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Chemical and Ultrastructural Characteristics of Mycobacterial Biofilms

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ABSTRACT

Mycobacterial biofilm is a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface, which constitutes a protected mode of growth that allows survival in hostile environment. Biofilms can be defined as communities of mycobacteria attached to a surface. It is clear that microorganisms undergo profound changes during their transition from planktonic (free-swimming) organisms to cells that are part of a complex, surface-attached community. These changes are reflected in the new phenotypic characteristics developed by biofilm mycobacteria and occur in response to a variety of environmental signals. The biofilm-forming microorganisms have been shown to elicit specific mechanisms for initial attachment to a surface, formation of micro colony leading to development of three-dimensional structure of mature biofilm. They differ from their free-living counterparts in their growth rate, composition and increased resistance to biocides, antibiotics and antibodies by virtue of up regulation and/or down regulation of approximately 40% of their genes. This makes them highly difficult to eradicate with therapeutic doses of antimicrobial agents. A greater understanding of mechanism of their formation and survival under sessile environments may help in devising control strategies.

Key words: Mycobacteria, biofilm, ultrastructure, SEM

INTRODUCTION

In the natural world more than 99% of bacteria survive as biofilms (Costerton *et al*., 1999) and according to NIH report more then 65% of all human infections are associated with biofilms formation (Spoering *et al*., 2001). Biofilm is a microbial derived sessile community of bacteria in which bacteria are attached to the substratum and produce an Extracellular Polymeric Substance (EPS) (Donlan and Costerton, 2002). Biofilm in bacteria give protection from a wide range of

environmental challenges, such as UV exposure (Espeland and Wetzel, 2001) metal toxicity (Teitzel and Parsek, 2003), acid exposure (McNeill and Hamilton, 2003), dehydration and salinity (Le Magrex-Debar *et al*., 2000), phagocytosis and several antibiotics and antimicrobial agents (Mah and O'Toole, 2001). It is approximated that the majority of all medical infections are caused by bacterial biofilms that colonize either non-biological or biological surfaces (Costerton *et al*., 1999; Hoiby *et al*., 2011; Romling *et al*., 2012). Abiotic surfaces such as medical devices are usually infected by biofilms. Examples include intravenous, endotracheal, Hickman and dialysis catheters, prosthetic heart valves, orthopaedic devices, tissue fillers, cardiac pacemakers and cerebrospinal fluid shunts. Certainly, 60-70% of all nosocomial infections are due to the presence of biofilms on implants (Bryers, 2008). The microorganisms most often associated with medical devices are the staphylococci particularly *S. epidermidis* and *S. aureus*, followed by the bacterium *P. aeruginosa* and additional of other environmental bacteria that opportunistically infect hosts compromised by invasive medical intervention, chemotherapy or a pre-existing disease state. In addition, biofilms associate with living biological surfaces, including those provided by the human body. In fact, biofilms play a significant role in human infections as diverse as dental caries, periodontitis, otitis media, chronic wounds, musculo skeletal infections, necrotizing fasciitis, biliary tract infection, osteomyelitis, bacterial prostatitis, native valve endocarditis, intra-amniotic infections, meloidosis, a wide range of nosocomial infections and Cystic Fibrosis (CF) pneumonia (Romling *et al*., 2012).

The majority of the mycobacteria which produce biofilm are NTMs and these organisms can produce localized disease in the lungs, lymph glands, skin, wounds or bone. Many species of mycobacteria form structured biofilm communities such as *M. avium*, *M. intracellulare* (Falkinham *et al*., 2001), *M. fortuitum* (Hall-Stoodley and Scott, 1998), *M. gordonae*, *M. abscessus*, *M. septicum* and *M. gilvum* (Korber *et al*., 1989) and recently, Ojha *et al*. (2008) reported biofilm formation in *M. tuberculosis* H37 Rv. At least two nontuberculous mycobacterial species, *M. ulcerans* and *M. avium,* have been recently reported to colonize in the host as multicellular communities (Marsollier *et al*., 2005; Carter *et al*., 2003). Infection of an aquatic insect, *Naucoris cimicoides*, by *M. ulcerans* produces a community of the pathogen encapsulated by an extracellular matrix. The most important thing was the matrix of *M. ulcerans,* multicellular structures was laden with the toxin mycolactone, which was required for the colonization and virulence of the pathogen (George *et al*., 1999). However, the formation of the extracellular matrix, the hallmark of biofilms, by *M. ulcerans* has a direct control on its virulence properties. Inference of biofilms in *M. avium* infection is demonstrated by the inability of the biofilm-defective mutant strain to invade and translocate the bronchial epithelial cells. Whereas the questions as to how, when and where *M. tuberculosis* forms biofilms *in vivo* remain open to explore.

Biofilms are composed microbial cells and EPS, may account for 50-90% of the total organic carbon of biofilms which can be measured as the primary matrix material of the biofilm. Biofilm-associated EPS is different, both chemically and physically, from the bacterial capsule (McKenney *et al*., 1998) but it is primarily composed of polysaccharides. The EPS is also highly hydrated because it can integrate large amounts of water into its structure by hydrogen bonding. Different bacteria produce differing amounts of EPS and the quantity of EPS increases with age of the biofilm. Mostly EPS may be mycolic acid and glycopeptidolipids (GPL) in mycobacteria and alginate (anionic polysaccharide) in *P. aeruginosa* (O'Toole *et al*., 2000). The GPLs are required for sliding motility, biofilm formation and for maintaining cell wall reliability in mycobacteria (Donlan, 2001). Whereas the mutant of GPL acetyl-transferase produced defective biofilms, it had no growth defect in planktonic form (Recht and Kolter, 2001). The GPL biosynthesis was induced

during multicellular growth of *M. avium*, suggesting that two species share the mechanisms for biofilm development (Yamazaki *et al*., 2006). The GPL, mycolyldiacylglycerol (MDAG) and Free Mycolic (FM) acids are the other two surface molecules known so far that have an important role in biofilm development of *M. smegmatis* (Chen *et al*., 2006). Therefore, lipids could probably have critical roles in intercellular and cell-to substratum interactions in mycobacterial biofilms. The development of *M. smegmatis* biofilms requires an abundance of intracellular iron, which is facilitated by induced activity of siderophore synthesis (Ojha and Hatfull, 2007). Interestingly, dependence on iron availability for biofilm formation is also found in *P. aeruginosa* (Banin *et al*., 2005). They influence bacterial colonization (O'Toole *et al*., 2000) induce the release of prostaglandin E2 and interfere with the interaction between mycobacteria and human monocytes/macrophages. Therefore, GPLs play an important role in both the physiology and the pathogenicity of mycobacteria. The EPS can associate with metal ions, divalent cations and other macromolecules (such as proteins, DNA, lipids and even humid substances). The biofilm is an extremely complex community of microorganisms and the cells are located in matrix-enclosed "Towers" and "Mushrooms". *Mycobacterium smegmatis* forms pellicle-like biofilms at air-liquid interfaces that involve sliding motility (Recht *et al*., 2000; Recht and Kolter, 2001) and undecaprenyl phosphokinase is necessary for biofilm and smegma formation (Rose *et al*., 2004). Open water channels are distributed between the microcolonies that contain the sessile cells (Lewandowski, 2000).

Scanning Electron Microscopy (SEM) is a rapid and suitable means of assessing the pattern of colonization as well as screening samples for major bacterial morphotypes (Samaranayake *et al*., 1996). The SEM resolve morphology and ultrastructure of bacteria and biofilms has been utilized as in several other studies (Lie, 1979; Nyvad and Fejerskov, 1987; Zee *et al*., 1997; Monsenego, 2000; Sukontapatipark *et al*., 2001). Hence, SEM was used to study the detailed surface structure and configuration of the material, which may lead to an understanding of the patterns of biofilm formation on different appliances.

