

Proteomic Changes in *Pseudomonas aeruginosa* Biofilm Cells after Adaptive Resistance Development

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Abstract

This work investigates the outer membrane (OM) proteomic alteration of *Pseudomonas aeruginosa* biofilm cells after adaptation to benzalkonium chloride (BC) and ciprofloxacin (CIP). Biofilms were formed in 6-well plates for 24 h being after submitted to 324 mg/L BC and 6.0 mg/L CIP, during 12 days. Cells were harvested, the OM proteins extracted and patterns compared. *P. aeruginosa* adaptation altered the total amount of six proteins, about 10% of the discriminated proteins on two-dimensional gels. Exposure to both antimicrobials generated a common down-regulation of three proteins, GroEL, the major capsid protein and the putative tail sheath protein. The type 4 fimbrial biogenesis outer membrane protein PiiQ precursor was over-expressed only in biofilm cells submitted to BC, while the probable bacteriophage protein and the hypothetical protein PA0537 were overexpressed in CIP exposed biofilm cells. The alterations in OMPs expression might be involved in the biofilm bacteria high resistance to antimicrobial agents.

Keywords: *Pseudomonas aeruginosa*; Biofilms; Proteome; Chemical adaptation

Introduction

The Gram-negative bacterium *Pseudomonas aeruginosa* is a major cause of infection in hospitalized, immunocompromised, and cystic fibrosis patients and is considered an intrinsic resistant organism [1]. *P. aeruginosa* resistance is often associated with its high ability to attach to a surface and to form biofilms [2]. In this type of communities cells are embed within a matrix of exopolymeric substances [3], in particular polysaccharides, proteins, and nucleic acids [4]. Though increased biofilm tolerance to antimicrobials is not yet fully understood, several mechanisms have been recognized as playing major roles: (i) slow microbial growth rate [5,6]; (ii) the emergence of persister cells; (iii) the diffusion barrier promoted by the biofilm matrix that prevents antimicrobials to reach their targets, and (iv) intercellular signals that alter the biofilm physiology, causing bacteria to produce molecular pumps that expel antibiotics from the cells and allow the community to tolerate the presence of the drug [7]. In order to effectively kill bacteria embedded in biofilms, high doses of antibiotics applied during long periods of time should be used, however, due to toxicity issues this is not feasible [8]. Anti-biofilm agents, like biocides, usually kill only external cell layers of biofilms, exposing the inner-entrapped biofilm cells to gradually reduced concentrations of the antimicrobial products and thus the enclosed protected bacterial cells persist and instigate biofilm regrowth. This exposure to supra-minimum inhibitory concentrations (MIC) frequently induce the development of adaptive resistance to antimicrobial agents [9]. It is now known that this type of resistance, caused by exposure to supra-MIC levels of antibiotics, can induce alterations in gene and / or protein expression [10]. This type of adaptive resistance to antimicrobials is a way of antimicrobial stress response and has been widely reported in planktonic growth and studied through phenotypic characterization and proteomic analyses [11-14]. Concerning biofilm adaptation, the increased resistance and the mechanisms at the membrane level of biofilm-entrapped cells to antimicrobial exposure have been scarcely studied. The proteins specifically recruited to cause biofilm resistance when compared with the free floating cells have rapidly emerged at the beginning of the

2000s [15-20]. Multivariate analyses demonstrated that the biofilm growth mode leads to a specific bacterial proteome in sessile bacteria as compared to their planktonic counterparts [19,21-25]. However, as recently pointed out by Seneviratne [26], data regarding biofilm antimicrobial-induced proteomic changes are sparse. The present study aimed at characterizing the proteomic changes, induced at the outer membrane level of *P. aeruginosa* cells that persist in biofilms exposed to benzalkonium chloride (BC) and ciprofloxacin (CIP). With this, it is intended to identify possible pathways associated with biofilm adaptation to antimicrobial agents (biocides and antibiotics) bringing know-how regarding the possible cross-resistance mechanisms observed in biofilms.

