Antibacterial Activity of Methyltiosulfonate and Its Complexes with Rhamnolipid and Trehalose lipid against Pseudomonas aeruginosa NBIMCC 1390.

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ABSTRACT

The possibility for potentiation of the antibacterial activity of methyltiosulfonate by biosurfactants (rhamnolipid and trehalose lipid) against Pseudomonas aeruginosa was studied. The analysis included examination of the changes in growth, morphology, and ultrastructure of bacterial cells. The results demonstrated that the bactericidal effect of methyltiosulfonate was well expressed. It was found that trehalose lipid had no antibacterial activity and no potentiative effect on methyltiosulfonate activity. The combination of methyltiosulfonate and rhamnolipid-biosurfactant had a strong synergistic-like effect. The presence of rhamnolipid - biosurfactant decreased significantly the minimal bactericidal concentration of the antimicrobial agent. Scanning electron microscopy showed considerable changes in the bacterial cell surfaces under the action of methyltiosulfonate alone and the methyltiosulfonate - rhamnolipid combination. Confocal microscopy after Live/Dead staining indicated lack of bacterial killing by rhamnolipid alone, increase of dead cell number after methyltiosulfonate application, and confirmed predominance of dead cells after simultaneous use of the two preparations. It was suggested that the biosurfactant provoked changes in the bacterial membrane organization that increased the access of methyltiosulfonate into the bacterial cell. The enhancement of antibacterial activity of methyltiosulfonate in the presence of biosurfactants significantly increased the therapeutic potential of this compound.

Keywords: Pseudomonas aeruginosa; methyltiosulfonate; biosurfactants; antibacterial activity; synergistic-like effect.

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INTRODUCTION

*Pseudomonas aeruginosa* is a Gram-negative bacterium that can cause disease in animals and humans. It is found in soil, water, skin flora, and most man-made environments throughout the world. It is an important opportunistic pathogen, highly resistant to a large number of antibiotics. This resistance to some extent is related with the permeability barrier function of the outer membrane. The bacteria control their membrane permeability to protect themselves against external toxic compounds such as antimicrobial agents.

A number of studies suggest that it is possible to modify the outer membrane of Gram-negative bacteria and to change the cell surface properties by addition of surface active compounds that can act as membrane permeabilizing agents [1-4].

Surfactants are amphiphilic molecules that have both hydrophobic and hydrophilic moieties. A variety of surfactants can permeabilize bacterial membranes and make them more susceptible to antibiotics or other antibacterial agents. Recently, a significant interest has been focused on the surfactants, produced by microorganisms - biosurfactants [5, 6]. Microbial surfactants have several advantages over chemical surfactants such as lower toxicity, higher biodegradability and structural diversity [7, 8]. Moreover, some biosurfactants have a potential as biologically active compounds and have applicability in the medical field [9]. Biosurfactants that have been studied extensively are the rhamnolipids, sophorolipids and trehalolipids.

The rhamnolipids from *Pseudomonas* species represent one of the most important classes of microbial surfactants with good potential for commercial exploitation [10, 11]. Their effect on the cell surface structures of microorganisms grounded their application in biotechnology, bioremediation and in biomedicine as well [12, 13]. Rhamnolipids are an alternative to synthetic medicines and antimicrobial agents and may be used as effective therapeutic agents [13-16]. Recent studies suggested that the rhamnolipid- biosurfactant provokes various changes in surface and intracellular structures of the bacterial cells [13, 17-19]. These changes, probably, may help overcome the effective protective cell barrier as the outer membrane of *Pseudomonas* sp., and thus decrease cell tolerance or resistance to antibiotics and other antibacterial agents [20].

The increased interest to trehalose lipids is due to their ability to lower interfacial tension and increase pseudosolubility of hydrophobic compounds that make them potential candidate for applications in a number of fields [21]. Trehalose lipids are found in *Rhodococcus* and other actinomycetes. Surfactants of rhodococci are glycolipids, in particular, trehalose lipids (trehalose mono- and dicorynomycolates) [22]. There are few reports in the literature on the interaction of trehalose lipids with membrane vesicles [23-25]. The examination of the interactions between a trehalose lipid from *Rhodococcus* sp. and dimyristoylphosphatidylglycerol membranes showed that biosurfactant incorporates into the phosphatidylserine bilayers thus suppose structural perturbations of the membrane affecting their function [24]. These results suggest that trehalose lipids may have potential application in the healthcare industry.

