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Persister cells in a biofilm treated with a biocide

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This study investigated the physiology and behaviour following treatment with *ortho*-phthalaldehyde (OPA), of *Pseudomonas fluorescens* in both the planktonic and sessile states. Steady-state biofilms and planktonic cells were collected from a bioreactor and their extracellular polymeric substances (EPS) were extracted using a method that did not destroy the cells. Cell structure and physiology after EPS extraction were compared in terms of respiratory activity, morphology, cell protein and polysaccharide content, and expression of the outer membrane proteins (OMP). Significant differences were found between the physiological parameters analysed. Planktonic cells were more metabolically active, and contained greater amounts of proteins and polysaccharides than biofilm cells. Moreover, biofilm formation promoted the expression of distinct OMP. Additional experiments were performed with cells after EPS extraction in order to compare the susceptibility of planktonic and biofilm cells to OPA. Cells were completely inactivated after exposure to the biocide (minimum bactericidal concentration, MBC = 0.55 ± 0.20 mM for planktonic cells; MBC = 1.7 ± 0.30 mM for biofilm cells). After treatment, the potential of inactivated cells to recover from antimicrobial exposure was evaluated over time. Planktonic cells remained inactive over 48 h while cells from biofilms recovered 24 h after exposure to OPA, and the number of viable and culturable cells increased over time. The MBC of the recovered biofilm cells after a second exposure to OPA was 0.58 ± 0.40 mM, a concentration similar to the MBC of planktonic cells. This study demonstrates that persister cells may survive in biocide-treated biofilms, even in the absence of EPS.

Keywords: biocide; persistence; phenotypic changes; biofilm recovery; biofilm resistance

Introduction

Microbial adhesion to surfaces and the consequent formation of biofilm has been documented in many different environments (eg Hall-Stoodley et al. 2004; Choi et al. 2010; Tang et al. 2011; Teodósio et al. 2011). Despite the unquestionable importance of biofilms and their effects on humans, present knowledge regarding the physiology and behaviour of sessile communities is still limited. A switch from planktonic to growth in biofilm form is believed to result in profound and complex phenotypic changes in bacteria (Sauer and Camper 2001). Some reports on the properties of bacteria present in biofilms indicate that growth on surfaces involves significant changes in gene transcription, including the establishment of new genetic traits (Christensen et al. 1998; O'Toole and Kolter 1998; Tremaroli et al. 2011).

One of the earliest observations related to the different characteristics observed in planktonic and biofilm cells was the increased resistance of cells in biofilms to antimicrobial agents and adverse environmental conditions (Drenkard and Ausubel 2002; McBain et al. 2002; Simões et al. 2008a; Ferreira et al. 2010). However, it is not known why and how bacteria, growing within a biofilm, develop increased resistance to antimicrobial agents. The persistent cell state is the most recent explanation for the lack of susceptibility of biofilms to antimicrobial agents (Lewis 2001, 2007; Sufya et al. 2003). The conventional explanation of transport limitation and chemical interaction with the extracellular polymeric matrix components of the biofilm does not always explain the recalcitrant properties of biofilms (Davies 2003). It has been known for many years that small numbers of persistent bacteria resist killing when exposed to antimicrobials. Persister cells, first described for *Staphylococcus* spp., were not completely inactivated by a lethal concentration of ampicillin (Bigger 1944). When the surviving bacteria were grown in antibiotic-free medium, they grew like the parent population, which was again susceptible to ampicillin. In a health context, chronic infections are seriously hampered by the presence within a bacterial population of a small

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number of cells capable of surviving very high doses of antibiotics. These so called persister cells give rise to a population with the same antibiotic susceptibility as the original population and thus have not undergone mutations that confer resistance (Balaban et al. 2004). Persisters have become the focus of scientific research as they are held responsible for recurring outbreaks of infections when the antibiotic pressure decreases (Balaban et al. 2004; Singh et al. 2009). There are multiple hypotheses about persistence, but the exact cause of the phenomenon remains elusive (Balaban et al. 2004; Shah et al. 2006; Lewis 2010). Persisters are a small subpopulation of bacteria that survive lethal concentrations of antibiotic without having antibiotic-resistance genes. Isolation of persisters from a normally dividing population is considered difficult due to their slow growth, low numbers and phenotypic shift when regrown in antibiotic free medium, they revert to the parent population.