Experimental Procedures

Strain and culture conditions

Pseudomonas aeruginosa (ATCC 10145 strain) was stored at -80 ± 2°C in 10% glycerol stocks. Prior to each experiment, bacterial cells were grown on Tryptic Soy Agar (TSA, Merck) plates for 24 h, at 37°C.

Antibacterial agents

Benzalkonium chloride (BC), a quaternary ammonium compound, widely used in clinical disinfectant formulations, was purchased from Calbiochem (Merck Biosciences, UK). Ciprofloxacin (CIP) is a broad-

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spectrum fluoroquinolone purchased from Fluka, used clinically to treat chronic *P. aeruginosa* infections.

Biofilm development

The methodology used for biofilms formation was based on the microtiter plate test developed by Stepanovic et al. [27]. Cell suspensions of *P. aeruginosa*, were diluted in TSB to obtain a final concentration around 10^7 cfu/ml. Two millilitres per well of the bacterial suspension were then transferred into sterile 6-well tissue culture plates (Orange Scientific, Braine-l'Alleud, Belgium). Plates were incubated aerobically on a horizontal shaker (120 rpm), at 37°C, during 24 h to form biofilms.

Biofilm adaptation

After biofilm formation, the content of each well was removed. After washing with 3 ml of ultra-pure (UP) sterilized water, 3 ml of TSB supplemented with 324 mg/L BC or 6.0 mg/L CIP were added. Every 24 h, the content of each well was removed and 2 ml of fresh medium with antimicrobials were added into each well. This procedure was repeated for 12 subsequent days. The content of each well was then removed and biofilms were washed twice with 3 mL of UP water. Control biofilms, i.e., non-adapted biofilms, were done for the same period of time using the same protocol but without antimicrobials. Attached bacteria were harvested by scraping with a sterile rubber scraper until the wells were visually clear, and bacteria were then resuspended in UP water. The resulting suspension was vigorously vortexed and stored at -80°C until needed.

Proteomics

Preparation and analysis of outer membrane protein (OMP) extracts: Bacterial cultures were centrifuged for 15 min at $3500 \times g$ and the pellets were suspended in 1 ml of (3-[(3-Cholamidopropyl) dimethylammonio]-1 propanesulfonate) (CHAPS). Cells were then disrupted by sonication (Ultrasonic Processor, Cole-Parmer, USA) using 10 W powers for 6 pulses of 10 s (separated by 2-s breaks) on ice. Unbroken cells and cell debris were removed from the resulting suspension by centrifugation at $7\,000 \times g$ for 10 min at 4°C. The supernatant was then centrifuged at $100,000 \times g$ for 1 h at 4°C, the formed pellet representing the total membrane fraction. The separation of the bacterial membranes was adapted from the protocol described by Winder et al. [28]. Membrane proteins were incubated in 25 ml of 2% (w/v) sodium lauryl sarcosinate solution at room temperature for 1 h, followed by centrifugation at $100,000 \times g$ for 1 h at 4°C. The resulting pellet, i.e., the outer membrane fraction, was resuspended in 1 ml of UP water. The protein amount was measured using the Bio-Rad protein assay (Bio-Rad, Hercules, CA).

Two-dimensional gel electrophoresis: OMPs patterns were analyzed by two-dimensional gel electrophoresis (2-DE). Two hundred micrograms of proteins were added to isoelectric focusing (IEF) buffer (final volume, 300 μ l) [29] 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 was carried out with Immobiline Dry Strips L (pH 4-7, Amersham Pharmacia Biotech). The second dimension was obtained by a 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 [30].

Gel analysis: Spot quantification was achieved by computing scanning densitometry (ProXPRESS 2D, PerkinElmer Sciex). Gels were analyzed using the Progenesis Samespot (Nonlinear Dynamics)

software. For each experimental condition, three 2-DE gels were matched together to form a reference image. The two reference gels were then matched together so that the same spot in different gels had the same number. Protein spots from the two bacterial populations were considered to display significant quantitative differences if they fulfilled the following criteria: p values 0.05 (t-test); detection threshold, average volume ≥ 20 ($n = 3$); differential tolerance, fold change ≥ 2 [23].

