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The effect of hydrodynamic conditions on the phenotype of *Pseudomonas fluorescens* biofilms

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Abstract

This study investigated the phenotypic characteristics of monoculture *P. fluorescens* biofilms grown under turbulent and laminar flow, using flow cells reactors with stainless steel substrata. The cellular physiology and the overall biofilm activity, structure and composition were characterized, and compared, within hydrodynamically distinct conditions. The results indicate that turbulent flow-generated biofilm cells were significantly less extensive, with decreased metabolic activity and a lower protein and polysaccharides composition per cell than those from laminar flow-generated biofilms. The effect of flow regime did not cause significantly different outer membrane protein expression. From the analysis of biofilm activity, structure and composition, turbulent flow-generated biofilms were metabolically more active, had twice more mass per cm^2, and higher cellular density and protein content (mainly cellular) than laminar flow-generated biofilms. Conversely, laminar flow-generated biofilms presented higher total and matrix polysaccharide contents. Direct visualisation and scanning electron microscopy analysis showed that these different flows generate structurally different biofilms, corroborating the quantitative results. The combination of applied methods provided useful information regarding a broad spectrum of biofilm parameters, which can contribute to control and model biofilm processes.

Keywords: *Biofilms, hydrodynamic conditions, laminar and turbulent flow, phenotypic characterisation,* Pseudomonas fluorescens

Introduction

Biofilm development, behaviour and population characteristics are strongly influenced by many environmental factors and by intrinsic biological properties (Sauer & Camper, 2001; Whiteley et al. 2001; Purevdorj et al. 2002). One of the most important factors affecting biofilm structure and behaviour is the velocity field of the fluid in contact with the microbial layer (Vieira et al. 1993; Stoodley et al. 1999a; 1999b; Hall-Stoodley & Stoodley, 2002; Pereira et al. 2002b; Purevdorj et al. 2002; Tsai, 2005). Hydrodynamic conditions will determine the rate of transport of cells, oxygen and nutrients to the surface, as well as the magnitude of the shear forces acting on a developing biofilm. Thus, these conditions significantly influence many of the processes involved in biofilm development (Vieira et al. 1993). The biofilm formation process, by itself, triggers profound cellular changes (Sauer & Camper, 2001; Whiteley et al. 2001). Sessile cells respond to external adverse environmental conditions differently from their planktonic counterparts. One of the earliest observations related to the different characteristics observed in planktonic and cells within biofilms is the increased resistance of biofilm cells to antimicrobial agents and adverse environmental conditions (Brown & Gilbert, 1993; Costerton et al. 1995; Pereira & Vieira, 2001; Roberts & Stewart, 2005; Simões et al. 2005a; 2005b). Simões et al. (2003a; 2003b; 2005a) have previously demonstrated that *P. fluorescens* cells were more easily inactivated when they grew under planktonic conditions than when the cells were entrapped in biofilms, and that biofilms formed under laminar flow conditions were more easily inactivated than those formed under turbulent flow conditions.

P. fluorescens cells are known to be good biofilm producers and are ubiquitous in industrial environments (Pereira et al. 2002a; 2002b; Simões et al. 2003a; 2005b; 2006). A detailed knowledge of the role of hydrodynamic conditions on sessile features

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and the reasons behind the distinct biofilm tolerance to antimicrobial agents is essential in order to create accurate strategies to control unwanted biofilms. The changes in cells of *Pseudomonas* spp. associated with biofilm formation have been previously studied with emphasis on quorum-sensing events, morphological aspects (image based conclusions) and induced genetic variation (Sauer & Camper, 2001; Whiteley et al. 2001; Purevdorj et al. 2002; Purevdorj-Gage et al. 2005). Concerning the influence of hydrodynamic stress on the biofilm phenotype, Pereira et al. (2002b), using a similar system to that used in this study, demonstrated by confocal laser scanning microscopy (CLSM) analysis that the flow regime under which the biofilms were formed significantly influenced their architecture. Purevdorj et al. (2002), comparing the hydrodynamic conditions and cell signalling on the structure and behaviour of *P. aeruginosa* biofilms, found that quorum-sensing events were diluted by the effects of hydrodynamic conditions. Other studies also evaluated the single and concomitant effects of varying nutrient conditions and hydrodynamics on biofilm physiology and behaviour (Bishop et al. 1997; Stoodley et al. 1999a; Wäsche et al. 2002; Garny et al. 2006). Physiological biofilm characterisation with final results as quantitative numeric values can represent useful information for both engineered and modelling systems, ranging from biofilm control in industrial processes to biofilm reactor systems (Pereira et al. 2002b; Simões et al. 2005b). In an engineering sense, the hydrodynamic conditions can be manipulated, as a control parameter.