The development of microbial antibiotic tolerance or resistance necessitates the search of new antimicrobial agents and novel strategies to fight against microbial infections [26]. In order to search for new preparations with antimicrobial action we studied biological activity of thiosulfonates RSO₂SR₁ compounds similar by structure to antibiotic allicin, the active antibacterial substance of garlic [27]. Allicin (CH₃=CH-CH₂SOSCH₂-CH=CH₂) is one of the esters of thiosulfonic acid, which are not stable enough. Antibacterial activity of many thiosulfonates exceeds manifold the allicin activity. Alkyl esters of thiosulfonic acids possess high level of antimicrobial activity [28]. Furthermore, the toxicity of alkyl esters of thiosulfonic acids is lower. The results of our preliminary studies showed that methylthiosulfonate has a strong bactericidal and fungicidal effect. What is more, the addition of insignificant amounts of rhamnolipid-biosurfactant which has no detectable antibacterial effect potentiated the antimicrobial’s efficacy [29]. Previously, we evaluated the antibacterial activity of rhamnolipid-biosurfactant within the range of its critical micelle concentration on the Gram-negative strain *P. aeruginosa* NBMCC 1390 and the results showed that the biosurfactant does not kill the strain but significantly increased the cell permeability [18]. Ortiz et al [24] reported that trehalose lipid interacts with phospholipids and affects the function of the membrane. We therefore hypothesized that the presence of a biosurfactant will result in potentiation of the antibacterial activity of methylthiosulfonate by increasing the permeability for the drug.
The present study focuses on the mode of action of the combination of biosurfactants (rhamnolipid or trehalose lipid) and methyltiosulfonate on *P. aeruginosa* NBIMCC 1390. The aim is to investigate: (i) the antimicrobial potential and permeabilizing effect of trehalose lipid; (ii) the possibilities to decrease the bactericidal concentration of methyltiosulfonate by combination with biosurfactants; (iii) the cell damage by fluorescence and scanning electron microscopy examinations.

**MATERIALS AND METHODS**

**Microorganism**

The strain *P. aeruginosa* NBIMCC 1390 (National Bank of Industrial Microorganisms and Cell Cultures, Sofia, Bulgaria) was used throughout this study. The culture was maintained at 4°C on Bacto agar (Difco) slants and transferred monthly.

**Culture medium and growth condition**

The cells of *P. aeruginosa* NBIMCC 1390 were grown in a mineral salts medium (MSM) [30], supplemented with 2mM CaCl₂, 0.5% casein hydrolysate (Fluka), 0.5% maltose, pH 7.2. Starter cultures were prepared by transferring the cells from agar slants to 2ml of MSM medium in 20ml flasks and cultured for 18 h at 37°C with agitation at 200 rpm.

The experimental cultures of 10 ml were inoculated with 1% (v/v) inoculums and incubated in 100-ml flasks until late exponential phase. Growth conditions were the same as those used for preparing the inoculum. Growth was monitored by measuring the absorption at 570 nm.

Protein was determined by the method of Bradford [31].

**Inhibitor (Antimicrobial agent)**

Methyltiosulfonate (MTS) was synthesized in the Laboratory of Biotechnology, Ukrainian Academy of Sciences (Lviv town), and provided by Dr. V. Lubenets [27]. The tested concentrations were between 1 µg ml⁻¹ to 120 µg ml⁻¹.

**Biosurfactant**

The biosurfactants - rhamnolipid (RL) and trehalose lipid (TL) used in the study are produced by *Pseudomonas* sp. PS-17 and *Rhodococcus erythropolis* respectively and were isolated and characterized in Laboratory of Biotechnology of Ukraine Academy of Sciences and provided by Dr. E. Karpenko [32].

**Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC)**

Antimicrobial activity was evaluated according to the minimum inhibitory concentration (MIC), the lowest concentration of an antimicrobial agent that inhibits the development of visible microbial growth after incubation at 37° C for 20 hours.

To determine the MBC, subcultures were made on inhibitors-free agar plates from each clear tube in the MIC test series, followed by incubation at 37°C for 20 hours.

**Microscopy**

For microscopic analyses, the cell suspensions were cultivated to early exponential phase and then incubated with 50µg ml⁻¹ MTS, RL 10µg ml⁻¹ and with a mixture of MTS (50µg ml⁻¹) and RL 10µg ml⁻¹ for 60 min at 37 °C with agitation at 200 rpm.

