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Effect of drugs on development of biofilm in *M.tuberculosis* H37 Rv and sensitive isolates

V. Kumar[1], Nishant Sharma[2] and Sonali Nagar[3]

[1] National JALMA Institute for Leprosy & Other Mycobacterial Diseases, (ICMR), Tajganj, Agra-282001
[2.3] Department of Biotechnology, Noida International University, Noida

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Abstract

A biofilm is an assemblage of microbial cells that is irreversibly associated with a surface and enclosed in a matrix of primarily polysaccharide material. However, our knowledge about the diverse components of extracellular matrix is still very limited, with almost nothing being known about the EPS of mycobacteria. The current perception is that multiple mechanisms operate in a concerted strategy of the resistance to antimicrobial reagents within a single biofilm community. Although the following mechanisms are partially evidential but still speculative, one important basis is the mechanisms depend on the multicellularity and cooperation between differentiated cells within the structure. First, the biofilm structure provides the chemical barriers. This mechanism may be more relevant for antimicrobial reagent that are bound or neutralized by the EPS substances Recently, there have been found cells of another distinct phenotype called persisters. Biofilm formation in slow growers mycobacterial specie *M. tuberculosis* sensitive and resistant isolates and H37Rv were taken for evaluating the effect of drugs on biofilm formation. Selected mycobacterial isolates were taken to observed the effect of first line and second line drugs on planktonic cells and mycobacterial. REMA plate method was used for observed the effect of drug on planktonic cells. Crystal violet microtiter plate assay method was used for observed the effect of drug on biofilm formation. The biofilm of *M.tuberculosis* H37Rv are capable of growing at higher drug concentrations (i.e. have higher MICs) than suspension cultures: the MIC of selected drug concentration that inhibited exponentially growing biofilm was found to be higher than the MIC for planktonic culture. Hence, our work provides the basis for a genetic dissection of the biofilm-associated drug resistance phenomenon uncovered in this work biofilm cells.

Keywords: *Biofilm, Antimycobacterial, Drug resistance, Ultrastructure, MIC*

Introduction

Mycobacterium means the fungus like bacteria. Two important species of genus *Mycobacterium* that cause human diseases, namely tuberculosis and leprosy, are *Mycobacterium tuberculosis* and *Mycobacterium leprae* respectively. In 2011, there were an estimated 8.7 million new cases of TB and 1.4 million people died from TB geographically and approximately 2 million were estimated to have occurred in India. It is estimated that about 40% of Indian population is infected with TB bacillus. It has been estimated that there were 310,000 incident cases of multidrug-resistant tuberculosis, caused by organisms resistant to at least Isoniazid and Rifampicin, among patients who were reported to have tuberculosis in 2011. More than 60% of these patients were in China, India, the Russian Federation, Pakistan, and South Africa. *M. tuberculosis* and many of the non tuberculosis mycobacteria such as *M. avium*. *M intracellulare*

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(Falkinham *et al.,* 2001), *M. fortuitum* **(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. A biofilm is an assemblage of microbial cells that is irreversibly associated with a surface and enclosed in a matrix of primarily polysaccharide material. Biofilms are composed primarily of microbial cells and exopolymeric substance (EPS). However, our knowledge about the diverse components of extracellular matrix is still very limited, with almost nothing being known about the EPS of mycobacteria. In contrast, most bacteria produced robust biofilms, mycobacteria do not produce exopolysaccharides. Given the important role of short-chain mycolic acids in developing the architecture of *Mycobacterial* biofilm and our observation that the lack of these mycolic acids coincides with defective extracellular matrix formation, it seems reasonable to suspect that mycolic acids may contribute to the process of matrix formation. The chemical components of the extracellular matrix can be polysaccharides, nucleic acids and proteins, extracellular DNA and possibly even the debris left behind by the dead cells **(Flemming *et al.,* 2000; Sutherland, 2001; Webb *et al.,* 2003; Whitchurch *et al.,* 2002; Yarwood *et al.,* 2004).** Human tuberculosis infections typically require treatment with multiple antibiotics for 6–9 months to avoid re-emergence of the disease **(Hopewell *et al*., 2006).** But why such extended treatment are required are not completely understood. Current antimicrobial strategies have been developed in order to control acute infections caused by planktonic bacteria. Prolonged and high-dose antibiotic therapy and the elimination of infected foreign body material are key components of the strategies. These strategies often fail to control bacteria in the biofilm mode of growth. When considering about biofilm community, a logical assumption is that any cells within the biofilm experience a slightly different environment from other cells within the same biofilm community. The current perception is that multiple mechanisms operate in a concerted strategy of the resistance to antimicrobial reagents within a single biofilm community. Although the following mechanisms are partially evidential but still speculative, one important basis is the mechanisms depend on the multicellularity and cooperation between differentiated cells within the structure. First, the biofilm structure provides the chemical barriers. This mechanism may be more relevant for antimicrobial reagent that are bound or neutralized by the EPS substances Recently, there have been found cells of another distinct phenotype called persisters. These cells might express a biofilm-specific resistance phenotype induced by the particular environmental factors. This concept is particularly interesting because the control of key biofilm associated genes would offer ways to overcome this resistance. In the case of mycobcacteria the effect of drug resistance for biofilm cell growths are not completely understood therefore the current studies carried out to affects antimicrobial resistance for the formation of a useful mono species biofilm, excluding possible variables such as interspecies interactions and communication which are often observed.

