Isoniazid resistance of exponentially growing Mycobacterium smegmatis biofilm culture

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Abstract

Biofilm growth of Mycobacterium smegmatis was found to be unaffected at an isoniazid concentration that inhibited growth of planktonic bacilli (i.e. at isoniazid minimum inhibitory concentration = 10 μg ml⁻¹). Significant growth (50% of drug-free control) of biofilms was observed at up to 40 μg ml⁻¹ and the MIC for biofilm growth showed an increase to up to 80 μg ml⁻¹ isoniazid. Thus, the biofilm growth modus appears to be a strategy for replicating bacilli to evade the onslaught of antibacterials.

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1. Introduction

Conventionally, drug resistance is defined as the ability of a microbe to grow in the presence of an elevated level of antibiotic (reflected in an increase in the minimum inhibitory concentration (MIC)). Biofilms, i.e. microbes attached to a surface, are usually considered to be drug-resistant. In most biofilm susceptibility studies, however, survival of cells in a preformed biofilm (rather than the ability of the biofilm to grow) is recorded. Accordingly, the reported ‘biofilm resistance’ usually describes an increased resistance of cells to killing (not growth inhibition) and this is what virtually all biofilms are good at [1].

Here, we asked whether mycobacterial biofilm cultures are drug-resistant in the conventional sense, i.e. whether biofilms can grow beyond drug concentrations that inhibit growth of planktonic cells. For this study we used the non-pathogenic and fast-growing mycobacterial model organism Mycobacterium smegmatis mc²155 [2]. Recently it was demonstrated that M. smegmatis is capable of forming biofilms on polyvinyl chloride surfaces and the first genes (involved in synthesis and transport of glycopeptidolipids) essential for biofilm formation were identified by Kolter and co-workers [3–5]. First we optimized a polyvinyl chloride-based biofilm culture system to grow reproducibly exponential biofilms within 1 day. Then we employed this culture system to investigate the inhibitory effects of isoniazid on biofilm growth. This frontline tuberculosis drug inhibits mycolic acid synthesis, and hence cell wall synthesis. Biofilms of the bacillus were found to grow significantly beyond the MIC for planktonic culture.

2. Materials and methods

2.1. Strain, cultivation and drugs

All experiments were conducted with M. smegmatis mc²155 [2]. Aliquots of seed stocks were kept at −70°C. All cultures were grown in biofilm medium (M63 salts minimal medium supplemented with 2% glucose, 0.5% casamino acids, 1 mM MgSO₄, and 0.7 mM CaCl₂ [5]) at 37°C. Biofilm cultures were grown in the 200-μl dishes of 96-well, U-bottom, polyvinyl chloride plates (Falcon, cat. no. 353911) with lids (Falcon, cat. no. 353913) as described by Kolter and co-workers [5] with two modifications. (i) To produce a biofilm growth curve from start to stationary phase within 1 day (rather than several days) incubation was at 37°C (as opposed to room temperature). (ii) To increase reproducibility of the growth curve inoculation was done with an exponentially growing pre-culture of defined cell density (rather than tooth-picking from a
colony). Briefly, exponential phase pre-cultures (with 0.05% Tween 80, to prevent clumping) were grown overnight in tissue culture flasks (OD_{600} = 0.2–0.3), washed in biofilm medium to remove Tween and resuspended in biofilm medium. The washed pre-culture was diluted to an OD_{600} of 0.005 and 100-µl aliquots (containing 10^7 cfu) were grown in dishes under gentle shaking at 100 rpm on an orbital shaker. Planktonic cultures were grown in the same way as described for biofilm cultures with the difference that the 200-µl dishes of 96-well, U-bottom, polystyrene (instead of polyvinyl chloride) plates with lids (Nunc, cat. no. 163320) were used as culture vessels.

Isoniazid and ofloxacin were from Sigma. Stock solutions were made in water. Drug solutions of appropriate dilutions were added as 10-µl aliquots to the culture dishes.

