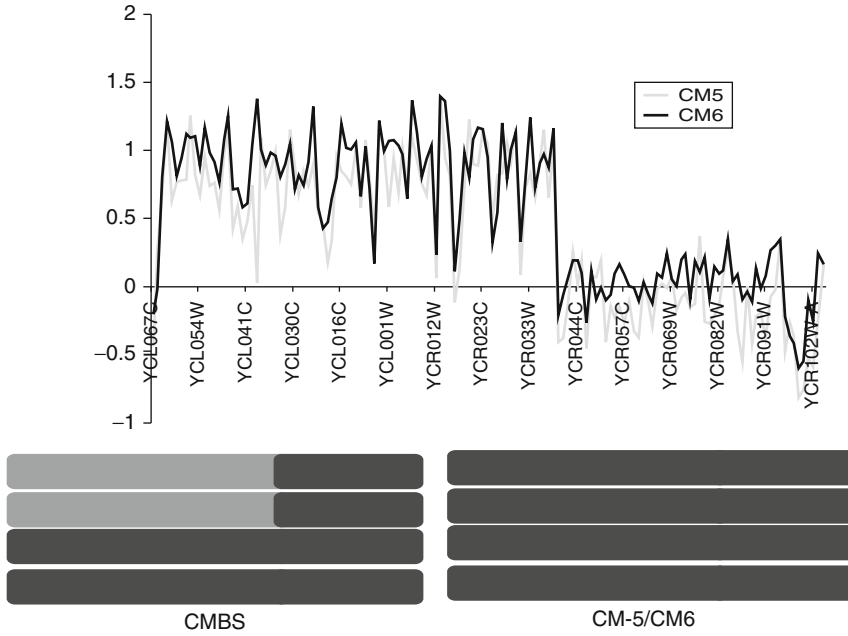

FIGURE 6.3 Ratio of hybridizations (Log_2 ROH) for genes on chromosome III in the stress-tolerant strains CM5 and CM6. DNA isolated from CM5 (grey) and CM6 (black) was competed with DNA from the parental strain for hybridization to *S. cerevisiae* microarrays. Putative composition of chromosome III in CMBS, CM5 and CM6: black blocks, *S. cerevisiae*-like, grey blocks, *S. bayanus*-like. Adapted from James *et al.* (2008).

chromosomes I, and VI. Significantly, a gradient of amplification of genes flanking the major ribosomal RNA gene cluster on chromosome XII was observed (Fig. 6.4). In *S. cerevisiae*, this locus consists of 100–200 copies of a 9.1-kb repeated unit (RDN1) containing the genes encoding for 5S, 5.8S, 25S and 18S rRNAs, respectively, as well as internal transcribed spacers (ITS1, ITS2), external transcribed spacers (5′ ETS, 3′ ETS) and non-transcribed spacers (NTS1, NTS2). The *S. pastorianus* Weihenstephan strain contains both *S.c* and *S.b* copies of chromosome XII, although there appears to have been a massive loss of rDNA copies in the *S.b*-like chromosome. Interestingly, two other lager yeast strains CBS-1513 (*S. carlsbergensis*) and CBS-1538 appear to have only *S.b*-like rDNA (Kodama *et al.*, 2006). Thus, the rRNA cluster seems to be highly dynamic in the lager yeast strains. What may be driving these dynamics is currently unclear, but there are high levels of transcription from this region as well as high levels of replication as each rDNA unit contains an autonomously replicating sequence (ARS). The collision of the replication and transcription machinery may stall replication fork progression

