Minireview

The biology of environmental mycobacteria

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Summary

Although the environmental mycobacteria are slow growing relative to other microorganisms in water and soil which would suggest that they are poor competitors, compensating factors permit survival, growth and persistence in natural and human-engineered environments. Factors such as the hydrophobic, lipid-rich impermeable envelope, biofilm formation, acid resistance, anaerobic survival and metabolism of recalcitrant carbon compounds permit survival and growth of the environmental mycobacteria in a wide range of natural and human-engineered habitats. High numbers of environmental mycobacteria are found in coastal swamps and estuaries and boreal, peat-rich forest soils and waters. The hydrophobic surface results in concentration of the environmental mycobacteria at interfaces (air–water and surface–water) and in aerosolized droplets ejected from water. The survival and growth in protozoa and amoebae permit environmental mycobacteria to persist in habitats subject to predation and likely led to survival and growth in phagocytic cells of animals. Finally, slow growth allows time for mycobacterial cells to adapt to changing conditions before loss of viability.

Biology: study of the structure, function, growth, physiology, origin, evolution, distribution and classification of living things. (King and Stansfield, 1997)

Introduction

Many of the environmental mycobacteria are opportunistic pathogens of humans, animals and birds (Falkinham, 1996; 2002; Wallace et al., 1998; Marras and Daley, 2002). Risk factors for human infection include chronic obstructive pulmonary disease (COPD), changes in lung and chest architecture, α-1-antitrypsin deficiency, cystic fibrosis, heterozygosity for CFTR mutations, gastric reflux disease and immunodeficiency due to HIV infection, cancer or chemotherapy (Marras and Daley, 2002; Kim et al., 2005; Koh et al., 2007; Thomson et al., 2007). The environmental mycobacteria are normal inhabitants of soil, natural waters and water in engineered systems, including households (Falkinham et al., 1980; 2001; Brooks et al., 1984; Kirchner et al., 1992; Iivanainen et al., 1997; 1999). Drinking water and potting soil have been shown to be sources of human infection by identity of DNA fingerprints of isolates from patients and their environment (von Reyn et al., 1994; De Groote et al., 2006; Falkinham et al., 2008). Recognition of the importance of environmental mycobacteria has been the inclusion of Mycobacterium avium on the US EPA’s ‘Candidate Contaminant List’; meriting further study and possible enactment of rules concerning allowable numbers in drinking water.

The most common environmental mycobacterial pathogens can be divided into two groups based on growth rate; the slowly growing species include: M. avium, Mycobacterium intracellulare, Mycobacterium kansasi, Mycobacterium marinum, Mycobacterium xenopi and Mycobacterium malmoense (particularly in Europe). The rapidly growing species include: Mycobacterium abscessus, Mycobacterium chelonae and Mycobacterium fortuitum. The rapidly growing mycobacterial species are commonly associated with nosocomial infections (Wallace et al., 1998), although it has been recognized that the prevalence of M. abscessus infections is increasing among patients with cystic fibrosis (Jönsson et al., 2007). Mycobacterium avium and M. intracellulare, the M. avium complex (MAC), cannot be distinguished by phenotypic tests, but are distinct species sharing only 70% DNA–DNA similarity (Baess, 1983). Although M. avium and M. intracellulare are recovered at approximately equal percentages from patients with pulmonary infection, almost all AIDS patients are infected with M. avium (Drake et al., 1988). A number of species have unique epidemiologic traits. For example, M. marinum causes infections in fish and skin granulomas in humans where infection is associated with occupation in the fishing industry or exposure to aquaria (Aubry et al., 2002). Mycobacterium xenopi infections are usually encountered in
outbreaks and associated with the presence in hot water distribution systems in buildings; consistent with that species ability to grow at 45°C (Sniadack et al., 1993). Five clonal subtypes of M. kansasii have been identified based on hsp-65 RFLP and 16S rRNA sequence (Alcaide et al., 1997). Although M. kansasii is considered an environmental opportunist, none of the major type associated with human infection (type I, 68%) had been isolated from the environment (Alcaide et al., 1997), suggesting that there might exist another, unidentified source for M. kansasii infection in humans. The prevalence of M. malmoense infections has steadily risen since its first description in 1977 and infections are commonly associated with damaged lungs due to infection or particulate inhalation (Zaugg et al., 1993). It is likely that the prevalence of M. malmoense infections is underestimated, as this mycobacterial species grows quite slowly on egg-based media (e.g. Lowenstein-Jensen), requiring up to 6–8 weeks for the appearance of colonies, unless pyruvate-containing media are employed (Zaugg et al., 1993).

