Persister cells in *Pseudomonas fluorescens* biofilms treated with a biocide

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Abstract
This study investigates the physiology and the behavior, after treatment with *ortho*-phtalaldehyde (OPA), of *Pseudomonas fluorescens* in both planktonic and sessile states. Steady-state biofilms and planktonic cells were collected from a bioreactor rotating system and their extracellular polymeric substances (EPS) were extracted using a non-invasive method. The cell physiology was compared in terms of respiratory activity, morphology, amount of cell proteins and polysaccharides and outer membrane proteins expression. Significant differences were found when comparing the physiological parameters analysed. Planktonic cells were more metabolically active, and were composed by a higher amount of proteins and polysaccharides than biofilm cells. Moreover, the biofilm formation process promoted the expression of distinct OMP. Additional experiments were performed in order to compare the susceptibility of planktonic and biofilm cells to OPA. That cells were completely inactivated after biocide exposure (minimum bactericidal concentration - MBC = 0.65 ± 0.20 mM – planktonic cells; MBC = 1.5 ± 0.25 mM – biofilm cells). After treatment, the potential of inactivated cells to recover from antimicrobial exposure was evaluated. Planktonic cells remained inactivated while cells from biofilms recovered (from an initial population of log 9.0 ± 0.21 cells/mL of inactivated bacteria it was verified a final population of log 3.3 ± 0.35 cells/mL of viable bacteria). This result indicates the possibility of existence of persister cells in the biofilm population and the extreme ability of biofilm bacteria to recover from inauspicious situations, even in the absence of EPS.

**Keywords:** Biofilm control; *ortho*-phtalaldehyde; persistence; phenotypic changes; recovery

INTRODUCTION
Microbial adhesion to abiotic surfaces and the consequent biofilm formation has been documented in many different environments (Hall-Stoodley *et al.* 2004). Despite the unquestionable importance of biofilms in microbial life style and their effects on human beings, our present knowledge about the physiology and behavior of sessile communities is still limited (Simões *et al.* 2008a). A switch from planktonic to growth in a 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 the growth on surfaces involve significant changes in gene transcription, including the establishment of new genetic traits (Christensen *et al.* 1998; O’Toole and Kolter, 1998).
Another important aspect associated with biofilms is related with the increased resistance of sessile cells to antimicrobial agents comparatively to their planktonic counterparts (McBain et al. 2002). In fact, bacteria in biofilms have intrinsic mechanisms that protect them from even the most aggressive environmental conditions, namely the exposure to chemical antimicrobials. Nevertheless, there is no answer to why and how bacteria, growing within a biofilm, develop increased resistance to antimicrobial agents. The persistent cell state is the newest explanation for biofilm insusceptibility to antimicrobials (Sufya et al. 2003; Lewis, 2007). It has been known for many years that small fractions of persistent bacteria resist killing when exposed to antimicrobials (Sufya et al. 2003; Keren et al. 2004; Lewis, 2007). This resistance mechanism was reviewed recently by Lewis (2007). Therefore, the conventional explanation of transport limitation and chemical interaction with biofilm EPS does not always explain the recalcitrant properties of biofilms. This study investigates the physiology and the behavior, after treatment with the aldehyde-based biocide ortho-phthalaldehyde (OPA), of Pseudomonas fluorescens in both planktonic and sessile states.

MATERIALS AND METHODS

**Microorganism and culture conditions**

*Pseudomonas fluorescens* (ATCC 13525) biofilms were developed in a bioreactor rotating system described in previous studies (Simões et al. 2008a; 2009). A 3.5 L bioreactor was continuously fed with growth medium and bacteria in the exponential phase of growth, supplied from an independent continuously operating 0.5 L chemostat. Biofilms were grown on stainless steel cylinders inserted in the bioreactor and rotating at a constant Reynolds number of agitation of 2400. The biofilms were allowed to grow for 7 d in order to obtain steady-state biofilms (Simões et al. 2009). The planktonic cells used through this study were collected from the 3.5 L bioreactor (dilution rate of 0.486 h⁻¹).

**Biofilm scraping**

The biofilm that covered the metal slides was entirely removed from the slides, using a stainless steel scraper according to the methodology described by Simões et al. (2009). Homogenised biofilm suspensions were then used to assess the respiratory activity, cellular size, proteins and polysaccharides content and OMP expression.

**Extracellular polymeric substances extraction**

Extraction of EPS from planktonic and biofilm cells was carried out using Dowex resin (50X 8, Na⁺ form, 20-50 mesh). The extracellular components were separated from the cells through centrifugation (Frøland et al. 1996).

**Bacterial characterization**

The respiratory activity of the bacterial samples was evaluated by measuring the rate of oxygen uptake in a biological oxygen monitor (BOM) during short-term assays, according to the procedure described by Simões et al. (2008a; 2009). The proteins were determined by the Lowry modified method using bovine serum albumin as standard and the polysaccharides by the phenol-sulphuric acid method (Dubois et al. 1956) using glucose as standard. The outer membrane proteins (OMP) were isolated and analyzed according to the method described by Simões et al. (2007).
Disinfection and antimicrobial recovery procedure
Planktonic and biofilm cells separated from the EPS were diluted to an adequate concentration and used to assess the minimum bactericidal concentration (MBC). The MBC of planktonic and biofilm cells was determined as the lowest concentration of OPA where no viability was detected according to Simões et al. (2007). After the OPA contact with the cells, the biocide was subjected to a process of neutralization with sodium bisulphite. The cells were transferred to fresh media and put in an orbital shaker (120 min⁻¹, 27 °C). After 24 h, bacteria were characterized in terms of viable cells counts using the Live/Dead BacLight bacterial viability kit, according to the procedure described by Simões et al. (2007).