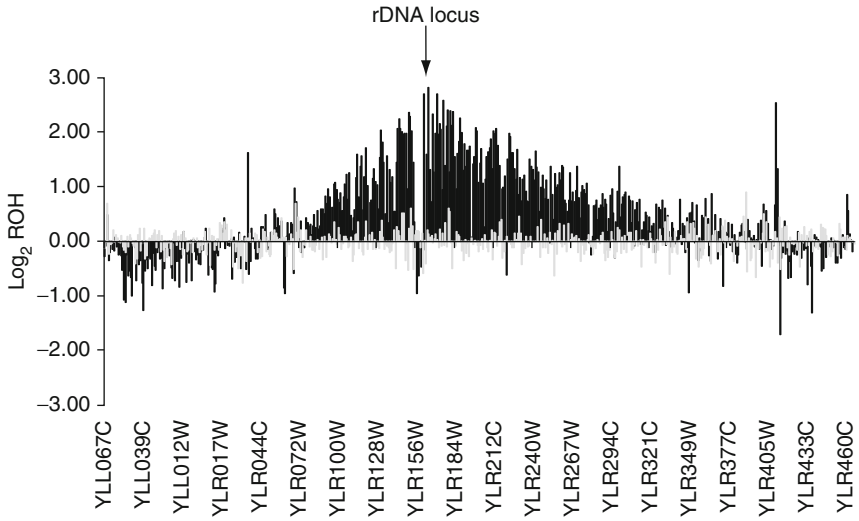

FIGURE 6.4 Gene amplification adjacent to the rDNA locus on chromosome XII. Ratios of hybridization (ROH) for genes flanking the rDNA locus. CGH was carried out using DNA isolated from lager yeast strain CM1051 at the beginning (day 1) or end of an 8-day fermentation in high maltose (22%), high temperature (20 °C): black bars. ROH values for day 1 DNA differentially labelled with Cy3 and Cy5: grey bars. The location of the rDNA locus is indicated by an arrow. Adapted from James *et al.* (2008).

leading to an increased possibility of generating DSBs, which may facilitate such gene amplification. This hypothesis is supported by data showing that deletion of the DNA-binding protein Fob1p in *S. cerevisiae* leads to transcription-mediated replication stalling radiating from the rRNA locus (Takeuchi *et al.*, 2003).

The yeasts fermented under the high sugar and high temperature conditions also showed a second cluster of gene amplification on chromosome I. This site is located in a region referred to as DUP240, which contains genes belonging to one of the largest gene families in yeast. The multigene family consists of at least 10 genes with a high level of nucleotide identity (from 50% to 98%), scattered on four chromosomes and arranged either as tandem repeats or as isolated genes (Despons *et al.*, 2006). Short repetitive DNA sequences identified in this cluster have been implicated in large chromosomal rearrangements observed at the tandem DUP240 loci on chromosome I through non-allelic recombination events (Leh-Louis *et al.*, 2004).

It should be noted that the study described earlier examined the chromosome structures in a population of yeast cells at the end of fermentation, and thus any observed changes reflect significant changes in the population as a whole. While gross chromosomal rearrangements

were not observed, such changes may be present but clonal selection would be required to detect such changes. In fact, karyotyping of DNA from individual colonies of lager yeasts, isolated at the end of a fermentation by pulse-field gel electrophoresis, revealed differences in the chromosomal profiles between colonies, suggestive of chromosomal rearrangements (U. Bond, unpublished data).

The finding that the lager yeast genomes are dynamic and susceptible to influence by the environment is of significance to the brewing industry as the stability of proprietary strains is of utmost importance to the industry. Since yeasts are harvested at the end of fermentations and repitched as many as seven times into subsequent fermentations, mixed populations of strains may emerge during the process. It is possible that dynamic changes in the genome may confer selective fitness on a sub-population of cells, which may generate novel sub-strains on subsequent repitching.

IX. GENE EXPRESSION PATTERNS OF LAGER YEASTS

The co-existence of the multiple genomes in the lager yeasts coupled with the general aneuploid nature of the genome raises many questions regarding the gene regulation in these species. For example, what are the consequences of the co-expression of homeologous genes? What effect does polyploidy have on gene expression patterns? Does gene dosage occur to prevent overexpression of certain proteins? Does the presence of the *S. bayanus* genome alter the expression of the *S. cerevisiae* genes and vice versa? Subtle changes in gene dosage resulting from competition of alleles for transcription factors or other factors required for RNA biogenesis may directly influence the adaptive capabilities of the species, for example expression of additional copies of the alcohol dehydrogenase gene (ADH1) in *S. cerevisiae* can lead to increased chronological and replicative life-span (Reverter-Branchat *et al.*, 2007).

