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Effects of rhamnolipid-biosurfactant on cell surface of *Pseudomonas aeruginosa*

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Summary

The effect of rhamnolipid-biosurfactant produced by *Pseudomonas* sp. PS-17 on cell surface structures of *Pseudomonas aeruginosa* NBIMCC 1390 was studied. The results demonstrated that the rhamnolipid at concentrations below and above CMC provoked a multi-component response of the bacterial cells without affecting their growth and viability. Above CMC, the rhamnolipid caused reduction of total cellular LPS content of 22%, which can be associated with an increase in cell hydrophobicity to 31% adherence. The rhamnolipid-biosurfactant at concentration below CMC did not affect the LPS component of the bacterial outer membrane but caused changes in OMP composition of *P. aeruginosa*. Examination of the OMP profiles revealed that the amount of major proteins (Opr F, Opr D, Opr J and Opr M) markedly decreased. To our knowledge this is the first report on the rhamnolipid-biosurfactant interactions with bacterial cells showing changes in outer membrane proteins of *P. aeruginosa*. In both concentrations, the biosurfactant caused changes in cell surface morphology. The results indicate that the rhamnolipid-biosurfactant from *Pseudomonas* sp. PS-17 has a potential application in the relatively new field of biomedicine.

Introduction

Pseudomonas aeruginosa is a Gram-negative bacterium living in soil and aqueous environments. It is an important opportunistic pathogen, highly resistant to a large number of disinfectants and antibiotics as a result of permeability barrier function of the outer membrane. The outer membrane of Gram-negative bacteria is composed of lipopolysaccharides (LPS), phospolipids and lipoproteins, covalently linked to the peptidoglycan layer through hydrophobic interactions (Sikkema et al., 1995) and also contains porins and efflux pump embedded in the LPS layer (Poole, 2001). In *P. aeruginosa*, four major (OprF, OprP, OprB, OprD) and two minor (OprC, OprE) outer membrane

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proteins (OMPs) were reported to act as porins (Denyer and Maillard, 2002).

A number of studies suggest that it is possible to modify the outer membrane of Gram-negative bacteria by mutation (Yoneyama et al., 1995; Nikaido, 2003; Ni and Chen, 2004) or by addition of chemical agents that can act as membrane permeabilizing agents (Daugelavičius et al., 2000; Denyer and Maillard, 2002; Longbottom et al., 2004). These processes result in changes of the cell surface properties as well as in increased permeability and hydrophobicity of the bacterial membrane. Changes in cell surface properties were observed also in presence of surfactants and biosurfactants, surface active compounds with amphiphilic structure (Zhang and Miller, 1994; Al-Tahhan et al., 2000; Möbius et al., 2001). The biosurfactants that were studied most extensively in terms of their industrial and environmental application are the rhamnolipids. Nevertheless little is known about their interaction with bacterial cells, although it is likely that their surface and membrane active properties play an important role (Lang and Wullbtandt, 1999; Singh and Cameotra, 2004).

The rhamnolipid-biosurfactant PS was produced by bacterial strain *Pseudomonas* sp. PS-17 (Shulga et al., 2000). The low parameters for surface and interfacial tensions and critical micelle concentration of the rhamnolipid-biosurfactant PS indicate its high surface activity.

The aim of this study was to investigate the effect of a rhamnolipid-biosurfactant PS produced by *Pseudomonas* sp. PS-17 on (i) growth and total protein release, (ii) cell surface properties, and (iii) chemical and structural changes in the cell surface of *P. aeruginosa* NBIMCC 1390.

Materials and methods

Biosurfactant

The rhamnolipid-biosurfactant PS used in this study was produced and purified from *Pseudomonas* sp. PS-17 in the Laboratory of Biotechnology, Ukrainian Academy of Sciences (Lviv town), and provided by Dr. E. Karpenko and Dr. A. Shulga.

