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Pseudomonas aeruginosa PAO1 Resistance to Zinc Pyrithione: Phenotypic Changes Suggest the Involvement of Efflux Pumps

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Abstract The aim of this study is to investigate the involvement of an efflux pump in the development of *Pseudomonas aeruginosa* resistance to zinc pyrithione (ZnPT). In the presence of efflux inhibitor carbonyl cyanide *m*-chlorophenyl-hydrazone (CCCP), the minimum inhibitory concentration of ZnPT for *P. aeruginosa* resistant cells is reduced significantly ($p < 0.05$). In addition, the concentration of ZnPT excluded by the resistant bacteria was reduced significantly ($p < 0.01$). However, the above reductions did not reach the levels measured for *P. aeruginosa* PAO1 sensitive strain. Furthermore, such changes in *P. aeruginosa* resistant cells were correlated with the overexpression of outer membrane proteins, reduced sensitivity toward imipenem ($p < 0.01$) and increased sensitivity toward sulphatriad and chloramphenicol ($p < 0.05$). In a continuation to a previous study, we conclude that *P. aeruginosa* resistance to ZnPT is multifactorial and involves induced efflux systems.

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Introduction

Pyrithiones are known to be potent antimicrobials and excellent chelating agents [10, 20].

The most well-known of these chelators is the zinc pyrithione (ZnPT) that is used in antidandruff shampoos, and is an effective antifungal agent [19, 20]. In addition, pyrithiones are widely applied in fuel industries as general preservatives and are used in cosmetic and pharmaceutical preparations intended for topical use [14]. These products exhibit high activity against a broad spectrum of microorganisms, compatible with most cosmetic ingredients, safe for topical use, biodegradable, and have high persistence [10, 20]. Scientists believe that bacteria cannot develop resistance to such a powerful biocide with multiple antimicrobial mechanisms [10] Previously, it has been shown that resistance of *Pseudomonas aeruginosa* strain PAO1 to ZnPT could be induced, and that this resistance was accompanied by loss of an outer membrane protein and increased insusceptibility toward other biocides [1]. Other authors have discussed the involvement of the CzcCBA efflux system in resistance of *P. aeruginosa* to zinc and its relation to imipenem resistance [4, 21].

It is known that the prolonged exposure to sub-lethal concentrations of biocides could select for mutants cell lines that overexpress efflux pumps [9, 12, 18]. Efflux systems use energy for the antiport of harmful substances, including antibiotics, numerous dyes, detergents, inhibitors, organic solvents, and biocides, out of the cell [2, 21].

Attacking the energy source will inhibit the efflux pump and allow the accumulation of harmful substances inside the bacteria to a toxic level that will reduce its tolerance to such substances. Efflux inhibitors are known to interfere with the energy building mechanisms of the cell, particularly affecting the energy levels of the bacterial membrane [11, 17]. The effect of efflux pump inhibition could be manifested by reducing minimal inhibitory concentrations (MIC) for certain antibiotics and biocides [2, 6] or increasing the intracellular accumulation of the harmful substance [5, 22, 23]. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) is an extremely effective proton motive force (PMF) inhibitor [11, 13, 17]. Hence, CCCP has been used as an active efflux blocker [5, 13, 23].

In this study, the aim is to investigate the involvement of an efflux pump in the development of resistance of *P. aeruginosa* PAO1 to the biocide ZnPT.

Materials and Methods

Bacterial Cultures and Chemicals

Stock cultures of *P. aeruginosa* PAO1 (NCIMB 10548) were obtained from NCIMB (Aberdeen, UK), and subcultured in R2A broth [26]. This culture was used as the ZnPT-sensitive strain throughout the experiment. ZnPT-resistant strains were obtained by passaging the ZnPT-sensitive strain in sub-MICs of ZnPT for ten successive passages [1]. Cells from the tenth passage (P10) were deemed ZnPT-resistant, and were maintained on R2A agar containing 1/4 MIC of ZnPT [1].

The ZnPT biocide was a kind gift from Zeneca Specialties (Manchester, UK), and was provided as stable powder in sealed containers. The biocide was prepared fresh by dissolving it in dimethylformamide. CCCP was purchased from Sigma and prepared by dissolving 10 mg ml^{-1} in methanol as a stock. Copper chloride dihydride was purchased from Fluka and prepared at the concentration required in distilled water.