The purpose of the present study was to provide an extensive physiological characterisation of monoculture *P. fluorescens* biofilms and their respective entrapped cells when developed under different hydrodynamic conditions. Quantitative values were assessed and compared for both turbulent and laminar flow-generated biofilms and their respective biofilm cells. This understanding will allow hydrodynamic conditions to be manipulated as a control parameter in order to improve strategies for biofilm control.

Materials and methods

Bacteria

Pseudomonas fluorescens ATCC 13525^{T} was obtained from the American Type Culture Collection and preserved in cryovials (Nalgene) at $-80 \pm 2^{\circ}$C. The growth conditions were $27 \pm 1^{\circ}$C, pH 7.0 (0.02 M phosphate buffer - KH_2PO_4; Na2HPO$_4$), and 5 g l^{-1} glucose, 1.25 g l^{-1} yeast extract and 2.5 g^{-1} l peptone as nutrients. All the medium components were purchased from Merck (VWR, Portugal).

Biofilm reactors

A continuous pure culture of *P. fluorescens* was grown in a 0.5 l glass chemostat (Quickfit, MAF4/41, England) at 27°C, aerated (air flow rate - 0.425 min^{-1}) and agitated with a magnetic stirrer (Heidolph Mr 3001). The reactor was continuously fed (Ismatec Reglo) with 10 ml h^{-1} of sterile medium containing 5 g l^{-1} glucose, 2.5 g l^{-1} peptone and 1.25 g l^{-1} yeast extract in 0.02 M phosphate buffer pH 7.0. The 0.5 l chemostat was used to continuously inoculate (10 ml h^{-1}) a 3.5 l reactor (Perspex) which was aerated (air flow rate - 0.243 min^{-1}) and agitated with a magnetic stirrer. This reactor was fed with a diluted nutrient medium consisting of 0.05 g l^{-1} glucose, 0.025 g l^{-1} peptone and 0.0125 g l^{-1} yeast extract in 0.02 M phosphate buffer (pH 7), at a flow rate of 1.7 l h^{-1}, which supported a bacterial cell density of approximately 6×10^{7} cells ml^{-1}. This diluted bacterial suspension obtained in the 3.5 l reactor was pumped up (Eheim Typ 1060 and Eheim Typ 1048 pumps), passing through the flow cell reactors and back to the reactor.

A flow cell reactor described by Pereira et al. (2002a) was used as the device for biofilm formation. This type of device offers a simple possibility for the study and characterisation of biofilms in a well-controlled and reproducible manner. The device consists of a semicircular Perspex duct (45 cm length and 1.6 cm of hydraulic diameter) with 10 apertures on its flat wall where several removable rectangular pieces of Perspex may be fitted. In the present study stainless steel (ASI 316) slides (1.75 × 1.25 cm) were glued onto one of the faces of these Perspex pieces. Biofilms were formed on the metal slides whose upper faces were in contact with the bacterial suspension circulating through the flow cell reactor. Each of the rectangular pieces could be removed separately without disturbing the biofilm formed on the others and without stopping the flow (Pereira et al. 2002a; 2002b; Simões et al. 2003a; 2003c; 2005b) with reproducible results. This was possible because the outlet ports are disposed on the round face of the flow cell between each two adjacent removable pieces of Perspex, thus allowing the diversion of the circulating flow from the point where the reactor was opened.