RESULTS AND DISCUSSION
Steady-state biofilms and planktonic cells were subjected to an EPS extraction using a non-invasive method (Frlenlnd et al. 1996). Their cell physiology was compared in terms of respiratory activity, amount of cell proteins and polysaccharides (Table 1), morphology (Figure 1) and OMP expression.

Table 1 Respiratory activity, total proteins and polysaccharides content of P. fluorescens planktonic and biofilm cells

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<tr>
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<th>Planktonic cells</th>
<th>Biofilm cells</th>
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<tr>
<td>Oxygen uptake rate</td>
<td>9.09 × 10⁻¹⁶ ± 3.24 × 10⁻¹⁷</td>
<td>2.50 × 10⁻¹⁸ ± 1.41 × 10⁻¹³</td>
</tr>
<tr>
<td>(mg O₂/cell.min)</td>
<td></td>
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<tr>
<td>Total proteins</td>
<td>578 ± 165</td>
<td>3.31 ± 0.151</td>
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<tr>
<td>(pg/cell)</td>
<td></td>
<td></td>
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<tr>
<td>Total polysaccharides</td>
<td>1803 ± 576</td>
<td>6.91 ± 2.50</td>
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<tr>
<td>(pg/cell)</td>
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Significant differences were found when comparing the parameters analysed. Planktonic cells were more metabolically active and were composed by a higher amount of proteins and polysaccharides than biofilm cells.

In order to observe the morphological changes triggered by the biofilm formation process planktonic and biofilm cells were stained with Live/Dead BacLight stains and observed under epifluorescence microscopy (Fig. 1).

Planktonic cells differ notably in length from biofilm cells. P. fluorescens in planktonic state has a spherical equivalent cell radius (Simões et al. 2008a) of 0.899 ± 0.06 μm, while biofilm cells have a spherical equivalent cell radius of 0.318 ± 0.05 μm.

The OMP profile of P. fluorescens planktonic and biofilm cells was assessed in order to characterize OMP expression of cells at different states (results not shown). It was found, as expected, that cells in different states differ significantly in the expression of the major OMP. One of the most important consequences of this result could be related with the significant 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 with phenotypic differences between the cells (Sufya et al. 2003; Simões et al. 2008b).
Additional experiments were performed in order to compare the susceptibility of planktonic and biofilm cells (without EPS) to OPA. This biocide is known to have effective antimicrobial properties and has well characterized mechanisms of antibacterial action (Simões et al. 2007). Planktonic and biofilm cells were completely inactivated with OPA (MBC = 0.65 ± 0.20 mM – planktonic cells; MBC = 1.5 ± 0.25 mM – biofilm cells). After antimicrobial treatment, the potential of inactivated cells to recover from antimicrobial exposure was evaluated. Inactivated cells were inoculated into fresh growth medium and their potential for recovery was evaluated 24 h after OPA treatment. Planktonic cells remained inactivated while cells from biofilms recovered (from an initial population of log 9.0 ± 0.21 cells/mL of inactivated bacteria it was verified a final population of log 3.3 ± 0.35 cells/mL of viable bacteria).

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 the increased biofilm resistance. These persistent bacteria are not believed to be mutants. Rather it has been hypothesized that they are phenotypic variants and can exist in both planktonic and sessile populations (Lewis, 2007). However, planktonic persisters are antimicrobial susceptible, conversely the biofilm persister cells are protected by the extracellular polymeric matrix (Lewis, 2007). This study shows the possibility of existence of persister cells in the biofilm population and the extreme ability of biofilm bacteria, even in the absence of EPS, to recover from inauspicious situations. The occurrence of persistent cells is a phenomenon already described for several bacteria, but, when exposed to standard antibiotics (Keren et al. 2004; Lewis, 2007). The potential of biofilm cells to recover from biocide treatment is remarkable in this study. In fact, biofilm bacteria may be injured to sublethal conditions and could be, therefore, undetectable through conventional viability assessment methodologies. However, this study demonstrates that biofilm bacteria may have the ability to recover from those injured conditions. This finding has significant implications in many systems where P. fluorescens are associated as biofilms and biocides are applied for their control.

Fig. 1 Epifluorescence photomicrograph of P. fluorescens planktonic (a) and biofilm (b) cells. X 1320 magnification, bar =10 μm.

CONCLUSIONS
The overall results indicate that P. fluorescens planktonic and biofilm cells display distinct physiological characteristics and behave differently after treatment with OPA.
The OPA effects on biofilm bacteria inactivation raises concerns about the potential impact of the increased use of inefficient chemical agents and the prevalence of events of resistance. In fact, to our knowledge this is the first report indicating the ability of biofilm to persist after exposure to an antimicrobial chemical acting on multiple biochemical targets of the cell.

REFERENCES