Antibiotic Susceptibility Testing

Susceptibility of overnight R2A cultures of ZnPT-resistant and ZnPT-sensitive strains to tetracycline, ampicillin, sulphatriad, streptomycin (Mast Diagnostics), chloramphenicol, ticarcylin, aztreonam, ciprofloxacin, imipenem, tobramycin, Pipracillin/Tazocin, amikacin (OXOID), ceftriaxone, meropenem, and ceftazidime (a kind gift from Dr. Gabby Phillips, Ninewells Hospital, and Dundee, UK) was tested using the disc diffusion method. Results were obtained by measuring the diameter of the zone of inhibition (mm).

Minimal Inhibitory Concentration of ZnPT

R2A broth (25 ml) was inoculated with ZnPT-sensitive or ZnPT-resistant strains of *P. aeruginosa* PAO1. ZnPT and CCCP [5] were added simultaneously with inoculation. Cultures were incubated in an orbital-shaking incubator (Gallenkamp INA-305) at 37°C overnight and used as inocula for MIC determination by the tube dilution method [3].

ZnPT Assay

ZnPT concentration in the supernatant media was measured using an assay adopted from Dinning et al. [7]. Briefly, ZnPT-resistant cells obtained from P10 were grown in fresh R2A broth containing 1/4 MIC of ZnPT (6 μg ml^{-1}). ZnPT-sensitive cells were grown overnight in fresh R2A broth free of ZnPT. Cells were harvested by centrifugation (2795*g*, 15 min) and washed twice in sterile normal saline. After the final centrifugation, cell density was adjusted to OD of 1.4 at 470 nm. CCCP (15 μg ml^{-1}) [5] was added to the cells. Cell suspensions were incubated for 5 min, followed by the addition of concentrations of the biocide equivalent to 90% of the previously determined MIC and incubated for another 5 min at room temperature. An aliquot (2 ml) of the cell suspension was removed and centrifuged for 3 min at 4025*g*. The supernatant was assayed for biocide content by mixing 1 ml of the supernatant with 5 ml of 1 mM copper chloride dihydride, incubated at room temperature for 5 min, and then OD at 318 nm was measured [7]. The ZnPT concentration was calculated from a constructed calibration curve. The experiment was performed in triplicate and repeated three times on three different days with a construction of new calibration curve every time.

Control Experiment for CCCP

Growth and growth rate of ZnPT-resistant strain of *P. aeruginosa* at hourly intervals were determined in the absence and presence of CCCP (15 μg ml^{-1}). Cultures were incubated in an orbital-shaking incubator (180 rpm, Gallenkamp INA-305) at 37°C. The experiment was performed in triplicate.

Outer Membrane Protein Profiles and Gel Image Analysis

Outer membranes of both ZnPT-sensitive and ZnPT-resistant strains were prepared using the method of Pugsley et al. [25]. Outer membrane preparations were assayed by SDS-PAGE [1] after protein quantification by bicinchoninic acid assay [27]. Gels were analyzed using

Phoretix imaging analysis software (non-linear Dynamics Ltd., Newcastle upon Tyne, UK). The Phoretix imaging system was used to detect the bands of all the outer membrane proteins in the samples and analyze them to allow for comparison between profiles of resistant and sensitive strains.

Data Analysis

All data are presented with mean ± SD. Comparisons between conditions or treatments were analyzed by one-way ANOVA followed by Wilcoxon-rank sum test to compare between two conditions or treatments.

Results

CCCP Effect on Cells

The growth and growth rate of ZnPT-resistant strain of *P. aeruginosa* in the presence of CCCP were not significantly different than in the absence of CCCP (Fig. 1). This indicates that the efflux inhibitor at the concentration used did not affect the growth or growth rate of ZnPT-resistant strain of *P. aeruginosa.*

MIC of ZnPT and ZnPT Assay

The MIC of ZnPT for the ZnPT-resistant strain was significantly higher ($p < 0.001$) than that of ZnPT-sensitive *P. aeruginosa* strains. Upon the addition of CCCP to the ZnPT-resistant strain, the MIC dropped significantly ($p < 0.05$) in comparison to the CCCP untreated ZnPT-resistant cells. However, the reduced MIC level was still significantly ($p < 0.05$) higher than the MIC level in the ZnPT-sensitive strain. The addition of CCCP to *P. aeruginosa* ZnPT-sensitive strain had no effect on the MIC (Fig. 2).