The Reynolds number was calculated as a function of the duct design, using the hydraulic equivalent diameter (D_h), defined as (Tosun et al. 1988):

$$D_h = 4 \times \text{Flow area/Wetted perimeter} \quad (1)$$

For the semicircular duct (Tosun et al. 1988):

$$D_h = 4 \times (\pi/8 \times d^2)/(\pi/2 \times d + d) \quad (2)$$

where d is the semicircular duct diameter (1.6×10^{-2} m $- p > 0.5$ when comparing Re with

and without the biofilm thickness associated with the diameter), corresponding to a D_h of 9.78×10^{-3} m (Pereira et al. 2002a).

The Reynolds number, based on the hydraulic diameter, was:

$$Re = (D_h \times u \times \rho)/\mu \quad (3)$$

where u is the flow velocity (m/s), ρ is the fluid density (Kg m^{-3}) and μ the fluid viscosity (Kg $m\ s^{-1}$). For this study, the fluid characteristics were considered from water at the operation temperature.

Two parallel similar flow cell reactors were used simultaneously in such a way that biofilms were formed under turbulent (Reynolds number – Re = 5200, $u = 0.532$ m s^{-1}) and laminar (Re = 2000, $u = 0.204$ m s^{-1}) flow conditions, respectively, in each flow cell. The biofilms were allowed to grow for 7 d to ensure that steady-state biofilms were used in every experiment (Pereira et al. 2002a).

Sampling of biofilms

The biofilms that covered the metal slides were removed using a stainless steel scraper and then resuspended into 10 ml of buffer composed by 2 mM Na_3PO_4, 2 mM NaH_2PO_4, 9 mM NaCl and 1 mM KCl, pH 7 and homogenised in a vortex (Heidolph, model Reax top) for 30 s with 100% power input, according to the methodology described by Simões et al. (2003b; 2005a). Homogenised biofilm suspensions were then used to assess, sequentially, the morphological characteristics, respiratory activity, biochemical composition, OMP expression and mass.

Respiratory activity assessment

The assays were performed in a model 53 Yellow Springs Instruments (Ohio, USA) Biological oxygen monitor (BOM) as previously described (Simões et al. 2003a; 2005c). This procedure has already been applied successfully in the assessment of the metabolic activity of *P. fluorescens* biofilms formed in several bioreactor systems (Simões et al. 2003c; 2005a; 2005b). In another study (Simões et al. 2005c), this methodology showed higher reliability for assessing the metabolic state of bacteria than conventional assessment of colony forming units on solid medium and microscopic techniques using viability staining The homogenised biofilm suspensions were placed in the temperature-controlled vessel of the BOM ($T = 27°C \pm 1°C$). Each contained a dissolved oxygen (DO) probe 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 $l^{-1} - 27°C$; 1 atm). The vessel was then closed and the decrease in the oxygen concentration monitored over time. The initial linear decrease observed corresponds to the endogenous respiration rate. To determine the oxygen uptake due to substrate oxidation, 50 μl of a glucose solution (100 mg l^{-1}) were introduced into each vessel. The slope of the initial linear decrease in the DO concentration, after glucose injection, corresponds to the total respiration rate. The difference between the two respiration rates gives the oxygen uptake rate due to glucose oxidation.

Biomass quantification

The dry biofilm mass accumulated on the slides was assessed by the determination of the total volatile solids (TVS) of the homogenised biofilm suspensions (after the respiratory activity determination) according to the Standard Methods (American Public Health Association [APHA], American Water Works Association [AWWA], Water Pollution Control Federation [WPCF], 1989), method number 2540 A – D. According to this methodology the TVS assessed at $550 \pm 5°C$ in a furnace (Lenton thermal designs) for 2 h was equivalent to the amount of biological mass (cells and EPS). The dry biofilm mass accumulated was expressed in terms of biofilm mass per slide surface area.