The genome sequence analysis identified multiple allelic forms of genes involved in maltose metabolism. For example there appears to be three copies of the MAL31 gene, which encodes an α-glucoside transporter. Two of these genes, the *S.c*-like LBYG0616 and the *S.b*-like LBYG03039, encode full-length proteins while a third the *S.b*-like gene LBYG09472 appears to be truncated. Likewise, two alleles of SUL1 (*S.c* and *S.b*-like) also appear to encode truncated forms of permeases, which are required for sulphate transport, while two other genes, *S.c.* and *S.b* forms of SUL2, can encode full-length forms of the permease. Thus, multiple isotypes of the same protein may be produced at any one time. The contribution of each of these alleles to the overall final biological activity may be quite complex.

Several transcriptome analyses have been carried to examine the gene expression patterns in these yeasts under fermentation conditions (Higgins *et al.*, 2003; James *et al.*, 2002, 2003; Olesen *et al.*, 2002). These early studies used only *S. cerevisiae* microarrays and therefore are confined to an analysis of the *S. cerevisiae* component of the lager yeast genome. Meta analysis of these databases is complicated by the varying experimental conditions used in each study. For example, some studies were carried out on nylon filters (Higgins *et al.*, 2003; Olesen *et al.*, 2002), others on glass microarray slides (James *et al.*, 2003; James *et al.*, 2002). Additionally, differences in yeast strains analyzed, growth conditions, time points and hybridization conditions make comparisons difficult. In most studies, transcriptome analysis has been carried out under stringent hybridization conditions that allow selective detection of the *S. cerevisiae* and not the *S. bayanus* complement of the genome. Despite the differences in experimental conditions, common trends in the databases are evident and thus it is possible generate a picture of the gene expression profiles during the fermentation process in lager yeasts. The derived databases of gene expression patterns in the lager yeasts can be compared to the substantial transcriptome databases of haploid strains of *S. cerevisiae* under a vast number of environmental conditions (http://www.yeastgenome.org) and (http://transcriptome.ens.fr/ymgv). No one dataset reflects the unique environmental conditions experienced by *S. pastorianus* during fermentation. For example, the majority of transcriptome analyses of *S. cerevisiae* have been carried out under aerobic conditions while standard fermentations are conducted under anaerobic conditions.

Two of the most comprehensive transcriptome analyses of the fermenting lager yeasts are described by Olesen *et al.* (2002) and James *et al.* (2003). The latter study used the *S. pastorianus* strain 6701, from the Guinness collection, fermented in a malt-wort (16% maltose) while the former used the production strain *S. carlsbergensis*, fermented in 14% malt-wort.

In James *et al.* (2003), the gene expression profile was examined on days 3 and 8 of fermentation comparing the expression patterns to those observed on day 1. The compiled data presented a picture of the metabolism of these cells and indicated that genes involved in sterol and fatty acid metabolism, cell transport, cell wall biogenesis, oxidative stress, peroxisome metabolism, protein degradation and oxidative phosphorylation were all upregulated as fermentation progressed. The most active period of transcription occurs in the early stages of fermentation. One of the surprising finding was the upregulation of genes involved in oxidative phosphorylation considering that the cells are grown under strict anaerobic conditions. The study by Olesen *et al.* (2002), also noted highest gene expression levels for genes involved in protein biosynthesis, glycolysis and lipid biosynthesis. In this study, gene expression levels were examined each day over 12 days of fermentation. Again, the period

showing the most active upregulation of genes occurred between days 1 and 3, although genes clusters of genes showing upregulation later in the fermentation process were also identified. Using gene ontology classifications, again the most active classes of genes induced early in fermentation were those involved in protein biosynthesis, ribosomal RNA biogenesis and glycolysis, while genes involved in transcription were induced mid-fermentation. A similar study by Higgins *et al.* (2003) also identified the same classes of genes being induced early in fermentation and specifically verified the upregulation of genes involved in fatty acid, lipid and sterol biosynthesis in the early stages of fermentation.