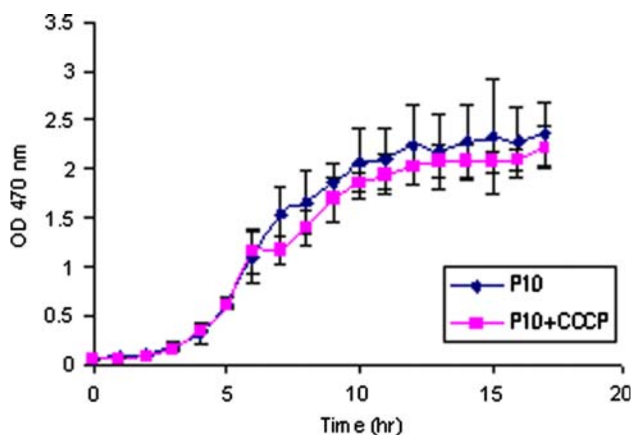

Fig. 1 The growth curves of *P. aeruginosa* (P10) resistant strain grown in R2A broth containing MIC/4 of ZnPT and the growth curve of the same cells with CCCP. Error bars are the standard deviation for each data point

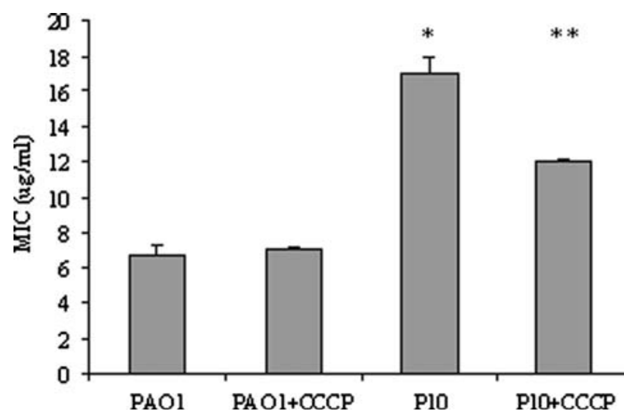

Fig. 2 MIC of ZnPT for *P. aeruginosa* sensitive (PAO1) and resistant (P10) strains without or with CCCP. Bars represent the standard deviation. Over all changes in MIC between all treatments were statistically significant ($p < 0.001$) and * indicates $p < 0.05$ when compared to PAO1, whereas ** means MIC of P10 + CCCP was significantly ($p < 0.05$) less than P10 but also significantly higher than PAO1 strain ($p < 0.05$)

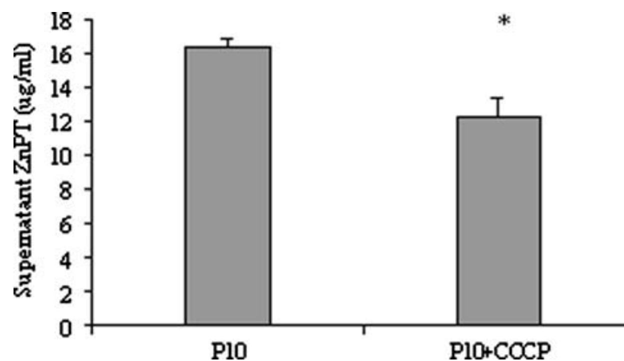

Fig. 3 The concentration of ZnPT excluded in the supernatant fluid of *P. aeruginosa* resistant (P10) strains without or with CCCP. Bars represent the standard deviation. * Concentration of ZnPT in P10 + CCCP was significantly ($p < 0.01$) less than that in P10

In the ZnPT assay, the level of ZnPT was significantly reduced from 16.4 ± 0.5 to 12.3 ± 1.2 μg ml^{-1} ($p < 0.01$) when the P10-resistant cells were exposed to CCCP for 5 min (Fig. 3).

Antibiotic Testing

The results of the sensitivity of *P. aeruginosa* ZnPT-sensitive and ZnPT-resistant cells to different antibiotics are shown in Table 1. ZnPT-resistant *P. aeruginosa* has shown a reduced susceptibility to the antibiotic imipenem ($p < 0.05$), and increased susceptibility toward sulphatriad ($p < 0.05$) and chloramphenicol ($p < 0.05$). The sensitivity for the other antibiotics remained unaffected (Table 1).