Material Methods:

(i) Biofilm formation by slow growing mycobacterial species:

Slow growers mycobacterial specie *M. tuberculosis* sensitive and resistant isolates and H37Rv were taken for evaluating the effect of drugs on biofilm formation. One loopful culture of *M. tuberculosis* from the LJ slopes were scraped and suspended in 300 µl of Middlebrook's 7H9 media with 0.5% (vol/vol) glycerol, 0.05% Tween 80 and 10% (vol/vol) OADC in a dilution bottle and vortexed to break clumps. The suspension in dilution bottle was made up to 5 ml with Middlebrook's 7H9 broth medium containing 0.5% (vol/vol) glycerol, 0.05% Tween 80 and 10% (vol/vol) OADC and incubated at 37^0C up to 7 days. After 7 days, 1 ml. of primary culture was used to inoculate 9 ml of Middlebrook's 7H9 media to prepare secondary culture. The secondary cultures were incubated for 7 days at 37°C. Culture from these bottles was centrifuged at 8000 RPM for 5 minutes at 4°C in oak ridge tube. Subsequently, the pellet was washed with different media such as Sauton media and MB7H9 media adjusted to wide pH range from 4.7, 5.7 and 7.4 as well as other condition which is glucose Oleic

acid, albumin, dextrose, catalase (OADC) and temperature for mycobacterial clinical isolates(MDR and sensitive) and H37 Rv to remove the Tween 80. The culture was diluted and matched with 0.5 × McFarland standard (10^8 CFU/ mL) and 1:10 serial dilutions were prepared.

Biofilm formation was determined as described previously (**O'Toole *et al.,* 2000**) by seeding 200 µl Sauton or 7H9 broth containing 1×10^7 bacteria in a PVC plastic 96-well microtitre plate(Axygen). The assay was determined by observing the ability of cells to adhere to the wells as reported previously **(Limia *et al.,* 2001**). After inoculation, plates were incubated at room temperature for two week and four week then 250µl of 1% crystal violet solution was added to each well (the cells stains by dye but not the PVC). The plates were incubated at room temperature for 30 minutes, rinsed vigorously four times with water, blotted on tissue paper and scored for biofilm formation. The crystal violet was solubilized in 200µl 95% ethanol and then 125µl was transferred to another microtiter dish.

(ii) Drug efficacy by REMA plate method for biofilm cells:

Selected mycobacterial isolates were taken to observed the effect of first line and second line drugs on planktonic cells and mycobacterial biofilm cells. REMA plate method was used for observed the effect of drug on planktonic cells. Crystal violet microtiter plate assay method was used for observed the effect of drug on biofilm formation.

