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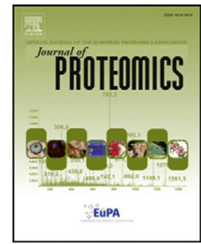

Proteomic approach to *Pseudomonas aeruginosa* adaptive resistance to benzalkonium chloride

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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*.
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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

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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.

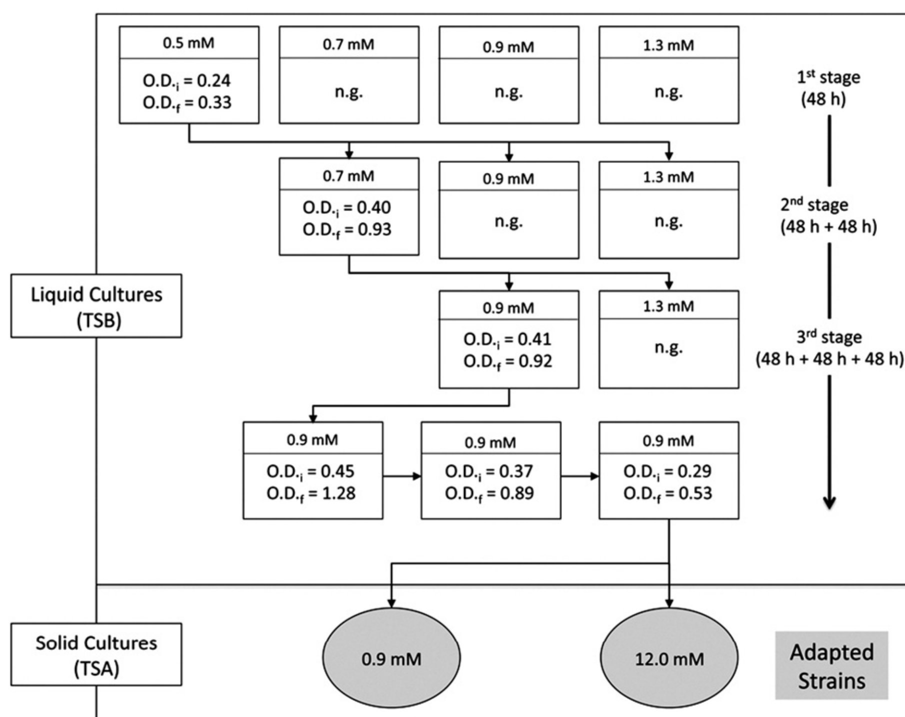

Fig. 1 – Summary scheme of the adaptation process to benzalkonium chloride; OD_i and OD_f are respectively the growth of bacteria observed by absorbance reading at $OD_{640\ nm}$ in the beginning and the end of each stage of adaptation. n.g. represents planktonic culture where no growth was observed.

2.6. *Gel analysis*

Spot quantification was achieved by computing scanning densitometry (ProXPRESS 2D, PerkinElmer Sciex). Gels were analyzed using the Progenesis Samespot (Nonlinear Dynamics). 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 [13].

2.7. *Protein identification*

Spots were excised from the polyacrylamide gel and sliced into small pieces. Selected spots complied with the following criteria: volume varying with the incubation condition and displaying a high (average) value with a low coefficient of variation. Gel plugs were then 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 μL 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 NCBInr protein database restricted to *P. aeruginosa* (February 2, 2007, version 4, 1,342,017 residues, 4243 sequences), using the MASCOT Daemon (version 2.1.3) 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 two 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.

2.8. *Bioinformatic tools for subcellular location*

For the identified proteins, and in particular for unknown proteins, the prediction of their location within the bacterial cell was obtained from the genome annotation of *P. aeruginosa* (accessible at http://www.pseudomonas.com/).

