

Emerging Concept and Technology on Mycobacterial Biofilm

Virendra Kumar^{1*}, Tarun Kumar Sachan² and UD Gupta³

¹National JALMA institute for leprosy and other mycobacterial Diseases, Indian Council of Medical Research, India

²Department of Electron Microscopy, National JALMA Institute for Leprosy and Other Mycobacterial Diseases, India

³Director in charge, National JALMA Institute for Leprosy and Other Mycobacterial Diseases, India

Abstract

Bacteria have a natural propensity to grow as sessile, matrix-encapsulated, multicellular communities called biofilms. Many Mycobacteria can develop biofilm, a multicellular structure largely combining bacteria and their Extracellular Polymeric Substances (EPS). Biofilms proceed through genetically programmed, distinct developmental stages signalled by intricate networks of communication among the constituent population and their environment. Biofilm-relevant infections are more persistent, resistant to most antibiotics, and more recalcitrant to host immunity. Non-Tuberculous Mycobacteria (NTM) are emergent pathogens whose importance in human health has been growing. *Mycobacterium tuberculosis*, the causative agent of tuberculosis, can develop biofilm, though whether *M. tuberculosis* can form biofilm within tuberculosis patients has yet to be determined. Most notable among these is an extraordinary level of tolerance to a variety of environmental stresses, including antibiotics. Although mycobacteria have long been observed to spontaneously form complex multicellular structures *in vitro*, it has only recently become apparent that these structures are not only formed through dedicated genetic pathways but are also tolerant to antibiotics. In this article, we review the recent advances in the understanding of mycobacterial biofilms *in vitro*. We further consider the possible linkage between biofilm-like lifestyles and characteristic persistence of mycobacterial infections against host-defence mechanisms as well as antibiotics.

Keywords: Biofilm; Mycobacteria; Ultra structural; Antibiotic resistance; Molecular; Gene regulation

Introduction

Mycobacterium tuberculosis, (*M. tuberculosis*) generally called M.TB or simply the tubercle bacillus, is a slightly. More than 60% of the patients were from China, India, the Russian Federation, Pakistan, and South Africa [1]. Species other than *M. tuberculosis* and *M. leprae* have been nominated "atypical mycobacteria" or "mycobacteria other than *M. tuberculosis*" (MOTT) in the past and are now called simply "Non-Tuberculous Mycobacteria" (NTM). The incidence of NTM infections surpasses that of TB infections in developed countries. Although infection may occur in virtually any organ, pulmonary infections are most common. *M. avium*, *M. kansasii*, and *M. abscessus* are the most frequently identified organisms causing lung disease. The isolation of an NTM organism does not necessarily equate with active infection; clinical, radiologic, and microbiologic parameters are all needed to

establish the diagnosis of infection. Eradication of disease with drug therapy requires prolonged combination therapy.

The authors concluded that the ability to switch from smooth non-cording to rough cording morphotypes may allow *M. abscessus* to make the transition between a colonizing phenotype and a more virulent invasive form. This is a very interesting hypothesis, supported by the findings who reported a case of acute respiratory failure involving a rough variant of *M. abscessus*. Another correlation can be made from these findings, between rough colonies and cording, since only rough colonies of *M. abscessus* formed microscopic cords, and cording *M. tuberculosis* and *M. marinum* strains displayed only rough colonies. *Mycobacterium* cell envelope researchers. This study improves our understanding of the biology of different species of the genus *Mycobacterium*, which could contribute to a better understanding of how *M. tuberculosis* and other mycobacteria cause disease. In the world more than 99% of bacteria survive as biofilms [2] and according to NIH report more than 65% of all human infections are associated with biofilms formation [3]. Biofilm is a microbial derived sessile community of bacteria in which bacteria are attached to the substratum and produce an Extracellular Polymeric Substance (EPS) [4]. Biofilm in bacteria give protection from a wide range of environmental challenges, such as UV exposure and metal toxicity [5] acid exposure [6] dehydration and salinity [7] phagocytosis and several antibiotics and antimicrobial agents [8]. It is approximated that the majority of medical infections are caused by bacterial biofilms that colonize either non-biological or biological surfaces [9]. 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 [10]. Biofilm is a microbial derived sessile community of bacteria in which bacteria are attached to the substratum and produce an Extracellular Polymeric Substance (EPS) 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 [11].

However, the formation of the extracellular matrix, the hallmark of biofilms, in *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. 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 [12]. Hence, SEM were used [13] 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

*Corresponding author: Virendra Kumar, Scientist 'D' & Head, Department of Electron Microscopy, National JALMA Institute for Leprosy and Other Mycobacterial Diseases, (ICMR) Tajganj Agra-282001, India, Email: vksjalma@gmail.com

Received: August 10 2017; Accepted: August 23 2017; Published: August 27 2017

morphology). The specific polysaccharide stain called Ruthenium red, coupling with osmium tetroxide fixative, was 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.

The growth of bacterial biofilms involves a developmental process that begins with surface attachment, followed by spreading, maturation and matrix synthesis [14]. This process may be affected by a variety of environmental factors [15] such as pH, iron, oxygen, ionic strength and temperature, and nutrient level. 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 planktonically growing counterparts. Biofilm infections are difficult to eliminate with antimicrobial treatment, and *in vitro* susceptibility tests show significant resistance of biofilm cells to killing [16,17]. Biofilms are highly resistant to antibiotics than planktonic cells. The planktonic cultures of Staphylococci clinical isolates have been found to be approximately 20-50 times more sensitive to antibiotics than their biofilms [18]. Likewise, biofilms of pathogenic *E. coli*, *P. aeruginosa* and Mycobacteria are 100-1000 times more tolerant to all tested antibiotics than their planktonic counterpart [19]. 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. 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 microarrays after studies [20] 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% to 38% 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* [21] *P. aeruginosa* [22] *B. Subtilis* [23] *V. Cholera* [24]. 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. *M. 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 [25]. 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 O22- and F2+ interacting to generate hydroxyl radicals in a Fenton's reaction [26]. 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.