Aldehydes belong to the group of electrophilically active agents which, due to the electron deficiency at the carbonyl carbon atom can react with nucleophilic cell entities and thus exert antimicrobial activity (Simões et al. 2011). *Ortho*-phthalaldehyde (OPA), an aromatic compound with two aldehyde groups, is a biocide approved by the Food and Drug Administration and which has been claimed to have an effective bactericidal character, and it has therefore been suggested as a replacement for glutaraldehyde for high-level disinfection (McDonnell and Russell 1999; Walsh et al. 1999). OPA has several potential advantages compared to glutaraldehyde, viz. it is virtually odourless, stable and effective over a wide pH range (3–9), it is non-irritant to the eyes and nasal passages, and does not require activation before use (Walsh et al. 1999). Moreover, microorganisms that have acquired resistance to glutaraldehyde have not yet gained cross-resistance to OPA (Walsh et al. 1999; Cabrera-Martinez et al. 2002). Simões et al. (2007a) proposed that the antimicrobial effects of OPA may be due to chemical interactions with membrane components, such as altering surface hydrophobicity and membrane permeability. In addition, the biocide might cross the cell membranes and interact with intracellular sites that are critical for antibacterial activity. For higher OPA concentrations (> 500 mg l^{-1}), the growth cycle is compromised, inducing cell elongation due to the lack of septation.

There are no reports regarding the existence of persister cells after exposure of biofilms to lethal concentrations of biocides or other antimicrobials acting on multiple cell targets. This study investigates the physiology and behaviour of *Pseudomonas fluorescens* in both planktonic and sessile states, after treatment with the aldehyde-based biocide OPA.

Materials and methods

Microorganism and culture conditions

Pseudomonas fluorescens (ATCC 13525^T) biofilms were developed in a bioreactor rotating system described in previous studies (Simões et al. 2008b, 2009). This device offers a simple approach to study and characterize biofilms in a well-controlled, real-time and reproducible manner, and to mimic industrial flow processes (Azeredo and Oliveira 2000). Bacterial growth conditions were 27°C $\pm$ 2°C and pH 7, with glucose as the main carbon source (growth medium contained glucose, 5 g l^{-1}; peptone, 2.5 g l^{-1} and yeast extract, 1.25 g l^{-1} in 0.02 M phosphate buffer, pH 7). A 3.5 l bioreactor was continuously fed with diluted growth medium (glucose, 50 mg l^{-1}; peptone, 25 mg l^{-1}; yeast extract, 12.5 mg l^{-1} in 0.02 M phosphate buffer pH 7) and bacterial cells in the exponential phase of growth, supplied from an independent continuously operating 0.5 l chemostat. Biofilms were grown on stainless steel (ASI 316) cylinders, with a surface area of 34.6 cm^2 (2.2 cm diameter; 5 cm length), inserted in the bioreactor and rotating at a constant Reynolds number of 2400. The biofilms were allowed to grow for 7 days in order to obtain steady-state biofilms (Simões et al. 2009). The planktonic cells used throughout this study were collected from the 3.5 l bioreactor (operating at a dilution rate of 0.486 h^{-1}).

Biofilm scraping

The biofilm that covered the cylinders was removed from the surface, using a stainless steel scraper as described by Simões et al. (2009). A volume of 20 ml of the biofilm suspension was homogenised by vortexing (Heidolph, model Reax top) for 30 s with 100% power input and then used to separate extracellular polymeric substances (EPS) and cells, according to the method described by Simões et al. (2011). The efficiency of the process of biofilm scraping and disaggregation was ascertained by staining the stainless steel cylinders with 4′, 6-diamidino-2-phenylindole (DAPI) and further visualization by epifluorescence microscopy (AXIOS-KOP; Zeiss), according to Simões et al. (2007b). DAPI is believed to be very specific for DNA and is thus used to quantify total bacteria (Saby et al. 1997). It was found that only *ca* 1 $\pm$ 0.3% of the total bacterial population remained adhered to the cylinder surface.