It is the objective of this minireview to introduce and briefly review topics of relevance to the biology of the environmental mycobacteria. The topics chosen cover those encompassed by the word biology: population biology, structure–function, adaptation, metabolism and genetics. Throughout, the theme is that a major determinant of environmental mycobacterial biology is the presence of a lipid-rich cell envelope that has costs and benefits. The major ramification of the hydrophobic envelope is that environmental mycobacteria are concentrated and grow at interfaces (e.g. air–water, water–particle and water–surface). The low permeability of the envelope to hydrophilic nutrients limits growth, but also results in resistance to a broad spectrum of antimicrobial agents (Rastogi et al., 1981; Jarlier and Nikaio, 1994; Taylor et al., 2000). Although the environmental mycobacteria grow slowly, their metabolic rates are comparable to faster-growing bacteria; the mycobacteria simply expend a great deal of energy on the synthesis of the thick, lipid-rich outer membrane. As it is the case that rapidly growing cells are killed by antimicrobial agents as a consequence of imbalances in metabolism (e.g. inhibition of cell wall synthesis without inhibition of cell growth) coupled with short times before reaching determinants of cell survival (Maaløe and Kjeldgaard, 1966), slow growing mycobacteria can adapt to changing conditions. Environmental mycobacteria are thereby able to adapt before critical stages of the cell cycle are reached.

**Population biology of mycobacteria**

**Mycobacterial population (clonal) variation**

Studies whose objective has been to identify possible sources of environmental mycobacteria have shown wide variation in DNA fingerprint patterns of isolates from the same habitat (water) and sample (e.g. M. avium and M. intracellulare; De Groote et al., 2006; Falkinham et al., 2008). In many instances, the variants are clonally related (Falkinham et al., 2008). Such clonal variation is also found among isolates collected from individual patients (von Reyn et al., 1995). Multiple isolates of M. avium sharing the same colony morphology were recovered from a single patient and antibiotic susceptibilities measured. In spite of the fact that the isolates were recovered from the same patient and formed colonies of identical morphology, the pattern of antibiotic susceptibilities differed quite markedly (von Reyn et al., 1995). Thus, selection of one colony for measurement of antibiotic susceptibility could lead to either excluding a drug from treatment as the strain’s minimal inhibitory concentration (MIC) was too high or including a drug to which members of the community were resistant. One ramification of such population variation is the necessity of recovery of multiple isolates from individual habitats and samples, including patients. Evidence of genetic variation suggests that as the environmental mycobacteria are relatively slow growing, there must be a lively frequency of mutation and transposition, as yet not systematically explored.

**Colony variation**

There is a long history of reports of colony variation among the environmental mycobacteria, particularly those of M. avium and M. intracellulare (M. avium complex or MAC). Using MAC as an example, isolates alternate between two types, smooth transparent and smooth opaque (McCarthy, 1970). Smooth transparent types are more commonly recovered from patients, are more virulent and antibiotic-resistant than smooth opaque types (Schaefer et al., 1970; Kajioka and Hui, 1978). The fast-growing, more hydrophilic opaque types appear to be selected by prolonged transfer in laboratory medium (J.O. Falkinham, unpublished). The frequency of appearance of the larger opaque variants among colonies of transparent isolates is easily measured at approximately 1/1000 (McCarthy, 1970), as the opaque colonies are easy to see and count against the background of small transparent colonies. Because small transparent types are difficult to find among larger opaque colonies, the frequency of transition can only be estimated; it occurs at approximately the same frequency. As colonies or cultures of MAC isolates are mixtures of colony types, caution must be exercised in interpretation of antimicrobial susceptibilities, as survival values will be influenced by the proportion of each colony variant. This is particularly the case in extrapolating MIC to antibiotic concentrations in patients (von Reyn et al., 1995) or disinfectant concentrations in drinking water distribution systems (Taylor et al., 2000).
mechanism of this genetic transition has not yet been identified, although preliminary evidence suggests that interconversion between colony types in *M. intracellulare* is accompanied by the transposition of an insertion sequence, IS1141, between different sites in the *M. intracellulare* genome (L.E. Via and J.O. Falkinham, unpublished). Our current focus is sequencing the DNA adjacent to the sites of transposition of IS1141, anticipating that genes involved in determining the morphology of colonies would be linked. Identification of the mechanism of colony variation (e.g. transposition) might lead to identification of genes or markers of virulence and of novel targets for chemotherapeutic intervention.