For scanning electron microscopy (SEM), the samples were fixed for 2 h in 4% glutaraldehyde in 0.2 M Na cacodylate, pH 7.2. Followed a 1 h post-fixation in 1% cacodylate-buffered OsO₄ and dehydration in graded...
ethanol series. The materials were vacuum-coated with gold. The observations were made on Lyra/Tescan scanning electron microscope.

For confocal laser scanning microscopy (CLSM), the fluorescent “LIVE/DEAD” dye combination, BacLight Bacterial Viability Kit (L7007), composed of the green-fluorescent nucleic acid stain (Syto 9®) and the red-fluorescent nucleic acid stain (propidium iodide, PI) was applied according to the producer’s instructions. The observations were made on laser scanning confocal microscope Nikon Eclipse TiU with a 60x plan apochromatic oil objective, at excitation wavelengths of 488 and 543 nm. Image acquisition and processing were done with EZ-C1 software. 20 confocal images were randomly taken for each of the examined samples.

Statistical analyses

All experiments were carried out in triplicate, the reported results are the averages of at least three measurements, and the coefficients of variations, expressed as the percentage ratio between standard deviations (SD) and the mean values, were found to be <10 in all cases.

RESULTS

Antimicrobial potential and permeabilizing effect of TL

The effect of different concentrations of TL on the growth and cell permeabilization of P. aeruginosa was studied and the results are shown in Figure 1. The effect on the cell permeability during growth was assessed by means of a quantity of the extracellular protein. The presence of the biosurfactant in the medium at concentrations below (10 μg ml⁻¹ and 30 μg ml⁻¹), close to (50 μg ml⁻¹) and above (100 μg ml⁻¹, 300 μg ml⁻¹ and 500 μg ml⁻¹) critical micelle concentration (CMC) did not alter significantly the growth compared to the control. No antibacterial activity of the biosurfactant was registered. A decrease in the amount of extracellular protein was recorded at all tested concentrations of TL, which was more significant at concentration 500 μg ml⁻¹ (Figure 1). This lowering in cell permeability was dose dependent for the concentration range investigated, showing a general decrease in protein release (compared to the control) with increasing of TL concentration.

![Figure 1: Effect of different concentrations of TL on growth (columns) and protein release (curve) of P. aeruginosa. Mean values are given with standard deviations of ≤ 10%.

The ability of biosurfactants to potentiate the effect of MTS

The studies on the effect of combination- TL or RL and MTS on the growth of Ps. aeruginosa were carried out to determine availability of synergistic-like effect. The established MIC of MTS is 70μg ml⁻¹ for P. aeruginosa (Figure 2). At this concentration, there was no visible growth in the test tubes but the number of
surviving colony forming units CFU/ml is $0.253 \times 10^8$. This means that the effect of MTS is bacteriostatic. Bactericidal activity was registered at concentration $80 \mu g ml^{-1}$ (Figure 2).

![Graph showing effect of different concentrations of MTS on growth by Pseudomonas aeruginosa in absence (dark grey bars) and in presence of rhamnolipid-biosurfactant (white bars). Mean values are given with standard deviations of ± 10%.

Figure 2: Effect of different concentrations of MTS on growth by Pseudomonas aeruginosa in absence (dark grey bars) and in presence of rhamnolipid-biosurfactant (white bars). Mean values are given with standard deviations of ± 10%.

In contrast to MTS, RL and TL were not effective as antimicrobial agent against P. aeruginosa even at high concentrations. However, the difference between the effects of MTS alone and in combination with the RL was significant. As shown in Figure 2, we observed an enhancement in antibacterial activity when MTS and RL were applied in combination. According to the experimental data, presented on Fig.2, MIC ($50 \mu g ml^{-1}$) and MBC particularly ($50 \mu g ml^{-1}$) are considerably lower in the presence of rhamnolipid-biosurfactant applied in concentrations below CMC ($10 \mu g ml^{-1}$). The combination of $30 \mu g ml^{-1}$ MTS and $10 \mu g ml^{-1}$ RL dramatically reduced the level of surviving CFU/ml compared to that $30 \mu g ml^{-1}$ MTS alone. Moreover, no CFU/ml was counted when cells were treated with combination $50 \mu g ml^{-1}$ MTS and $10 \mu g ml^{-1}$ RL. These results suggest that RL potentiates the effect of MTS thus makes it markedly more effective against P. aeruginosa.