Extraction of EPS

Extraction of EPS from planktonic and biofilm cells was carried out using Dowex resin (50X 8, NA^+ form, 20–50 mesh, Fluka-Chemika). Prior to extraction, the Dowex resin was washed with extraction buffer (2 mM Na_3PO_4, 2 mM NaH_2PO_4, 9 mM NaCl and 1 mM

KCl, pH 7) and 50 g of Dowex resin per g of volatile solids were added to the biofilm or planktonic cells. EPS extraction was performed with planktonic or biofilm cells by stirring (VWR, VMS-C7 Advanced; stirrer length – 2.5 cm) the Dowex resin at 400 rpm for 4 h at a temperature of 4°C, according to Frølund et al. (1996). The extracellular components were separated from the cells by centrifugation (3777 *g*, 5 min). The separation of the EPS matrix without damage to cells is an important prerequisite. ATP was used as an indicator of cell lysis (Simões et al. 2005a), and no ATP release was detected during the extraction process.

To guarantee that all EPS were removed through the extraction procedure, bacteria were also stained with acridine orange (AO) (Merck 15931.0025) 0.003% (w/v), according to the procedure described by Simões et al. (2003). AO is a metachromatic dye, when it intercalates with DNA it fluoresces green, and when it interacts with RNA it fluoresces red. AO is capable of binding not just nucleic acids, but also humic substances, acidic polysaccharides, glucosaminoglycans, galactosaminoglycans, liposomes and phospholipids (Paul 1982). However, it binds to nucleic acids at specific excitation and emission wavelengths (500 nm and 526 nm, respectively) (Panda and Chakraborty 1997; Zhang and Fang 2001). By changing the microscopic filter (Chroma 61000-V2 DAPI, FITC, TRITC, Texas Red and DAPI/FITC/TRITC), it was possible to detect different biological components on the polycarbonate filter before EPS extraction. After EPS extraction, only cells were detected.

A final volume of 20 ml of bacteria (planktonic and those derived from biofilms) at a cell density of $1.0 \times 10^{13} \pm 9.8 \times 10^{11}$ cells ml^{-1} was obtained and used for physiological characterization (10 ml) and antimicrobial tests (10 ml).

Bacterial respiratory activity assessment

Respiratory activity assays were performed in a model 53 Yellow Springs Instruments (Ohio, USA) biological oxygen monitor (BOM), as previously described (Simões et al. 2005b). A volume of 10 ml ($1.0 \times 10^{13} \pm 9.8 \times 10^{11}$ cells ml^{-1}) of bacterial suspension (planktonic or biofilm) was placed in two temperature-controlled BOM vessels (27°C ± 2°C). Each vessel contained a probe (Yellow Springs Instruments) to measure the dissolved oxygen (DO), connected to a DO meter. Once inside the vessel, the samples were aerated for 30 min to ensure oxygen saturation ($[O_2] = 9.2$ mg l^{-1}, 1 atmosphere). Afterwards, the vessel was closed and the decrease in the oxygen concentration monitored over time. The initial linear decrease observed corresponded to the endogenous respiration rate. To determine oxygen uptake due to substrate oxidation, 50 μl of a glucose solution (100 mg l^{-1}) were injected into each vessel. The slope of the initial linear decrease in the DO concentration, after glucose addition, corresponded to the total respiration rate. The difference between the two respiration rates (total respiration rate and endogenous respiration rate) represents the oxygen uptake rate due to glucose oxidation and was expressed as mg O_2 $cell^{-1}$ min^{-1}. After respiratory activity assessment, the same bacterial cells were used to characterize their outer membrane expression, protein and polysaccharide content, and cellular size.

At least three independent experiments were performed for each condition tested.