Protein identification: Spots excised from the polyacrylamide gel complied with the following criteria: volume varying with the incubation conditions and displaying a high (average) value with a low coefficient of variation. Gel plugs were dried using a SpeedVac centrifuge. Trypsin digestion was performed using an automatic digester (MultiPROBE II, PerkinElmer Sciex). After lyophilization, the peptide extracts were resuspended in 10 μ l of 0.2% formic acid / 5% acetonitrile.

Peptides were enriched and separated using a lab-on-a-chip technology (Agilent, Massy, France) and fragmented using an on-line XCT mass spectrometer (Agilent). The fragmentation data were interpreted using the Data Analysis program (version 3.4, Bruker Daltonic). For protein identification, MS / MS peak lists were extracted and compared to the NCBI protein database restricted to *P. aeruginosa* (Version 4, 1,342,017 residues, 4243 sequences), using the MASCOT Daemon search engine. All searches were performed with no fixed modification and allowed for carbamidomethylation, oxidation and a maximum of one missed trypsin cleavage. MS / MS spectra were searched with a mass tolerance of 1.6 Da for precursor ions and 0.8 Da for fragment ions, respectively. If a protein was characterized by 2 peptides with a fragmentation profile score higher than 25 the protein was validated. When one of the criteria was not met, peptides were systematically checked and / or interpreted manually to confirm or cancel the MASCOT suggestion.

Bioinformatic tools for subcellular location: The prediction of identified proteins location, and in particular for unknown proteins, within the bacterial cell, was realized from the genome annotation of *P. aeruginosa* (accessible at <http://www.pseudomonas.com>).

Results and Discussion

Biofilm proteins extraction

Biofilm proteomic investigations generally comprise the characterization of the alterations of the protein patterns between planktonic and sessile organisms, and during the different developmental biofilm stages [23,31,32]. In the present study, the alterations of the OM proteome of sessile *P. aeruginosa* cells after adaptation for 12 days to supra-MIC of two antimicrobials were characterized. It has been shown that permeability changes caused by antimicrobials exposure can lead to bacterial adaptive resistance to antimicrobial agents [33]. Particularly, biocides are known to induce alterations in bacterial OM, fatty acid changes as well as changes in active efflux [12]. Regarding adaptive resistance induced by fluoroquinolones like CIP, some studies [12,34] showed that the main adaptive resistance mechanism is related with the dysregulation of genes encoding the MexAB efflux pump, and that more generally, the cell envelope plays a key role in this type of bacterial resistance [35-38]. Often performed on planktonic cultures [12], such studies have been rarely performed on biofilm organisms. High doses of antimicrobials were here used in order to ensure the selection of *P. aeruginosa* biofilm cells, which were able to survive upon a long exposure to these agents.

About 600 proteins were discriminated with $p < 0.05$ and fold > 2

(Figure 1). Due to the small number of gels that could be analysed for each condition (3 gels), it was also considered the q value ($q \leq 0.05$) to guarantee that no false positives were mistakenly analysed. The protein expression of the biofilm-growing bacteria challenged by BC and CIP is listed in Table 1 and shown in Figure 2. The main difficulty during sample preparation was related with the reduced number of bacteria that developed adaptive resistance and survived within the stressed biofilm and, in consequence the reduced concentration of OMPs obtained in each extraction. Additionally, the difficulties felt during due to the presence of biofilm matrix within the suspension of bacterial cells may also lead to sample contamination. This difficulty became even higher when biofilms were developed under chemical stress since more extracellular polymeric substances were secreted by bacteria in order to defend the surviving cells from the aggression [2,5]. Consequently, an increase in sample contaminants was observed when