To determine the MIC (Minimal Inhibitory Concentration) of first line drug such as Streptomycin, Isoniazid, Rifampicin, Ethambutol, Pyrazinamide, the sensitive isolates and H37 Rv were taken to observe the effects of second line drug such as Ofloxacin, Kanamycin, Ethanamide and Moxifloxacin by REMA (**Resazurin Microtiter Assay Plate**) method. Briefly, 100 µl volume of Middlebrook 7H9 broth **(Difco, USA**) was dispensed in each well of a 96-well culture plate (**Nunc, Denmark**). Two fold increasing concentrations of selected drugs 1-256 µg/ml Streptomycin, 0.0156 to 4 µg/ml Isoniazid, 0.25 to 64 µg/ml of Rifampicin, 0.093 to 24 µg/ml Ethambutol, 0.78 to 200 µg/ml Pyrazinamide were taken to determine MIC in *M. smegmatis*, *M. fortuitum*, H37 Rv, and sensitive isolates. Concentration 0.125-16 µg/ml Ofloxacin, 2-256 µg/ml Kanamycin, 0.125 -16 µg/ml Ethanamide, 0.0624 to 8 µg/ml Moxifloxacin in *M. avium,* and resistant isolates. Perimeter wells of the plates were filled with sterile distilled water to avoid dryness of medium during incubation. Growth from LJ slope was scrapped into fresh and autoclaved beaded bijou bottle containing 400µl 7H9 media with OADC growth enrichment & vortexes for 1 minutes. Standard bacterial suspension (matched with no. 1 McFarland standard) was prepared and diluted 1:10 in 7H9 broth. Then 100 µl inoculums was used to inoculate each well of the plate described by **(Palomino *et al.,* 2002, Martin *et al.,* 2003).** Bacterial suspension containing no drug used as a positive control and sterile MB7H9 media without inoculums used as a negative control were also included for each plate with isolates**.** Plates were sealed properly and incubated at 37ºC for seven days. 0.02% resazurin (**Sigma**) solution (25 µl) was added to each well and plates were re-incubated for an additional 2 days. The change in colour from blue to pink indicated the growth of bacteria, and the MIC was read as the minimum selected drug concentration that prevented the colour change in resazurin.

(iii) Electron microscopy of biofilm:

Biofilm was grown in tissue culture flasks as described above was fixed in a solution of 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for overnight at 40 ^{0}C. The samples were rinsed in the same buffer and dehydrated by increasing concentrations of ethanol (30 %, 70 %, and 90% and 100 %). The samples were coated with gold film sputtering and used for image analysis by SEM (Hitachi S 3000 N).

Results

The biofilm of slow grower *M. tuberculosis* H37Rv **(Figure 5.5)** sensitive and multiple drug resistant isolates were found attached to the sides of the flask and some maturation was observed after 3 weeks of incubation, but there was a little change in the biofilm appearance over the subsequent 2 weeks of incubation. It was observed that the amount of biofilm