Materials and Methods

Bacterial strains

The clinical isolates of mycobacterial species were taken for the study of mycobacterial biofilm. The *M. fortuitum*, and *M. tuberculosis* H37RV *M. smegmatis* were obtained from Repository Centre of NJIL

& OMD Agra and *M. avium* was obtained from Tuberculosis Research Centre Chennai. The Planktonic cell growths of *M. smegmatis*, *M. fortuitum*, *M. avium* and *M. tuberculosis* H37RV were made in Middle brook 7H9 broth supplemented with 0.05% Tween 80 and 2% glucose. For the development of biofilm of these mycobacterial species, firstly remove the tween 80 by two to three time washing with the media. The different conditions such as pH, temperature and growth supplement OADC enrichment as well as Sauton's and MB7H9 media were analysed. *M. smegmatis*, and *M. fortuitum* as fast growers and *M. avium*, and *M. tuberculosis* H37Rv as slow growers were studied for biofilm formation under different conditions. One loopful culture of these mycobacteria from the Lowenstein Jensen media (LJ) slopes was scraped and suspended in MB7H9 media and incubated till mid log phase growth. The mid log phase culture from these bottles was centrifuged at 8000 RPM for 5 minutes at 4°C. The pellets were washed with Sauton's medium and MB7H9 medium of different pH range to remove the Tween 80. These cultures were then diluted with MB7H9 and Sauton's and matched with 0.5 x McFarland standards (108 CFU/ml) and 1:10 serial dilutions were prepared.

Fourier transforms infrared (ftir) analysis

FTIR spectroscopy was carried out with ethanol precipitated and dried EPS samples. EPS was precipitated using three volumes of 100% cold ethanol and incubated in ice for 2 hour. The precipitates were centrifuged at 17500 xg for 20 minutes at 4°C and dried in an oven for overnight at 50°C to performed FTIR spectroscopy.

Protein concentration: The total protein concentration was measured by adding 12% Trichloroacetic acid (TCA) to EPS solution and the mixture was incubated on ice for 30 minutes and centrifuged at 15,000xg for 20 minutes. The TCA precipitates were then washed twice with 10 ml acetone and resuspended in 2ml of 2-N-Morpholinoethanesulfuric Acid (MES) buffer pH 5.0. The protein content was estimated using Bradford protein method with BSA as the calibration standard. The protein content was measured by reading out the absorbance at 595nm in nanophotometer (Implen, Germany).

DNA concentration: Total DNA content was estimated from EPS solution after extraction with 3 volumes of 100% cold ethanol. The mixture was incubated in ice for 2 hours and then DNA was recovered by centrifuging at 17,500xg for 20 minutes at 4°C. The DNA content was measured by reading out the absorbance at 260nm in nanophotometre.

Scanning Electron Microscopy (SEM): The mycobacterial biofilm developed in microtiter dish as described above were fixed in a solution of 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, overnight at 40C. The samples were rinsed once in the same buffer and dehydrated by increasing concentrations of ethanol (30%, 50%, 70%, 90% and 100%). The samples were dried in a fume hood were fixed on to stubs with conductive self-adhesive carbon tapes, coated with gold film sputtering and used for analysis with SEM (S3000- N). The ultrastructural picture shown by SEM described as thin, thick, and thicker of the biofilm.

Expression study using microarrays

Designing of DNA chip: To investigate the expression profile of 59 selected genes (related to biofilm formation in mycobacteria) in biofilm and planktonic cells of *M. tuberculosis* and *M. avium*, a DNA chip was designed at microarray facility, Department of microbiology and molecular biology, NJIL&OMD, Agra. The targeted genes were selected on the basis of functional classification of Mycobacterial genes and 70 mer oligos were design by using oligopicker software. Specificity of the designed oligos was checked by NCBI BLAST. The oligos were purchased from Bioserve Biotechnologies, Hyderabad India.

Real time PCR: The over expressed genes during microarray experiments, were further studied by Real-Time Reverse Transcription PCR assay and also their over expressions were reconfirmed in

M. tuberculosis MDR and *M. avium* isolates. RNA was extracted from biofilm cells developed under different conditions such as glucose, OADC and drug inducing. cDNAs were generated from the RNA samples for real time PCR and expression of selected genes were measured using the SYBR Green method. All gene expression values were normalised to housekeeping gene 16S rRNA.

Molecular identification: The identification of *M. smegmatis* MC2 155, *M. fortuitum*, *M. avium* and *M. tuberculosis* was done by PCR-RFLP techniques, (Figure 1) and molecular typing method such as spoligotyping and the octal code compared with spol DB4 Data base (Figure 2).

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. qRT-PCR has a large dynamic range and may be used to verify gene expression data obtained from microarrays. The microarray analysis of biofilms [27] 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 [28] 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 planktonically. 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.

Results and Discussion

Formation and composition of biofilm

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.

Biofilms when consists primarily of microbial cells and Extracellular Polymeric Substance (EPS) may account for 50% to 90% of the total organic carbon of biofilms. Biofilm is “city of microbes” [29] 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 [30]. 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 [31]. The lipid, found in biofilm is Glycopeptidolipids (GPLs) are a class of glycolipids produced by several (NTM) such as *M. abscessus* and *M. chelonae*, [32].