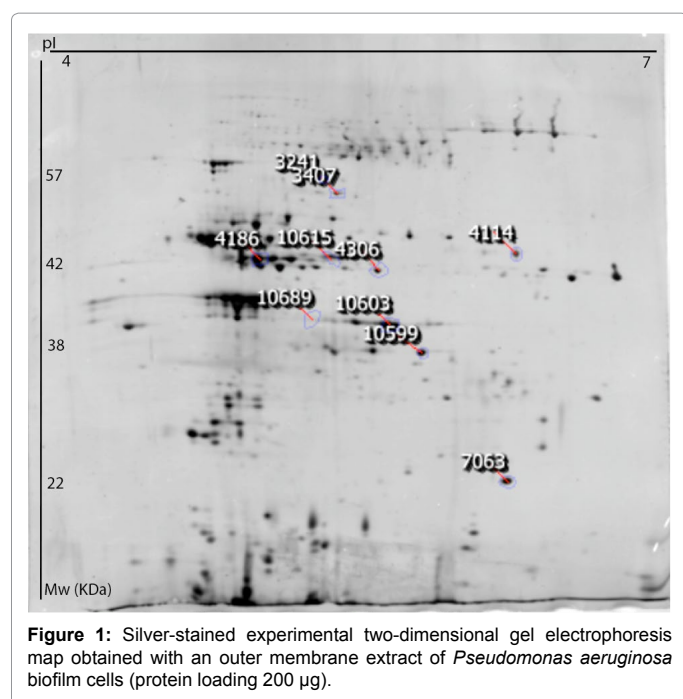


Figure 1: Silver-stained experimental two-dimensional gel electrophoresis map obtained with an outer membrane extract of *Pseudomonas aeruginosa* biofilm cells (protein loading 200 µg).

biofilms were developed under stress compared with control biofilms. Ten modified spots were common to the two adapted populations. All these spots were down-regulated both in BC- adapted and CIP-adapted biofilm cells. The identification of the corresponding proteins is given in Table 1. The low percentage of modified OMPs may suggest that this specific bacterial adaptation induced small alterations at OMP level of the biofilm-adapted organisms. This observation may witness the over-expression of stress proteins by sessile cells even in the absence of antimicrobials, due to environmental conditions prevailing within the matrix [32].

Down-regulated proteins

Biofilm exposure to BC and CIP generated a down-regulation of four same proteins: GroEL, the predicted major capsid protein, the putative tail sheath protein, and a hypothetical protein PA0537 (Table 1).

GroEL was found in several spots (3241, 3407, 4114), as well as putative tail sheath protein (4186, 4306), pointing out some translational modifications [37]. Posttranslational modifications of GroEL have already been observed both in the membrane, cytoplasmic and periplasmic compartments [14,39]. Indeed, though this protein is predominantly cytoplasmic, it has been found in all the cellular compartments [1,40]. It has been shown that this protein facilitates the folding process of membrane associated proteins [41], and participates in bacteria adhesion to tissues [42]. Though downregulated in sessile bacteria adapted to both antimicrobials, its amount decreased more in BC than in CIP cells. The downregulation of this chaperon protein is surprising considering its role in bacterial adaptation. In a study exposing *Escherichia coli* cells to a shock concentration of trimethoprim, this chaperonin complex was 1.4 fold times over-expressed [43].

The major capsid protein and putative tail sheath proteins, that were also under-expressed in BC and CIP adapted cells (Figure 2 and Table 1), have unknown function(s) yet and are encoded by genes that are part of a previously described genomic island (GI), Pagi-6 [44]. This GI is an example of a prophage that has undergone multiple recombination and deletion events resulting in *P. aeruginosa* altered virulence [45]. The putative tail sheath protein has been also associated with membrane vesicles (MV). Membrane vesicles are naturally released from the bacterial outer surface and are constituted of OM proteins, lipopolysaccharides and periplasmic components. *P. aeruginosa* MV