The growth of bacterial biofilms involves a developmental process that begins with surface attachment, followed by spreading, maturation and matrix synthesis (O'Toole *et al*., 2000). This process may be affected by a variety of environmental factors (O'Toole and Kolter, 1998) such as pH, iron, oxygen, ionic strength and temperature and nutrient level. Excess available carbon and the limitation of nitrogen, potassium or phosphate support EPS (Sutherland, 2001). In *M. avium* more amount of biofilm formation was reported in MB7H9 with OADC enrichment and at 28°C (Johansen *et al*., 2009). *Mycobacterium smegmatis* developed more amount of biofilm at 37 and 42°C as compared to 30°C. However, *M. fortuitum* developed more amount of biofilm at 30°C as compared to 37 and 42°C. *Mycobacterium tuberculosis* H37 Rv, sensitive and resistant isolates not developed biofilm at 30 and 42°C and strong biofilm was developed at 37°C (Kumar *et al*., 2015). Biofilm forming bacteria undergo a developmental program in response to environmental factors that lead to the expression of new phenotypes that distinguishes these attached cells from their plank tonically growing counterparts. Biofilm infections are difficult to eliminate with antimicrobial treatment and in vitro susceptibility tests show significant resistance of biofilm cells to killing (Gilbert *et al*., 1997; Maira-Litran *et al*., 2000). Biofilms are highly resistant to antibiotics than planktonic cells. The planktonic cultures of clinical isolates of Staphylococci have been found to be approximately 20-50 times more sensitive to antibiotics than their biofilms (Saginur *et al*., 2006). Likewise, biofilms of pathogenic *E. coli*, *P. aeruginosa* and Mycobacteria are 100-1000 times more tolerant to all tested antibiotics than their planktonic counterpart (Ceri *et al*., 1999). Several factors

have been recommended to account for biofilm tolerance slow growth. The presence of an exopolysaccharide matrix can slow the flow of antibiotics. Slow growth undoubtedly contributes to resistance to killing by antimicrobials, multidrug resistance pumps also represent a generalized resistance mechanism and have been considered as an additional candidate for a resistance mechanism. Despite decades of research on antibiotic resistance in bacteria, a comprehensive understanding of biofilm-specific antibiotic resistance is lacking. which display an increased resistance to antimicrobial agents (Costerton *et al*., 1995; Hoiby *et al*., 2010). Given the heterogeneous nature of biofilms (Stewart and Franklin 2008), it is likely that multiple mechanisms of resistance and/or tolerance act together to provide an overall high level of protection against natural and synthetic antimicrobial agents. The familiar mechanisms of antibiotic resistance, such as efflux pumps, modifying enzymes and target mutations (Walsh, 2000) do not seem to be responsible for the protection of bacteria in a biofilm. Even sensitive bacteria that do not have a known genetic basis for resistance can have profoundly reduced susceptibility when they form a biofilm. When bacteria are dispersed from a biofilm they usually rapidly become susceptible to antibiotics (Williams *et al*., 1997), which suggests that resistance of bacteria in biofilms is not acquired via mutations or mobile genetic elements.

On the other hand, the development of microarrays and the ability to study expression of the entire genome of an organism grown under two conditions, has launched the post genomic era of biofilm research and generated a wealth of new information. The process in biofilm formation involves changes in gene expression profiles. But a comparison of the differentially expressed gene sets identified in several recent DNA microarray studies (Schembri *et al*., 2003; Whiteley *et al*., 2001; Wen and Burne, 2002) reveals that no common expression pattern for biofilms has yet emerged. However, in different studies different genes are found up and down-regulated, in varying numbers ranging from 1.0-38.0% of the total genome. One explanation for these apparent discrepancies is that DNA microarrays provide a sensitive but transient picture of gene expression and that gene expression does not necessarily directly correlate with phenotype and these have been described for several prokaryotes, including *E. coli* (Ren *et al*., 2004), *P. aeruginosa* (Waite *et al*., 2005, 2006), *B. subtilis* (Ren *et al*., 2004), *V. cholera* (Moorthy and Watnick, 2005), *X. fastidiosa* (De Souza *et al*., 2004), *T. maritima* (Pysz *et al*., 2004), *S. aureus* (Beenken *et al*., 2004), *C. jejuni* (Sampathkumar *et al*., 2006) and *S. pyogenes* (Cho and Caparon, 2005).

Genes that have been associated with biofilm in addition to microarray analysis of the *M. smegmatis* transcriptome shows that iron-responsive genes-especially those involved in siderophore synthesis and iron uptake-are strongly induced during biofilm formation. *Mycobacterium smegmatis* induces a number of genes in both biofilms and stationary growth phases that are involved in stress management. For example, the universal stress response proteins Msmeg3816, Msmeg3950 and Msmeg3957 are induced in at least one of the biofilm samples (Ojha and Hatfull, 2007). The *lex A* and *rad A* (Msmeg2743 and Msmeg6041, respectively) are induced in biofilm formation, suggesting the possibility that they are responding to oxidative damage to DNA. This could also be associated with the requirement for iron uptake, with O^{-2} and F^{2+} interacting to generate hydroxyl radicals in a Fenton's reaction (Andrews *et al*., 2003). It is thus clear that very limited information about mycobacterial genes that may be playing part in biofilm formation in mycobacteria thus exist. Such studies would be very important.

Quantitative reverse transcriptase real time PCR (qRT-PCR) can be used effectively to quantify the number of RNA transcripts of specific genes from bacteria growing in biofilms. The qRT-PCR has a large dynamic range and may be used to verify gene expression data obtained from

microarrays. The microarray analysis of biofilms (Schembri *et al*., 2003) also revealed differential expression of genes under oxygen and nutrient-limiting conditions and of genes associated with enhanced heavy-metal resistance. A DNA microarray analysis of *Pseudomonas aeruginosa* detected only 1% of genes as differentially expressed in the biofilm growth mode, with 0.5% of the genes being activated and about 0.5% being repressed (Whiteley *et al*., 2001) assigned the differentially regulated genes to motility, attachment, translation, metabolism, transport and regulatory functions and found that temperate phage genes were the most highly activated. Bacteria growing in biofilms often express a different subset of genes compared to the same strains growing plank tonically.

Biofilm formation is a highly effective and ubiquitous strategy for the pathogen to proliferate as a stress-tolerant community in protected host niches, with limited invasion from the immune system. Thus, biofilm infections can potentially pose significant diagnostic and therapeutic challenges in clinical settings. The factors which affect the biofilm development are not yet completely understood. However, many species are known to form biofilms, little is known about the genetic requirements, patterns of gene expression, or the nature of the extracellular matrix of mycobacteria. Therefore, the present study has been designed to record the extent to which a single change in growth condition affects the formation of a useful mono species biofilm, excluding possible variables such as interspecies interactions and communication which are often observed. A more comprehensive understanding of processes connected with biofilm development in different stress conditions is expected to lead to new knowledge that would help in developing novel and effective control strategies for prevention of biofilms in clinically relevant situations in mycobacterial diseases and hence would stimulate new thinking that would be of help in improvement in patient management.

History: Antoni van Leeuwenhoek, initially observed microorganisms on tooth surfaces and can be credited with the discovery of microbial biofilms. The "Bottle effect" was examined (Costerton *et al*., 1999) in marine microorganisms, i.e., bacterial growth and activity were significantly enhanced by the incorporation of a surface to which these organisms could attach. Donlan (2001) has observed that the number of bacteria on surfaces was dramatically higher than in the surrounding medium (in this case, seawater). Biofilms may form on a variety of surfaces, including living tissues, in dwelling medical devices, industrial or potable water system piping or natural aquatic systems. The water system biofilm is highly complex, containing corrosion products, clay material, fresh water diatoms and filamentous bacteria.

Yet, a detailed examination of biofilms would await the electron microscope, which allowed high-resolution microscopy at much higher magnifications than light microscope. The scanning and transmission electron microscopy were used (Jones *et al*., 1969) to observe biofilms on trickling filters in a wastewater treatment plant and showed them to be composed of a variety of organisms (based on cell morphology). The specific polysaccharide- stain called Ruthenium red, coupling with osmium tetraoxide fixative, were used by researchers to show that the matrix material surrounding and enclosing cells in these biofilms were polysaccharide. Much of the work in the last two decades has relied on tools such as Scanning Electron Microscopy (SEM) or standard microbiologic culture techniques for biofilm characterization. Two major thrusts in the last decade have dramatically impacted the confocal laser scanning microscope to characterized biofilm at ultrastructure level and examination of the genes involved in cell adhesion and biofilm formation.

Process of biofilm formation: Biofilm-forming microorganisms have been shown to elicit specific mechanisms for initial attachment to a surface, microcolony formation, development of a three-dimensional community structure and maturation and detachment.

Attachment of bacteria for biofilm formation: The solid-liquid interface between a surface and an aqueous medium (e.g., water, blood) provides an ideal environment for the attachment and growth of microorganisms. A clear picture of attachment cannot be obtained without considering the effects of the substratum, conditioning films forming on the substratum, hydrodynamics of the aqueous medium, characteristics of the medium and various properties of the cell surface. Each of these factors will be considered in detail. Surface and interface binding of cells of microbial cells is driven by cell surface hydrophobicity (Bendinger *et al*., 1993). It follows that the environmental mycobacteria, whose cell surface hydrophobicity is the highest among the bacteria (Oss *et al.,* 1975), are more likely attached to surfaces or interfaces than suspended in water. Mycobacteria form biofilm on rough surface as compared to smooth surface. Adetunji *et al*. (2014a) reported *M. bovis* and *M. tuberculosis* produced more amount of biofilm in cement surface as compared to ceramic and stainless steel surface. Once mycobacterial cells colonize a surface, other microorganism might be able to attach to form a mixed biofilm community. Cells, particularly mycobacterial cells with their lipid-rich, thick outer membrane, can bind or sequester compounds, effectively taking them out of solution. 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. Biofilm formation occurs under both high and low shear conditions (Lehtola *et al*., 2007). 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. 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*., 1998; Carter *et al*., 2003). 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 (Fig. 1).