Apart from being the source of energy, lipids constitute the integral part of cell wall and cytoplasmic membranes and thus, in turn, regulate various cellular processes. Recent demonstration of the close interrelationship between lipids and pathogenesis of mycobacteria portends new insight into the lipid metabolism of this organism. Phospholipids represented by phosphatidylethanolamine, phosphatidylinositol mannosides and cardiolipins constitute about 25 % of total lipids and 3-7 % of total dry weight of mycobacteria. A number of studies suggested that GPLs may play an important role in these processes. Interestingly, [33] 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. *M. 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. 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

Restriction analysis of *hsp65* gene in *M. tuberculosis*, *M. smegmatis*, *M. fortuitum*

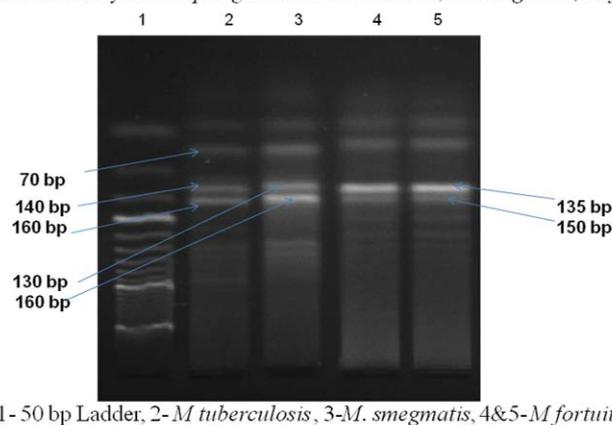


Figure 1: Identification of various Mycobacterium by PCR-RFLP techniques.

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 [34]. An association between biofilm formation and virulence has also been observed and 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 [35]. These mutants were defective in their GPL biosynthetic pathways and suggesting a role for GPLs in epithelial cell invasion as well as in biofilm formation. The mycobacterial cell envelope has an extremely extraordinary structure. 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. 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 [36]. 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.

Eps-key factor of biofilm formation

the production of EPS by bacteria upon adhesion to surfaces has indeed been hailed as the “hallmark” of biofilm formation [37] 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 [38] 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 visualised using CLSM (for more details of the specific probes for each component, please refer to [39]). CLSM has played an important role in shaping our understanding of the spatial organisation and formation of micro-domains within biofilms [40]. Fourier-Transformed Infrared (FTIR) spectroscopy is another popular non-destructive technique for monitoring time resolved EPS accumulation in biofilms (Figure 3). 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 biofilm directly on the attenuated total reflectance crystal [41] or by growing biofilms on surfaces of interest like stainless steel and plastics [42]. 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 visualised from



Figure 2: Identification of various Mycobacterium by spoligotyping and octal code.

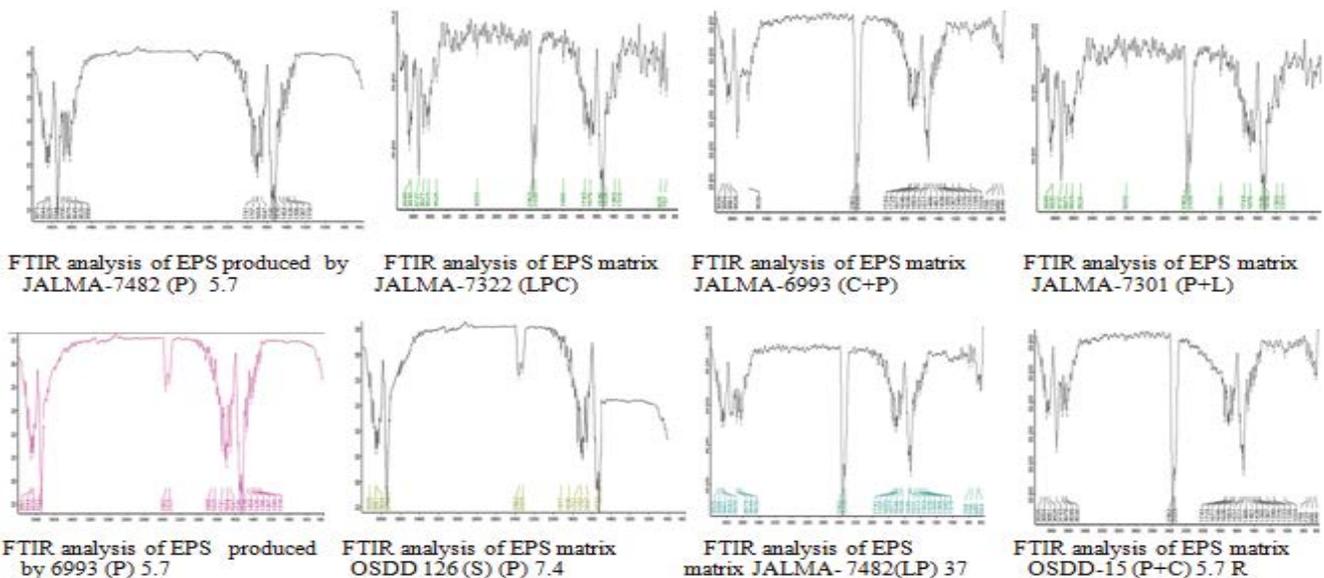


Figure 3: Biochemical analysis of biofilm by FTIR showing Protein, Carbohydrates and Glycopeptidolipid present in high amount.

CLSM than from reflectance micro-FTIR spectroscopy, still (Figure 3) FTIR can provide useful information about the functional groups in EPS that play an adhesive and cohesive role in the maintenance of biofilms [43]. FTIR spectroscopy of EPS extracted from disrupted biofilms has also been carried out [44]. 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 [45].