Microorganism

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

Culture medium and growth conditions

The cells of *P. aeruginosa* were grown in a mineral salts medium (MSM) (Spizizen, 1958) supplemented with $CaCl_2$, 2 mM; casein hydrolysate (Fluka), 0.5%; maltose, 0.5%; pH 7.2. Inoculum was prepared by transferring the cells from agar slants to 2 ml of MSM medium in 20-ml flasks and cultivated for 18 h at 37 °C with agitation at 200 rpm. The experimental culture of 20 ml was inoculated with 1% (v/v) inoculum and incubated in 250-ml flasks until late exponential phase (20 h). Growth conditions were the same as those used for preparing the inoculum. The growth was monitored by measuring the absorbance at 570 nm. Extracellular protein content was determined by the method of Bradford (1976).

Cell surface hydrophobicity

Cell hydrophobicity was measured by microbial adherence to hexadecane according to the method of Rosenberg et al. (1980). The cells were washed twice and resuspended in PUM buffer, pH 7.1 (22.2 g $K_2HPO_4 \cdot 3H_2O$, 7.26 g KH_2PO_4, 1.8 g urea, 0.2 g $MgSO_4 \cdot 7H_2O$ and distilled water to 1000 ml) to an initial absorbance of the cell suspension at 550 nm of 0.5–0.6. The cell suspension (1.2 ml) after the addition of hexadecane (0.2 ml) was vortexed in a test tube at high speed for 2 min and equilibrated for 1 h. The optical density of the bottom aqueous phase was then measured at 550 nm. Hydrophobicity was expressed as the percentage of cells adhered to the hydrocarbon and calculated as follows: $100 \times$ (1 − OD of the aqueous phase/OD of the initial cell suspension).

Isolation and analysis of LPS

Total LPS content in whole-cell lysates was determined by the thiobarbituric acid assay of 2-keto-3-deoxyoctonic acid (KDO) adapted from the method of Osborn et al. (1972). A standard curve was prepared by using purified LPS obtained from *P. aeruginosa* (Calbiochem). Cells were prepared for KDO assay as follows: 0.1 ml samples of culture were washed twice with distilled water, and the cell pellet was lysed by dissolving in 50 ml of lysing buffer and heating at 100 °C for 10 min (Hitchcock and Brown, 1983).

For LPS isolation, a modified version (Skurnik et al., 1995) of the protocol devised by Hitchcock

and Brown (1983) was used. The cells were grown till late log phase. The cell content of 1.5 ml cell suspension with an OD at 540 nm of 1.7 was harvested by centrifugation in a microcentrifuge (13,000*g*; 3 min), and then resuspended in 100 μl lysis buffer (2% deoxycholate [DOC], 4% 2-mercaptoethanol, 10% glycerol, and 0.002% bromophenol blue in 1 M Tris–HCl buffer (pH 6.8). Lysates were heated at 100 °C for 10 min. Then, 40 μg of proteinase K was added, and the suspension was incubated at 55 °C for at least 2 h. Samples were stored at −20 °C until analyzed by DOC-PAGE.

DOC-PAGE

DOC-PAGE was performed according to the system of Laemmli (1970) modified by Komuro and Galanos (1988) with DOC as detergent. The separation gel contained final concentration of 13% acrylamide, 0.5% DOC, and 375 mM Tris–HCl (pH 8.8). The stacking gel contained 4% acrylamide, 0.5% DOC, and 125 mM Tris–HCl (pH 6.8). LPS samples (0.1%, w/v) were prepared in the sample buffer [0.25% DOC, 175 mM Tris–HCl (pH 6.8), 10% glycerol, and 0.002% bromphenol blue]. The electrode buffer was prepared of DOC (2.5 g l^{-1}), glycine (14.4 g l^{-1}), and Tris (3.0 g l^{-1}). Electrophoresis experiments were performed at a constant current of 30 mA. After the electrophoresis run, the gel was soaked in an aqueous solution containing 40% ethanol and 5% acetic acid with gentle shaking. LPS bands were visualized by silver staining as described by Tsai and Frash (1982).

