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Proteomic approach to *Pseudomonas aeruginosa* adaptive resistance to benzalkonium chloride

Idalina Machado[a,*], *Laurent Coquet*[b], *Thierry Jouenne*[b], *Maria Olívia Pereira*[a]

[a]*IBB — Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal*

[b]*BRICs Group, Laboratory "Polymères, Biopolymères, Surfaces", UMR 6270 CNRS, FR 3038 PISSARO Proteomic Platform, University of Rouen, France*

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ABSTRACT

This study aimed to assess the membrane modifications in *Pseudomonas aeruginosa* after continuous exposure to increasing doses of benzalkonium chloride (BC). Two different concentrations were used, 0.9 and 12.0 mM.

Proteomic investigations revealed that the range of the outer membrane proteome alterations following continuous exposure is very low, i.e. about 10% and BC concentration dependent. Adapted cells revealed different expressions of key proteins frequently reported as involved in acquired resistance mechanisms. Porins (OprF and OprG) and lipoproteins (OprL and OprI) were underexpressed when the higher adaptation concentration (12 mM) was used.

Some of these membrane alterations have been described as involved in the acquired resistance to antibiotics, suggesting possible common mechanisms between these two types of resistance.

Biological significance

Results obtained after *P. aeruginosa* adaptation to benzalkonium chloride suggest that the bacterial adaptation to BC do not mobilize complete outer membrane systems. Though, we showed that adaptive resistance to BC promoted some changes in proteins previously described as involved in antibiotic resistance. These results contribute to the assumption that there are common resistance mechanisms, between adaptive and acquired resistance of *P. aeruginosa*.

1. Introduction

Pseudomonas aeruginosa has long been regarded as an antimicrobial-resistant organism. The major causes of this bacteria intrinsic resistance [3] are its low outer membrane permeability [1] responsible for preventing the access of some agents to their sites of action and the presence of efflux pumps responsible for the extrusion of many antibiotics [2]. There are two well-described mechanisms of resistance, intrinsic and acquired, both characterized by an irreversible phenotype and independent of the presence of the antibiotic or the adverse environmental conditions [3]. However, there is a third mechanism, adaptive resistance that is not so well understood. It is mainly characterized by reversible phenotypic changes that occur at the cell level. These changes allow the bacteria to grow in adverse conditions [3], but once the external stress is removed, the organism reverts to its wild-type susceptibility [3,4].

Antimicrobial products, like benzalkonium chloride, that are frequently used to eradicate bacteria, can alter the

* *Corresponding author at:* CBQF/Escola Superior de Biotecnologia, Universidade Católica Portuguesa, 4200-072 Porto, Portugal. Tel.: +351 225580046.
E-mail address: imachado@porto.ucp.pt (I. Machado).

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environment and can trigger adaptive resistance mechanisms [5,6]. Some reports suggest that the widespread use of biocides and disinfectants in hospitals, and to a lesser extent at home, even where there is a clear benefit, could act as a selective pressure for antimicrobial-resistant bacteria [7,8]. The shift of bacterial adaptive resistance mechanisms due to the exposure to antimicrobial agents encompasses some alterations of the outer membrane protein systems [3].

Membrane proteins are central to cell life because they are in the interface between the intra and extra-cellular compartments of the cell [9] and play important roles in various cellular processes such as cell adhesion, metabolites and ion transport, and endocytosis host immune responses. Thus, membrane proteins are very important for pharmacological action and represent potential targets for vaccine development [10].

This work aims to identify the proteomic modifications endorsed by the induction of adaptive resistance of *P. aeruginosa* to different adaptation concentrations of benzalkonium chloride. This knowledge will give some insights in the understanding of the parameters involved in sanitation failure and adaptive resistance acquisition.

2. Experimental procedures

2.1. Strain and culture conditions

P. aeruginosa (ATCC 10145) was preserved in criovials (Nalgene) at −80 ± 2 °C. Prior to each experiment, *P. aeruginosa* bacterial cells were grown on Triptic Soy Agar (TSA, Merck Biosciences) plates for 24 h, at 37 °C. Adapted *P. aeruginosa* strains were preserved in criovials and cultured in TSA supplemented with benzalkonium chloride (BC), in a final concentration of 0.9 mM and 12.0 mM for 24 h at 37 °C.

2.2. Antibacterial agent

Benzalkonium chloride (BC), a quaternary ammonium compound, was purchased from Calbiochem (Merck Biosciences).