Although non-destructive, in situ monitoring techniques are available, the more commonly employed strategy is to analyse 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 [46]. A detailed biochemical analysis reveals that the polysaccharide components can either contain homopolysaccharides like cellulose in *Salmonella typhimurium* [47] or charged heteropolysaccharides that can either be polyanionic like in the case of alginate *Pseudomonas aeruginosa* [48] and colonic acid in *Escherichia coli* [48] or polycationic in the case of the intercellular adhesion of *Staphylococcus aureus* [49]. 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 [50]. 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 [51]. The biochemical significance of the immobilisation 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 [52]. The active role of enzymes like peptidases, polysaccharases and phosphatases has been confirmed within biofilms and these enzymes increase the bioavailability of nutrients in the surrounding environment [53]. 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 [54]. Generally, the production of sugar-binding peptides, lectins, is also thought to contribute to the structural integrity of the biofilms. 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. 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. 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 [55]. The outcome of such research has spurred investigations into novel surface coatings that reduce bio adhesion [56]. In addition to physicochemical characterisation, 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 [57]. 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. 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 [58]. Finally, both the desirable effects of biofilms such as environmental detoxification and the undesirable effects of biofilms such as biofouling of surfaces and insulation of the walls of heat exchangers are directly linked to the

sorptive properties of the matrix components. The stabilisation and concentration of extracellular enzymes within biofilms help the biofilms function as powerhouses of degradation of xenobiotics and organic polymers. On the other hand, the increased metal ion binding by the metallo proteins within biofilms contributes to the bio corrosion of surfaces. The colligative properties of the EPS polymers [59] insulate the walls of heat exchangers against convective heat transfer and reduce the efficiency of industrial processes.

Microtiter plate assay and analysis of mycobacterial biofilm

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 pseudomonads, *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.

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 [60]. The relative resistance of mycobacteria to most of the toxic heavy metals and oxyanions [61] 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. 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 [62]. Hence, experimental methods must be developed separately to measure the adherence and biofilm growth. Several examples of mycobacterial adaptation and existence was most notably survival as a consequence of exposure to anaerobiosis [63] starvation, temperature and elevated antibiotic disinfectant resistance of biofilm-grown cells [64]. 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 and *M. avium* cells grown in amoebae were more readily phagocytosed and were more virulent [65]. 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 additionally, 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. Other mycobacteria, specifically *M. kansasii*, *M. chelonae*, *M. fortuitum* and *M. phlei* [66] were also form biofilms on surfaces, including high density polyethylene and silastic rubber. It is likely that the high cell surface

hydrophobicity of mycobacteria contributes to biofilm formation. Such a predilection for attachment would also lead to colonization of catheter surfaces. Growth of biofilms by *M. Avium*, *M. tuberculosis* and numerous other species of mycobacteria, including *M. fortuitum*, *M. marinum* and *M. smegmatis* 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.

Ultrastructural analysis of mycobacterial biofilm

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. SEM represents a rapid and convenient means of assessing the pattern of colonization as well as screening samples for major bacterial morphotypes. 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. 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 per cent of its original volume [67]. Thus, care was taken in interpretation of the photomicrographs. No software has to date been developed to quantitatively analyse 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 [68]. The use of quantitative image analysis also allows for the determination of live/dead cell counts and their distribution within the biofilm. These along with concurrent investigations into the microbiological identification of bacterial species involved in biofilm formation warrant further study. The use of SEM to resolve the morphology and ultrastructure of bacteria and biofilms has been utilized in several other studies. 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 microcolonies 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. On the other hand, 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. 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. 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. The pH, temperature and nutrient composition are crucial factors for the growth of mycobacterial biofilm. *M. 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. After 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 addition, the presence of OADC *M. tuberculosis* produce more amount of biofilm (Figure 4).