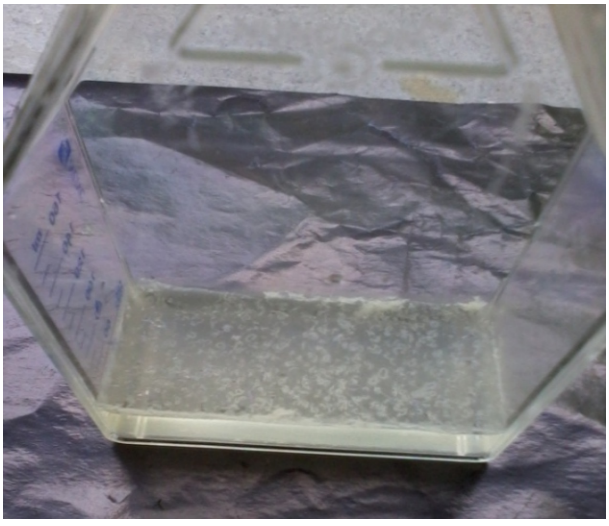

Fig 1. Biofilm of multi drug resistnt isolates developed in tissue culture flask

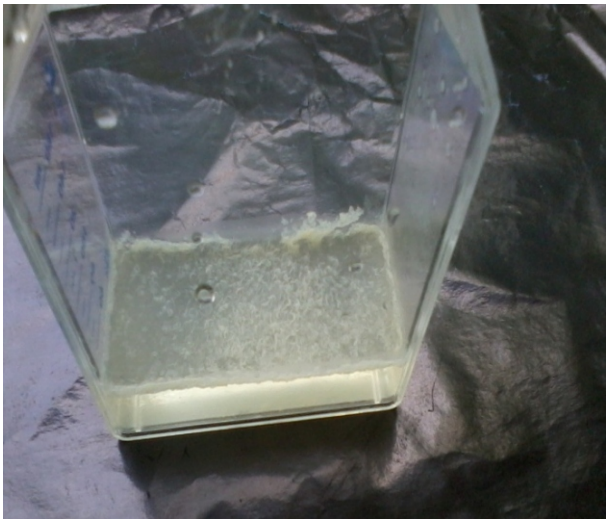

Fig 2. Biofilm of sensitive isolates developed in tissue culture flask

development was time dependent and the effect was most apparent on the maturation stages after two and 3 weeks, and after that also the bacteria remained competent to attach itself to the sides of the polystyrene bottles. The thickness of biofilm was more in H37Rv rather than MDR **(Figure 1)** and sensitive isolates **(Figure 2).**

Ultrastructure characteristics of M. tuberculosis H37Rv biofilm:

This is the first report of the ultrastructural observation of the biofilm of *M.tuberculosis*. The bifilm of *M.tubercuolosis* was found to be depicted distinctly different morphological features as compared to non tuberculosis mycobacteria. The rock shaped and globular deposits were evident and distributed across the entire surface but seemed to be concentrated around the raised edges of the furrowed surface. (**Figure 3.**). SEM analysis revealed irregular and smooth crystalline structures, appeared to be calcifications of biofilm material or the formation of microcrystalline structures. The surfaces were not completely smooth but exhibited micro abrasions and irregularities, which may contribute to bacterial adhesion. The overall surface configuration, with its furrowed corrugated facade would appear to make the appliance more conducive to bacterial and biofilm accumulation.

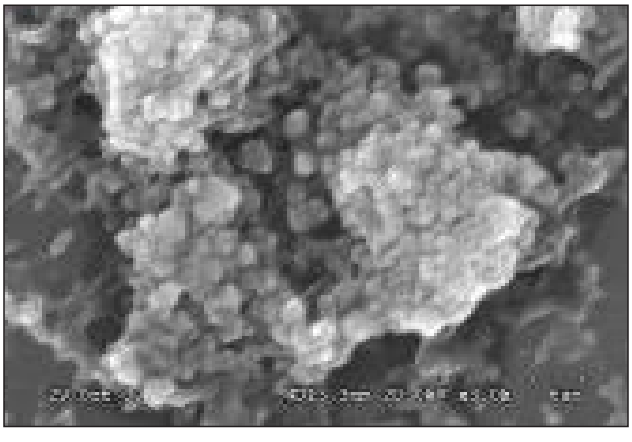

Fig 3. Ultrastructural of biofilm of *M. tuberculosis* rock shaped and globular deposits were and distributed across the entire surface.(scale bar 50 µm)

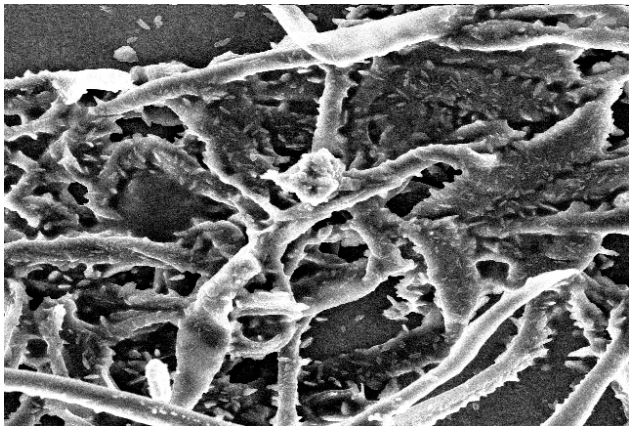

Fig 4. Net like structure of *M.tuberculosis* multiple drug resistant isolates at low magnification (scale bar 10 µm).

Table 1. Summary of concentration of drug for inhibition (little) of biofilm cells of H37Rv and sensitive isolates.