2.2. Monitoring of growth

Biofilm growth was monitored via crystal violet staining of the cell material [4,5]. The wells were rinsed twice with water, and 120 µl of a 1% solution of crystal violet was added. Plates were incubated at room temperature for 30 min and rinsed with water three times. Quantification of biofilm formation was performed by extracting the biofilm-associated crystal violet with ethanol. 200 µl ethanol was added per dish for 1 h and the contents of eight dishes were pooled for measuring absorbance of crystal violet at 570 nm. To determine cfu of biofilm culture, the biofilm was washed once and then dissolved by adding biofilm medium containing 0.5% Tween 80, pipetting up and down 10 times, incubation for 15 min on an orbital shaker at 100 rpm, and finally pipetting up and down 10 times. Cfus were determined by plating appropriate dilutions and colony count. Crystal violet staining of the dish after Tween 80 treatment confirmed that virtually the entire biofilm had been removed from the surface. Growth of planktonic culture was determined by turbidity measurement at OD_{600} and colony count after plating of appropriate dilutions.

3. Results

3.1. Growth of M. smegmatis biofilm cultures on polyvinyl chloride dishes

First, we established conditions to reproducibly generate exponentially growing biofilm cultures within 1 day. 200-µl dishes of polyvinyl chloride 96-well plates were used as culture vessels. Biofilm growth was determined by measuring photometrically the increase in cell-bound crystal violet after staining of the biofilm at various time points and extraction of the dye. Variation of temperature, aeration and inoculum size showed that growth at 37°C, with shaking at 100 rpm and inoculation of the dishes with 100 µl of an exponentially growing (planktonic) pre-culture diluted to OD_{600} = 0.005 resulted in reproducible biofilm growth curves. Fig. 1A (BF-PVC) shows that biofilm growth under these conditions was detectable by the crystal violet assay after 3 h. Exponential biofilm growth (generation time of 3 h) was observed for 15 h post inoculation.

To confirm that increase in crystal violet staining indeed correlates with increase in cell number, cfus were determined. The mild detergent Tween 80 was used to ‘dissolve’ the biofilm at various time points during exponential growth and the resulting suspension was plated for colony counting. Fig. 1C (BF-PVC) shows that biofilm cfu increased exponentially with a generation time of 3 h, consistent with the increase in cell-bound crystal violet.

The polyvinyl chloride culture dish contained, in addition to the biofilm bacilli growing attached to the surface, a subpopulation of planktonic cells in the liquid phase. Fig. 1B (PC-PVC) shows that no increase in turbidity (and cfu, data not shown) in the liquid phase of the culture was observed. To determine whether the planktonic subpopulation in the dish is required for the growth of the biofilm (or whether biofilm growth can be driven ‘internally’), the planktonic cells were removed after 3, 6, 9, and 12 h post inoculation, the dishes were washed and 100 µl of (cell-free) fresh medium was added. The effect of the removal of the planktonic subpopulation from the culture on the growth of the biofilm was determined by measuring the biofilm after 24 h post inoculation. Removal of the planktonic subpopulation had little or no effect on biofilm growth (data not shown). This suggests that biofilm growth (once the surface is ‘colonized’ by planktonic cells) is predominantly driven internally, i.e. by replicating cells within the film and not via attachment of planktonic cells from the liquid phase to the film. The same results were obtained when the planktonic subpopulation was removed via filtration and the used (rather than fresh) medium was re-introduced to the dishes (data not shown).

3.2. Growth of planktonic cultures in polystyrene dishes

To carry out a comparative analysis of the inhibitory effects of drugs on the growth of biofilm and planktonic culture we also established a 96-well plate-based culture system to grow exponential planktonic cultures in 200-µl dishes. To do this we made use of the finding that M. smegmatis does not form biofilms on polystyrene surfaces. We characterized growth of planktonic culture in dishes of polystyrene 96-well plates and demonstrated the absence of biofilm formation on this surface. Polystyrene dishes were inoculated with exponentially growing planktonic pre-culture as described above for the seeding of polyvinyl chloride dishes and growth of the planktonic culture was monitored by turbidity measurement and cfu determination. Fig. 1B (PC-PS) shows that the planktonic culture grew exponentially with a generation time of 3 h up to 24 h post inoculation (see Fig. 1C (PC-PS) for cfu).
Fig. 1A (BF-PS) shows that biofilm formation was negligible for bacilli grown in polystyrene dishes. Taken together, we have established a culture system based on polyvinyl chloride and polystyrene dishes for the growth of biofilm and planktonic culture, respectively. Both biofilm and planktonic cultures showed a similar generation time of about 3 h (Fig. 1C).