Protein number ^a	Protein	Accession number	Subcellular Localization ^b	M (kDa)	pI	N ^o of matching peptides	Mas-cot Score	ANOVA (p)	Normalized volumes mean (S.D.) ^c				
									TSB	BC	CIP		
3241	Chain A, Crystal Structure of the Asymmetric Chaperonin Complex GroELGROES (ADP)7	38491472	C	57.3	4.85	10	363	3,303e-4	5.79 (0.047)	5.05 (0.25)	-	5.32 (0.16)	-
3407	GroEL	6225121	C	56.6	4.85	6	293	3,845e-4	6.16 (0.063)	5.62 (0.063)	-	6.00 (0.21)	-
4114	GroEL	6225123	C	56.6	4.85	3	162	1,214e-4	6.40 (0.032)	5.51 (0.21)	-	6.08 (0.22)	-
4186	Putative tail sheath protein	148807411	OM	41.9	5.00	3	156	1,873e-4	6.98 (0.099)	6.61 (0.18)	-	6.39 (0.066)	-
4306	Putative tail sheath protein	148807411	OM	57.3	5.00	1	47	1,190e-4	6.52 (0.18)	5.75 (0.14)	-	6.47 (0.19)	-
7063	Hypothetical protein PA0537	15595734	U	22.1	8.63	9	462	2,038e-6	6.66 (0.06)	5.68 (0.057)	-	6.35 (0.16)	-
10599	Predicted major capsid protein	148807393	U	37.8	5.83	7	371	2,930e-4	6.6 (0.012)	5.4 (0.56)	-	5.25 (0.21)	-
10603	Predicted major capsid protein	148807393	U	37.8	5.83	10	455	4,328e-4	6.93 (0.20)	5.84 (0.13)	-	5.81 (0.49)	-
10615	Bacteriophage protein	15595819	C	41.2	5.27	1	49	4,966e-4	6.35 (0.26)	5.73 (0.32)	-	6.69 (0.12)	+
10689	Type IV fimbrial biogenesis outer membrane protein PilQ precursor	254244078	OM	77.3	5.56	8	317	3,751e-4	6.0 (0.18)	6.6 (0.14)	+	5.59 (0.35)	-

^a Protein numbers refer to those in Figure 1

^b Localization prediction according to genome annotation, PSORTdb 2.0 and www.pseudomonas.com

^c Symbols represent differential expression of proteins in adapted strains compared with reference strain: (-) down regulated, (+) up regulated

Table 1: Identification of selected proteins whose amount was changed in *P. aeruginosa* biofilm-cells adapted to BC and CIP.

are a component of the matrix of the *P. aeruginosa* mature biofilm and contain some virulence factors, including proteases, phospholipase C, alkaline phosphatase, and antibacterial factors [46]. However, the fact that *P. aeruginosa* biofilm cells underexpressed this protein after induced adaptive resistance might suggest membrane degradation (and perhaps the release of vesicles) in adapted cells. The hypothetical protein, PA0537, was also down-regulated in adapted sessile cells (Figure 2 and Table 1).

Overexpressed proteins

The type 4 fimbrial biogenesis outer membrane protein PilQ precursor was over-expressed in biofilms adapted to BC and under-expressed in CIP-adapted cells (Figure 2 and Table 1). PilQ is essential for *P. aeruginosa* pilus formation particularly for type IV pilus [47], and is consequently involved in the bacterial motility and the adhesion [47]. Nde et al. [47] reported the downregulation of the gene *pilQ* after *P. aeruginosa* exposure for 60 min to chlorhexidine diacetate (CHX). According to this author, the mechanism of action of CHX in *P. aeruginosa* involves changes in outer membrane permeability. BC and CHX having similar action at the membrane level, however, the discrepancy observed here might be due to the sessile growth mode in this work in opposition to the planktonic growth reported by Nde et al. [47]. The probable bacteriophage protein was overexpressed in biofilms adapted to CIP (Figure 2 and Table 1). As the putative tail sheath protein, this protein is a MV protein in *P. aeruginosa*. Several studies