3. Results and discussion

3.1. *Antimicrobial adaptation*

The stability of BC adapted cells (A0.9 and A12 cultures) was determined after three transfers in TSB. Regarding the de-adaptation process, adapted strains lost their adaptive resistance, as they were unable to grow in TSA supplemented with BC after 10 passages in TSA. Despite being easily attained, adaptive resistance can be lost if bacteria are sub-cultured in the absence of the selection pressure [17]. This seems to be a specific characteristic of bacterial adaptation achieved by

continuous exposure. This feature was also reported by Gilleland et al. [18] who confirmed the instability of *P. aeruginosa* resistance as the strains readily revert to susceptible during several transfers in the absence of antibiotic. In order to keep the bacterial adaptive resistance, and to assure that this condition was kept for proteomic studies, A0.9, and A12, *P. aeruginosa* cultures were maintained in TSA supplemented with 0.9 and 12.0 mM of BC, as referred in the Experimental procedures section.

3.2. Outer-membrane protein patterns

Due to their interfacial position in cells, membrane proteins play important roles in various cellular processes including signal transduction, metabolites and ions transport, and cell adhesion [19]. The membrane is also a protective barrier and constitutes more than half of all known drug targets [20]. Also, its implication in adaptive resistance acquisition in *P. aeruginosa* is one of the most impressive features of this cellular component. However, membrane proteins are notorious complex to analyze via 2-DE due to extraction from the membrane difficulties and their inherently hydrophobic nature [21]. The hydrophobicity of these proteins makes them difficult to solubilize for isoelectric focusing (IEF) and they often precipitate at their isoelectric point (pI) in the immobilized pH gradient (IPG), reducing transfer to the second-dimension gel. The incorporation of the zwitterionic detergent ASB-14 in the 2-D sample solution prior to 2-DE has previously been shown to improve the solubility of the membrane proteins compared with sample solutions containing CHAPS or SB 3-10 [22]. Protein hydrophobicity and solubilization as well as sample contamination with cytoplasmic proteins revealed to be the major difficulties regarding sample preparation and proteome analysis. Also, it could have occurred some protein loss during extraction. All together, these issues may be the reason underlying the reduced number of outer membrane proteins differentially expressed in this work.

Bacteria adaptive resistance to antimicrobials occurs mainly at the outer membrane level [3,23] therefore, changes induced by BC on *P. aeruginosa* OMP pattern were studied. Some proteins were found as mass isoforms. Those isoforms exhibited different apparent molecular masses and pI values and witness posttranslational modifications [24]. The major constitutive porin OprF was found as three isoforms, confirming previous observation [19]. It was also the case of OprL and OprG. The range of the membrane proteome alterations following adaptation was very low (about 10%) i.e. only 21 on 206 spots were discriminated on 2-DE gels and BC concentration dependent.

Protein identification showed that some of these spots correspond to cytosolic proteins, e.g. HemB, Ndk, PyrB, SucC, Tsf, PA4352 and PA 5339 (Table 1). The presence of these proteins in samples obviously points out some cytoplasmic contaminations.

Four OMPs where differentially expressed in 0.9 mM BC adapted cells when compared with non-adapted organism, whereas seven OMPs were differentially expressed in 12 mM adapted organisms (Table 1). The number of outer membrane proteins that were differentially expressed after BC treatment was surprisingly low if it is considered (i) that antimicrobial action of BC occurs mainly at the bacterial outer membrane level [23] and (ii) the strategic role of the outer membrane in the cell adaptive resistance to environmental stresses.

OprF was accumulated by the bacteria adapted to 0.9 and 12 mM BC. This OMP is non-covalently linked to peptidoglycan and is involved in the maintenance of *P. aeruginosa* shape, having homology to another structural outer membrane protein, the *Escherichia coli* OmpA [25]. It has been shown that the N-terminal domains of OprFs from *Pseudomonas* strains are able to form ion channels in planar lipid bilayers similar to those induced by the OmpA N-terminal transmembrane domain [26]. Besides that, OprF is also a major environmental sensor [27,28], with an important role in adaptation to hyperosmotic conditions [28]. However, the role of OprF in antibiotic resistance remains controversial [29]. It has been suggested that loss of this protein may be involved in the multiple-antibiotic resistance phenotype [30,31].

Bacteria adapted to the higher BC concentration accumulated the probable outer membrane protein PA10141 whose function is yet unknown.