![SEM images of P. aeruginosa. (a) untreated control, (b) RL-treated, (c) MTS-treated and (d) MTS plus RL-treated bacteria. Dark arrows point to membrane vesicles released at the bacterial surface; white arrows point to deep infolds of the bacterial surface. Asterisks mark two adherent bacteria (c) or a broken cell (d). Scale bar = 1 µm.

Figure 3: SEM images of P. aeruginosa. (a) untreated control, (b) RL-treated, (c) MTS-treated and (d) MTS plus RL-treated bacteria. Dark arrows point to membrane vesicles released at the bacterial surface; white arrows point to deep infolds of the bacterial surface. Asterisks mark two adherent bacteria (c) or a broken cell (d). Scale bar = 1 µm.
We also examined the combination between TL and MTS for synergistic-like effect. The data do not indicate a potentiative effect of studied biosurfactant. The concentration of TL to be tested was chosen below, close to and above its CMC. When the biosurfactant was added in all tested concentrations to MTS no increased activity of methyltiosulfonate was noted. This coincides well with the evidence of decreased instead of increased permeability of the cells (see Figure 1).

Microscopic analysis

The effects of the applied treatments on the morphology and the surface relief of *P. aeruginosa* were examined by SEM. Control cells of the strain were c.a. 0.5 μm thick and had length varying between 1 and 2 μm. The surface of untreated bacteria was even with only occasional membrane vesicles released from the surface (Figure 3a). RL treatment resulted in a bit fluffier cell surface and increase of the number of associated membrane vesicles (Figure 3b). The effect of MTS treatment alone was aberrant morphology, swelling and sticking of bacterial cells (Figure 3c).

![Image of microscopic analysis](image_url)

Figure 4: Estimation of *P. aeruginosa* cell vitality by CLSM using the Live/Dead viability kit. (a) control untreated and (b) bacteria treated for 60 min in the presence of 10 μg ml⁻¹ RL. (c, d) MTS treatment; image (d) represents the area in image (c) but with the red signal turned off. (e, f) Simultaneous application of RL and MTS; image (f) represents the area in image (e) with the red signal turned off. Scale bar = 4 μm.
The SEM examination of bacteria subjected to combined RL plus MET treatment did not show significant differences from the MTS alone treated group. There occurred aberrant cells, infolds on the bacterial surface and parts of broken bacteria (asterisks, Figure 3d).

The changes in membrane permeability of P. aeruginosa cells in presence of RL, MTS and the combination of MTS and RL were examined by CLSM using a Live/Dead staining kit (Figure 4).

The images obtained for both untreated control and rhamnolipid-treated bacteria were characterized by green fluorescence alone indicating penetration only of the vital stain, Syto 9®, into the cells. Controls were represented by distinct individual bacterial cells (Figure 4a). The rhamnolipid-treated microorganisms were often stuck in groups (Figure 4b) which implied changes in surface characteristics, yet no permeation of the “Dead”-stain PI was noted. After MTS treatment, groups of adherent cells occurred which were colored with either red or green (Figure 4c). The red staining showing the penetration of PI indicated that a significant number of cells were with disrupted permeability barrier. Nevertheless a substantial proportion of the bacteria still remained permeable only to the live-cell label, the green-fluorescent Syto 9®. This is clearly seen for example on Figure 4 (d), which shows the same image as in Figure 4 (c) but with the red signal turned off. Unlike this, in the MTS-rhamnolipid combination PI coloring predominated (Figure 4e) and only occasional cells were green and supposedly vital (Figure 4f).

**DISCUSSION**

The worldwide development of resistance to antibacterials necessitates new antimicrobial agents and investigation of novel antimicrobial strategies. One of the novel approaches is to use synergistic or synergistic-like association of two or more agents in order to increase their efficacy. There are many reports for synergistic action of antimicrobials and conventional antibiotics (33; 34), for antibiotics synergy interaction [35, 36], and a few - for synergistic combination of biosurfactant and antibiotic [15, 37- 39] The results in the recent work demonstrated that the new synthetic derivative of allicin (MTS) designed to be more active and more stable in comparison with the natural product has a strong antibacterial activity against P. aeruginosa. Furthermore, we demonstrated the ability of RL to potentiate its effect. Rhamnolipid does not show antimicrobial activity against P aeruginosa but presented a synergistic-like effect in combination with MTS. The bacterial resistance could be due to decrease of the membrane permeability in response to antimicrobial agents thus preventing penetration of these compounds into cells. The molecular entities responsible for the drug resistance are most often associated with the bacterial surface and are therefore potentially subjected to the action of biosurfactants. Many authors reported that surfactants provoked structural changes in bacterial membrane and alteration in cell surface properties. [13, 17, 18, 24, 40] We hypothesized that the biosurfactants (TL and RL) may act as permeabilizing agent and thus lead to increasing the activity of MTS by forming pores in the outer membrane and thus facilitating its entrance.

