Effect of Exogenous Spermine on Biofilm Formation in Mycobacteria by Stimulating the Synthesis of Glycopeptidolipids

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Abstract. Biofilm formation is of great interest by its ability to increase bacterial tolerance to antibiotics that represent a serious problem for modern medicine. Among mycobacteria, which are also capable of forming biofilms, there are pathogens of socially dangerous infections, including tuberculosis. Basing on these data, the strains of *Mycolicibacterium smegmatis* mc^2 155 were chosen as the objects of this study, including the parent strain without deletions and its mutants with one (Δrel_{Msm}) and double ($\Delta rel_{Msm}\Delta relZ$) chromosomal deletions of the genes responsible for the synthesis of alarmone synthetase enzymes. Biofilms of mutant strains exhibited defects in biofilm formation. We have shown that the integrity, hydrophobicity, and the level of biomass of surface mycobacterial biofilms are dependent on the amount of glycopeptidolipids (GPL) in cells. The level of GPL depends on the activity of alarmone synthetases. The biogenic polyamine spermine is able to enhance the production of GPLs, restoring the integrity of biofilms of mutant strains. It is possible that this effect of spermine is caused by the influence on the activity of mycobacterial alarmone synthetases, which makes promising the further studying the molecular mechanisms of its action.

1 Introduction

Bacterial biofilms are one of the most important objects of research, the interest to which has been growing in a few recent years. This is due to the fact that the most of bacteria for the most part are found in single or multispecies biofilms [1], which gives them advantages over the planktonic cells [2, 3].

Mycobacteria are able to form biofilms near the bottom or on the surface of liquid media (pellicle) [4]. At the same time, biomass of the biofilms is localized near the bottom many-fold more inferior to surface ones. That leads to increased interest in their studying. The properties of bacterial biofilms depend on many factors, such as the composition of the matrix, the presence of signaling molecules in the environment, and the nature of the cell surface. For mycobacteria, the properties of the cell surface are largely conditioned by the

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quantitative and qualitative diversity of lipids, in particular glycopeptidolipids (GPL), in their envelopes. The increase in the incidence of mycobacteriosis determines the high interest to studying of mycobacterial biofilms [5, 6].

Cells in biofilms become more resistant to various stressors, including antibiotic impacts [7], so the biofilm formation can be considered as a coordinated response of microorganism's population to negative environmental factors. In bacterial cells, the regulation of stress response is under the control of special molecules – alarmones. One type of alarmones is guanosine tetraphosphate (ppGpp). In mycobacteria, two genes, rel_{Msm} and *relZ*, are known to responsible for the synthesis of this alarmone [8]. Therefore, it can be assumed that mycobacterial alarmones are able to participate in the regulation of the biofilm formation process.

Bacterial response to stress can be induced not only by the antibiotic substances, but also by various signaling molecules, for example, biogenic polyamines, aliphatic polycations synthesized by both pro- and eukraotic organisms [9]. Bacteria can synthesize putrescine, cadaverine, and spermidine, while multicellular organisms, including human beings, produce spermine, which concentration is reaching millimolar concentrations [10]. Spermine has the strongest positive charge as compared to other biogenic polycations, and, therefore, can have the strongest effect on metabolic processes. It can be assumed that spermine can also be perceived by bacteria present in the host organism as a signal of interspecies communication, initiating the launch of the adaptive mechanisms.

The aim of this work is to determine the role of spermine in the regulation of GPL concentration during biofilm formation in mycobacteria depending on the presence of alarmone synthetases.

2 Materials and methods

Strains and growth media. The strains of *Mycolicibacterium smegmatis* mc2 155 were the objects of this study. The parental strain without gene deletions, indicated on the graphs as "WT", was used as a control. Strains with a single deletion of the rel_{Msm} gene and a double deletion of the rel_{Msm} and *relZ* genes were designated as experimental ones. Cell cultures for the experiment were grown in Middlebrook 7H9 medium with the addition of ampicillin (1:1000) and tween-80 (1:100) in tubes for 24 hours in a thermostatically controlled shaker (37°C, 200 rpm), after they were transferred to a flask with fresh medium and cultured under similar conditions to an optical density of 2.0 (600 nm).

Biofilm cultivation. The inoculating cells were washed from Tween-80 and diluted with fresh medium to OD600 = 0.1. Spermine was first added to the media at final concentration 2 mM. The diluted culture was poured into the preliminary weighed plastic 5 ml Petri dishes. The cultures in dishes were grown for 72 hours, and then the liquid with planktonic cells was removed without damaging the surface biofilms. The dishes were dried in a thermostat for 24 hours, afterwards they were reweighed to the constant dry weight of the biofilm.