Microcolony formation: After the bacteria adhere to the inert surface/living tissue, the association becomes stable for microcolony formation. Bacteria begin to multiply while emitting chemical signals that 'intercommunicate' among the bacterial cells. Once the signal intensity exceeds a certain threshold level, the genetic mechanisms underlying exopolysaccharide production are activated. In this way, the bacteria multiply within the embedded exopolysaccharide matrix, thus giving rise to the formation of a microcolony (McKenney *et al*., 1998).

Formation of three-dimensional structure and maturation: During the attachment phase of biofilm development, perhaps after microcolony formation, the transcription of specific genes takes place. These are required for the synthesis of EPS. Attachment itself can initiate synthesis of the extracellular matrix in which the sessile bacteria are embedded, followed by formation of water-filled channels. It has been proposed that these channels constitute primitive circulatory systems, delivering nutrients to and removing waste products from the communities of cells in the microcolonies.

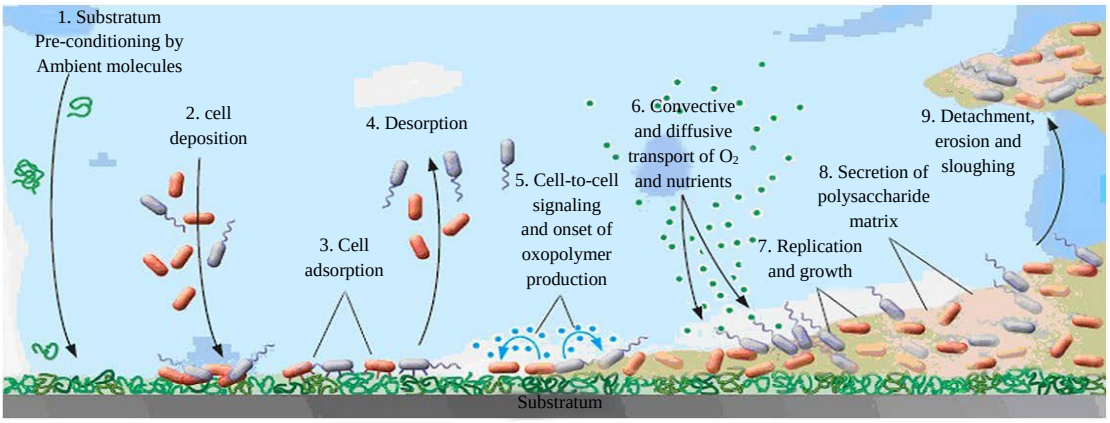

Fig. 1: Progressions leading to biofilm formation (Simoes *et al*., 2010)

Dispersion of biofilm: Biofilm cells may be disseminated either by shedding of daughter cells from actively growing cells, detachment as a result of nutrient levels or quorum sensing, or shearing of biofilm aggregates (continuous removal of small portions of the biofilm) because of flow effects. The mechanisms underlying the process of shedding by actively growing cells in a biofilm are not clearly defined. The surface hydrophobicity characteristics of newly divided daughter cells are spontaneously dispersed from either *E. coli* or *P. aeruginosa* biofilms differ substantially from those of either chemostat-intact biofilms or resuspended biofilm cells (Gilbert *et al*., 1993). Hydrophobicity was lowest for the newly spread cells and steadily increases upon continued incubation and growth. 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 mycobacterial cells. 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 500-10,000 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 the site of concentration of organic materials (Harvey and Young, 1980). Therefore, 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 or the particulate fraction of water (Falkinham *et al*., 2001).

Composition of biofilm: Biofilms are consists primarily of microbial cells and Extracellular Polymeric Substance (EPS) may account for 50.0-90.0% of the total organic carbon of biofilms. Biofilm is "City of microbes" (Watnick and Kolter, 2000) and EPS represent the "house of the biofilm cells." The EPS determine the instant conditions of life of biofilm cells, living in this

microenvironment by affecting porosity, density, water content, charge, adsorption properties, hydrophobicity and mechanical stability (Flemming and Wingender, 2002). The EPS are biopolymers of microbial origin in which biofilm microorganisms are rooted. In fact, archaeal, bacterial and eukaryotic microbes produce the biopolymers and commonly belief, EPS is certainly more than only polysaccharides. They contain, in addition, a wide variety of proteins, glycoproteins and glycolipids and in some cases, amazing amounts of extracellular DNA (e-DNA). However, the composition of mycobacterial biofilms is significantly different from that of other bacteria, containing an extracellular matrix rich in lipids and mycolic acid rather than polysaccharides (Ojha *et al*., 2010). The lipid, found in biofilm is glycopeptidolipids (GPLs) are a class of glycolipids produced by several (NTM) such as *M. abscessus* and *M. chelonae* (Ripoll *et al*., 2007).

A number of studies suggested that GPLs may play an important role in these processes. Martinez *et al*. (1999) found that *M. avium* 2151 smooth strains spread more than rough morphotypes, suggesting a role for GPLs in motility. Other studies have implicated role of GPLs in *M. smegmatis* sliding motility and biofilm formation. *Mycobacterium smegmatis* transposon mutants defective in mycobacterial peptide synthetase (mps) mps and GPL membrane transport proteins, such as those encoded by gap gene, lacked GPL expression and were nonmotile compared to the GPL-producing parent strains and some of these mutants were also defective in biofilm formation on PVC plastic (Recht *et al*., 2000). The model also proposed that GPL-defective mutants had more hydrophilic products exposed, such as polysaccharides, thus decreasing their motility due to an increase in friction. Though, this model implies that the GPL carbohydrate moieties would have only limited exposure to the environment, an unlikely prospect as published data supports exposure of the carbohydrate moieties on the bacterial surface (Kolk *et al*., 1989). An association between biofilm formation and virulence has also been observed. Carter *et al*. (2003) tested a number of *M. avium* strains originally isolated from AIDS patients for their ability to form biofilms on PVC plastic. They found that all strains could form biofilms but to varying degrees and that all expressed GPLs. Fascinatingly, the *M. avium* strain A5 was able to bind to and translocate across epithelial cells; however, biofilm-defective mutants were diminished in this capacity relative to the wild-type strain (Yamazaki *et al*., 2006). These mutants were defective in their GPL biosynthetic pathways. Yamazaki *et al*. (2006) suggesting a role for GPLs in epithelial cell invasion as well as in biofilm formation. The mycobacterial cell envelope has an extremely extraordinary structure (Brennan, 2003). One of its most striking features is the presence of very long-chain (C 70-C 90) fatty acids known as mycolic acids, that are usually anchored to the envelope through covalent linkage to arabinogalactan. While mycolic acids come with numerous baroque decorations, their general structure contains an invariant C-26 fatty acid that is condensed with a usually much longer and variable fatty acid through the action of a polyketide-synthase-like enzyme (Portevin *et al*., 2004). Mycolic acids contribute to the overall structure and characteristics of the mycobacterial envelope, providing a permeability barrier that is largely responsible for the ability of these organisms to resist many common therapeutic agents (Brennan, 2003; Ojha *et al*., 2005). The mycolic acid profiles of *M. smegmatis* were significantly different in free-living (planktonic) bacteria as compared to bacteria associated with biofilms. A mycobacterial mutant lacking GroEL1 was unable to develop architecturally complex biofilms and was also defective in the production of mycolic acids. Importantly, this defect was most apparent during biofilm formation, when the shorter-chain mycolic acids accumulate.