OprL and OprI were down-regulated in adapted *P. aeruginosa* for the two BC concentrations tested. Lipoproteins, like OprL and OprI have been shown to play a role in many fundamental cellular processes and in the pathogenesis. In *P. aeruginosa*, microarray analysis revealed that there is a prominent induction of lipoprotein-encoding genes during mucoid conversion [32,33].

OprL is a peptidoglycan-associated protein with strong immunogenicity and a hallmark for the identification of *P. aeruginosa*. OprL is the *Pseudomonas* homolog of Pal (peptidoglycan-associated lipoprotein), which is part of the *E. coli* Tol-Pal system. OprL is required to maintain OM integrity [34,35] and cell morphology [36–38].

OprI is a structural lipoprotein, essentially studied for its low variability between *P. aeruginosa* strains, which makes it useful as a phylogenetic marker [39], as a potential vaccine [40] and as an epidemic detection marker in the case of cystic fibrosis [41]. As an integral OM component, OprI plays a role in cell shape and membrane fluidity maintenance [28]. According to Linares [42], the exposure of *P. aeruginosa* to tobramycin and ciprofloxacin also promoted the down-regulation of the OprI gene.

Bacteria adapted to BC 12 mM strain also under-expressed OprG, OprE3 and the probable OMP PA2760 with unknown function. These OMPs are frequently described as involved in antibiotic resistance as porin and multidrug efflux [43]. OprG expression is highly dependent on growth conditions [44], including high growth temperatures and magnesium availability. In particular, this protein has been suggested to be involved in low-affinity iron uptake due to the correlation between its expression and the iron concentration in the medium [45].

This protein has been associated with changes in LPS in *P. aeruginosa* [46] and is reported as downregulated in hyperosmotic conditions [28]. The association of bacterial adaptive resistance to antimicrobial agents and the expression of OprG protein is yet not clear. According to Loughlin [6], this relation does not exist. This author evaluated the effect of BC in *P. aeruginosa* protein patterns, revealing an accumulation of OprG, in opposition to what it is observed in the present study. Some studies [43,47] suggested a link between the presence of OprG and *P. aeruginosa* resistance to antibiotics because increased resistance to kanamycin, and tetracycline was associated with the down-regulation of OprG [43].

OprE3 is a hypothetical protein with homology to the *E. coli* protein, b0681 that functions as a porin of the outer membrane.

Table 1 – Identification of the selected outer membrane proteins whose amount was differentially expressed in *P. aeruginosa* cells adapted to 0.9 mM and 12 mM of benzalkonium chloride.

Locus tag	Protein	Peptide sequence	No. of matching peptides		Location [a]	Gene name	Gene ID	Behavior in adapted strains [b]	
			A0.9	A12				A0.9	A12
PA3190	Putative binding protein component of ABC sugar transporter	R.SVLDPSFQK.D	**8**	**3**	P	gltB	882901	–	–
PA0766	Serine protease MucD precursor	R.GQLSIPDLEGLPPMFR.D + Oxidation (M)	5	5	P	mucD	879207	+	+
PA3655	Elongation factor Ts	K.LTDAAPLVEAR.E	12	**6**	C	tsf	5358722	–	–
PA3988	Hypothetical protein PA3988	R.GLGDAQFALK.E	14	**18**	U	–	878911	–	+
PA2800	Hypothetical protein PA2800	K.NLANNLLQAK.F	11	**8** [c]	OM	vacJ	879842	+	–
PA5339	Hypothetical protein PA5339	R.YFTQPYPAR.A	5	**6**	C	–	877913	+	+
PA2951	Electron transfer flavoprotein alpha-subunit	K.NYSHVLAPATTNGK.N	22	**10**	U	etfA	882932	+	+
PA4671	50S ribosomal protein L25/general stress protein Ctc	K.LLENEAAFSHVIALNVGGAK.E	**9**	–	C	–	881395	+	=
PA4495	Hypothetical protein PA4495	K.LAAETTSVLNAAVADAR.K	7	–	U	–	881066	+	=
PA1777	Major porin and structural outer membrane porin OprF precursor	K.VKENSYADIK.N	**17** [c]	**17** [c]	OM	oprF	878442	+	+
PA0973	Peptidoglycan associated lipoprotein OprL precursor	R.ALDVHAKDLK.G	**11** [c]	7	OM	oprL	882991	–	–
PA2853	Outer membrane lipoprotein OprI precursor	R.LTATEDAAAR.A	**3**	7	OM	oprI	879851	–	–
PA5489	Thiol:disulfide interchange protein DsbA precursor	K.LPADVHFVR.L	6	**5**	P	dsbA	877731	+	+
PA1041	Hypothetical protein PA1041	R.DLHFAFDSSK.V	–	**10**	OM	–	881756	=	+
PA4385	GroEl	K.MLVGVNVLADAVK.A + oxidation (M)	–	**5**	C	groEL	881348	=	-
PA1588	Succinyl-CoA synthase beta-subunit	K.IILSDSNVK.A	**7**	–	C	sucC	882016	–	=
PA3807	Nucleoside diphosphate kinase	K.MVQLSER.E + oxidation (M)	**7**	–	C	ndk	879892	+	=
PA0402	Aspartate transcarbamylase	R.ELLTEILDTADSFLEVGAR.A	**7**	–	C	pyrB	878267	+	
PA5243	Delta-aminolevulinic acid dehydratase	K.YASAYYGPFR.D	–	**9**	C	hemB	879701	=	+
PA4067	Outer membrane protein OprG precursor	K.AGDFIIR.G	–	**13** [c]	OM	oprG	879793	=	–
PA2760	Probable outer membrane protein precursor (OprE3)	K.SINVFGGK.Y	–	**9**	OM	–	882719	=	–