Bacterial protein and polysaccharide quantification

The amount of protein from planktonic and biofilm cells was determined using the modified Lowry method (Sigma, Portugal), with bovine serum albumin (BSA) as the standard (Sigma). The procedure is essentially the Lowry method (Lowry et al. 1951) as modified by Peterson (1979). Polysaccharides were quantified by the phenol-sulphuric acid method of Dubois et al. (1956), with glucose as the standard. Prior to quantification of protein and polysaccharide, the cells were disrupted by a 2 min sonication on ice (Vibracell, 60 W).

Bacterial OMP isolation

The outer membrane proteins (OMP) were isolated according to the method described by Winder et al. (2000). Planktonic or biofilm cells were suspended in 25 mmol Tris and 1 mmol $MgCl_2$ buffer (pH = 7.4). The bacterial suspension was sonicated for 2 min (Vibracell, 60 W) on ice to promote cell lysis. After sonication the solution was centrifuged (7000 *g*, 10 min, 4°C) in order to remove non-lysed cells. The supernatant was collected and N-lauroylsarcosine (Sigma) was added to obtain a final concentration of 2% (w/v), in order to solubilize the inner membrane proteins. This solution was left on ice for 30 min. After this, the solution was centrifuged (17000 *g*, 1 h, 4°C) to recover the OMP. The pellet containing the OMP was resuspended in 1 ml of deionised water and stored at −20°C until required.

Quantification and analysis of OMP

The protein content of the OMP samples was determined using the Bicinchoninic Acid Protein Assay Kit (BCA) (PIERCE Cat. No. 23225) with BSA as standard, according to Simões et al. (2006). This procedure was followed so the same OMP

concentration for different samples was inserted into the gel cassettes.

The OMP samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at a constant current of 10 mA, as reported by Laemmli (1970), with 12% (w/v) acrylamide. After electrophoresis, the proteins were stained with Coomassie blue and silver to reveal protein profiles.

Antimicrobial susceptibility of planktonic and biofilm cells

Planktonic and biofilm cells separated from the EPS were diluted to a concentration of $1.0 \times 10^9 \pm 7.6 \times 10^7$ cells ml^{-1} and used to assess the minimum bactericidal concentration (MBC) in 50 ml flasks with 20 ml of bacterial suspension. OPA (obtained as a powder from Sigma) solutions were prepared in 0.02 M phosphate buffer (pH 7). In order to assess the MBC, a range of OPA concentrations were tested, starting at 0.05 mM. After OPA contact with the cells, the biocide was neutralized with sodium bisulphite (0.5% w/v, Sigma) for 10 min. Control experiments showed there was no interference between sodium bisulphite at the concentration used and the viability and culturability of *P. fluorescens* ($P > 0.05$). The MBC of planktonic and biofilm cells was determined as the lowest concentration of OPA where no viable cells was detected after a 30 min exposure, according to Simões et al. (2005b; 2007a). Viability was determined with the Live/Dead *B*acLight bacterial viability kit (Invitrogen) to estimate both viable and total counts of bacteria. *Bac*Light is composed of two nucleic acid-binding stains: SYTO 9™ and propidium iodide (PI). SYTO 9™ penetrates all bacterial membranes and stains the cells green, while PI only penetrates cells with damaged membranes and the combination of the two stains produces red fluorescent cells.

Bacteria were diluted to a concentration such that 30–250 cells were visible per microscope field of view, then filtered through a Nucleopore® (Whatman) black polycarbonate membrane (pore size 0.22 μm), stained with 250 μl of SYTO 9™ solution and 250 μl of PI solution from the Live/Dead kit, and left in the dark for 15 min. A microscope (AXIOSKOP; Zeiss), fitted with fluorescence illumination and a 100× oil immersion fluorescence objective, was used to visualise the stained cells. The optical filter combination consisted of a 480 to 500 nm excitation filter, in combination with a 485 nm emission filter. Bacterial images were recorded digitally as micrographs (AxioCam HRC; Zeiss). ScanPro 5 (Sigma) was used to quantify the number of cells and to measure the equivalent spherical cell radius (determined through cell length and width measurements; Walker et al. 2005) as an estimate of cell size (Simões et al. 2008c). The mean number of cells was determined from counts of a minimum of 20 fields of view (FOV), for each independent experiment.