Extracellular polymeric substance is key factor of biofilm: The production of EPS by bacteria upon adhesion to surfaces has indeed been hailed as the "Hall mark" of biofilm formation (Stoodley *et al*., 2002); however, the mechanism of interaction between the EPS components

resulting in a stable matrix is, to date, a fertile field of research. Extensive research undertaken in the past few decades has focused on understanding the adhesive and cohesive properties of these biopolymers. The analytical techniques used for studying the EPS components can be broadly classified into two types: non-destructive techniques and techniques that study the EPS extracted from disrupted biofilms. Confocal Laser Scanning Microscopy (CLSM) is the most popular, non-destructive technique used (Neu *et al*., 2010) to monitor the time-resolved accumulation of various EPS components within biofilms. In this technique, the different components of the EPS can be identified visually by the addition of fluorescent probes. For instance, localisation of proteins using fluorescein isothiocyanate labelling, polysaccharides with Calcofluor white or concanavalin A labelling and nucleic acids using SYTOX Blue labelling can be visualized using CLSM (for more details of the specific probes for each component, please refer to Adav *et al*. (2010). The CLSM has played an important role in shaping our understanding of the spatial organization and formation of micro-domains within biofilms (Lawrence *et al*., 2007). Fourier-transformed infrared (FTIR) spectroscopy is another popular non-destructive technique for monitoring time resolved EPS accumulation in biofilms. In this technique, the accumulation of various EPS-associated functional groups and conformational changes in the EPS polymers can be monitored either by growing the biofilms directly on the attenuated total reflectance crystal (Delille *et al*., 2007; Quiles *et al*., 2010) or by growing biofilms on surfaces of interest like stainless steel and plastics (Pink *et al*., 2005; Bosch *et al*., 2006; Cheung *et al*., 2007; Ojeda *et al*., 2008). A microscope attached to the FTIR (micro-FTIR) can also aid in the analysis of micro-domains within biofilms, including the EPS matrix. Although, greater detail regarding the spatial distribution of EPS can be visualized from CLSM than from reflectance micro-FTIR spectroscopy, still FTIR can provide useful information about the functional groups in EPS that play an adhesive and cohesive role in the maintenance of biofilms (Geoghegan *et al*., 2008). The FTIR spectroscopy of EPS extracted from disrupted biofilms has also been carried out (Eboigbodin and Biggs, 2007; Tapia *et al*., 2009; Liang *et al*., 2010; Karunakaran and Biggs, 2011). The use of Raman spectroscopy and surface-enhanced Raman spectroscopy, as discussed further in the next section, has also been explored for the analyses of EPS samples (Ivleva *et al*., 2009).

Although, non-destructive, in-situ monitoring techniques are available, the more commonly employed strategy is to analyze the EPS obtained by the inevitable disintegration of biofilms. Results from simple calorimetric assays for total amount of proteins and carbohydrates and subsequent calculation of protein-carbohydrate ratios suggest that, generally, a predominance of protein components rather than polysaccharides leads to the greater stability of flocs and biofilms (Sheng *et al*., 2010). A detailed biochemical analysis reveals that the polysaccharide components can either contain homopolysaccharides like cellulose in *Salmonella typhimurium* (Zogaj *et al*., 2001) or charged heteropolysaccharides that can either be polyanionic like in the case of alginate *Pseudomonas aeruginosa* (Evans and Linker, 1973; Wozniak *et al*., 2003) and colonic acid in *Escherichia coli* (Danese *et al*., 2000) or polycationic in the case of the intercellular adhesin of *Staphylococcus aureus* (Gotz, 2002). A current understanding establishes that the interaction of exopolymers with inorganic substituents like divalent cations (e.g., Ca^{2+} and Mg^{2+}) and metal centres on surfaces serve to further influence the physical properties and enhance the mechanical stability of flocs and biofilms (Biggs *et al*., 2001; Geoghegan *et al*., 2008; Krstgens *et al*., 2001). A largely metabolic role is reserved for the extracellular proteins present within biofilms and the predominance of protein components in biofilms has led to the idea that the EPS matrix could possibly function as an efficient external digestive system (Flemming and Wingender, 2010). The

biochemical significance of the immobilization of extracellular enzymes within the polysaccharide matrix has been verified in the case of retention of extracellular lipase by alginate residues within *P. aeruginosa* biofilms (Mayer *et al*., 1999). The active role of enzymes like peptidases, polysaccharases and phosphatises has been confirmed within biofilms and these enzymes increase the bioavailability of nutrients in the surrounding environment (Romani *et al*., 2008; Neu and Lawrence, 2009). The interaction between the protein component and the EPS polysaccharides can also be of structural significance as in the case of the secreted TasA protein and exopolysaccharides in *Bacillus subtilis* biofilms (Branda *et al*., 2006). Generally, the production of sugar-binding peptides, lectins, is also thought to contribute to the structural integrity of the biofilms (Neu and Lawrence, 2009). A recent work on the detailed biochemical analysis of the protein components of EPS attributes a small yet significant contribution of polycationic peptides in maintaining the structural integrity of *Bacillus cereus* biofilms (Karunakaran and Biggs, 2011). The concept that the physical interaction between the polymers in the matrix through electrostatic forces, van der waals interaction, polar interactions and hydrogen bonding influences the mechanical stability of the biofilm has also been demonstrated by rotational viscosimetry (Mayer *et al*., 1999). The EPS molecules, by a process called polymer bridging, have also been found to play an important role in overcoming the electrostatic repulsion between the bacterium and the surface, thus ensuring firm, irreversible attachment of the bacteria to the surface (Neu and Marshall 1990; Karunakaran and Biggs, 2011). The outcome of such research has spurred investigations into novel surface coatings that reduce bioadhesion (Yuan *et al*., 2009; Khoo *et al*., 2009). In addition to physicochemical characterization, the regulation of EPS production at the genetic level has been studied in detail in several organisms. For instance, in *B. subtilis*, the matrix is composed of exopolysaccharides, produced by genes encoded on a single operon (eps operon) and an extracellular protein, TasA (Kearns *et al*., 2005). The eps operon and the genes involved in the production and processing of TasA have been demonstrated to be under the control of several transcriptional regulators (Kearns *et al*., 2005). The reduction of biofilm formation and maturation has been confirmed visually in the appropriate deletion mutants (Kearns *et al*., 2005). Recently, the translational control of EPS production has also been shown to occur in *B. subtilis* (Irnov and Winkler, 2010). Likewise, the control of EPS production by the intracellular levels of cyclic-diguanosine monophosphate is well studied (Jenal and Malone, 2006). An extensive analysis of the extracellular proteomes of the *B. cereus* group of organisms has revealed the role of the pleiotropic regulator, PlcR, in regulating extracellular protein production (Gohar *et al*., 2002, 2008; Oosthuizen *et al*., 2002). Finally, both the desirable effects of biofilms such as environmental detoxification (Flemming and Wingender, 2010) and the undesirable effects of biofilms such as biofouling of surfaces and insulation of the walls of heat exchangers (Flemming and Wingender, 2001) are directly linked to the sorptive properties of the matrix components. The stabilization and concentration of extracellular enzymes within biofilms help the biofilms function as powerhouses of degradation of xenobiotics and organic polymers (Flemming and Wingender, 2010). On the other hand, the increased metal ion binding by the metallo proteins within biofilms contributes to the biocorrosion of surfaces (Neu and Lawrence, 2009). The colligative properties of the EPS polymers (Keiding *et al*., 2001) insulate the walls of heat exchangers against convective heat transfer and reduce the efficiency of industrial processes (Flemming and Wingender, 2001). Although investigations into the external environment of biofilms (e.g., EPS) have incorporated techniques from various fields like microscopy, spectroscopy, biochemistry, surface science and genetics (Fig. 2) and have succeeded in providing the research