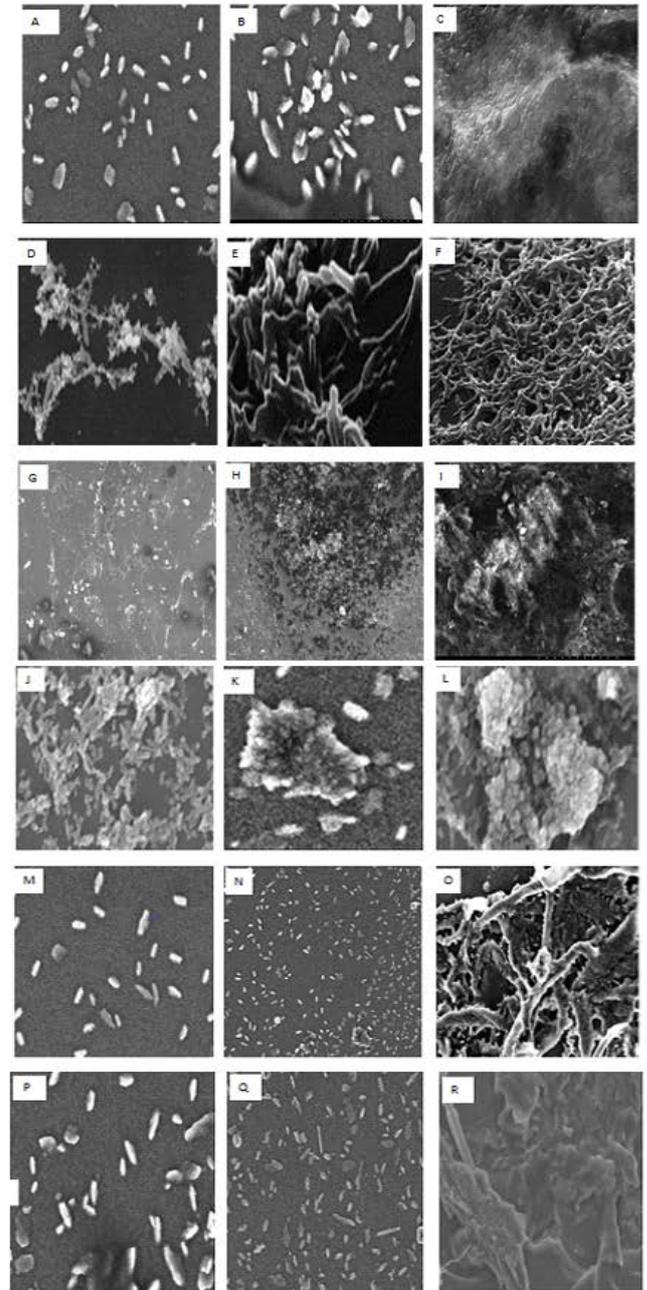


Figure 4: Scanning Electron micrographs showing various forms of ultrastructural analysis of mycobacterial biofilm in different conditions of Temperature, OADC, and Glucose studied. *M. smegmatis* (A,B,C), *M. fortuitum* (D,E,F); *M. avium* (G,H,I); *M. tuberculosis* H37Rv, (J,K,L) *M. tuberculosis* MDR isolates. (M,N,O), And *M. tuberculosis* sensitive isolates. (P,Q,R). Note some of them are abundant EPS rich aggregates that are apparent in biofilms.

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. Various nosocomial infections [69] such as those related to the use of central venous catheters, urinary catheters, prosthetic heart valves and orthopaedic devices 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; 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 [70]. There is no generic barrier to the diffusion of solutes the size of antibiotics through the biofilm matrix, which is mostly water [71]. 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. pneumoniae* but not a biofilm formed by the lactamase-positive wildtype strain of the same micro-organism. In the wild strain 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. These positively charged agents bind to negatively charged polymers in the biofilm matrix [72]. 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 [73]. 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, which could directly antagonise the action of an antibiotic. Aminoglycoside antibiotics are clearly less effective against the same micro-organism in anaerobic than in aerobic conditions [74]. 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 [75]. This alternative possibility is strengthened by direct experimental visualisation of metabolically inactive zones within continuously fed biofilms. Additionally, the osmotic environment within a biofilm might be altered, leading to induction of an osmotic stress response. Such a response could contribute to antibiotic resistance by changing the relative proportions of pouring 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 [76]. Additionally, most bacteria in the biofilm, but not all, are rapidly killed by antibiotics. 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.

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. This process is accompanied by changes in gene expression profiles, and these have been described for several prokaryotes, including *E. coli*, *P. aeruginosa*, *Bacillus subtilis* [77], *Vibrio cholerae*, and *Staphylococcus aureus*. 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. Second, there is no single core biofilm regular present in these bacteria, although induction of stress responses is common. 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. The first descriptions of specific genes that are up- or down-regulated in biofilm bacteria were made using transcriptional lac Z reporter-gene fusions [78] and led to the belief that bacterial attachment initiates the expression of a set of genes that culminates in a biofilm phenotype. 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. 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 synthesis gene contributes to synthesis of the immunomodulatory phenolic glycolipids. The five domains of Pks1, annotated as acyl transferase, dehydrogenase, enoyl reductase, 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. 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 (mycP1), and complex lipid biosynthesis (*pks1*). 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. 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 *lsr2* 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. 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 lipooligosaccharides [79].

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 *tntp* and *mps* revealed their involvement in biofilm formation upon seeding of the bacterium on Polyvinyl Chloride (PVC) plates. The *tntp* gene is highly conserved between *M. smegmatis* and *M. avium*, with both organisms having genes encoding one small (*tntpA*) and two large (*tntpB* and *tntpC*) 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 [80]. 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 tmpC (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. The genomic differences, described [81] 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 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. These gradients make it difficult to replicate the environmental conditions affecting biofilm cells in a single culture of planktonic cells. The expression profiling was used 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 [82] and for directing the development of endospores. Furthermore, 40 genes responsive to glucose concentration were found in the study by concluded that glucose inhibits biofilm formation through the catabolite control protein CcpA. 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 CsrA; disruption of *csrA* significantly decreased biofilm formation.

Real time pcr analysis for quantitative gene expression in biofilm

the 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. 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 nm and 521 nm, respectively, and are compatible for use with real-time cycler [83]. 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 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. 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 [84]. 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. However, in *M. smegmatis* the role of iron in biofilm development and the gene involving the induction of iron was studied by 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.

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. Anti-biofilm 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 nongrowing 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 characterised, 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, [85] chemical reactions that block biofilm matrix synthesis and analogues of microbial signalling molecules that interfere with cell-to-cell communication, required for normal biofilm formation. 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.

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.

Acknowledgement

We are highly thankful for funding from the Department of Biotechnology Govt. of India Grant No. BT/PR10435/Med/30/85/2008.