**Impact of mycobacteria on microbial populations**

In as much as the environmental mycobacteria are widely distributed in the environment, they have impacts on the local microbiome. Surface and interface binding of cells of microbial cells is driven by cell surface hydrophobicity (van Loosdrecht *et al.*, 1987; Bendinger *et al.*, 1993). It follows that the environmental mycobacteria, whose cell surface hydrophobicity is the highest among the bacteria (van Oss *et al.*, 1975), are more likely attached to surfaces or interfaces than suspended in water. Not only does that direct sampling strategies for isolation of mycobacterial populations, but it also suggests that mycobacteria may be biofilm ‘pioneers’. I suggest that mycobacteria are the first colonizers of natural (e.g. particulates, rocks and plants) and engineered (e.g. pipes and water filters) surfaces (Rodgers *et al.*, 1999). The relative resistance of mycobacteria to most of the toxic heavy metals and oxyanions (Falkinham *et al.*, 1984) would also contribution of surface colonization (e.g. zinc-coated, galvanized pipes). Once mycobacterial cells colonize a surface other microorganism might be able to attach to form a mixed biofilm community.

Mycobacteria are also capable of protecting their accompanying microbiome from toxicity by heavy metals. Cells of a strain of *Mycobacterium scrofulaceum* that are resistant to the heavy metal mercury (Hg) can reduce Hg^{2+} to insoluble HgO that is rapidly lost from solution by volatilization (Meissner and Falkinham, 1984). Such volatilization not only protects the mycobacterial cells from Hg toxicity, but the microbial population coexisting in the same habitat is also protected. Volatilization is not the only mechanism demonstrated to reduce the level of toxic heavy metals from environmental habitats. Cells, particularly mycobacterial cells with their lipid-rich, thick outer membrane, can bind or sequester compounds, effectively taking them out of solution. If compounds are out of solution, cells are usually unaffected. The *M. scrofulaceum* strain that volatilizes Hg^{2+} also sequesters cadmium (Cd^{2+}) and copper (Cu^{2+}) in the cell envelope as sulfides in a sulfate-dependant process (Erardi *et al.*, 1987; 1989). Consequently, in the presence of adequate levels of sulfate Cd and Cu sulfides are precipitated, again protecting both mycobacterial cells and their accompanying populations from toxicity by those heavy metals. Coupled with heavy metal removal from habitats, evidence of the metabolism of a variety of toxic pollutants (e.g. anthracene and vinyl chloride) suggests that mycobacterial colonization of toxic waste dumps (Wang *et al.*, 2006) could lead to reduction of pollutant concentrations in two ways. First, mycobacteria would catalyse the degradation of recalcitrant compounds. Second, mycobacterial colonization would lead to invasion of other, faster-growing microorganisms as mycobacterial cells de-toxify the habitat.

**Mycobacteria and protozoa and amoebae**

Environmental mycobacteria are capable of surviving phagocytosis by protozoa and amoebae and even grow as endosymbionts (Cirillo *et al.*, 1997; Strahl *et al.*, 2001). As protozoa and amoebae are grazers of microorganisms in biofilms and mycobacteria are preferentially found in biofilms in natural and engineered habitats (Brooks *et al.*, 1984; Falkinham *et al.*, 2001), slow growing mycobacteria populations would be decimated via protozoan predation. Thus, there is strong selection for intracellular survival. Further, this would also select for survival and growth of mycobacteria in human and animal phagocytic cells (e.g. macrophages).