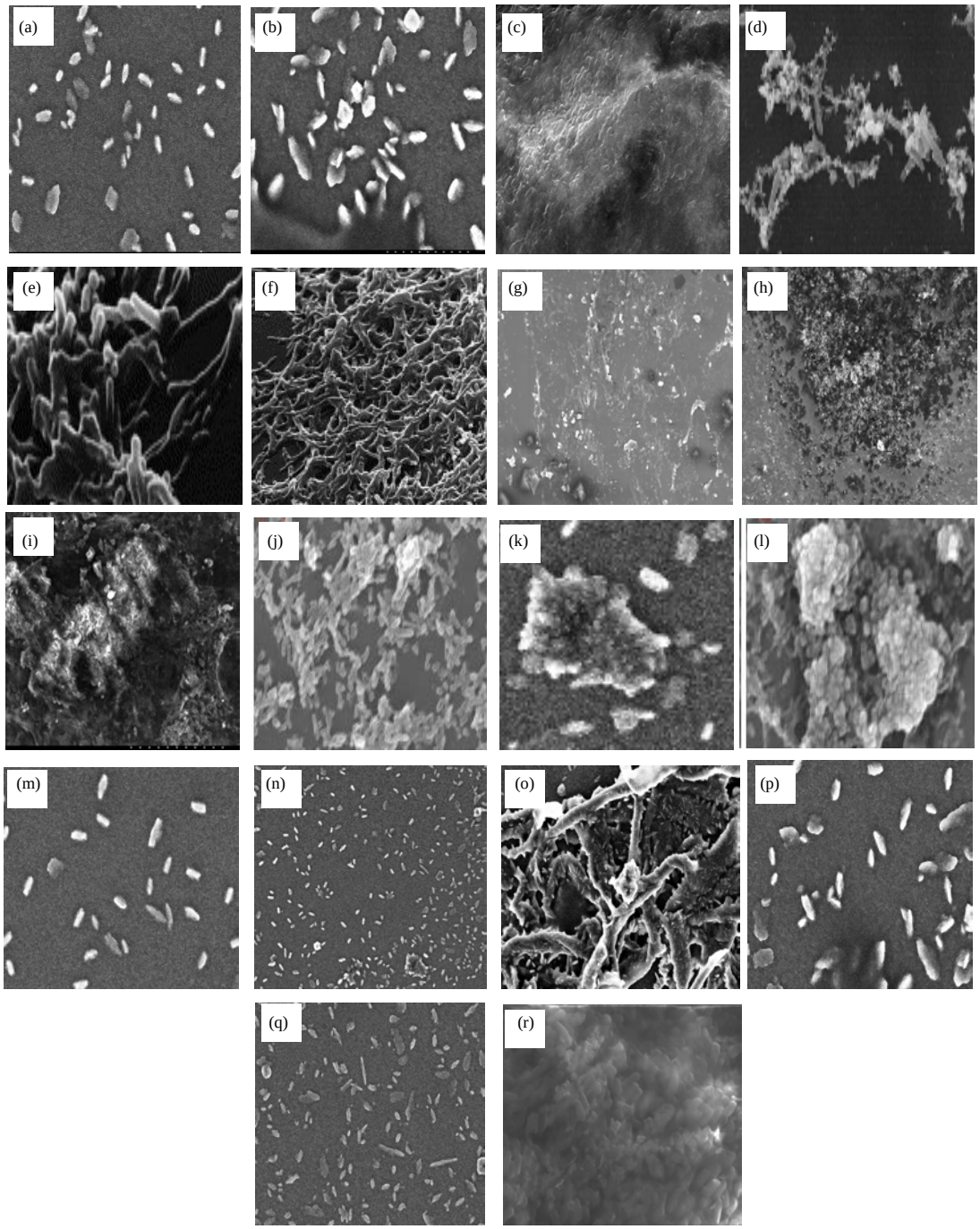

Fig. 2(a-r): Electron micrographs showing the various forms of ultrastructural analysis of mycobacterial biofilm in different conditions of Temperature, OADC and Gluce studied. *M. smegmatis* developed weak (a) Moderate, (b) Strong, (c) Biofilms, *M. fortuitum* developed weak, (d) Moderate, (e) Strong, (f) Biofilms, *M. avium* developed weak, (g) Moderate, (h) Strong, (i) Biofilms, *M. tuberculosis* H37Rv developed weak, (j) Moderate, (k) Strong, (l) Biofilms, *M.tuberculosis* MDR isolates developed weak, (m) Moderate, (n) Strong, (o) Biofilms, *M.tuberculosis* sensitive isolates developed weak, (p) Moderate, (q) Strong and (r) biofilms

community with a wealth of information, a few limitations exist. The limitations arise due to the fact that no consensus exists on the EPS extraction techniques and the complete recovery of all components of the EPS from a biofilm remains a challenge. The composition of the matrix polymers can be easily perturbed by changes in cellular metabolism and consequently can change the physical forces that stabilize the biofilms. Therefore, research into extracellular polymers when supplemented with an understanding of the nature of the physical interaction between the bacterial cell surface and the exopolymers, coupled with an appreciation of cellular metabolism, can lead to a much better integrated understanding of EPS interaction, regulation and control within biofilms.

Microtiter plate assay for study of biofilm formation: This is simple microtiter dish assay used for the assessment of the biofilm formation on the wall and/or bottom of a microtiter dish. The nature of the assay makes it useful for genetic screens, as well as testing biofilm formation by multiple strains under various growth conditions. Variants of this assay have been used to assess early biofilm formation for a wide variety of microbes, including but not limited to *Pseudomonas, V. cholerae*, *E. coli*, Staphylococci, Enterococci, Mycobacteria and fungi. The extent of biofilm formation is measured using the dye Crystal Violet (CV). A number of other colorimetric and metabolic stains have been reported for the quantification of biofilm formation using the microtiter plate assay. The low cost and flexibility of the microtiter plate assay has made it a critical tool for the study of biofilms. This method can be modified for use with a wide variety of microbial species. Motile microbes typically adhere to the walls and/or bottoms of the wells, whereas, non-motile microbes typically adhere to the bottom of the wells. The optimal conditions for biofilm formation (i.e., growth medium, temperature, time of incubation) must be determined empirically for each microbe.

Biofilm formation in mycobacteria: Environmental mycobacterial pathogens can be divided into two groups based on growth rate; the slowly growing species include: *M. avium*, *M. intracellulare*, *M kansasii*, *M. marinum*, *M. xenopi* and *M. malmoense* and fast growing mycobacteria such as *M. smegmatis*, *M. fortuitum*. Environmental mycobacteria are widely distributed in the environment; they have impacts on the local microbiome. The 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, 2002) contributed for surface colonization (e.g., zinc coated, galvanized pipes). The impermeable, hydrophobic, lipid outer membrane should be thought of as a double-edged sword.

In addition, 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). In several publications, surfaces are incubated in the continual presence of (growing or non-growing) mycobacterial cells. In those conditions it is impossible to separate the contributions of newly adherent cells and growth of adherent cells to increase in the number of cells on surfaces (Hall-Stoodley *et al*., 1998). Hence, experimental methods must be developed separately to measure the adherence and biofilm growth. Several example of mycobacterial adaptation and existence was most notably survival as a consequence of exposure to an aerobiosis (Dick *et al*., 1998), starvation (Archuleta *et al*., 2005), acid (Bodmer *et al*., 2000), temperature (Scammon *et al*., 1964) and elevated antibiotic disinfectant resistance of biofilm-grown cells (Steed and Falkinham, 2006; Falkinham,

2008). The feature shared by adaptations to resistance, acid and intracellular growth is that prior growth leads to increased survival under stressful conditions. For example, *M. avium* cells developed in medium of high acidity (e.g., pH 3-5) were better able to grow at low pH (Bodmer *et al.,* 2000) and *M. avium* cells grown in amoebae were more readily phagocytosed and were more virulent (Cirillo *et al*., 1997). Growth of cells of *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). Esteban *et al*. (2008) showed that low nutrient conditions decrease biofilm development in *M. fortuitum* and *M. chelonae*. Measurement of numbers of *M. avium* in drinking water systems all over the world have shown that the majority of *M. avium* cells are in biofilms on pipe surfaces and low numbers are recovered from bulk water (Falkinham *et al*., 2001). Other mycobacteria, specifically *M. kansasii* (Schulze-Robbecke and Fischeder, 1989). *Mycobacterium chelonae*, *M. fortuitum* (Hall-Stoodley and Scott, 1998) and *M. phlei* (Bardouniotis *et al*., 2001) were also form biofilms on surfaces, including high density polyethylene and silastic rubber. It is likely that the high cell surface hydrophobicity of mycobacteria (Oss *et al*., 1975) contributes to biofilm formation. Such a predilection for attachment would also lead to colonization of catheter surfaces. Growth of biofilms by *M. avium* (Falkinham, 2008), *M. tuberculosis* and numerous other species of mycobacteria, including *M. fortuitum* (Hall-Stoodley and Scott, 1998), *M. marinum* (Hall-Stoodley *et al*., 2006) and *M. smegmatis* (Recht *et al*., 2000) form biofilm on liquid air interface. The biofilm on the medical device, on the other hand, appears to be composed of a single, coccoid organism and the associated Extracellular Polymeric Substance (EPS). The formation of bacterial biofilms involves a developmental process that begins with surface attachment, followed by spreading, maturation and matrix synthesis (O'Toole *et al*., 2000).