Assay of outer membrane proteins

Exponentially grown cells were harvested by centrifugation at 7000*g* for 10 min at 4 °C, suspended in 30 mM Tris–HCl (pH 8.0), and then broken with a sonicator (MSE, UK) for 2 min. Unbroken cells were removed by centrifugation at 4 °C. Membranes were pelleted by centrifugation at 100,000*g* for 1 h at 4 °C and suspended in the same buffer. The inner membrane was solubilized by adding of sodium *N*-lauroylsarcosinate to the suspension at a final concentration of 1%. This was followed by incubation for 30 min at 30 °C. The outer membrane was pelleted by centrifugation at 18,000*g* for 40 min at 30 °C and suspended in the buffer (Masuda et al., 1995). The outer membrane fractions were analyzed by SDS-PAGE as reported by Laemmli (1970) with 10% (w/v) acrylamide. Samples for SDS-PAGE were treated with 2% SDS–5% 2-mercaptoethanol at 100 °C for 5 min or at 37 °C for 10 min, and then were subjected to electrophoresis at a constant current of 25 mA at 4 °C.

Transmission electron microscopy (TEM)

The bacterial cells were prepared for electron microscopy using the method of Sabatini et al. (1960) with 6% glutaraldehyde for 2 h and osmium postfixation overnight at room temperature. This procedure was followed by dehydration with alcohol (in increasing concentrations) and embedding in Durcupan. Staining was carried out using the technique of Raynolds (1963). Thin sections were examined with a Zeiss electron microscope (model 10C).

Results

Effect of rhamnolipid PS on bacterial growth and protein release

The effect of biosurfactant concentration on the growth and protein release of *P. aeruginosa* was studied and the results are shown in Fig. 1. The presence of rhamnolipid-biosurfactant in the medium at concentrations below CMC (50 μg ml^{-1}) and above CMC (100 μg ml^{-1}, 300 μg ml^{-1}) did not alter the growth compare to the control. The increase in the amount of extracellular protein registered at all tested concentrations of biosurfactant-rhamnolipid was more significant at concentrations above CMC (Fig. 1).

Effect of rhamnolipid PS on cell surface hydrophobicity

The cells of *P. aeruginosa* NBIMCC 1390 exhibited low values of cell surface hydrophobicity as

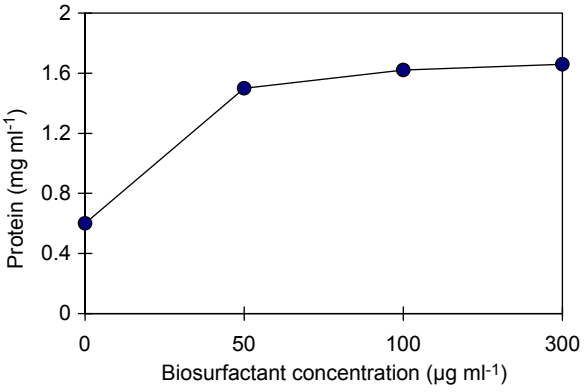

Figure 1. Effect of rhamnolipid-biosurfactant on protein release by *Pseudomonas aeruginosa* NBIMCC 1390. Mean values are given with standard deviations of ⩽10%.

measured by BAHT assay. The cell surface hydrophobicity did not exceed 21% adherence. The addition of rhamnolipid-biosurfactant in concentrations below and above CMC to the growth medium caused slight increase in cell surface hydrophobicity during the late exponential phase (Fig. 2).

Total cellular LPS content

To determine the effect of rhamnolipid-biosurfactant on the release of LPS from the cell surface total LPS content of the cells grown to the late exponential phase was measured. The content of LPS in control cells was determined to 7.6 $\mu g\,ml^{-1}$. The LPS content of cells grown in presence of rhamnolipid-biosurfactant in concentrations above CMC (100 and 300 $\mu g\,ml^{-1}$) was reduced with 1.6 $\mu g\,ml^{-1}$ of cell suspension compare to the control cells, or almost 22% of the total LPS content of the cells (Fig. 2).

DOC-PAGE revealed that the LPS band of cells grown in presence of rhamnolipid-biosurfactant at concentration 300 $\mu g\,ml^{-1}$ (line 3) was less intense than those of the control cells (line 2), as well as at the cells grown in presence of rhamnolipid-biosurfactant at concentrations below CMC (line 4) (Fig. 3). Obviously, the presence of rhamnolipid-biosurfactant in growth media at concentration above CMC resulted in a decrease of the total amount of LPS.