Culturability was assessed in terms of colony forming units (CFU) on Plate Count Agar (PCA; Merck, VWR), according to previous methods (Simões et al. 2005b; Ferreira et al. Forthcoming 2011). At least, three independent experiments were performed for each conditions tested.

Antimicrobial recovery – detection of persister cells

After the OPA neutralization process, cells were centrifuged (3777 *g*, 5 min), transferred to 20 ml of fresh medium ($1.0 \times 10^9 \pm 9.8 \times 10^7$ cells ml^{-1}) and put in an orbital shaker (120 rpm, 27°C). Bacterial viability and culturability were characterized over time (immediately after antimicrobial exposure, and 12, 24, 36 and 48 h after treatment).

Cells with the ability to recover from the antimicrobial treatment (after the 48 h recovery period) were also used to assess their susceptibility to a second dose of OPA (MBC) and to characterize their physiological properties (respiratory activity, protein and polysaccharide content, cell size and OMP expression), according to the methods described above.

Control experiments were also performed with planktonic and biofilm cells not exposed to OPA. Those experiments demonstrated no effects in terms of viability and culturability when cells where exposed to phosphate buffer (without OPA) for 30 min. A typical batch growth curve was obtained for the recovery experiments with non-treated cells (results not shown). At least, three independent experiments were performed for each conditions tested.

Statistical analysis

Data from bacterial physiological characterization and from antimicrobial tests are given as means ± standard deviation (SD). At least three independent experiments were performed for each condition tested. The data were analyzed by the nonparametric Kruskal–Wallis test, based on a confidence level ≥ 95%.

Results and discussion

Significant differences were found between planktonic and biofilm cells. Planktonic cells were more metabolically active ($P < 0.05$) and contained a greater ($P < 0.05$) amount of protein and polysaccharide than biofilm cells (Table 1). Planktonic cells differed in length from biofilm cells ($P < 0.05$). Planktonic and

Table 1. Respiratory activity, and total protein and polysaccharide content of planktonic and biofilm cells of *P. fluorescens*.

	Planktonic cells	Biofilm cells
Respiratory activity (mg O_2 cell^{-1} min^{-1})	$9.1 \times 10^{-16} \pm 3.2 \times 10^{-17}$	$2.5 \times 10^{-18} \pm 1.4 \times 10^{-19}$
Total protein (pg cell^{-1})	578 ± 165	3.31 ± 0.151
Total polysaccharide (pg cell^{-1})	1803 ± 576	6.91 ± 2.50

Results are shown as mean ± SD of at least three independent experiments.

biofilm cells had a spherical equivalent cell radius of 0.899 ± 0.06 μm and 0.318 ± 0.05 μm, respectively. The differences in composition and physiology are a consequence of the biofilm formation process. When a cell switches to the biofilm mode of growth, it undergoes a phenotypic shift in composition and behaviour (An and Parsek 2007). The presence of dwarf cells is common in biofilms (Donlan and Costerton 2002; Simões et al. 2007b). This phenotypic shift is emphasized by an increase in environmental stress, particularly shear stress (Simões et al. 2007b).