James *et al.* (2003) also identified a series of genes that are downregulated as fermentation proceeded. The largest categories were predictably those required for protein biosynthesis, transcription and rRNA processing, nuclear architecture, cell secretion, cell cycle and glycolysis with the exception of genes involved in alcohol and aldehyde metabolism which were generally upregulated. Since fermentations were carried out in a maltose-based wort medium, it is interesting to note that with the exception of the MAL 11 gene, which was upregulated on day 3 compared to day 1, the other genes involved in maltose metabolism (MAL 12, MAL 13, MAL 31, MAL 32, MAL 33, YDL247W, and YJR160C) showed no increase in mRNA levels. All MAL genes were downregulated on day 8. This gene expression profile agrees well with lack of cellular biogenesis as cell enters strict anaerobic conditions after day 3 of fermentation when metabolism is repositioned to alcohol production and survival.

One of the salient findings of all the transcriptome studies is the altered expression of so many genes of currently no known function. Some or many of these genes may be contributing to survival in the unusual environmental conditions experienced by these cells, as many of these have not been explored in gene expression analysis of *S. cerevisiae*.

More recently, Minato *et al.* (2009) have carried out an extensive microarray gene expression analysis in a lager yeast strain. The arrays consist of both *S. cerevisiae* and lager-specific information generated from expressed sequence tag (ESTs) analysis of cDNAs derived from RNA expressed during fermentation (Yoshida *et al.*, 2007). The study identified 976 gene probes capable of discriminating between *S.c*-type and Lager-type genes. Of these, 600 (60%) showed similar gene expression patterns while 40% showed differential gene regulation. The latter group was subdivided into nine groups based on expression patterns. From the initial analysis of the data, there appears to be no overall apparent relationship between the clustered genes and few appear to be specific to the fermentation process. Of the seven genes encoding isoenzymes of alcohol dehydrogenase (ADH 1–6), only one (ADH3) is differentially regulated. None of the genes encoding proteins required for maltose metabolism are listed as being differentially regulated.

X. EXPRESSION COMPARED TO HAPLOID *S. CEREVISIAE*

While the microarray analyses described can provide a picture of the fermenting cell, these studies do not address the issue of the influence of polyploidy on the patterns of gene expression. In an initial attempt to examine the effects of polyploidy on gene expression in lager yeasts and to determine if the presence of the *S. bayanus* genome is influencing the expression of the *S. cerevisiae* genes, the transcription pattern of a haploid *S. cerevisiae* strain has been compared to that of the *S. pastorianus* strain grown under identical fermentation conditions (James *et al.*, 2002). The results of these microarray experiments revealed that there was very little in common between the gene expression patterns of the two yeast species. Using the compiled transcriptome databases for *S. cerevisiae* mentioned earlier, a meta-analysis was conducted to compare the expression profiles. The patterns observed for the haploid *S. cerevisiae* strain were similar to those observed previously for *S. cerevisiae* grown to stationary phase and in the presence of antimycin D. These commonalities most likely reflect the high cell densities and anaerobic conditions experienced by cells during fermentation. Remarkably, the *S. pastorianus* strain showed a unique pattern of expression that did not resemble the patterns observed under any experimental condition for *S. cerevisiae*, indicating that the co-expression of the *S. bayanus* genome coupled with the existence of polyploidy greatly affects the observed gene expression patterns.

While the expression studies have provided a broad picture of the metabolic activity of fermenting yeasts and provide a greater insight into gene expression patterns in the lager yeasts, many more questions regarding gene regulation remain unanswered. For a complete understanding of the complex patterns of gene expression in lager yeasts, future studies will need to take into account the wide variations of chromosome composition, including variations in gene copy number of *S. cerevisiae*-like and *S. bayanus*-like genes in different strains of *S. pastorianus* and the dynamic nature of the genomes in response to changing environmental conditions.

XI. CONCLUSIONS

The recent availability of a complete genome sequence for the lager yeast *S. pastorianus* has allowed an in-depth analysis of the genome structure and composition of these species. The sequence analysis has confirmed the complex and hybrid nature of the genome of these yeasts and broadly confirms the prior conclusions drawn from CGH analyses and the classical studies using single chromosome transfer techniques. The findings that the genomes of these yeasts are dynamic and can undergo

chromosomal rearrangements, telomere loss or regional amplification in response to environmental stresses adds an additional layer of complexity to the analysis of the genomes of these yeasts and gene expression patterns during fermentation.

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