References

- World Health Organization Report. 2012. Global tuberculosis control - surveillance, planning, financing for TB control: WHO Geneva, Switzerland.
- Costerton JW, Stewart PS, Greenberg EP. 1999. Bacterial biofilms: a common cause of persistent infections. *Science*. 284: 1318-1322.
- Spoering AL, Lewis K. 2001. Biofilms and planktonic cells of *Pseudomonas aeruginosa* have similar resistance to killing by antimicrobials. *Journal of Bacteriology*. 183: 6746-6751.
- Donlan RM and Costerton JW. 2002. Biofilms: Survival mechanisms of clinically relevant microorganisms. *Clinical microbiology reviews*. 15: 167-193.
- Teitzel GM, Parsek MR. 2003. Heavy metal resistance of biofilm and planktonic *Pseudomonas aeruginosa*. *Applied and Environmental Microbiology*. 69: 2313-2320.
- McNeill K, Hamilton IR. 2003. Acid tolerance response of biofilm cells of *Streptococcus mutans*. *FEMS Microbiology Letter*. 221: 25-30.
- Debar L ME, Lemoine J, Gelle MP, Jacquelin LF, Choisy C. 2000. Evaluation of biohazards in dehydrated biofilms on foodstuff packaging. *International Journal of Food Microbiology*. 55: 239-234.
- Mah TF, O'Toole GA. 2001. Mechanisms of biofilm resistance to antimicrobial agents. *Trends in Microbiology*. 9: 34-39.
- Costerton JW, Lewandowski Z, Caldwell D, Korber D, Lappin-Scott HM. 1995. Microbial biofilms. *Annual Review of Microbiology*. 49: 711-745.
- Bryers JD. 2008. Medical Biofilms. *Biotechnology and Bioengineering*. 100: 1-18.
- Sachan T, Kumar V, Singh S, Gupta S, Chaubey KK, Jayaraman S, et al. 2015. Chemical and Ultrastructural Characteristics of Mycobacterial Biofilms. *Asian J Ani Vet Adv*. 10: 592-622.
- Kumar V, Sachan T, Sharma P and Rawat KD. 2014. Ultrastructural morphologic changes in mycobacterial Biofilm in different extreme conditions. *Ultra Pathol*. 1-11.
- Jones HC, Roth IL, Saunders WM. 1969. Electron microscopic study of a slime layer. *Journal of Bacteriology*. 99: 316-325.
- Sukontapatipark W, El-Agroudi MA, Selliseth NJ, Thunold K, Selvig KA. 2001. Bacterial colonization associated with fixed orthodontic appliances. A scanning electron microscopy study. *European Journal of Orthodontics*. 23: 475-484.
- O'Toole GA, Kolter R. 1998. The initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. *Molecular Microbiology*. 28: 449-461.
- Gilbert P, Das J, Foley I. 1997. Biofilm susceptibility to antimicrobials. *Advances in Dental Research*. 11: 160-167.
- Kumar V, Nishant Sharma, Sonali Nagar. 2016. Effect of drugs on development of biofilm in *M. tuberculosis* H37 Rv and sensitive isolates. *Ind Res J Genet Biotech*. 8: 120-126.
- Saginur R, Stdenis M, Ferris W, Aaron SD, Chan F, Lee C, et al. 2006. Multiple combination bactericidal testing of staphylococcal biofilms from implant-associated infections. *Antimicrobial Agents and Chemotherapy*. 50: 55-61.
- Schembri MA, Kjaergaard K, Klemm P. 2003. Global gene expression in *Escherichia coli* biofilms. *Molecular Microbiology*. 48: 253-267.
- Whiteley M, Bangera MG, Bumgarner RE, Parsek MR, Teitzel GM, Lory S, et al. 2001. Gene expression in *Pseudomonas aeruginosa* biofilms. *Nature*. 413: 860-864.
- Ren D, Bedzyk LA, Setlow P, Thomas SM, Ye RW and Wood TK. 2004. Gene expression in *Bacillus subtilis* surface biofilms with and without sporulation and the importance of yveR for biofilm maintenance. *Biotechnology and Bioengineering*. 86: 344-364.
- Waite RD, Papakonstantinou A, Littler E and Curtis MA. 2005. Transcriptome analysis of *Pseudomonas aeruginosa* growth: comparison of gene expression in planktonic cultures and developing and mature biofilms. *Journal of Bacteriology*. 187: 6571-6576.
- Waite RD, Paccanaro A, Papakonstantinou A, Hurst JM, Saqi M, Littler E, et al. 2006. Clustering of *Pseudomonas aeruginosa* transcriptomes from planktonic cultures, developing and mature biofilms reveals distinct expression profiles. *BMC Genomics*. 7: 162.
- Moorthy S and Watnick PI. 2005. Identification of novel stage-specific genetic requirements through whole genome transcription profiling of *Vibrio cholerae* biofilm development. *Molecular Microbiology*. 57: 1623-1635.
- Ojha A, Hatfull GF. 2007. The role of iron in *Mycobacterium smegmatis* biofilm formation: the exochelin siderophore is essential