The outer membrane protein composition

The OMP composition of *P. aeruginosa* cells grown in presence and absence of rhamnolipid-biosurfactant was analyzed by SDS-PAGE. The amount of

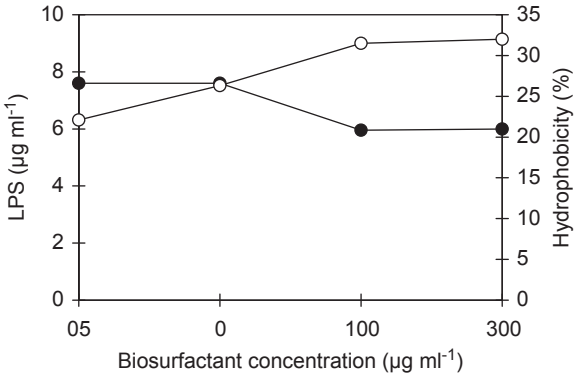

Figure 2. Effect of rhamnolipid-biosurfactant on cell hydrophobicity (○) and LPS content (●) in outer membrane of *Pseudomonas aeruginosa* NBIMCC 1390. Mean values are given with standard deviations of ⩽10%.

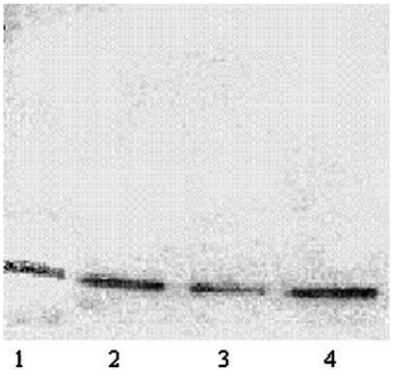

Figure 3. DOC-PAGE of outer membrane LPS of *Pseudomonas aeruginosa* NBIMCC 1390. Line 1, LPS of *Pseudomonas aeruginosa* (Calbiochem); line 2, LPS of cells grown in absence of rhamnolipid; line 3, LPS of cells grown in presence of 300 $\mu g\,ml^{-1}$ rhamnolipid; line 4, LPS of cells grown in presence of 50 $\mu g\,ml^{-1}$ rhamnolipid.

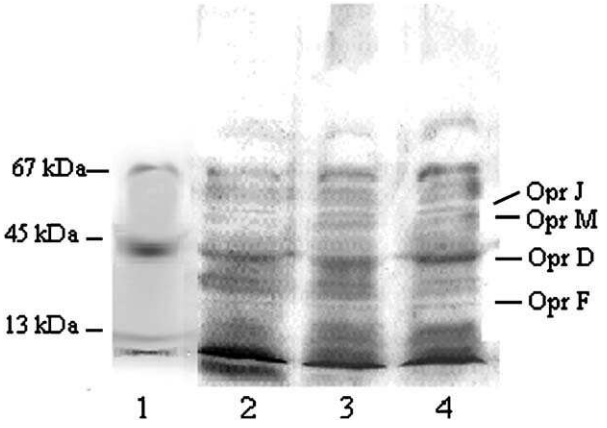

Figure 4. SDS-PAGE of outer membrane proteins (OMPs) of *Pseudomonas aeruginosa* NBIMCC 1390. Line 1, protein markers: citochrome C (13 kDa), ovalbumine (45 kDa), bovine albumine (67 kDa); line 2, OMPs of cells grown in presence of 50 $\mu g\,ml^{-1}$ rhamnolipid; line 3, OMPs of cells grown in presence of 300 $\mu g\,ml^{-1}$ rhamnolipid; line 4, OMPs of cells grown in absence of rhamnolipid.