Isolation of GPL and TLC. The isolation of GPL was carried out by thin layer chromatography (TLC), in accordance with previously described methods [11–13]. The culture was grown in a flask for the same time as biofilms, after which it was adjusted to OD600 = 1.5. The cells were washed from the medium and precipitated in a centrifuge, afterward they diluted in 600 µl of a solvent of chloroform:methanol (2:1). Then, the samples were kept in an ultrasonic bath at a temperature of 56°C for 2 hours, the supernatant was removed, and 600 µl of distilled water was added. The organic phase was then taken up, washed and dried. For chromatography, the samples were diluted in a solvent of chloroform:methanol (9:1), applied to aluminum plates with silica gel 60, and eluted in a similar solvent. The chromatographic plates were dried, sprayed with 10% sulfuric acid in

methanol and charred at 120°C for 90 seconds. The dried TLC plates were photographed, the image was inverted to increase the contrast using the free version of Photoshop CC 2015.5 (Adobe, USA), after brightness of the area around the spots in the GPL profile was measured (without background brightness). The indicators were compared relative to the control. Statistical analysis was performed with help of Statistica 7.0 program (StatSoft, Inc. USA). The amount of GPL was compared with the results obtained earlier [11-13].

3 Results and discussion

During the studying of mycobacterial biofilm formation we have found that mutant strains with deletions of the rel_{Msm} and *relZ* genes exhibited the disturbances in biofilm formation. The biofilms of mutant strains were fragmented and settled at dish bottom. These disturbances were not observed when spermine was added to the medium. Moreover, the biomass of the surface biofilms increased when the strains were grown with spermine (Fig. 1).

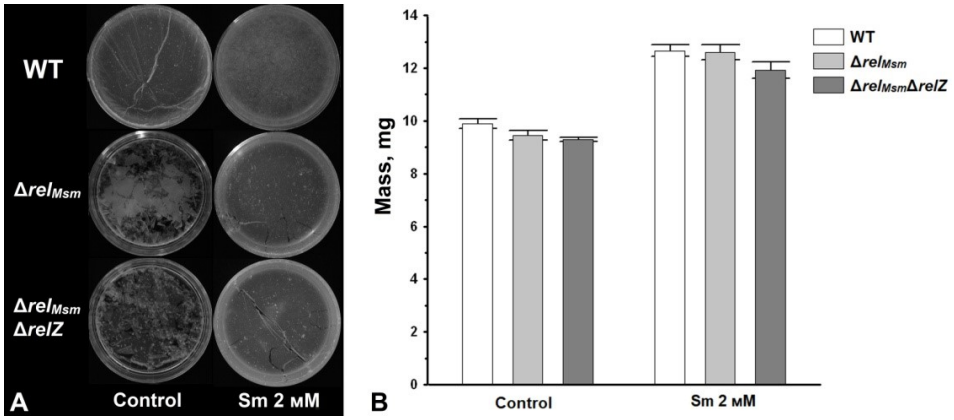

Fig. 1. Dependence of *M.smegmatis* surface biofilm formation on the presence of spermine in the medium. A – Surface biofilms without spermine (left) and with spermine (right). B – Comparison of the mass of surface biofilms grown with spermine and without it in the culture medium. Control – without spermine, Sm 2 мМ – with 2 мМ spermine.

Disappearance or restoration of pellicle biofilms was dependent on the contents of hydrophobic molecules in mycobacterial cell walls. Therefore, we studied the most common hydrophobic molecules that were present in the cell walls of nontuberculous mycobacteria - glycopeptidolipids (GPL). In the process of the studying we found that the amount of GPL in mutant strains upon completion of biofilm growth was less than in the parental strain without deletions. At the same time, the decrease in the amount of GPL was directly proportional to the number of deletions in the strain (Fig. 2).