Table 1 Zones of inhibition (mm) for ZnPT-sensitive and ZnPT-resistant *P. aeruginosa* PAO1 when exposed to various antibiotics

Antibiotics	ZnPT-sensitive strain	ZnPT-resistant strain
Amikacin	21.6 ± 0.0	20.0 ± 0.1
Ampicillin	R[a]	R
Aztreonam	26.3 ± 0.0	25.3 ± 0.2
Ceftazidime	23.6 ± 0.1	23.0 ± 0.1
Ceftriaxone	19.0 ± 0.1	19.3 ± 0.1
Chloramphenicol	10.3 ± 0.1	12.6 ± 0.1**
Ciprofloxacin	35.0 ± 0.1	34.0 ± 0.1
Imipenem	20.6 ± 0.7	14.0 ± 1.5**
Meropenem	41.3 ± 0.2	41.3 ± 0.2
Pipracillin/Tazocin	27.0 ± 0.0	26.0 ± 0.1
Streptomycin	17.3 ± 0.2	17.6 ± 0.2
Sulphatriad	18.0 ± 0.4	26.0 ± 0.4**
Tetracycline	R	R
Ticarcylin	23.6 ± 0.1	24.0 ± 0.1
Tobramycin	21.0 ± 0.0	20.0 ± 0.1

[a] R = No zone of inhibition

** $p < 0.05$

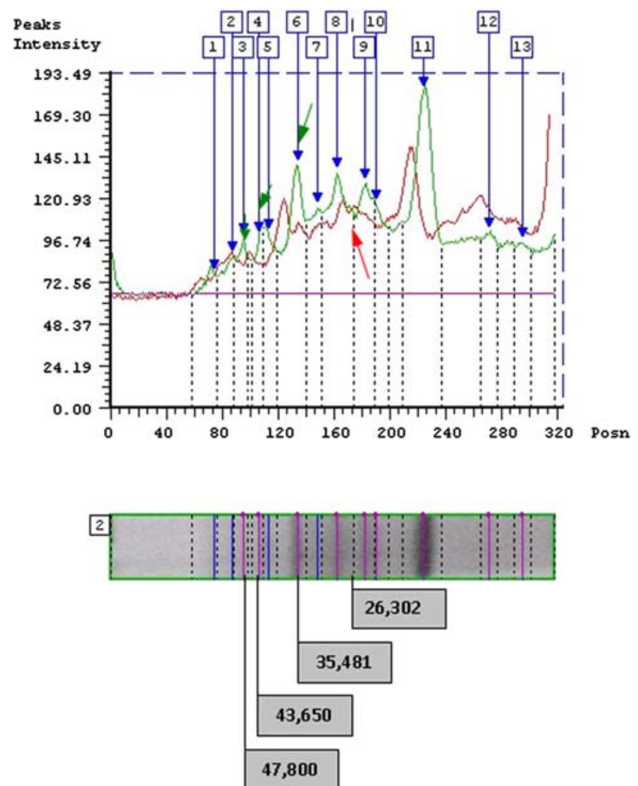

Fig. 4 Comparison between the peak analysis of the outer membrane proteins of ZnPT-resistant *P. aeruginosa* adapted to ZnPT (6 μg ml^{-1}; *green line*) and the peak analysis of the outer membrane proteins of ZnPT-sensitive *P. aeruginosa* (6 μg ml^{-1}; *red line*). The *upper graph* indicates the peak intensity in relation to the pixel position. *Blue arrows* point at peaks that represent the bands in the lower lane image. *Numbers in boxes* on the graph indicates band position. *Numbers in boxes* on the image indicates molecular weight of the specific bands. The *green arrow* indicates the protein band that appears to be overexpressed in outer membranes of ZnPT-resistant cells. The *red arrow* indicates missing protein in the outer membranes of ZnPT-resistant cells

Outer Membrane Protein Profile

Peak intensity profiles of both wild-type *P. aeruginosa* PAO1 strain and the ZnPT-resistant *P. aeruginosa* strain have shown alterations in the outer membrane protein expression. Overexpression of proteins was detected, specifically, three high molecular weight proteins (35.5, 43.6, and 47.8 kDa) in the outer membrane of ZnPT-resistant *P. aeruginosa* strain (Fig. 4). These, however, were not overexpressed in the outer membrane of the ZnPT-sensitive strain (Fig. 4).