Ultrastructural analysis of biofilms of mycobacterial isolates developed in different conditions: The use of SEM to resolve the morphology and ultrastructure of bacteria and biofilms has been utilized in several other studies (Nyvad and Fejerskov, 1987; Monsenego, 2000; Sukontapatipark *et al*., 2001). Ultrastructurally the microbial biofilm community is unique while some structural attributes can generally be considered universal. Biofilms are not a continuous monolayer surface deposit rather, biofilms are very diverse, containing micro-colonies of bacterial cells encased in an EPS matrix and separated from other microcolonies by interstitial voids (water channels). Liquid flow occurs in these water channels, allowing diffusion of nutrients, oxygen and even antimicrobial agents (Lewandowski, 2000). On the other hand, Hall-Stoodley and Scott (1998), observed microcolony branching cell, void and channel between microcolony and developed patches of aggregate cells formed biofilm with heterogeneous morphology in *M. chelonae*. The SEM image of biofilm of *M. chubuense*, *M. gilvum*, *M. obuense*, *M. fortuitum* and *M. vaccae* showed curved structures arranged in a definite order and voids were clearly visible with long fibre and short fibre (Agusti *et al*., 2008). However, *M. fortuitum* exhibited heterogeneous morphology with a mycelial-like texture while *M. avium* and *M. tuberculosis* showed crystalline and globular structure.

Biofilm development has been suggested to be a property of mycobacteria which might depend on the nutrients present in the medium (Esteban *et al*., 2008). The strength of biofilm development is reported to be dependent on various factors like contact surface, pH, temperature, humidity, nutrient availability, contact time of the bacteria with the surface, growth stage, surface hydrophobicity and textures of surface etc., which affect the attachment and colonization of the bacteria for biofilm formation (James *et al*., 1995). The pH, temperature (Johnson *et al*., 2009) and nutrient composition (Carter *et al*., 2003) are crucial factors for the growth of mycobacterial biofilm.

Mycobacterium tuberculosis is restricted for growth in acidic pH. Nontuberculous mycobacterial species, may grow in soil or aquatic environments, are much more acid tolerant and in fact *M. kansasii*, *M. scrofulaceum*, *M. avium* and *M. chelonae, M. fortuitum* grow well at pH 6.0 and 7.0 (Piddington *et al*., 2000). According to these observations we designed our experiments to see the effects of different biotic and abiotic factors, temperature, pH and OADC enrichment and effect of glucose on the development of biofilm. In the presence of OADC *M. tuberculosis* produce more amount of biofilm (Kumar *et al*., 2015). In this study the selected mycobacteria developed a different amount of biofilm weak, moderate and strong in different condition such as temperature, OADC, glucose and different pH and different time points such as first week and the second week for fast growers, *M. smegmatis* and *M. fortuitum* and second week and fourth week for slow growers, *M. avium* and *M. tuberculosis and* in sensitive and resistant isolates in Sauton's and MB7H9 media. Mycobacteria produced different amount of biofilm which we have observe by SEM and depicted in Fig. 2. Two strains of *Mycobacterium bovis* had similar ability to form biofilms on the three surfaces. More biofilms were developed in media containing 5% liver extract. Biofilm mass increased as incubation time increased till the 3rd week. More biofilms were formed on cement than on ceramic and stainless steel surfaces. Treatment with hot water at 85°C reduced biofilm mass, however, sanitizing treatments at 45°C removed more biofilms than at 28°C. However, neither treatment completely eliminated the biofilms. The choice of processing surface and temperatures used for sanitizing treatments had an impact on biofilm formation and its removal from solid surfaces (Adetunji *et al*., 2014b).

Electron microscopy for biofilm formation: The SEM was employed in the present study to visualize the general morphology and the detailed structural or ultrastructural features of biofilm formed on the tiles. The use of SEM to determine morphology and ultrastructure of bacteria and biofilms has been utilized in several other studies (Lie, 1979; Nyvad and Fejerskov, 1987; Zee *et al*., 1997; Monsenego, 2000; Sukontapatipark *et al*., 2001). The SEM represents a rapid and convenient means of assessing the pattern of colonization as well as screening samples for major bacterial morphotypes (Samaranayake *et al*., 1996). Hence, SEM was used to investigate the detailed surface structure and configuration of the material, which may lead to an understanding of the patterns of biofilm formation on different humid surfaces. There are however some drawbacks inherently associated with SEM as the identification of morphological features of the object is largely based on visual morphology (Sukontapatipark *et al*., 2001). The preparation of specimens for SEM to remove the water content also introduces the possibility of deformation, shrinkage and the inclusion of artefacts, all of which may distort the biofilm from its original state. The bacterial extracellular matrix is particularly susceptible to distortion by dehydration due to its aqueous content and may condense to as little as 1% of its original volume (Fischer *et al*., 1988). Thus, care was taken in interpretation of the photomicrographs. No software has to date been developed to quantitatively analyze the morphological features obtained through SEM images, which is another drawback of this technology. Other advanced imaging modalities, which were not used, could be considered by future investigators to overcome the latter drawbacks. These include environmental SEM, an analogue of SEM, which utilizes a hydrated specimen chamber, eliminating the need for dehydration and coating of specimens to reduce electron absorption, which should reduce sample distortion (Slayter and Slayter, 1992; Surman *et al*., 1996). Confocal laser scanning microscopy also allows inspection of aqueous live biofilm samples with relatively less distortion (Wood *et al*., 2000). The use of quantitative image analysis also allows for the determination of live/dead cell counts and

their distribution within the biofilm (Arweiler *et al*., 2004). These along with concurrent investigations into the microbiological identification of bacterial species involved in biofilm formation warrant further study.

Antimicrobial resistance of biofilm formation: Biofilm formation is important because this mode of growth is associated with the chronic nature of the subsequent infections and with their inherent resistance to antibiotic chemotherapy. Periodontitis and chronic lung infection in cystic fibrosis patients are examples of diseases that are generally acknowledged to be associated with biofilms (Darveau *et al*., 1997; Singh *et al*., 2000). Various nosocomial infections such as those related to the use of central venous catheters (Passerini *et al*., 1992), urinary catheters (Morris *et al*., 1999), prosthetic heart valves, (Hyde *et al*., 1998) and orthopaedic devices (Gristina *et al*., 1994) are clearly associated with biofilms that adhere to the biomaterial surface. These infections share common characteristics even though the microbial causes and host sites vary greatly. The most important of these characteristics is that bacteria in biofilms evade host defences and withstand antimicrobial chemotherapy. The mechanisms of resistance to antibiotics in bacterial biofilms are beginning to be elucidated. Mah and O'Toole (2001) shows three main hypotheses. The first hypothesis is the possibility of slow or incomplete penetration of the antibiotic into the biofilm. Measurements of antibiotic penetration into biofilms in vitro have shown that some antibiotics readily permeate bacterial biofilms (Stewart, 1996). There is no generic barrier to the diffusion of solutes the size of antibiotics through the biofilm matrix, which is mostly water (Stewart, 1998) However, if the antibiotic is deactivated in the biofilm, penetration can be profoundly retarded. For example, ampicillin can penetrate through a biofilm formed by a β-lactamase-negative strain of *K. pneumonia* but not a biofilm formed by the β-lactamase-positive wild type strain of the same micro-organism (Anderl *et al*., 2000). In the wildstrain biofilm, the antibiotic is deactivated in the surface layers more rapidly than it diffuses. Antibiotics that adsorb into the biofilm matrix could also have a retarded penetration, which might account for the slow penetration of aminoglycoside antibiotics (Kumon *et al*., 1994; Shigeta *et al*., 1997). These positively charged agents bind to negatively charged polymers in the biofilm matrix (Gordon *et al*., 1988). The second hypothesis depends on an altered chemical microenvironment within the biofilm. Microscale gradients in nutrient concentrations are a well known feature of biofilms. Findings from studies with miniature electrodes have shown that oxygen can be completely consumed in the surface layers of a biofilm, leading to anaerobic niches in the deep layers of the biofilm (De Beer *et al*., 1994). Concentration gradients in metabolic products mirror those of the substrates. Local accumulation of acidic waste products might lead to pH differences greater than 1 between the bulk fluid and the biofilm interior (Zhang and Bishop, 1996) which could directly antagonize the action of an antibiotic. Aminoglycoside antibiotics are clearly less effective against the same micro-organism in anaerobic than in aerobic conditions (Tack and Sabath, 1985). Alternatively, the depletion of a substrate or accumulation of an inhibitive waste product might cause some bacteria to enter a non-growing state, in which they are protected from killing. Penicillin antibiotics, which target cell-wall synthesis, kill only growing bacteria (Tuomanen *et al*., 1986). This alternative possibility is strengthened by direct experimental visualisation of metabolically inactive zones within continuously fed biofilms (Xu *et al*., 2000). Additionally, the osmotic environment within a biofilm might be altered, leading to induction of an osmotic stress response (Prigent-Combaret *et al*., 1999). Such a response could contribute to antibiotic resistance by changing the relative proportions of porins in a way that reduces cell envelope permeability to antibiotics. A third and still speculative mechanism of antibiotic resistance is that a subpopulation

of micro-organisms in a biofilm forms a unique and highly protected, phenotypic state-a cell differentiation similar to spore formation. This hypothesis is lent support by findings from studies that show resistance in newly formed biofilms, even though they are too thin to pose a barrier to the penetration of either an antimicrobial agent or metabolic substrates (Cochran *et al*., 2000. Das *et al*., 1998). Additionally, most bacteria in the biofilm but not all, are rapidly killed by antibiotics (Brooun *et al*., 2000). Survivors, which might consist of 1% or less of the original population, persist despite continued exposure to the antibiotic. The hypothesis of a spore-like state entered into by some of the bacteria in a biofilm provides a powerful and generic, explanation for the reduced susceptibility of biofilms to antibiotics and disinfectants of widely different chemistries.