By analogy to *Legionella*, intracellular mycobacteria in protozoa and amoebae are shielded from harsh environmental conditions. For example, protozoa and amoebae were shown to protect intracellular *M. avium* cells from antibiotics (Miltner and Bermudez, 2000). However, it is important to consider the susceptibility of *Legionella* and mycobacteria to disinfectants. *Legionella* are as susceptible to chlorine as other Gram-negative bacteria such as *Escherichia coli*. In contrast, mycobacteria are 100-fold more resistant to chlorine (Taylor *et al.*, 2000). Consequently, mycobacteria may not benefit from possible ‘protection’ provided by protozoa or amoebae. However, growth in protozoa and amoebae may benefit mycobacterial survival during starvation. Many protozoa and amoebae can encyst as a consequence of starvation and intracellular mycobacteria survive encystment (Steiner *et al.*, 1998; Strahl *et al.*, 2001). Survival of the causative agent of Johne’s disease in cattle, namely *M. avium* sub-species *paratuberculosis* in encysted amoebae, has been proposed as a mechanism for its survival and transport from dairy farms to water supplies (Mura *et al.*, 2006). Further, it would be understood that any environmental mycobacterium would be protected from disinfection if present in cysts of protozoa or amoebae.
Recent evidence suggests that a symbiotic relationship may exist between *M. avium* and protozoa and amoebae. It has been shown that a number of *Tetrahymena* species are incapable of growth in culture media when inoculated at low density (\(<1000\) cells ml\(^{-1}\); Schousboe and Rasmussen, 1994). This inoculum density effect can be overcome by incorporation of lipids in the culture medium (Schousboe and Rasmussen, 1994). Driven by this observation, we reasoned that mycobacterial cells could provide the required lipids and permit the growth of *Tetrahymena* cells from low inoculum density. *Tetrahymena pyriformis* cells carrying *M. avium* cells were able to grow from low inoculum density, whereas cells not carrying *M. avium* failed to grow (J.O. Falkingham, unpublished). Further, *T. pyriformis* cells carrying *M. avium* grew more rapidly than cells that did not carry intracellular *M. avium* (J.O. Falkingham, unpublished). Those data are consistent with a symbiotic association and show that there is strong selection for intracellular survival of environmental mycobacteria. Whether macrophage also benefit from the presence of lipids provided by intracellular mycobacteria has yet to be determined.

There is one other interesting anecdotal observation concerning the intracellular growth or survival of mycobacteria in protozoa and amoebae. That concerns the loss of acid fastness and cultivability of intracellular mycobacteria. Acid fastness is a staining characteristic of the mycolic acid-rich bacteria; stain fixed in the outer membrane with heat cannot be extracted with acidic alcohol solutions. During cultivation of amoebae or protozoa infected with mycobacteria, the detection of mycobacteria as acid-fast cells decreases to the point where no acid-fast cells are observed, yet colonies can be isolated and species-specific DNA sequences (e.g. IS900) can be amplified by PCR. That suggests substantial, perhaps, adaptive changes in mycobacterial cell architecture, particularly involving the outer membrane.

**Mycobacterial structure–function**

**Mycobacterial envelope composition**

Mycobacterial cells differ from Gram-positive and Gram-negative architectures by having a thin peptidoglycan surrounded by a thick, lipid-rich outer membrane (Brennan and Nikaido, 1995; Daffe and Draper, 1998). Depending upon the species, the fatty acid chains can be upwards of 60–80 carbons long. As a consequence, mycobacterial cells are hydrophobic and impermeable. The fatty acid tails are arrayed perpendicular to the cell surface and contain \(180^\circ\) bends at approximately position 28 (Brennan and Nikaido, 1995; Daffe and Draper, 1998). The outer membrane is a true membrane as shown by the presence of freeze fracture planes (Nikaido *et al.*, 1993; Christophersen *et al.*, 1999; Hoffman *et al.*, 2008). Further, it would be understood that mycobacterial cells have a periplasm, the space bound by the cytoplasmic and outer membranes. Unfortunately, the covalent linkage between the peptidoglycan and the outer membrane (Brennan and Nikaido, 1995; Daffe and Draper, 1998) complicates the development of methods for the removal of the mycobacterial outer membrane to analyse the contents of the mycobacterial periplasm.