Discussion

Significant increase in MIC of ZnPT occurred as *P. aeruginosa* adapted to ZnPT [1]. In this study the MIC of ZnPT for ZnPT-resistant strain dropped significantly when the efflux inhibitor CCCP was added to the media. However, the drop in MIC never reached the value of the MIC of the ZnPT-sensitive strain (Fig. 2). Moreover when CCCP is added to the growth medium of ZnPT-resistant cells the concentration of ZnPT in the supernatants is reduced significantly in comparison with the concentration measured in the supernatants of resistant cells that are not exposed to the CCCP (Fig. 3). These results indicate the presence of an efflux pump. The inhibition of the efflux pump by the PMF blocker CCCP indicates that it belongs to an H^+ antiporter superfamily [17]. This pump may be responsible for the cross resistance exhibited by the ZnPT-resistant strain toward cetrimide and sodium pyrithione [1] and chlorhexidine (results not shown).

ZnPT resistance is also accompanied by significant resistance toward the antibiotic imipenem and increased susceptibility toward chloramphenicol and sulphatriad, as seen in Table 1. Imipenem resistance in *P. aeruginosa* is known to be associated with the loss of the outer membrane OprD that is required for the diffusion of that antibiotic into the cell and the overexpression of the MexEF-OprN pump [16]. However, the outer membrane protein reported missing in an earlier study by this group as a result of ZnPT-induced resistance [1] had a molecular weight around 36 kDa which indicates that it is not the OprD (46 kDa). On the other hand, the increased susceptibility to chloramphenicol and sulphatriad rules out the involvement of the MexEF-OprN, MexCD-OprJ, and the constitutive efflux pump MexAB-OprM, since the latter two antibiotics

are substrates of these efflux pumps [15, 24]. The non-involvement of MexAB-OprM is emphasized here by: (i) the unchanged MIC for ZnPT upon the addition of CCCP to the sensitive strain (Fig. 2), (ii) the unaffected sensitivity of meropenem, which is a substrate of the MexAB-OprM [24] (Table 1).

Zinc resistance in *P. aeruginosa* was attributed to the heavy metal efflux pump CzcCBA, which is under the control of the two-component regulatory system CzcRS [4, 21]. We do not rule out the involvement of the CzcCBA system because the presence of Zn^{2+} ions seems to have induced it. However, the downregulation of OprD protein that was mentioned by Perron et al. [21] was not observed. Furthermore, the CzcCBA efflux system in *P. aeruginosa* pumps Zn^{2+} out of the cell [4, 21]. While in this study, the perthiolate ion was detected through its ability to chelate copper [7] indicating the efflux of pyrithione and not the Zn^{2+} alone.

SDS-PAGE and Phoretix analysis have shown the overexpression of outer membrane proteins and the repression of others. The exact identities of these proteins are not elucidated yet. It is known that efflux pumps use outer membrane proteins for the antiport of its substrates [5, 24]. Accordingly overexpressed proteins in the outer membrane of ZnPT-resistant cells do indicate the presence of the outer membrane components of efflux pumps and or of regulatory systems.

Our results lead us to the conclusion that the resistance to ZnPT in *P. aeruginosa* is multifactorial and involves alterations in the outer membrane in the form of decreased synthesis of a protein and overexpression of others, as well as, the induction of more than one efflux system. *P. aeruginosa* is known to exhibit an arsenal of efflux pumps that acts as a line of defense against antimicrobials. Perron et al. [21] have characterized two almost similar heavy metal RND-efflux systems CzrCBA and CzcCBA with minor variations in the N-terminus of their components. The two systems export Zn^{2+} out of the cell. Here we report the presence of an efflux system that confers resistance to ZnPT and other biocides. Although our results are preliminary and need further investigation, it suggests a co-regulation between more than one efflux system that results in: (i) the increased resistance to imipenem accompanied by increased susceptibility to chloramphenicol and sulphatriad, (ii) the cross resistance to other biocides, and (iii) the overexpression of several outer membrane proteins. Such co-regulation is seen when the bacteria is subjected to stress [10]. Exposure of bacteria to a powerful antimicrobial like ZnPT that has multiple antimicrobial mechanisms [8, 10] is considered stressful.

ZnPT is a good candidate for use as a preservative for topical antiseptics [10]. Sub-inhibitory concentrations of ZnPT that could be reached upon the usage of antidandruff shampoos or skin antiseptics or others might predispose for emergence of ZnPT-resistant *P. aeruginosa*.

The spread of such resistant strains in hospital communities might be a serious problem.

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