The OMP profile of planktonic and biofilm cells was assessed in order to characterize OMP expression of cells at different states (Figure 1). The results showed that cells at different states differed significantly in the expression of the major OMP and are in accordance with previous reports (Costerton et al. 1995; Coquet et al. 2002; Wang et al. 2003; Seyer et al. 2005). The major OMPs of 44 ± 1 kDa, 24 ± 2 kDa and 28 ± 2 kDa observed in planktonic cells were under-expressed by the biofilm cells. Also, the major OMP found in biofilm cells (32 ± 1 kDa, 21 ± 1 kDa) was under-expressed by planktonic cells. The molecular weights of the major OMP expressed are apparently similar to those of OprF (32 ± 1 kDa), OprH (21 ± 1 kDa), OprE (44 ± 1 kDa), FliC (24 ± 2 kDa) and OprG (28 ± 2 kDa) (Kragelund et al. 1996; Brimer and Montie 1998; von Götz et al. 2004; Seyer et al. 2005). It is known that genes involved in production of flagella have been found to be down-regulated following initial adhesion (Sauer and Camper 2001). Von Götz et al. (2004) demonstrated that highly adherent cells of *P. aeruginosa* down-regulated the most important structural proteins of the bacterial flagellum, so the perceptible under-expression of protein FliC in biofilm cells is in agreement with these observations. Whiteley et al. (2001) proposed that the reduction in flagella may help to stabilize the three-dimensional structure of the mature biofilm. The presence of the major constitutive porin OprF was also found in biofilm cells. OprF, a major OMP in *Pseudomonas* spp., is a non-specific porin that plays a role in the maintenance of cell shape and is required for growth in adverse environments (Brinkman et al. 1999; Seyer et al. 2005). This porin is also involved in cell–cell interactions and adhesion (Rebière-Huët et al. 2002; Seyer et al. 2005). Yoon et al. (2002) described the upregulation of OprF with maturation of *P. aeruginosa* biofilm under anaerobic conditions. In the context of microbial growth control, the outer membrane plays a significant role as it forms an adaptative barrier to the external environment, protecting the bacterial cell constituents from environmental changes and damaging substances (eg biocides) while allowing the selective uptake of nutrients (Nikaido 1996). One of the most important consequences of this result could be related to the role of the bacterial membrane transport systems in the provision of resistance to antimicrobial agents. Scenarios of antimicrobial resistance are extremely common in biofilm populations and are frequently related to the

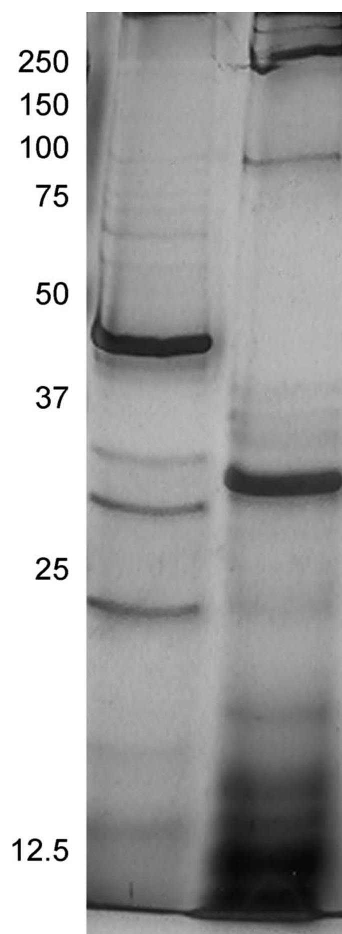

Figure 1. Profile of OMP of *P. fluorescens* planktonic (left column) and biofilm (right column) cells. Numbers on the left represent molecular weights in kDa. This figure is representative of OMP analysis from three independent experiments.

role of biofilm phenotype (Sufya et al. 2003; Simões et al. 2009).

Additional experiments were performed to compare the susceptibility of planktonic and biofilm cells (without EPS) to OPA, which has effective antimicrobial properties and a well characterized mode of action. OPA interacts with membrane components, promoting alteration of surface hydrophobicity and membrane permeability (Simões et al. 2007a). At high concentrations (>500 mg l^{-1}), OPA might cross the cell membranes and interact with intracellular sites, the growth cycle being compromised resulting in cell elongation due to the lack of septation (Simões et al. 2007a). Planktonic and biofilm cells were both inactivated by OPA (Figure 2, AII and BII) as assessed by Live/Dead *Bac*Light viability staining (MBC = 0.55 ± 0.20 mM – planktonic cells; MBC = 1.7 ± 0.30 mM – biofilm cells). The MBC of planktonic and biofilm cells were significantly different ($P < 0.05$). This difference in susceptibility of planktonic and biofilm cells (even in the absence of EPS) seems to be related to their distinct physiology (Table 1). *In vitro* studies have shown that bacteria growing in a biofilm can become 10–1000 times more resistant to antimicrobial agents compared to planktonic bacteria of the same strain (Amorena et al. 1999; Simões et al. 2005a).