The composition and structure of the mycobacterial outer membrane is a major determinant of growth, physiology, ecology and virulence of these opportunistic pathogens. Although rates of transport of hydrophilic compounds are quite low compared with other bacteria and limit growth, mycobacteria are resistant to antimicrobial agents of all types, particularly hydrophilic agents (Rastogi *et al.*, 1981; Nikaido *et al.*, 1993; Brennan and Nikaido, 1995; Taylor *et al.*, 2000). Hydrophobicity also drives the attachment of mycobacterial cells to surfaces, thus preventing the washing out and dilution of cells in flowing systems (rivers and drinking water distribution systems). Hydrophobicity also drives the concentration of environmental mycobacteria at air–water interfaces where organic compounds are also concentrated, providing nutrient (Harvey and Young, 1980). In fact, the best places to sample for mycobacteria are surfaces and particulate fractions of waters. The impermeable, hydrophobic, lipid outer membrane should be thought of as a double-edged sword: limiting growth while providing antimicrobial resistance and the occupation of surface habitats.

**Ramifications of envelope composition on mycobacterial physiological ecology**

My colleague, Bruce Parker, coined the term physiological ecology to describe those physiological features of a microorganism that are determinants of its ecology and hence epidemiology. In drinking water systems, disinfection (e.g. chlorine) reduces numbers of the majority of microorganisms, leaving the disinfectant-resistant environmental mycobacteria a clear field for consumption of nutrients and surface attachment in the absence of competition. Residence and growth in drinking water distribution systems and household plumbing is enhanced by hydrophobicity-driven surface attachment. Attached mycobacteria are less likely to be washed out at high flow rates and growth and biofilm formation lead to even higher levels of antimicrobial resistance. For investigators, cell surface hydrophobicity of mycobacteria results in cell aggregation, a troublesome problem in growing cultures.
Mycobacterial biofilm formation

As expected from the high surface hydrophobicity, mycobacteria readily form biofilms. Biofilm formation by *M. avium* required divalent cations, is higher when cells are in high and low nutrient conditions, and is inhibited by humic acid (Hall-Stoodley et al., 1999; Carter et al., 2003). Interestingly, medium from biofilm-forming cells of *M. avium* induced the formation of biofilm, suggesting that quorum sensing is involved in mycobacterial biofilm formation (Carter et al., 2003). Biofilm formation occurs under both high and low shear conditions (Lehtola et al., 2007). Mycolates of *Mycobacterium smegmatis* (Ojha et al., 2005) and glycopeptidolipids (GPL) are required for biofilm formation in *M. avium* on polyvinylchloride (PVC), but not plastic or glass surfaces (Freeman et al., 2006). A surprising discovery was that mutants of *M. avium* deficient in biofilm formation were also deficient in epithelial cell invasion (Yamazaki et al., 2006). It is particularly important that interpretation of biofilm formation rates include consideration of whether the separate steps of surface adherence and growth on surfaces are separated. In many publications, surfaces are incubated in the continual presence of (growing or non-growing) mycobacterial cells. Under those conditions it is impossible to separate the contributions of newly adherent cells and growth of adherent cells to the increase in the number of cells on surfaces. Under those conditions, accumulation is the correct term (Hall-Stoodley et al., 1999). Experimental methods must be developed to separately measure adherence and biofilm growth.