Extraction of extracellular polymeric substances (EPS)

Extraction of biofilm EPS was carried out using Dowex resin (50X 8, NA^+ form, 20 – 50 mesh, Fluka Chemika, Switzerland, Cat. No. 44445), according to the methods of Frølund et al. (1996). The biofilm suspension coming from the respiratory activity assessment was diluted with a volume of 10 ml of buffer (2 mM Na_3PO_4, 2 mM NaH_2PO_4, 9 mM NaCl and 1 mM KCl, pH 7), resulting in a 20 ml biofilm suspension. Additionally, 50 g of Dowex resin per g of volatile solids (Simões et al. 2005b) were added to the biofilms and the extraction took place at 400 min^{-1} for 4 h at 4°C. The extracellular components were separated from the cells by centrifugation (3777 ***g***, 5 min).

Epifluorescence microscopy analysis

The cells separated by centrifugation from the EPS (present in the supernatant), after the EPS extraction procedure, were stained with 4′,6-diamidino-2-phenylindole – DAPI (Sigma, Portugal, Cat. No. D-9542), a DNA binding stain, as described by Saby et al. (1997). Cells separated from the extracellular products were diluted such as to give 30 – 250 cells per microscopic field. Thereafter, the bacterial suspensions were microfiltrated through a Nucleopore® (Whatman) black polycarbonate membrane (pore size 0.22 μm) and then stained with 400 μl of DAPI

at 0.5 μg ml^{-1} and left in the dark for 5 min. A Zeiss (AXIOSKOP) microscope fitted with fluorescence illumination was used with a 100X oil immersion fluorescence objective to visualise the cells. The optical filter combination for optimal viewing of stained preparations consisted of a 359 nm excitation filter in combination with a 461 nm emission filter. The micrographs were obtained using a microscope camera (AxioCam HRC, Carl Zeiss). A program path (AxioVision, Carl Zeiss Vision) was used for image acquisition and processing.

In order to assess the cellular morphological characteristics, biofilms were stained with Live/Dead *Bac*Light bacterial viability kit (Molecular Probes Cat. no. L-7012, Leiden, The Netherlands) according to the procedure described by Simões et al. (2005c). These stains (component A – Syto 9 and component B – propidium iodide) were also used for determining cell size.

A program path (Sigma Scan Pro 5) involving object measurement and data output was used to quantify the number of cells and to measure the equivalent cell radius to estimate cell size (Walker et al. 2005). The average equivalent spherical radii of turbulent and laminar flow-generated biofilm cells were determined through cell lengths and widths measurements.

The mean number of cells was determined through counts of a minimum of 20 microscopic fields per membrane.

Protein and polysaccharide assays

Total protein concentrations were determined using the Lowry modified method (Sigma-Protein Kit Cat. No. P5656), using bovine serum albumin as standard and the total polysaccharide concentration by the phenol-sulphuric acid method of Dubois et al. (1956), using glucose (Merck) as standard. Protein and polysaccharide assays were performed using biofilm suspensions before EPS extraction (total constituents) and with cells (cellular constituents) and EPS (matrix constituents) after extraction. The final values were assessed as mass of proteins/polysaccharides per biofilm dry mass.

Outer membrane proteins isolation and analysis

The outer membrane proteins (OMP) were isolated according to the method described by Winder et al. (2000). Sessile cells were harvested by centrifugation (3777 ***g***, 5 min, 4°C). The pellet was suspended in 25 mM Tris and 1 mM $MgCl_2$ 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, Portugal) was added to give a final concentration of 2% (w/v), in order to solubilise the inner membrane proteins. This solution was left on ice for 30 min. The solution was then 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. All the components used for OMP isolation were purchased from Sigma.

The proteins content of each sample was determined by Bicinchoninic Acid Protein Assay Kit (BCA) (BCA - PIERCE Cat. No. 23225) and standardised to 240 ± 10 μg ml^{-1} for each gel. Afterwards, the OMP samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), as reported by Laemmli (1970), with 12% (w/v) acrylamide. Electrophoresis was performed at a constant current of 10 mA. After electrophoresis, the proteins were Coomassie blue and silver stained for protein profile detection (Simões et al. 2006). All the components for electrophoresis were purchased from BioRad (Portugal).