S.No.	Bacterial Species	Streptomycin	Isonizid	Rifampicin	Ethambutol	Pyrazinamide
1.	H37Rv	32	2	2	24	400
2.	JAL-6993	32	8	16	48	200

Ultrastructural analysis of Multi Drug Resistant isolates:

Selected multiple drug resistant isolates were taken for ultrastructural observation of biofilms by SEM revealed unique structural features (**Figure 4.)** running through the biofilm were cords of twisted material. Larger structures consisting of wrapped sheets were also present inside the biofilm. When specimen preparation led to breaks in this structure, the biofilm core was exposed and consisted of small numbers of bacteria embedded in a matrix of fibers and particulate matter aggregating on the fibers. In other parts of the biofilm, the fibers were more apparent and formed irregular, net-like structures. At higher magnification it was possible to see that the fibers were organized into ordered networks of periodic nets. At ultrahigh magnification nets contained few bacteria and were covered by thin sheets of material similar to that observed around the bacteria embedded in the particulate matter.

Drug efficacy for M. tuberculosis (H37Rv and sensitive isolates):

The MIC of streptomycin, isoniazid, rifampicin, ethambutol, pyrazinamide were 4.0 µg/ ml, 0.125 µg/ ml, 0.062 µg/ ml, 6 µg/ ml,50 µg/ ml for planktonic cells and for biofilm of *M.tuberculosis* H**37**Rv MIC was higher as compared to planktonic cells. Such as 32µg/ ml for streptomycin, 2µg/ ml for isoniazid, 2µg/ ml for rifampicin, 24 µg/ ml for ethambutol, and 400 µg/ ml for pyrazinamide. *M. tuberculosis* sensitive isolates was showed inhibitory effect (MIC) for streptomycin, isoniazid, rifampicin, ethambutol, pyrazinamide at concentration 8 µg/ml, 0.062 µg/ml, 0.062 µg/ml, 1.5 µg/ml and 50 µg/ml and for biofilm cells the MIC was 32, 8, 16, 48, 200 respectively.

Similar experiment had done in *M. tuberculosis* H37Rv, sensitive isolate biofilm with addition of streptomycin, isoniazid, rifampicin drug with inhibitory concentration and MDR isolates. The result was described in Fig. 5 & 6, H37Rv, sensitive and resistant isolates respectively.

Discussion

We demonstrate here for the first time that ultrastructural of biofilm cultures of a Mycobacterium tuberculosis H37Rv. The picture clearly showed the microcolony and extracellular polymeric substances of biofilm. The biofilm of *M.tuberculosis* H37Rv are capable of growing at higher drug concentrations (i.e. have higher MICs) than suspension cultures: the MIC of selected drug concentration that inhibited exponentially growing biofilm was found to be higher than the MIC for planktonic culture. Current chemotherapy for TB largely relies on drugs that inhibit bacterial metabolism with a heavy emphasis on inhibitors of the cell wall synthesis. According to their mode of action, first and second line TB drugs can be grouped as cell wall inhibitors (isoniazid, ethambutol, ethionammide, cycloserine), nucleic acid synthesis inhibitors (rifampicin, quinolones), protein synthesis inhibitors (streptomycin, kanamycin) and inhibitors of membrane energy metabolism (pyrazinamide). The current TB drugs can be divided into two categories: bacteriostatic (inhibit the growth of bacteria) and bactericidal (kill the susceptible bacteria*)* drugs. However, the distinction between static and cidal drugs is only relative, because some static drugs can be cidal under some conditions (such as with higher drug concentrations, smaller inoculum, or change in bacterial physiological status. Why do the bacilli, when growing as a surface-attached culture, show resistance when compared to bacilli growing in suspension. The biofilm literature offers essentially three possible explanations for biofilm-associated drug resistance: (i) slower biofilm growth, (ii) decreased drug penetration into the biofIm due to architecture and/or extracellular matrix of the biofilm, (iii) resistance mechanisms expressed specifically in biofilm bacilli but not in planktonic cells. The