Mycobacterial aerosolization

Hydrophobicity is also a determinant of aerosolization; the transfer of cells from water to air that is one route of acquisition of mycobacteria leading to lung infection. Hydrophobic cells attach to air bubbles rising in the water column and when the bubbles reach the surface and burst, a crater is formed whose walls are enriched in mycobacteria cells. As a result of the collapse of the crater, water droplets approximately 1/10 the diameter of air bubbles are ejected to heights of 10–20 cm. As the droplets are composed of water making up the crater, they are enriched up to 10 000 times in mycobacterial numbers compared with the concentration of cells in the bulk water (Parker et al., 1983). Depending upon the species, strain and colony type (e.g. transparent versus opaque), enrichment factors in ejected droplets (i.e. the ratio of cell number in the droplets divided the number in the bulk suspension) range from 500 to 10 000 for mycobacteria (Parker et al., 1983). Enrichment factor values correlate with cell surface hydrophobicity as measured by hexadecane adherence (Rosenberg, 1984). Thus it should not be surprising that aerosols collected near bodies of water contain mycobacteria (Wendt et al., 1980). Some of the droplets are of a size capable of entering the bronchi and alveoli of the human lung (Parker et al., 1983). Collection of mycobacterial (and other hydrophobic microbial cells and hydrophobic chemicals) by rising air bubbles leads to their enrichment at the air–water interface. It is to be understood that the air–water interface (i.e. ‘surface slick’) is also the site of concentration of organic materials (Harvey and Young, 1980). Thus, mycobacterial cells are concentrated at the air–water interface along with substrates for growth. It follows that a good strategy for collection of mycobacteria from bodies of waters is to collect aerosolized droplets (Wendt et al., 1980), the surface microlayer (Harvey and Young, 1980) or the particulate fraction of water (Falkinham et al., 2001).

Mycobacterial adaptation

One advantage of the slow growth of mycobacteria is that their cells are capable of adaptation to changing conditions. Implicit in that statement is understanding that microbial death brought about by antimicrobial agents or environmental stresses (e.g. oxygen deprivation) is due to unbalanced growth, a term first coined by Maaløe and Kjeldgaard, (1966). Death of microbial cells occurs as a consequence of inhibition of one cellular process (e.g. cell wall synthesis by penicillin) while DNA, RNA and protein synthesis continue unabated. Further, rapidly growing cells are more susceptible to antimicrobial agents or environmental stresses compared with slowly growing cells. It is important to point out the fact that because mycobacteria have only one or two ribosomal RNA (rRNA) operons (Bercovier et al., 1986), their ability to adapt may be limited in time. It has been shown that cells of *E. coli* with reduced numbers of rRNA operons are less able to rapidly react to increased nutrient availability (upshifts) as demonstrated by the time needed to arrive at a new, higher, growth rate (Stevenson and Schmidt, 2004).

There exist several examples of mycobacterial adaptation: most notably survival as a consequence of exposure to anaerobiosis (Dick et al., 1998), starvation (Archuleta et al., 2005), acid (Bodmer et al., 2000) and temperature (Scammon et al., 1964), and elevated antibiotic and disinfectant resistance of biofilm-grown cells (Steed and Falkingham, 2006; Falkingham, 2007). A gradual reduction in oxygen concentration, generated by allowing *M. smegmatis* cells to consume oxygen in a closed culture, leads to condition where the cells are viable for long periods of time (Dick et al., 1998). This adaptation to long-term survival was originally discovered by Larry Wayne in *Mycobacterium tuberculosis*, who sought to discover the basis for the long-term survival of *M. tuberculosis* in patients (i.e. dormancy). It would be expected that environmental...
mycobacteria would be able to survive long periods of anaerobiosis if oxygen concentrations gradually fell before the onset of anaerobiosis. Cells of \textit{M. avium} have been shown to enter a dormant stage upon starvation (Archuleta et al., 2005). Unfortunately, the molecular and genetic basis for the induction of dormancy has not been elucidated. The feature shared by adaptations to resistance to acid and intracellular growth is that prior growth leads to increased survival under those stressful conditions. For example, \textit{M. avium} cells grown in medium of high acidity (e.g. pH 3–5) were better able to grow at low pH (Bodmer et al., 2000) and \textit{M. avium} cells grown in amoebae were more readily phagocytosed and were more virulent (Cirillo et al., 1997). Growth of cells of \textit{M. intracellulare} at 42°C resulted in cells that were more virulent for chickens compared with cells grown at 37°C (Scammon et al., 1964).