3.3. Growth-inhibitory effect of isoniazid on planktonic and biofilm cultures

Exponentially growing planktonic and biofilm cultures (grown in polystyrene and polyvinyl chloride dishes, respectively) were exposed to increasing concentrations of isoniazid. The drug was added to the dishes at the beginning (0 h, Fig. 2A), and during early (6 h, Fig. 2B) and late (12 h, Fig. 2C) exponential phase. The effect of isoniazid on growth was monitored by measuring turbidity of the planktonic cultures and crystal violet staining of the biofilm cultures after 24 h of drug exposure. The isoniazid dose–response curves for planktonic cultures curves shown in Fig. 2A–C (PC-PS) demonstrate as expected the strong inhibitory effect of isoniazid on suspension cultures, independent of the age of the exponentially growing culture. The MIC was about 10 µg ml⁻¹. In contrast, little or no inhibitory effect on biofilm growth was observed when isoniazid was added at 10 µg ml⁻¹ to exponentially grow-
ing biofilms at 6 and 12 h post inoculation (Fig. 2B,C (BF-PVC)). Furthermore, the biofilm cultures showed marked growth (50% of drug-free control) for isoniazid concentrations as high as four times the MIC for planktonic culture (Fig. 2C (BF-PVC)). The MIC for biofilm growth was found to be as high as 80 µg ml⁻¹, i.e. eight times the MIC for planktonic growth (Fig. 2C). Taken together, the comparative analyses of the growth-inhibitory effects of isoniazid on planktonic and biofilm culture revealed a markedly reduced susceptibility of the biofilm culture to the drug.

Is the observed phenotypic drug resistance of exponentially growing biofilms against isoniazid specific to this drug or does it indicate development of general drug resistance? To answer this question the effect of the fluoroquinolone ofloxacin (a replication inhibitor) on exponentially growing planktonic and biofilm cultures was determined in the same way described for isoniazid (i.e. at 0, 6 and 12 h post inoculation). The shape of the dose–response curves and the MIC for ofloxacin (4 µg ml⁻¹) were found to be independent of the mode of growth and independent of the age of exponentially growing cultures (data not shown). This demonstrates that the observed resistance of *M. smegmatis* biofilm culture for isoniazid is specific for this drug and does not reflect the development of general drug resistance in biofilm bacilli.

4. Discussion

We demonstrate here for the first time that biofilm cultures of a *Mycobacterium* are capable of growing at higher drug concentrations (i.e. have higher MICs) than suspension cultures: the MIC of isoniazid that inhibited exponentially growing biofilm was found to be up to eight times higher than the MIC for planktonic culture. 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 biofilm due to architecture and/or extracellular matrix of the biofilm, (iii) resistance mechanisms expressed specifically in biofilm bacilli but not in planktonic cells (reviewed in [1]). The generation times of biofilm and planktonic *M. smegmatis* cultures were found to be similar. Hence, 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 fast-growing *M. smegmatis* 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.

Our present work demonstrates that *M. smegmatis* has a second phenotypic (as opposed to genetic) drug resistance strategy to evade the onslaught of antibacterials. Previously, we reported that the bacillus is capable of undergoing a hypoxia-induced dormancy response in which the organism exits from the cell cycle and differentiates into a defined quiescent form that is resistant to *killing* by drugs [6]. The work reported here shows that the bacterium is furthermore capable of “differentiating” into a multicellular, surface-attached form that is resistant to *growth inhibition* by antimycobacterials.

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