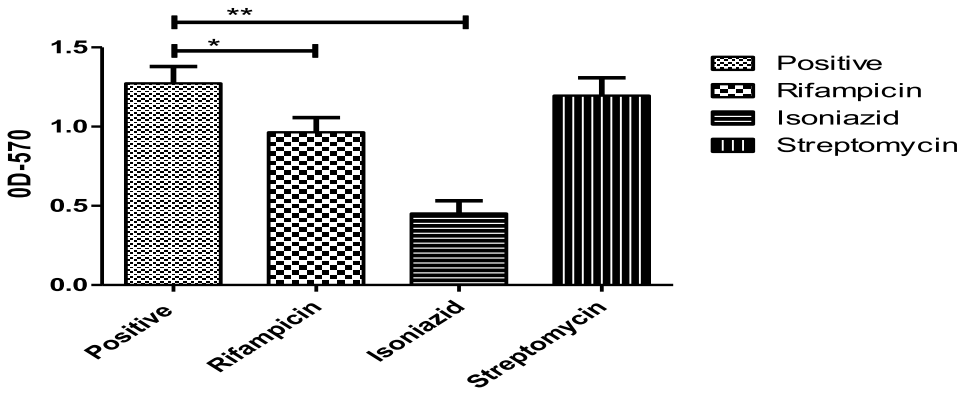

Fig 5. Effect of rifampicin, isoniazid and streptomycin on the ability of *M. tuberculosis* H37Rv to form biofilm.(*P value 0.0022* ** and (*P value 0.0411**)*

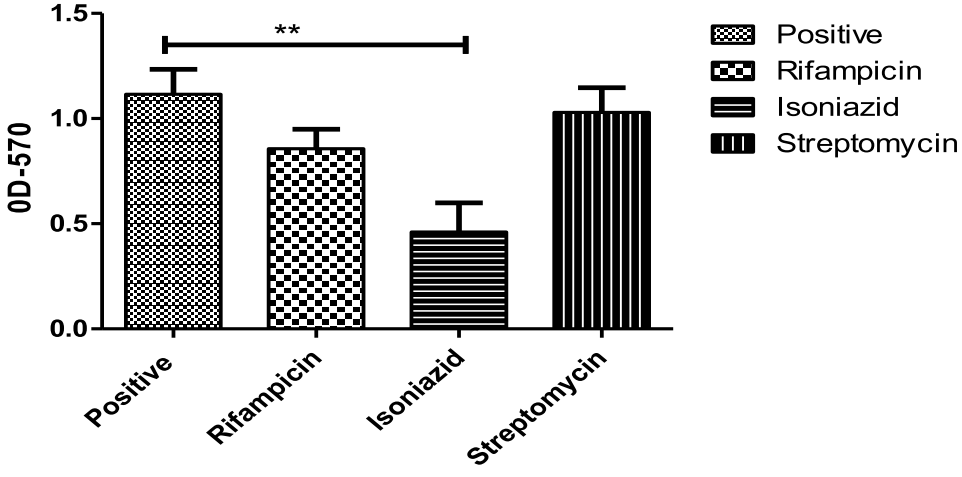

Fig 6. Effect of rifampicin, isoniazid and streptomycin on the ability of *M. tuberculosis* sensitive isolates to form biofilm.(*P value 0.0043***)

generation times of biofilm and planktonic cells of *M.tuberculosis* H37 Rv and sensitive isolates JALMA-7426 cultures were found to be similar. Isoniazid shows specific activity of *M.tuberculosis* and sensitive isolates. As we have previously reported the component of mycobacterial biofilm is mycolic acid and glycopeptidolipid and isoniazid is a potent inhibitor of cell wall mycolic acid and other multiple effects on DNA, lipids, carbohydrates metabolism (**Suzuki *et al.,* 1995).** The another important possibility is slower growth of the biofilm culture as a mechanism for the increase in MIC appears not to play a role. This leaves drug penetration problems and expression of (unknown) biofilm-specific resistance mechanisms as possible causes for the observed resistance against isoniazid. We used the genetically tractable and slow growing *M,tuberculosis* and sensitive isolates.for this study. Hence, our work provides the basis for a genetic dissection of the biofilm-associated drug resistance phenomenon uncovered in this work. Isolation of mutant strains that have lost the ability to form a biofilm at elevated isoniazid levels (but retain this ability under drug-free conditions) is currently under way. The work reported here shows that the bacterium is furthermore capable of 'differenting' into a multicellular, surface-attached form that is resistant to growth inhibition by antimycobacterials. A more comprehensive understanding of processes connected with biofilm development in different drug concentration will lead to new knowledge that would help in developing novel and effective control strategies for prevention of biofilms and improvement in patient management.

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