OMPs obtained from cells, grown in presence of 300 $\mu g\,ml^{-1}$ rhamnolipid-biosurfactant (line 3, Fig. 4) was comparable to those in the control cells (line 4, Fig. 4). In contrast, the OMP membrane banding patterns of cells grown in presence of rhamnolipid-biosurfactant at concentration below CMC (line 2, Fig. 4) were less intense than those observed with the control cells. The amount of proteins with an apparent molecular mass of 54 kDa (Opr J) and 50 kDa (Opr M) decreased. Reduction in the amounts of the Opr D (45 kDa) and Opr F (38 kDa) was also registered as well as the decrease of the amounts of all other OMPs

Transmission electron microscopy (TEM)

Morphological changes of *P. aeruginosa* NBIMCC 1390 cells in the presence of rhamnolipid-biosurfactant were examined by TEM. Ultrastructural studies revealed that biosurfactant effects were directed mainly on cell surface structures and in lesser degree on inner cell structures of the bacterial cells (Fig. 5). Compare to the control cells (Fig. 5A), in thin sections of cells grown in presence of rhamnolipid-biosurfactant at concentrations below CMC the cell surface structures became smooth, thin and indistinct (Fig. 5B). A disruption of the surface membranes in certain zones was observed in the cells cultivated in presence of the rhamnolipid-biosurfactant at concentrations above CMC (Fig. 5C). The cytoplasmic content in contact with the disrupted cell membrane became translucent. Furthermore, the release of electron-dense amorphous material was observed (Fig. 5D). These morphological changes did not, however, affect the cell viability.

Discussion

In the present work, we studied the effect of rhamnolipid-biosurfactant on bacterial cell growth and protein release, cell surface properties, chemical and structural changes in the cell surface of *P. aeruginosa* NBIMCC 1390. The results demonstrated that the addition of rhamnolipid-biosurfactant to the growth medium at concentrations of 50–300 $\mu g\,ml^{-1}$ did not affect the growth but increased significantly the levels of extracellular proteins. The increased amount of extracellular