Localization prediction, gene code and function according to genome annotation, PSORTdb 2.0 and www.pseudomonas.com. Matching peptides in bold indicate a statistically significant difference between *Pseudomonas aeruginosa* ATCC 10145 and the adapted strain ($p < 0.05$).

[a] OM: outer membrane; P: periplasm; EC: extracellular; C: cytoplasm.

[b] Symbols represent differential expression of proteins in adapted strains compared with the reference strain: (–) down regulated, (+) up regulated, (=) no change.

[c] Indicates proteins that are identified in more than one spot.

The accumulation of OprE3, an anaerobically induced porin [48] by biofilm *P. aeruginosa* cells has been reported [13].

The conserved hypothetical protein PA 2800, whose location is predicted in the outer membrane, was accumulated by the BC 0.9 mM BC adapted cells, but underexpressed by the BC12 mM adapted ones. The function of this protein is yet unknown.

Some cytoplasmic proteins were identified (Table 1) suggesting a contamination by cytoplasmic proteins. Though predominantly cytoplasmic, GroEL has also been localized in the membrane and periplasm [49,50] and in extracellular medium [51]. GroEL might be involved in the folding of membrane-associated proteins [48] and in cell adherence [52]. The overproduction of this chaperon highlights bacterial adaptation to BC.

A periplasmic protein with important functions in *P. aeruginosa* virulence, MucD, was also overexpressed by adapted cells. MucD belongs to an operon of alginate regulatory genes algT(U) > mucA > mucB > mucC > mucD [53]. Besides its role in alginate production, and consequently in mucoidy, this protein is also a close homolog of the *E. coli* periplasmic serine protease HtrA (DegP), a protein thought to remove misfolded proteins in the periplasm and required for resistance to high temperatures and oxidative stress in *E. coli* [54,55].

4. Conclusion

A proteomic approach was herein used to characterize the alterations of the outer membrane subproteome of *P. aeruginosa* after adaptation to benzalkonium chloride. Results showed that few OMPs exhibited a change in their amount, suggesting that the bacterial adaptation to BC does not mobilize complete outer membrane systems though its antimicrobial action occurs mainly at the bacterial outer membrane level. However, this work showed that *P. aeruginosa* adaptive resistance to BC, promoted some changes in proteins previously described as involved in antibiotic resistance. These results can consequently suggest possible common resistance mechanisms, between adaptive and acquired resistance of *P. aeruginosa* when facing external chemical pressures. Moreover, it may herein advance that, different expressions of some key proteins found during this work, can suggest common resistance mechanisms between antimicrobial products and antibiotics.

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