Mycobacterial cells readily adhere to surfaces and form biofilms (Carter et al., 2003). Interestingly, cells grown in biofilms are transiently more resistant to disinfectants (Steed and Falkinham, 2006) and antibiotics (Falkinham, 2007). Cells grown in biofilms can be released (e.g. scraped or vortexed) from surfaces and the cell suspensions exposed to antimicrobial agents without the protection of layers of cells as in biofilms. Such biofilm-grown, but suspended cells are more resistant to antimicrobial agents than cells grown in suspension, but of reduced resistance compared with cells grown and exposed to antimicrobial agents in biofilms (Steed and Falkinham, 2006; Falkinham, 2007). However, the resistance of biofilm-grown but suspended cells is transient; after 24 h growth in medium as suspended cells, their susceptibility is equal to that of cells grown in suspension (Steed and Falkinham, 2006; Falkinham, 2007). In the absence of attachment and growth in a biofilm, mycobacterial cells eventually revert back to the susceptibility of suspension-grown cells. The adaptation to antimicrobial resistance as a consequence of biofilm growth has important ramifications. In pipes, cells growing on surfaces in drinking water distribution systems and in households are more resistant to disinfection. Further, cells released from the biofilm would be transiently of intermediate disinfectant resistance. If methods to identify the contact time for killing 99.9% of cells employ suspension-grown cells, the resulting estimates would direct the use of too low a concentration of disinfectant. Likewise, selection of concentrations of antibiotics for killing or inhibiting the growth of mycobacterial cells should not be based on those of suspension-grown cells, but rather upon cells grown in biofilms. Further, mycobacterial cells released from biofilms would be transiently of higher resistance to antibiotics. Finally, it is quite likely that one contributing factor to the lack of correlation between in\textit{vitro} measurement of MIC of mycobacteria and patient response is due to the possibility that MIC values are of cells grown in suspension, not in biofilms.

All of the foregoing examples meet the definition of an adaptation, a physiological state maintained by conditions. It is not due to mutation. First, all members of the population, not just the rare mutant, express the novel phenotype. Second, the trait (e.g. high virulence in chickens) is only maintained if the cells are maintained under the inducing condition (e.g. growth at 42°C). It is likely that the slow growth of mycobacteria assists these examples of adaptation. Slow growth with normal rates of metabolism means that the induction of novel traits can occur before events are triggered that lead to cell death. What is interesting in the case of adaptive antimicrobial resistance is the nature of the inducing condition, namely biofilm growth. As it is likely that the majority of mycobacterial cells in the environment and infected animals are attached to surfaces, investigations into the adaptations of mycobacterial cells to biofilm growth should be a primary research goal.

\textbf{Mycobacterial metabolism and metabolites}

\textbf{Mycobacterial growth requirements}

I would caution mycobacteriologists to be wary of exclusively using medium developed for the cultivation of \textit{M. tuberculosis} (e.g. Lowenstein-Jensen and Middlebrook 7H9, 7H10 and 7H11) for the environmental mycobacteria and, further, for drawing inferences concerning their growth requirements. For example, a majority of the environmental mycobacteria can grow on a minimal salts medium at almost the same rate as they grow on M7H9 or M7H10 (Tsukamura, 1967). The environmental mycobacteria are neither fastidious nor auxotrophic, with the possible exception of fatty acid auxotrophy. It is important that investigators remain aware of the possibility that some of the environmental mycobacteria may require an exogenous source of fatty acids for growth (McCarthy and Ashbaugh, 1981). For many experiments, fatty acid auxotrophy is not relevant because egg- (Lowenstein-Jensen) or oleic acid-based (Middlebrook) media are used. In minimal defined media, fatty acids are added (e.g. palmitic) with albumin or as a detergent (e.g. Tween 80) as fatty acids alone are toxic. However, inclusion of any compound promoting detergency in mycobacterial media is to be avoided as they substantially increase the permeability of cells to hydrophilic compounds, particularly disinfectants and antibiotics. Their widespread presence in both natural and engineered habitats belies the suggestion that they require complex media for their isolation and cultivation. Further, the high concentration of components may actually hinder growth; for example, \textit{M. avium}, \textit{M. intracellulare} and \textit{M. scrofulaceum} are oligotrophic (George et al., 1980), able to grow at levels of assimilable organic carbon (AOC) of
and metabolism of recalcitrant compounds. Difficulties in cultivation are likely contributors to the lack of mycobacterial isolates whose degradation of recalcitrant compounds has been measured are recovered from polluted sites, as reported (Wang et al., 1991; Burback and Perry, 1993; Kirschner et al., 1999). The majority of mycobacterial isolates whose degradation faces. As noted above, mycobacterial cells grow slowly because much energy is channelled into synthesis of long-chain lipids, but their rate of metabolism and oxygen consumption are as high as rapidly growing bacteria such as E. coli. Thus, they would be good candidates for membership in consortia of pollutant-degrading microorganisms.