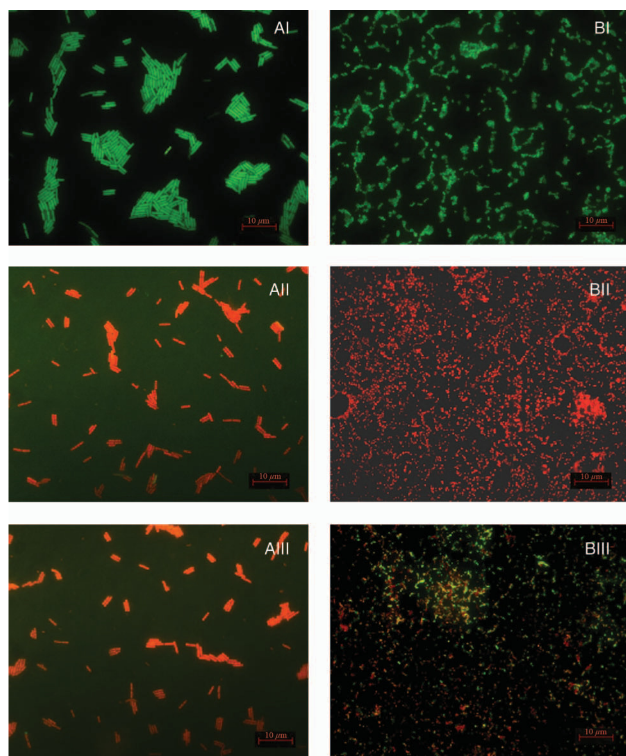

Figure 2. Epifluorescence photomicrographs of homogenized suspensions of *P. fluorescens* planktonic (A) and biofilm (B) cells before (I), after (II) and 24 h (III) later following OPA treatment. Scale bars = 10 μm. Cells stained green indicate live cells (AI and BI); cells stained red indicate non-viable cells (AII, AIII and BII). Analysis 24 h after treatment with OPA demonstrates that all planktonic cells are non-viable (AIII), but a high proportion of biofilm cells stain green indicating they are viable (BIII). The photomicrographs are representative of at least 20 microscope fields per independent experiment. At least 3 independent experiments were performed.

The potential of non-viable cells to recover was evaluated after antimicrobial treatment. Cells were inoculated into fresh growth medium and their potential for recovery was evaluated over time (Figure 2, AIII and BIII; Table 2). There were no viable or culturable planktonic cells during the 48 h period of analysis. However, biofilm cells recovered in viability and culturability 24 h after OPA treatment. The numbers of viable cells in biofilms increased significantly 36 h ($P < 0.05$) and 48 h ($P < 0.05$) after OPA treatment. Viability data were corroborated with CFU counts. Culturable cells were only detected for bacteria derived from biofilms and 24 h after OPA treatment (Table 2). Also, 24 h post-OPA treatment, the number of culturable cells increased significantly ($P < 0.05$) over time. Based on the 24, 36 and 48 h sampling times, only 2.1 ± 0.32% of the viable cells were culturable ($P < 0.05$). Several reasons may account for this difference: (i) presence of starved or injured cells or potentially viable but non-culturable cells (VBNC) (Banning et al. 2002) that are not able to initiate cell division at a sufficient rate to form colonies; (ii) inadequate culture conditions; (iii) aggregation of bacteria that can lead to the formation of one colony from more than one cell, thereby underestimating the

Table 2. Viable and culturable cells obtained from antimicrobial recovery experiments: immediately after OPA treatment (0), 12, 24, 36 and 48 h later.