Multicellular nature of biofilm defence: All three main hypotheses of biofilm resistance to antibiotics depend on the multicellular nature of biofilms (O'Toole *et al*., 2000). An antimicrobial agent cannot slowly or incompletely penetrate the biofilm unless the microorganisms form aggregates that affect its diffusion. Local variations in the concentrations of microbial substrates and products develop only when a cluster of cells reaches a critical size and the bacteria exert their combined metabolic activity. The small population of cells that differentiate into a dormant and protected state depend on their growing neighbours to propagate the genome and their neighbours depend on them to reseed the community in the event of catastrophic killing. The fact that all these antibiotic resistance mechanisms are inherently multicellular helps to explain why bacteria dispersed from biofilms rapidly revert to a susceptible phenotype. Researchers investigating bacterial biofilms are beginning to discuss biofilm formation in terms of developmental biology. Recent results lend support to the idea of biofilm formation as a multicellular developmental process. We now know that specific gene products are required for the initial association of bacteria with a surface. Dozens of new genes are turned on and others are turned off as bacteria move onto a surface, suggesting a pathway of differentiation. Motility seems to be critical in the early stages of biofilm formation. Coordinated by unknown cues, bacteria use flagellar, twitching and gliding motility mechanisms to grow together in nascent clusters. The further organization of the biofilm into complex structures is regulated by the exchange of chemical signals between cells in a process known as quorum sensing. Add to these observations the capacity for bacteria in biofilms to collectively withstand antimicrobial treatments that would kill a lone cell and the case for multicellularity in biofilms is compelling. The recognition of biofilm formation as a multicellular developmental process is important because this insight will allow new approaches for treatment of the persistent infections stemming from biofilms.

Gene regulation by attached biofilm cells: The formation of bacterial biofilms involves a developmental process that begins with surface attachment, followed by spreading, maturation and matrix synthesis (O'Toole *et al*., 2000). This process is accompanied by changes in gene expression profiles and these have been described for several prokaryotes, including *E. coli* (Schembri *et al*., 2003), *P. aeruginosa* (Whiteley *et al*., 2001), *Bacillus subtilis* (Stanley *et al*., 2003), *Vibrio cholerae* (Moorthy and Watnick, 2005), *Xylella fastidiosa* (De Souza *et al*., 2004), *Thermatoga maritima* (Pysz *et al*., 2004), *Staphylococcus aureus* (Beenken *et al*., 2004). The many important findings generated from this, first, bacterial biofilms are likely composed of heterogeneous populations of cells experiencing different microenvironments and possibly expressing different subsets of genes and there are large variations in planktonic cells' growth conditions (Lazazzera, 2005). Second, there is no single core biofilm regulon present in these bacteria, although induction of stress

responses is common (Beloin and Ghigo, 2005). Third, a substantial portion of genes differentially expressed in biofilms are also expressed in stationary phase cells. Fourth, genes of unknown function comprise a high proportion of genes differentially expressed in biofilms. Finally, different sets of genes are expressed at different stages throughout the course of biofilm development (Waite *et al*., 2005). The first descriptions of specific genes that are up- or down-regulated in biofilm bacteria were made using transcriptional lac Z reporter-gene fusions (Davies *et al*., 1993) and led to the belief that bacterial attachment initiates the expression of a set of genes that culminates in a biofilm phenotype (Costerton *et al*., 1995). That major fractions of the bacterial genome could be involved in or affected during biofilm formation was shown in *E. coli* in a genome-wide screen using random chromosomal insertions of a lacZ reporter gene fusion construct (Prigent-Combaret *et al.,* 1999). The bacterial biofilms encounter higher osmolarity conditions, greater oxygen limitation and higher cell density than in the liquid phase. Different genes such as *pks*, a polyketide synthase gene contributes to synthesis of the immunomodulatory phenolic glycolipids. The five domains of *pks*1, annotated as acyltransferase, dehydrogenase, enoylreductase, ketoreductase and acyl carrier protein and the single domain of *pks* 15, annotated as a keto-acyl synthase, catalyze the elongation of *p*-hydroxybenzoic acid with malonyl coenzyme. A units to form *p*-hydroxyphenylalkanoic acid, intermediate product of the PGL backbone. Many supplementary genes likely contribute to pellicle biofilm formation in *M. tuberculosis*. The mutants emphasize a variety of functions important to biofilm production and maintenance, including nitrogen metabolism (Rv0021c and *nirB*), cell surface protease activity (*mycP*1) and complex lipid biosynthesis (*pks*1). In *M. abscessus mmpL4b* gene, a gene coding for a membrane protein which has been found to play an essential role in GPL expression by NTM (Recht *et al*., 2000). Changes in GPL expression associated with rough/smooth phenotypic variation are accompanied by other changes which influence biofilm forming capability, sliding motility, immune stimulatory activity and the ability to replicate in macrophages. The gene *lsr*2 orthologs have been identified in all sequenced mycobacterial genomes and homo logs are found in many actinomycetes. Although, its precise function remains unknown, *M. smegmatis*, as well as *M. avium*, has been shown to produce a biofilm or a biofilm-like structure (Martinez *et al*., 1999). The outermost layers of the *M. smegmatis* and *M. avium* cell walls contain glycopeptidolipid (GPL), whereas the outermost layer of *M. tuberculosis* is made of phenolic glycolipids, dimycocerosate and lipo-oligosaccharides (Ortalo-Magne *et al*., 1996).

Molecular mechanism of biofilm formation in mycobacteria: Recent studies recommend that the *M. smegmatis* biofilm is associated with a GPL present on the cell wall and indirect evidence indicates a similar role in *M. avium*. Transposon inactivation of the GPL gene clusters in *M. smegmatis* decreased the production of biofilm and the deletion of the genes *tmtp* and *mps* revealed their involvement in biofilm formation upon seeding of the bacterium on polyvinyl chloride (PVC) plates (Recht and Kolter, 2001). The *tmtp* gene is highly conserved between *M. smegmatis* and *M. avium*, with both organisms having genes encoding one small (*tmtpA*) and two large (*tmtpB* and *tmtpC*) putative transmembrane transport proteins in the same operon. The proposed function involves the transport of the precursor of GPL from the inner membrane. The *mps* genes are identified as *pstA*, B and C, constituting the GPL gene clusters in *M. avium*. The peptide synthetase (*mps*, Mps protein) has a role in the initial step of GPL synthesis, i.e., in the assembly of the lipopeptide core and acceptor of acyl-Phe, which is modified by sequential addition of threonine, alanine and alaninol (Billman-Jacobe *et al*., 1999). The lipopeptide core may subsequently be glycosylated with rhamnose and 6-deoxytalose, resulting in the nonspecific GPL

(nsGPL). The acetyltransferase (*atf1*) acetylates on 6-deoxytalose in the cell wall and the putative *tmtpC* (Tmtpc) protein transports it to the outermost layer of the cell wall. However, the roles of GPLs in biofilm formation are still not well defined. The genetic determinant of biofilm formation in *M. avium* has not been clearly acknowledged. Furthermore, *M. avium* strains produced more biofilm when inoculated in water than in MB 7H9 broth on a PVC surface. During biofilm formation, microorganisms rarely come into contact with a clean surface and normally colonize a surface that has been modified following the absorption of molecules from the environment, such as water and proteins, etc. The *M. avium* 101 and 104 strains belong to serotype 1 while *M. avium* A5 and strain 109 belong to serotype 4. Krzywinska and Schorey (2003) described the genomic differences, especially in GPL gene clusters, between *M. avium* 104 (the strain from which the genome sequence is available) and *M. avium* A5. The GPL was highly conserved upstream of the GPL clusters methyl transferase B (*mtf*B), glycosyl transferase A (*gtf*A), rhamnosyl transferase A (*rtf*A), *mtf*C, *mtf*D and dehydrogenase A (*dhg*A). DNA microarrays of all the open-reading frames of an organism are typically used to determine which genes are controlled by a particular transcription factor or environmental signal.