- in limiting iron conditions for biofilm formation but not for planktonic growth. *Molecular Microbiology*. 66: 468-483.
26. Andrews SC, Robinson AK, Rodriguez-Quinones F. 2003. Bacterial iron homeostasis. *FEMS Microbiology Review*. 27: 215-237.
27. Williams I, Venables WA, Lloyd D, Paul F, Critchley I. 1997. The effects of adherence to silicone surfaces on antibiotic susceptibility in *Staphylococcus aureus*. *Microbiology*. 143: 2407-2413.
28. Recht J, Martinez A, Torello S and Kolter R. 2000. Genetic analysis of sliding motility in *Mycobacterium smegmatis*. *Journal of Bacteriology*. 182: 4348-4351.
29. Watnick P and Kolter R. 2000. Biofilm, city of microbes. *Journal of Bacteriology*. 182: 2675-2679.
30. Flemming HC and Wingender J. 2002. Extracellular polymeric substances: structure, ecological functions, technical relevance. Bitton G, editor. In: *Encyclopedia of environmental microbiology*. Wiley, New York. 3: 1223-1231.
31. Ojha AK, Trivelli X, Guerardel Y, Kremer L, Hatfull GF. 2010. Enzymatic hydrolysis of trehalose dimycolate releases free mycolic acids during mycobacterial growth in biofilms. *Journal of Biological Chemistry*. 285: 17380-17389.
32. Ripoll F, Deshayes C, Pasek S, Laval F, Beretti JL, Biet F, et al. 2007. Genomics of glycopeptidolipid biosynthesis in *Mycobacterium abscessus* and *Mycobacterium chelonae*. *BMC Genomics*. 8: 114.
33. Martinez A, Torello S and R Kolter. 1999. Sliding motility in mycobacteria. *Journal of Bacteriology*. 181: 7331-7338.
34. Kolk AH, Evers R, Groothuis DG, Gilis H, Kuijper S. 1989. Production and characterization of monoclonal antibodies against specific serotypes of *Mycobacterium avium* and the *Mycobacterium avium-Mycobacterium intracellulare, Mycobacterium scrofulaceum* complex. *Infection and Immunity*. 57: 2514-2521.
35. Yamazaki Y, Danelishvili L, Wu M, Macnab M, Bermudez LE. 2006. *Mycobacterium avium* genes associated with the ability to form a biofilm. *Applied and Environmental Microbiology*. 72: 819-825.
36. Brennan PJ. 2003. Structure, function, and biogenesis of the cell wall of *Mycobacterium tuberculosis*. *Tuberculosis (Edinb)*. 83: 91-97.
37. Stoodley LH, Brun OS, Polshyna G, Barker LC. 2006. *Mycobacterium marinum* biofilm formation reveals cording morphology. *FEMS Microbiology Letter*. 257: 43.
38. Neu TR, Manz B, Volke F, Dynes JJ, Hitchcock AP, Lawrence JR. 2010. Advanced imaging techniques for assessment of structure, composition and function in biofilm systems. *FEMS Microbiol Ecol*. 72: 1-21.
39. Adav SS, Lin JCT, Yang Z, Whiteley CG, Lee DJ, Peng XF, et al. 2010. Stereological assessment of extracellular polymeric substances, exo-enzymes, and specific bacterial strains in bio aggregates using fluorescence experiments. *Biotechnol Adv*. 28: 255-280.
40. Lawrence JR, Swerhone GDW, Kuhlicke U, Neu TR. 2007. *In situ* evidence for microdomains in the polymer matrix of bacterial microcolonies. *Can J Microbiol*. 53: 450-458.
41. Delille A, Quiles F, Humbert F. 2007. In situ monitoring of the nascent *Pseudomonas fluorescens* biofilm response to variations in the dissolved organic carbon level in low-nutrient water by attenuated total reflectance-Fourier transform infrared spectroscopy. *Appl Environ Microbiol*. 73: 5782.
42. Pink J, Smith-Palmer T, Chisholm D, Beveridge TJ, Pink DA. 2005. An FTIR study of *Pseudomonas aeruginosa* PAO1 biofilm development: interpretation of ATR-FTIR data in the 1500–1180 cm⁻¹ region. *Biofilms*. 2: 165-175.
43. Ojeda JJ, Romero-Gonzalez ME, Bachmann RT, Edyvean RGJ, Banwart SA. 2008. Characterization of the cell surface and cell wall chemistry of drinking water bacteria by combining XPS, FTIR spectroscopy, modelling, and potentiometric titrations. *Langmuir*. 24: 4032-4040.
44. Eboigbodin KE, Ojeda JJ, Biggs CA. 2007. Investigating the surface properties of *Escherichia coli* under glucose controlled conditions and its effect on aggregation. *Langmuir*. 23: 6691-6697.
45. Ivleva NP, Wagner M, Horn H, Niessner R, Haisch C. 2009. Towards a nondestructive chemical characterization of biofilm matrix by Raman microscopy. *Anal Bioanal Chem*. 393: 197-206.
46. Sheng GP, Yu HQ, Li XY. 2010. Extracellular polymeric substances (EPS) of microbial aggregates in biological wastewater treatment systems: a review. *Biotechnol Adv*. 28: 882-894.
47. Zogaj X, Nimtz M, Rohde M, Bokranz W, Romling U. 2001. The multicellular morphotypes of *Salmonella typhimurium* and *Escherichia coli* produce cellulose as the second component of the extracellular matrix. *Mol Microbiol*. 39: 1452-1463.
48. Evans LR, Linker A. 1973. Production and characterization of the slime polysaccharide of *Pseudomonas aeruginosa*. *J Bacteriol*. 116: 915-924.
49. Gotz F. 2002. Staphylococcus and biofilms. *Mol Microbiol*. 43: 1367-1378.
50. Biggs CA, Ford AM, Lant PA. 2001. Activated sludge flocculation: direct determination of the effect of calcium ions. *Water Sci Technol*. 43: 75.
51. Flemming HC, Wingender J. 2010. The biofilm matrix. *Nat Rev Microbiol*. 8: 623-633.
52. Mayer C, Moritz R, Kirschner C, Borchard W, Maibaum R, Wingender J, et al. 1999. The role of intermolecular interactions: studies on model systems for bacterial biofilms. *Int J Biol Macromol*. 26: 3-16.
53. Romani AM, Fund K, Artigas J, Schwartz T, Sabater S, Obst U. 2008. Relevance of polymer matrix enzymes during biofilm formation. *Microbiol Ecol*. 56: 427-436.
54. Branda SS, Chu F, Kearns DB, Losick R, Kolter R. 2006. A major protein component of the *Bacillus subtilis* biofilm matrix. *Mol Microbiol*. 59: 1229-1238.
55. Neu TR, Marshall KC. 1990. Bacterial polymers: physicochemical aspects of their interactions at interfaces. *J Biomat Appl*. 5: 107-133.
56. Yuan SJ, Liu CK, Pehkonen SO, Bai RB, Neoh KG, Ting YP, et al. 2009. Surface functionalization of Cu-Ni alloys via grafting of a bactericidal polymer for inhibiting biocorrosion by *Desulfovibrio desulfuricans* in anaerobic seawater. *Biofouling*. 25: 109-125.
57. Kearns DB, Chu F, Branda SS, Kolter R, Losick R. 2005. A master regulator for biofilm formation by *Bacillus subtilis*. *Mol Microbiol*. 55: 739-749.
58. Gohar M, Kstad OA, Gilois N, Sanchis V, Kolsto AB, Lereclus D. 2002. Two-dimensional electrophoresis analysis of the extracellular proteome of *Bacillus cereus* reveals the importance of the PlcR regulon. *Proteomics*. 2: 784-791.
59. Keiding K, Wybrandt L, Nielsen PH. 2001. Remember the water- a comment on EPS colligative properties. *Water Sci Technol*. 43: 17.
60. Rodgers MR, Blackstone BJ, Reyes AL and Covert TC. 1999. Colonisation of point of use water filters by silver resistant non-tuberculous mycobacteria. *Journal of Clinical Pathology*. 52: 629.