2.3. Induction of BC adaptive resistance in P. aeruginosa

Adaptive resistance was induced by subculturing *P. aeruginosa* in Triptic Soy Broth (TSB) supplemented with increasing BC concentrations, according to the adaptive procedures described by Machado et al. [11]. Briefly, 5 mL of an overnight culture (1×10^7 CFU/mL) was added to flasks containing 45 mL of TSB supplemented with BC at final concentrations of 0.5, 0.9, 1.3 and 2.0 mM. Cultures were then incubated at 37 °C for 48 h on a horizontal shaker (120 rpm). Bacterial growth was monitored by optical density measurement at 640 nm (OD_{640}). Every two days, 5 mL of the bacterial culture, supplemented with the highest BC concentration for which bacterial growth was observed, was used to inoculate 45 mL of TSB containing BC in a final concentration 0.2 mM higher than the one that exhibited growth. At the end of the third growth cycle in increased BC solutions, no significant bacterial load was observed. Bacteria were then subcultured in the presence of the maximum BC concentration that allowed growth for another three complete cycles. At the end of the adaptation process, culture purity was checked by spreading aliquots on to *Pseudomonas* spp. selective agar (Cetrimide agar base, DIFCO).

The stability of the BC-adapted strains was later determined by continuous subculture every 24 h for 10 passages in TSA and subsequent evaluation of the adapted strains ability to maintain their growth in TSA supplemented with BC. To preserve the BC-adapted strains, Petri dishes were prepared with TSA supplemented with BC at a final concentration of 0.9 mM, and 12.0 mM (referred as adapted *P. aeruginosa* strains A0.9 and A12, respectively) (Fig. 1). Bacteria from the step-wise training were preserved in the BC-enriched TSA. Adaptation processes were performed in three independent replicates.

2.4. Proteomics

2.4.1. Preparation and analysis of outer membrane protein (OMP) extracts

Crude outer membrane extracts were prepared from bacterial pellets following the spheroplast procedure described by Mizuno and Kageyama [12].

For protein extraction, and for each adapted strain obtained in each different adaptation process, standardized cell suspensions were prepared in three separate occasions in a minimal salt medium (MSM medium, pH 7.5) [13]. The bacterial cell concentration of each suspension was estimated by OD_{640} referred to a calibration curve [14]. The bacterial planktonic cultures were prepared in 800 mL of MSM medium by adjusting the final cell concentration to 1×10^7 CFU/mL and were allowed to grow at 37 °C for 20 h, at 150 rpm. Bacterial cultures (collection and adapted strains) were harvested for 15 min at 3500 ×*g* and washed twice with 20% (w/v) sucrose solution. Cells were suspended in a digestion solution: 9 mL 2.0 M sucrose solution; 10 mL 0.1 M Tris–HLCl pH 7.8, at 25 °C; 0.8 mL 1% (w/v) Na–EDTA, pH 7.0; and 1.8 mL 0.5% (w/v) lysozime. The mixture was incubated for 1.5 h at 37 °C in the presence of DNAse and RNAse (5 μg/mL; Sigma). Spheroplasts were collected by centrifugation (20 min at 10,000 ×*g*) and outer membranes were then pelleted (120,000 ×*g* for 1 h at 4 °C) and resuspended in 1 mL of sterile ultra-pure (UP) water. The protein amount was measured using the Bio-Rad protein assay (Bio-Rad, Hercules, CA). Protein extraction experiments were performed three times per condition.

2.5. Two-dimensional gel electrophoresis

Outer membrane protein patterns were analyzed by two-dimensional gel electrophoresis (2-DE). Two hundred micrograms of proteins was added to isoelectric focusing (IEF) buffer (final volume, 300 μL) [15], with the following composition: 5 M urea, 2 M thiourea, 1% amidosulfobetaine-14 (ASB-14), 2% w/v DTT and 2% v/v carrier ampholytes 4–7 NL. The first-dimension gel separation was carried out with Immobiline Dry Strips L (18 cm, pH 4–7, Amersham Pharmacia Biotech). The second dimension was obtained by SDS-PAGE using a 12.5% (w/v) polyacrylamide resolving gel (width 16 cm, length 20 cm, thickness 0.75 mm). After migration, proteins were visualized by silver nitrate staining [16]. For each extraction, two gels were obtained per condition.

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