Mycobacterial antibiotic production

One neglected area of mycobacterial study is their possible synthesis of antibiotics. Antibiotic production would be one way to ensure survival and access to nutrients in mixed microbial populations. This would be especially the case for slowly growing mycobacteria that are poor competitors. Antibiotic production might be even more important for mycobacteria as they are preferentially found in biofilms, where higher interacting microbial populations are found. In fact, a significantly higher proportion of particulate-associated bacteria produce antibiotics compared with free-living bacteria (Long and Azam, 2001). Investigation of the genomics of the environmental mycobacteria has just begun, but already it is clear that their genomes are relatively large and possible of encoding genes for antibiotic production. However, I have found only one report of mycobacterial antibiotic production (Obgoltseva et al., 1991). The absence of antibiotic-producing mycobacteria from the catalogues of drug and biotechnology companies may be due, again, to their slow growth and the fact that they are perceived as poor experimental microorganisms. Although mycobacterial slow growth might suggest they would not be useful for antibiotic production via fermentation, antibiotic-synthesis genes can be cloned and expressed in more rapidly growing microorganisms.

Mycobacterial plasmids

Mycobacterial plasmids

Studies of plasmids have not been among the major topics of mycobacterial research. However, the available literature suggests they may be of significant to genomics, physiology and ecology. First, identical plasmids have been found in M. avium, M. intracellulare and M. scrofulaceum (Jucker and Falkinham, 1990), showing that these three species are not reproductively isolated. Further, mycobacterial plasmids are relatively large; the smallest reported is approximately 12 kb and the largest 500 kb (Meissner and Falkinham, 1986). It is not unusual to find plasmids carrying 20–30% (1–2 Mb) of the chromosomal DNA of a cell (Meissner and Falkinham, 1986). Thus, they likely contribute to mycobacterial physiology and behaviour. Genes for heavy metal resistance (Meissner and Falkinham, 1984), restriction endonucleases (Crawford et al., 1981), morpholine (Waterhouse et al., 1991) and phenanthrene degradation (Guerin and Jones, 1988), and conjugal DNA transfer (Kirby et al., 2002) have been identified on plasmids. Discovery of conjugal transfer genes is consistent with the fact that homologous plasmids have been found in M. avium, M. intracellulare and M. scrofulaceum, distinct species. One likely reason for the paucity of mycobacterial plasmid studies has to do with the difficulty in their isolation, as our experience has taught us. Fortunately, the advent of rapid genome sequencing will provide us with a better picture of the role of mycobacterial plasmids in the genetics of mycobacteria.

Future trends

Although great strides have been made in investigations of the environmental mycobacteria a great deal remains to be elucidated. Over the past 20 years the number of Mycobacterium species has jumped to now over 100 (Tortoli, 2003). In the absence of evidence of person-to-
person transmission, most are likely environmental in origin. The majority of research on the environmental mycobacteria has focused on the major pathogens: M. kansasii, M. marinum, M. malmoense, M. avium, M. intracellulare, M. abscessus, M. chelonae and M. fortuitum. The presence of members of the M. avium complex in drinking water has led to promotion of the US EPA’s ‘Candidate Contaminant List’. However, although environmental surveys have been performed and reported, we lack markers for virulence. Without virulence markers, risk analysis is impossible as it is not known which isolates (and their number) are potential pathogens.

Continued study of biofilm formation by the environmental mycobacteria offers the possibility of answering questions, not only about the habitats of mycobacteria, but genes that contribute to virulence and adaptation to that interfacial lifestyle. As cited above, growth in biofilms leads to increased virulence and antibiotic resistance. Recently, it has been shown that addition of the universal quorum-sensing compound AI-2 to suspensions of M. avium leads to increased biofilm formation (Geier et al., 2008). Additionally, the oxidative stress response was also induced by AI-2, suggesting that the effect of AI-2 on biofilm formation is oxidative stress response from samples is increased by incubation under Avium.

References