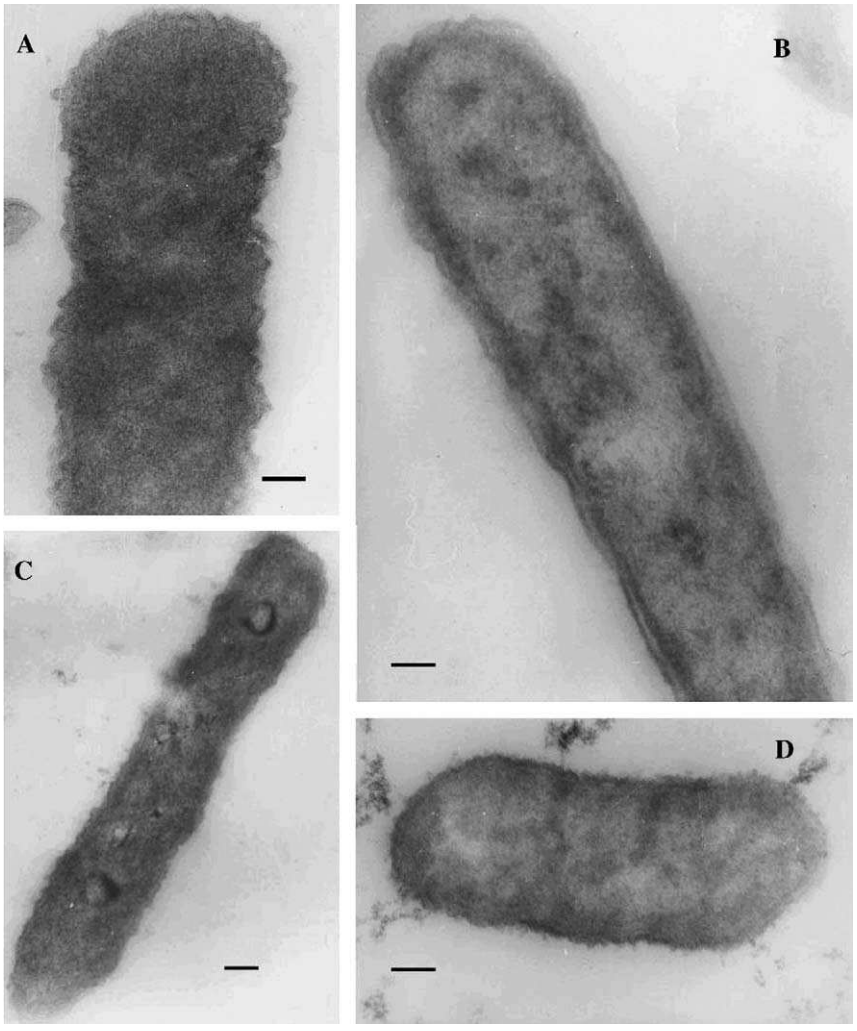

Figure 5. Ultrathin sections of cells of *Pseudomonas aeruginosa* NBIMCC 1390 grown in absence of rhamnolipid-biosurfactant (A), in presence of 50 $\mu g\,ml^{-1}$ rhamnolipid-biosurfactant (B), and in presence of 300 $\mu g\,ml^{-1}$ rhamnolipid-biosurfactant (C, D). Bars 0.1 μm.

protein was dependent on the biosurfactant concentration and was probably due to increased cell permeability.

It was found that in presence of rhamnolipid-biosurfactant at concentration above CMC the cell surface became more hydrophobic. The results suggest that the enhanced cell hydrophobicity is associated with a reduction of the total cellular LPS content. LPSs are the main component of the outer membrane of Gram-negative bacteria and are responsible for the cell inpermeability characteristics (Denyer and Maillard, 2002). LPS play an important role in bacterial resistance to antimicrobial agents (Russell and Furr, 1986; Russell, 1995). The quantity and type of LPS has a profound effect on the interactions between the microbial cell and its environment (Makin and Beveridge, 1996). Al-Tahhan et al. (2000) observed a loss of LPS in *P. aeruginosa* strains treated with rhamnolipid at low concentrations and this resulted in increased cell surface hydrophobicity. We observed similar changes with *P. aeruginosa* NBIMCC 1390 grown in presence of rhamnolipid-biosurfactant but in concentrations above CMC. The release of LPS from the cell surface may be due to solubilization of the outer membrane by binding of aggregated biosurfactant to the membrane followed by removal of the LPS component.

Our results showed that the rhamnolipid-biosurfactant at concentration below CMC did not affect the LPS component of the bacterial outer membrane but caused changes in the OMP composition of *P. aeruginosa* NBIMCC 1390. Examination of OMP profiles revealed that the amount of major proteins (Opr F, Opr D, Opr J and Opr M) markedly decreased. Bacterial OMPs are important virulence factor and play a significant role in the pathogenesis of infection diseases as well as in antibiotic resistance. The results of this research demonstrate that rhamnolipid-biosurfactant in concentrations below CMC caused a decrease in the amount of important OMPs, which may lead to alterations in pathogenic properties and antibiotic sensitivity of *P. aeruginosa* NBIMCC 1390. The observed effect of the rhamnolipid-biosurfactant on the protein composition was probably due to binding of biosurfactant monomers to outer membrane causing changes in the membrane organization. The reduction in the amount of OMPs may decrease the compactness of the outer membrane leading to it becoming smoother, thinner and indistinct as was demonstrated by TEM. The disruption of the outer membrane observed in certain zones allows for increased passage of hydrophobic compounds. Still, these changes did not affect the cell viability.

In conclusion, the results of the present study reveal that the changes in the bacterial cell surface structures of *P. aeruginosa* NBIMCC 1390 in presence of rhamnolipid-biosurfactant concern different components of the outer membrane and are dependent on the rhamnolipid concentration. The biosurfactant in concentrations below CMC has an effect on OMPs, while in concentrations above CMC the LPS layer was affected. In both cases the biosurfactant caused changes in the cell surface morphology. To our knowledge, this is a first report that shows changes in the bacterial OMPs in presence of a rhamnolipid-biosurfactant. The understanding of the biosurfactant effects on the cell surfaces can contribute to the use of biosurfactants not only in bioremediation technologies but also in different biomedical applications.

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