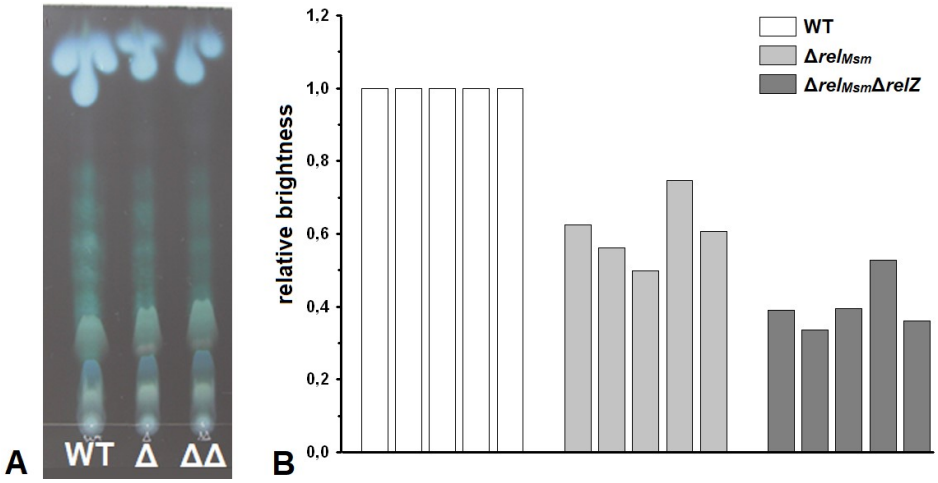

Fig. 2. Comparison of the amount of HPL in the cell walls of *M. smegmatis* strains. A – GPL profiles on aluminum plates after TLC. B – Brightness values relative to the parent strain (WT). Δ – strain with rel_{Msm} deletion, ΔΔ strain with rel_{Msm} and *relZ* double deletion.

In dishes with spermine addition we have found the restoration of the surface biofilms in the mutant strains with simultaneous increase in the levels of GPL occurring in the cell walls. At the same time, the amount of HPL in the cells of the parental strain was also increased (Fig. 3).

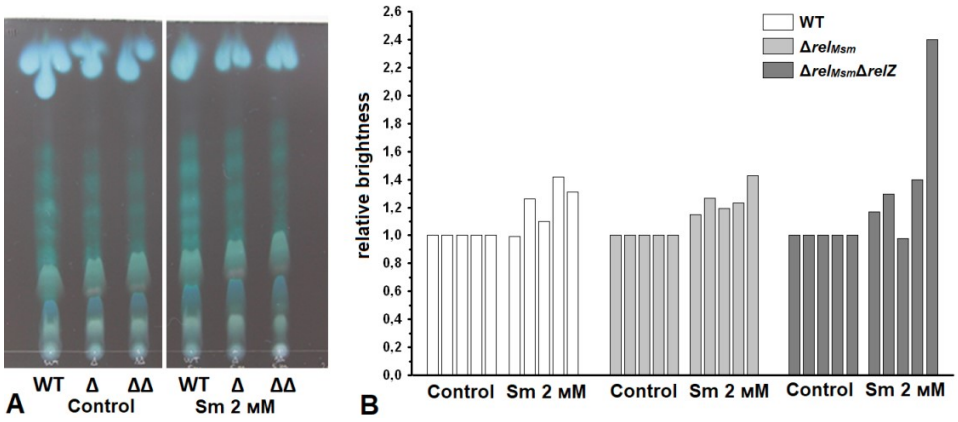

Fig. 3. Effect of spermine on the amount of GPL in the cell walls of *M.smegmatis* strains. A – GPL profiles on aluminum plates after TLC. B – Brightness values relative to the parent strain (WT). Δ – strain with rel_{Msm} deletion, ΔΔ strain with rel_{Msm} and *relZ* double deletion, Control – without spermine, Sm 2 мМ – with 2 мМ spermine.

An increase in the amount of GPL in cells can be the cause of the increasing in biomass of surface biofilms. It is possible that an increase in the hydrophobicity can lead to the cell retention in the biofilms and prevent their earlier destruction caused by the natural biofilm growth cycle.

4 Conclusions

Previously, we have assayed the effect of GPL on sliding motility of mycobacteria and didn't find some direct relationships between the amount of GPL in cell walls and the ability to cell sliding [14]. However, this study first demonstrates the presence of the relationship between the activity of mycobacterial alarmone synthetase genes and biofilm formation. The alarmone syntetase gene's deletion led to a decrease in the number of GPL in the cells of the mutant strains proportionally to the number of deletions. Deficiency of GPL was phenotypically characterized by fragmentation of pellicle biofilms and loss of their hydrophobicity. Therefore, the product of the studied genes, alarmone ppGpp, is involved in the regulation of the GPL content of the cell wall and indirectly affects the biofilm formation of *M.smematis*.

Polyamine spermine promotes the increase in the levels of GPL in mycobacterial cells, independently on the presence of the rel_{Msm} and *relZ* genes. An increase in the amount of GPL restores the integrity of biofilms in mutant strains and increases the biofilm biomass in all studied strains. This fact confirms the relationship between GPL and biofilm formation. The previously undescribed mechanism that we propose for GPL regulation with the participation of spermine is carried out through the interaction of positively charged amino groups of spermine with negatively charged bacterial DNA that modulates gene expression and contributes to the increasing in the activity of alternative alarmone synthetases. The final mechanism of GPL regulation with the participation of spermine needs in further research.

Acknowledgment

The work was supported by a grant from the Russian Science Foundation № 18-73-10156.

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