61. Falkinham JO. 2002. Nontuberculous mycobacteria in the environment. *Clinics in Chest Medicine*. 23: 529-551.
62. Stoodley HL, Keevil CW and Lappin-Scott HM. 1998. *Mycobacterium fortuitum* and *Mycobacterium chelonae* biofilm formation under high and low nutrient conditions. *Journal of applied microbiology symposium supplement*. 85: 60-69.
63. Dick T, Lee BH and Murugasu-Oei B. 1998. Oxygen depletion induced dormancy in *Mycobacterium smegmatis*. *FEMS Microbiology Letter*. 163: 159-164.
64. Steed KA and Falkinham JO. 2006. Effect of growth in biofilms on chlorine susceptibility of *Mycobacterium avium* and *Mycobacterium intracellulare*. *Applied and Environmental Microbiology*. 72: 4007-4100.
65. Cirillo JD, Falkow S, Tompkins LS and Bermudez LE. 1997. Interaction of *Mycobacterium avium* with environmental amoebae enhances virulence. *Infection and Immunity*. 65: 3759-3767.
66. Bardouniotis E, Huddleston W, Howard C, Olson ME. 2001. Characterization of biofilm growth and biocide susceptibility testing of *Mycobacterium phlei* using the MBEC™ assay system. *FEMS Microbiology Letters*. 203: 263-267.
67. Fischer W, Han Bel I, Paradies HH. 1998. First result of microbial induced corrosion of copper pipes. sequence CAC Tiller K, editor. In: microbial corrosion proceedings of the 1st EFC workshop sintra Portugal. 300-327.
68. Slayter EM, Slayter HS. 1992. Light and electron microscopy. Cambridge university press.
69. Arweiler NB, Hellwig E, Sculean A, Hein N, Auschill TM. 2004. Individual vitality pattern of in situ dental biofilms at different locations in the oral cavity. *Carries Research*. 38: 442-447.
70. Stewart PS. 1996. Theoretical aspects of antibiotic diffusion into microbial biofilms. *Antimicrobial Agents and Chemotherapy*. 40: 2517-2522.
71. Kumar V, Goyal J, Bhardwaj N. 2016. Drug sensitivity assay on development of biofilm in Non-tuberculosis Mycobacteria. *Ind Res J Genet & Biotech*. 8: 101-111.
72. Gordon CA, Hodges NA, Marriott C. 1988. Antibiotic interaction and diffusion through alginate and exopolysaccharide of cystic fibrosis derived *Pseudomonas aeruginosa*. *Journal of Antimicrobial Chemotherapy*. 22: 667-674.
73. de Beer D, Stoodley P, Roe F, Lewandowski Z. 1994. Effects of biofilm structure on oxygen distribution and mass transport. *Biotechnology and Bioengineering*. 43: 1131-1138.
74. Zhang TC, Bishop PL. 1996. Evaluation of substrate and pH effects in a nitrifying biofilm. *Water Environment Research*. 68: 1107-1115.
75. Tuomanen E, Cozens R, Tosch W, Zak O, Tomasz A. 1986. The rate of killing of *Escherichia coli* by beta lactam antibiotics is strictly proportional to the rate of bacterial growth. *Journal of general microbiology*. 132: 1297-1304.
76. Cochran WL, McFeters GA, Stewart PS. 2000. Reduced susceptibility of thin *Pseudomonas aeruginosa* biofilms to hydrogen peroxide and monochloramine. *J Appl Microbiol*. 88: 22-30.
77. Stanley NR, Britton RA, Grossman AD, Lazazzera BA. 2003. Identification of catabolite repression as a physiological regulator of biofilm formation by *Bacillus subtilis* by use of DNA microarrays. *Journal of Bacteriology*. 185: 1951-1957.
78. Davies DG, Charabarty AM, Geesey GG. 1993. Exopolysaccharide production in biofilms: substratum activation of alginate gene expression by *Pseudomonas aeruginosa*. *Applied and Environmental Microbiology*. 59: 1181-1186.
79. Ortalo-Magne A, Lemassu A, Laneelle MA, Bardou F, Silve G, Gounon P, et al. 1996. Identification of the surface exposed lipids on the cell envelopes of *Mycobacterium tuberculosis* and other mycobacterial species. *Journal of Bacteriology*. 178: 456-461.
80. Jacobe HB, McConville MJ, Haites RE, Kovacevic S, Coppel RL. 1999. Identification of a peptide synthetase involved in the biosynthesis of glycopeptidolipids of *Mycobacterium smegmatis*. *Molecular Microbiology*. 33: 1244-1253.
81. Krzywinska E, Schorey JS. 2003. Characterization of genetic differences between *Mycobacterium avium* subsp. *avium* strains of diverse virulence with a focus on the glycopeptidolipid biosynthesis cluster. *Veterinary Microbiology*. 91: 249-264.
82. Piggot PJ and Hilbert DW. 2004. Sporulation of *Bacillus subtilis*. *Current Opinion in Microbiology*. 7: 579-586.
83. Smith CJ, Osborn AM. 2009. Advantages and limitations of quantitative PCR (Q-PCR)-base approaches in microbial ecology. *FEMS Microbiol Ecology*. 67: 6-20.
84. Shemesh M, Tam A and Steinberg D. 2007. Expression of biofilm-associated genes of *Streptococcus mutans* in response to glucose and sucrose. *Journal of Medical Microbiology*. 56: 1528-1535.
85. Nemoto K, Hirota K, Ono T, Murakami K, Murakami K, Nagao D, et al. 2000. Effect of varidase (streptokinase) on biofilm formed by *Staphylococcus aureus*. *Chemotherapy*. 46: 111-115.