	Viable (cells ml^{-1})					Culturable (CFU ml^{-1})				
Time (h)	0	12	24	36	48	0	12	24	36	48
Planktonic	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Biofilm	n.d.	n.d.	$5.0 \times 10^3 \pm 13$	$1.3 \times 10^6 \pm 3.2 \times 10^4$	$3.2 \times 10^9 \pm 5.5 \times 10^7$	n.d.	n.d.	$1.3 \times 10^2 \pm 21$	$2.0 \times 10^4 \pm 9.8 \times 10^2$	$6.3 \times 10^7 \pm 5.6 \times 10^6$

Results are shown as mean ± SD of at least 3 independent experiments, n.d. = not detected (<1 CFU ml^{-1} and zero viable cells ml^{-1}).

total number of cells. Ericsson et al. (2000) also considered that the method of cultivation on a solid medium is often inadequate due to the failure of the bacterial cell to reproduce on standard nutrient agar plates. Nevertheless, this may not mean that the cells were non-viable. Cells can be viable but lack the ability to divide. Gião et al. (2009) showed that *Legionella pneumophila* cells in biofilms can lose culturability without losing viability. Hence, alternative approaches such as epifluorescence microscopy with viability-indicator stains provide reliable information about the antimicrobial effects of biocides (Simões et al. 2005b; Ferreira et al. 2011).

The recovered bacteria (after the 48 h recovery period) were used to assess their ability to survive a second dose of OPA. The MBC for those recovered cells was 0.58 ± 0.40 mM, a concentration similar to the MBC of planktonic cells ($P > 0.05$). This result is in accordance to Balaban et al. (2004) who found that a fraction of a genetically homogeneous microbial population may survive exposure to antibiotic treatment. However, unlike resistant mutants, cells regrown from such a persistent state remained sensitive to the antibiotic. Furthermore, the cells recovered from biofilms were characterized in terms of cell size, metabolic activity, protein and polysaccharide content and OMP expression, and were found to have similar characteristics ($P > 0.05$, for all the parameters characterized) to those described for planktonic cells (results not shown). This result clearly demonstrates that the phenotypic switch from the planktonic to biofilm state is reversible. Biofilm persisters, when freely suspended and after a certain period of time, can acquire the physiology and behaviour of susceptible planktonic cells.

Physiological adaptation of microorganisms induces the development of intrinsic resistance. Biofilms are the leading example of physiological adaptation and are one of the most important sources of bacterial resistance to antimicrobial products. The persistent cell state is the newest explanation for increased biofilm resistance (Lewis 2010). However, the persister cells are not believed to be mutants. Rather it has been hypothesized that they are phenotypic variants that can exist in both planktonic and biofilm populations (Lewis 2007). However, planktonic persisters are susceptible to antimicrobial agents, whilst conversely, biofilm persister cells are protected by EPS (Lewis 2007). This study shows the possibility of the existence of persister cells in the biofilm population and the extreme ability of biofilm bacteria to recover from unfavourable situations, even in the absence of EPS. The occurrence of persistent cells is a phenomenon already described for several bacteria when exposed to standard antibiotics (Lewis 2001; Korch et al. 2003; Keren et al. 2004; Shah et al. 2006; Singh et al. 2009). This study demonstrates that injured biofilm bacteria may have the ability to recover and has significant implications for many systems where cells of *P. fluorescens* in biofilms are controlled by biocides.

The overall results indicate that *P. fluorescens* planktonic and biofilm cells display distinct physiological characteristics and behave differently after treatment with OPA. Biofilm bacteria (in the absence of EPS) were more resistant to OPA than planktonic cells and had persister cells able to resist antimicrobial treatment. When freely suspended for significant periods ($\geq$ 48 h) persister cells from biofilms acquired properties similar to planktonic cells and did not resist a second dose of biocide. Knowledge of bacterial tolerance and adaptation to biocides will help to improve the efficiency of antimicrobials used for biofilm control (Gattlen et al. 2010). To the authors' knowledge this is the first report indicating the ability of biofilm cells (without EPS) to persist after exposure to an antimicrobial chemical that acts on multiple biochemical targets of the cell. Further studies are in progress in order to ascertain the possibility of the presence of persister cells in biofilm populations exposed to large spectrum antimicrobials, particularly surfactants.

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