Thus, DNA microarray studies have been found to be extremely useful for comparison of two samples to identify differentially expressed genes. The choice of samples for comparison to determine genes controlled by a transcription factor is rather easy. However, the sample against which to compare biofilm cells is far less clear. The difficulty in performing DNA microarray analysis of biofilms stems from the fact that they comprise a heterogeneous population of cells, even for a single species biofilm. Cells in a biofilm have been shown to have heterogeneous growth rates (Werner *et al*., 2004) indicating that there is a concentration gradient of nutrients. Oxygen concentration drops significantly in the depths of the biofilm that are furthest from the oxygenated liquid-biofilm interface (Yu *et al*., 2004). These gradients make it difficult to replicate the environmental conditions affecting biofilm cells in a single culture of planktonic cells. Stanley *et al*. (2003) used expression profiling to identify transcriptional regulators that were affected during biofilm formation in *B. subtilis*, by extrapolating from the expressed genes expressed to their regulators. Using this indirect approach, several transcription factors were identified; including Spo0A and the starvation-activated transcription factor H. Spo0A was previously shown to be required for biofilm formation (Piggot and Hilbert, 2004) and for directing the development of endospores. Furthermore, 40 genes responsive to glucose concentration were found in the study by Stanley *et al*. (2003) concluded that glucose inhibits biofilm formation through the catabolite control protein C cpA. The role of glucose in biofilm formation has also been proposed for *S. mutans* and *E. coli* when grown under stagnant batch growth conditions. In the case of *E. coli*, the availability of glucose affected biofilm formation through the carbon storage regulator C srA; disruption of csrA significantly decreased biofilm formation.

Real time PCR analysis for quantitative gene expression in biofilm: Real-time PCR can be used to estimate the number of copies of a target gene in a sample and is reported to be more sensitive than conventional qualitative PCR. Real-time PCR is based on the detection and quantification of a fluorescent reporter, whose emission is directly proportional to the quantity of amplicons generated during the PCR. The fluorescent reporter used in this study was SYBR Green I, a nonspecific double-stranded DNA-binding dye. The SYBR® Green I binds all double-stranded DNA molecules, emitting a fluorescent signal of a defined wavelength on binding. The excitation and emission maxima of SYBR Green I at 494 and 521 nm, respectively and are

compatible for use with real-time cycler (Smith and Osborn, 2009). Detection takes place in the extension step of real-time PCR. Signal intensity increases with increasing cycle number due to the accumulation of PCR product. Use of fluorescent dyes enables analysis of many different targets without having to synthesize target-specific labelled probes. However, nonspecific PCR products and primer-dimers were also contributed to the fluorescent signal. Therefore, high PCR specificity is required when using SYBR Green I.

Quantification of the primary target sequences of an unknown concentration is determined from the Ct values and can be described either in relative or in absolute terms. In relative quantification, changes in the unknown target are expressed relative to a co amplified steady state (normally housekeeping) gene. Any variation in the presence (or expression) of the housekeeping gene can potentially mask real changes or show artificial changes in the abundance of the gene of interest. RT-Q-PCR amplifications can be conducted using either a one-step or a two-step reaction. In a one-step RT-Q-PCR, both the RT reaction and the Q-PCR are carried out successively in a single tube. The RNA is first reverse transcribed, with all resultant c DNA serving as templates in the subsequent Q-PCR amplification. In addition to the reduced risk of contamination and the convenience of setting up only a single reaction, a further advantage of this method is that all the resulting c DNA produced is used to quantify the target RNA sequence.

Quantification of early colonizer micro organisms in biofilm by Real Time PCR has been reported in several studies (Shemesh *et al*., 2007; Guilbaud *et al*., 2005). The expression of genes known to be involved in biofilm development at different concentration of glucose and sucrose by *S. mutans,* for comparison with the expression of genes in planktonic cells was also assessed with Real-time PCR (Shemesh *et al*., 2007). However, in *M. smegmatis* the role of iron in biofilm development and the gene involving the induction of iron was studied by Ojha and Hatfull (2007) with the help of expression analysis using Real Time PCR. Therefore, the above mentioned studies is expected to help in identifying important and transiently expressed genes for understanding the mechanism of biofilm formation with respect to ultrastructural and genomic analysis. Recently, Chern *et al*. (2015) reported qPCR for the rapid detection of *M. avium, M. intracellulare* and MAP can be used to provide data supporting drinking water biofilms as potential sources of human exposure. Study characterized two qPCR assays targeting partial 16S rRNA gene sequences of MA and MI and use these assays, along with two previously reported MAP qPCR assays (IS900 and Target 251), to investigate *Mycobacterium* occurrence in kitchen faucet biofilms. MA and MI qPCR assays demonstrated 100% specificity and sensitivity when evaluated against 18 non-MA complex, 76 MA and 17 MI isolates. Both assays detected approximately 1,000 cells from a diluted cell stock inoculated on a sampling swab 100% of the time. DNA analysis by qPCR indicated that 35.3, 56.9 and 11.8% of the 51 kitchen faucet biofilm samples contained MA, MI and MAP, respectively. The qPCR can be alternative to culture for detection of MA, MI and MAP in microbiologically complex samples.

Potential for new therapies: More work is needed to fully elucidate antibiotic resistance mechanisms in biofilms and develop new therapeutic strategies but we have enough evidence to make some observations and suggestions. Clearly, there are multiple resistance mechanisms that can act together. Antibiofilm therapies might have to thwart more than one mechanism simultaneously to be clinically effective. Heterogeneity is a common theme of these resistance mechanisms; micro-organisms in a biofilm exist in a broad spectrum of states. First, cells might be

exposed to different concentrations of antibiotic depending on their spatial location. Second, gradients in the concentration of microbial nutrients and waste products crisscross the biofilm and alter the local environment, which leads to a broad range of growth rates of individual microbial cells. Third, a small proportion of cells in a bacterial biofilm might differentiate into a highly protected phenotypic state and coexist with neighbours that are antibiotic sensitive. The proliferation of states that arises when these three types of heterogeneity are crossed means that any given antimicrobial agent might be able to kill some of the cells in a biofilm but is unlikely to effectively target all of them. Most or all the antibiotics in current use were identified on the basis of their activity against growing cultures of individual cells. New screens of existing and potential antibiotics that select for activity against non-growing or biofilm cells might yield antimicrobial agents with clinical efficacy against biofilm infections. As genes that mediate biofilm resistance to antibiotics are identified and their gene products characterized, these will become targets for chemotherapeutic adjuvants that could be used to enhance the effectiveness of existing antibiotics against biofilm infections. Because biofilm resistance depends on aggregation of bacteria in multicellular communities, one strategy might be to develop therapies that disrupt the multicellular structure of the biofilm. If the multicellularity of the biofilm is defeated, the host defences might be able to resolve the infection and the efficacy of antibiotics might be restored. Potential therapies include enzymes that dissolve the matrix polymers of the biofilm (Nemoto *et al*., 2000), chemical reactions that block biofilm matrix synthesis (Yasuda *et al*., 1993) and analogues of microbial signalling molecules that interfere with cell-to-cell communication, required for normal biofilm formation (Parsek *et al*., 2000). As the genetic basis for biofilm development emerges, the gene products identified as required for multicellular colony formation will become a potential target for chemotherapy. In other words, we believe that treatment strategies will target the formation of multicellular structures rather than essential functions of individual cells. We will learn to treat the persistent infections associated with biofilms when the multicellular nature of microbial life is understood. Xiang *et al*. (2014), summarized the factors involved in the development and dispersal of mycobacterial biofilms, as well as underlying regulatory factors and inhibitors against biofilm to deepen our understanding of their development and to elucidate potential novel modes of action for future antibiotics. Key factors in biofilm formation identified as drug targets represent a novel and promising avenue for developing better antibiotics.

CONCLUSION

A combination of genetic and molecular techniques in conjunction with direct microscopic visualization, has been used to initiate investigations into the molecular mechanisms that control biofilm development. Based on these and earlier studies, biofilm formation can be viewed as a well-regulated developmental process that results in the formation of a complex community of organisms. Biofilm formation is not itself necessarily a virulence factor, because many non-tuberculosis organisms produce biofilms that cause disease. However, biofilm formation by certain pathogens appears to facilitate the survival of these pathogens in the environment and the host. This might be due to the accumulation and dispersal of a sufficient number of pathogens for an infective dose, which is not typically found in a bulk fluid. Additionally, the heterogeneous microenvironments that occur within biofilms might promote a differentiated population of phenotypic and genotypic variants of microorganisms that promises survival in the face of changing

environmental conditions and might also facilitate infection. We think that the investigation of biofilm development will yield insights into pathogenicity, virulence and the prevention of certain deadly mycobacterial infections.

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