have suggested that the most highly activated genes in sessile bacteria are those of temperate bacteriophages [48,49]. These take part in the diversification and phenotypic variation, including the production of small colony variants phenotypes [50]. Biofilms were developed under antimicrobial stress which may regulate proteins related with phages that may be involved in the population diversity found within biofilms [51], and can influence bacteria phenotype, in particular regarding adhesion potential, hydrophobicity and swimming and twitching motilities [52]. All these described features can increase the ability of a biofilm to survive under stress conditions [5].

Conclusion

A proteomic approach was here used to characterize the alterations of the outer membrane proteome of *P. aeruginosa* biofilm cells after BC and CIP adaptation. The regulation of OMPs is a cause of the adaptive process. This study demonstrated that proteins involved in pili and vesicles formation, and phage related proteins exhibited different amount in BC and CIP adapted sessile cells as compared with non-adapted counterparts. Also, the differential expression of some OM proteins after exposure to both antimicrobials was observed, revealing a possible link between the bacteria response within biofilms when exposed to different external pressures. Further proteome studies will provide more comprehensive information on protein variations and reveal biofilm resistance mechanisms, allowing the identification of candidate target proteins for novel antibiotics and defining some possible cross-resistance mechanisms associated with both antimicrobial products.

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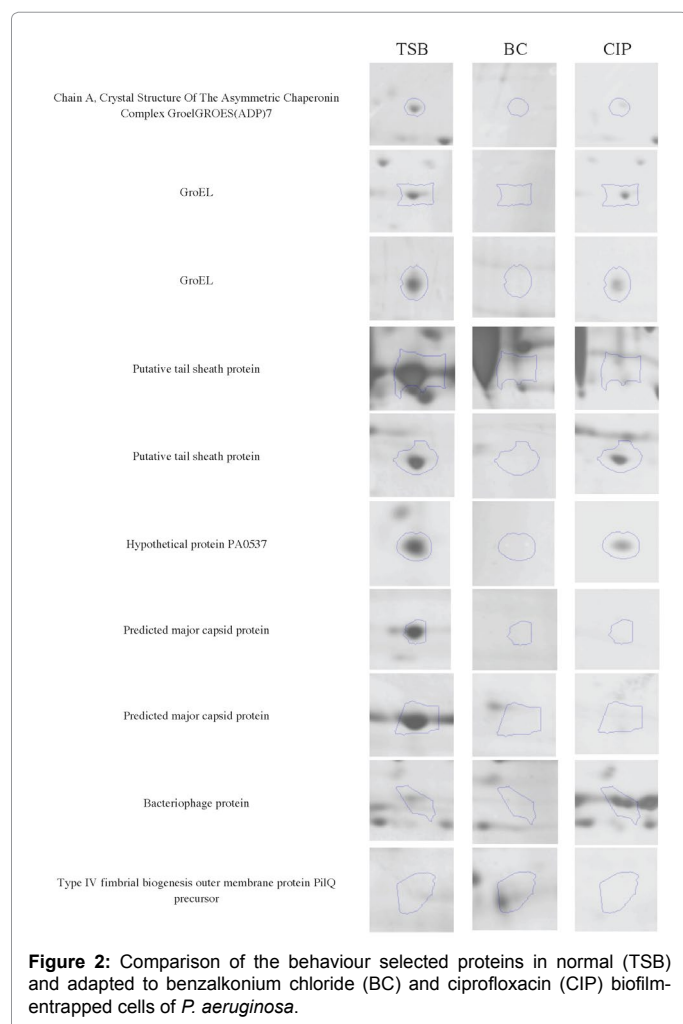


Figure 2: Comparison of the behaviour selected proteins in normal (TSB) and adapted to benzalkonium chloride (BC) and ciprofloxacin (CIP) biofilm-entrapped cells of *P. aeruginosa*.

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