In this report we established the ability of RL to potentiate the effect of MTS. In the case of TL, the results demonstrated an absence of antimicrobial activity. Furthermore, general decrease in protein release with increasing of TL concentration was registered, indicating a decrease of membrane permeability of P. aeruginosa cells. These results explain also its inability to increase the antibacterial effect of MTS.

The data obtained in this study demonstrated that the bactericidal effect of MTS on P. aeruginosa is well expressed. However, the addition of RL contributed for the decrease of MBC of MTS. The difference between the effects of MTS alone and in combination with the RL was significant - MBC lowered from 80 μg ml⁻¹ to 50 μg ml⁻¹. Obviously, it is related with the higher protein leakage as a result of permeabilization with RL [4, 18]. Probably, the decrease in the effective concentrations of MTS, that are able to suppress the microbial growth completely, is due to the increased penetration of the MTS into the bacterial cells as a consequence of bacterial membrane damage. The results of our previous studies revealed that the changes in the presence of RL concern different components of the outer membrane of P. aeruginosa cells and depend on the rhamnolipid concentration [18, 19]. At concentration below CMC the biosurfactant effect was directed predominantly to outer membrane proteins, provoking modifications in outer membrane organization. Membrane proteins play important roles in various cellular processes and have a key role in the antibiotic resistance. One of the mechanisms for the control of penetration of antimicrobial agents in Gram-negative bacteria is by the use the tripartite efflux pumps, which included and an outer membrane proteins [41]. In P. aeruginosa the pump mexA-mexB-oprM has a significant role in antibiotics efflux [42- 44]. This major
multidrug efflux pump is composed of the MexB antiporter, the MexA periplasmic protein, and the OprM outer membrane protein [43]. The results reported by many researchers support the idea that over-expression of mexA-mexB-oprM contribute to increased resistance of P. aeruginosa [42, 43, 45]. Masuda et al. [46] established that the cross-resistance to meropenem, cepham, and quinolones is associated with overproducing of the outer membrane protein OprM. Cavallo et al. [47] also reported that overproduction of OprM is involved in the resistance of French P. aeruginosa isolates to β-lactams. In this investigation we observed an enhancement in antibacterial activity when MET and RL were applied in combination. Moreover, our previous results showed that the amount of protein OprM (involved in antibiotic resistance) markedly decreased in presence of RL [18], suggesting that this is a reason for significant increase of susceptibility of the strain to MTS.

SEM observations showed that RL treatment resulted in only minor changes - a bit more uneven cell surface and increase of the number of associated membrane vesicles. The changes in the cell surface morphology are probably due to the decrease in levels or loss of important outer membrane proteins [18, 48]. The observed ultrastructural changes of Ps. aeruginosa in the presence of rhamnolipid-biosurfactant are related to changes in the microbial cell surface properties and may lead to alterations in the sensitivity of the strain to MTS. Nevertheless, the rhamnolipid-biosurfactant does not affect the cell growth and viability.

The synergistic-like effect of RL and MTS was well demonstrated in this research by CLSM. The fluorescence method allowed monitoring of the bacterial viability in relation to membrane integrity or damage. The application of the surface-active substance alone was however not sufficient to cause cell-barrier damage that can result in permeation for propidium iodide into the bacterial cells. The predominance of propidium iodide-permeable non-viable cells in the samples treated with the rhamnolipid-MTS combination coincides with the other experimental results in this study and illustrates well the synergistic-like effect of the combination of the two preparations.

In conclusion, the bactericidal effect of the tested MTS was well expressed. Moreover, the MBC level was significantly reduced by the addition of RL. RL has a strong potentiative effect. RL targets mainly the outer membrane of the P. aeruginosa cells, changing the surface properties thus probably providing easier access of the antimicrobial agent to the cell. Additionally, RL did not affect the cell growth and viability. In fact, it has been demonstrated that the biosurfactant is needed in very low concentration (10 µg ml⁻¹) in this combination.

Altogether our results confirm that the use of the synergistic-like combination of rhamnolipid and antimicrobial agents is a good approach for production of novel, more effective antimicrobial preparations and has increasing potential for future antibacterial therapies.

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