Scanning electron microscopy (SEM)

Several stainless steel slides covered with biofilms were observed by SEM. The SEM inspections always comprised the observation of at least 15 fields of each biofilm-covered slide. Prior to SEM observations, biofilm samples were gradually dehydrated in ethanol (Merck) series to 100% (15 min each in 10, 25, 40, 50, 70, 80, 90 and 100% v/v), and dried in a desiccator for 3 d. The samples were sputter-coated with gold and examined with a Leica S360 scanning electron microscope at 10 – 15 kV. The slides were not fixed because fixation procedures involve the use of chemicals that tend to react with some of the components of the biological matrix, as documented by Azeredo et al. (1999), hence modifying the real biofilm structure. SEM observations were documented through the acquisition of at least 20 representative microphotographs.

Statistical analysis

The data were analysed using the statistical program SPSS version 14.0 (Statistical Package for the Social Sciences). The mean and standard deviation (SD) within samples were calculated in all cases. Because low sample numbers contributed to uneven variation, the nonparametric Wilcoxon test (for paired comparisons) was used. Statistical calculations were based on confidence level equal or higher than 95% ($p < 0.05$ was considered statistically significant).

Results

Characterisation of cells grown within turbulent and laminar flow-generated biofilms

Table I shows the cellular metabolic (respiratory) activity and the amount of total proteins and polysaccharides per cell. The results were established per amount of TVS that provided the amount of biological mass, resulting in accurate comparative measures.

Cells within laminar flow-generated biofilms were more metabolically active than those from turbulent biofilms ($p < 0.01$). Also, cells within laminar biofilms had a higher total protein ($p < 0.05$) and polysaccharide ($p < 0.01$) content than turbulent flow-generated biofilm cells (Table I).

In order to observe the morphological changes triggered by the distinct environmental hydrodynamic conditions, sessile cells were stained with Live/Dead *Bac*Light stains and observed under epifluorescence microscopy (Figure 1).

From Figure 1 it can be observed that sessile cells from both biofilms were in a viable state; green cells (the proportion of non-viable cells from 20 representative fields surveyed was $<0.05\%$ of the total community for both biofilms; $p > 0.1$). Cells within turbulent and laminar flow-generated biofilms appear to differ from each other, notably in length. *P. fluorescens* cells in a planktonic state had an equivalent cell radius of approximately 1 μm (results not shown), while cells from laminar flow-generated biofilms had an intermediate shape (equivalent cell radius $= 0.408 \pm 0.03$ μm) and cells from turbulent flow-generated biofilms were the smallest (equivalent cell radius $= 0.201 \pm 0.01$ μm). A statistical analysis of the sessile cellular size results revealed that they were significantly different ($p < 0.05$).

Table I. Respiratory activity, total proteins and polysaccharides content of turbulent and laminar flow-generated biofilm cells.

Cellular characteristics	Turbulent	Laminar
Respiratory activity ($mgO_2\ cell^{-1}\ min^{-1}$)	6.40×10^{-19} ($\pm 1.99 \times 10^{-20}$)	4.94×10^{-17} ($\pm 8.20 \times 10^{-19}$)
Total proteins ($pg\ cell^{-1}$)	1.25 ($\pm$0.38)	6.69 ($\pm$2.36)
Total polysaccharides ($pg\ cell^{-1}$)	0.105 ($\pm$0.026)	9.30 ($\pm$3.28)

Data are means of 5 experiments (95% confidence interval of are given in parenthesis).

Outer membrane protein (OMP) expression of turbulent and laminar flow-generated biofilm cells

Turbulent and laminar flow-generated biofilm cells exhibited comparable OMP, expressing similar major OMP (Figure 2). The main difference is that OMP extracted from the turbulent flow-generated biofilm cells (lane 1) increased in expression when compared with the laminar flow-generated biofilm cells (lane 2), particularly for the low weight protein bands. This property is arguably related to the distinct characteristics of cells within turbulent and laminar flow-generated biofilms. The major OMP found in both sessile cells had apparent molecular weights of 32 ± 3 kDa and 21 ± 2 kDa. In view of the similarity in the OMP profiles of turbulent and laminar flow-generated biofilm cells as assessed by SDS-PAGE, further proteomics analysis, such as 2-D electrophoresis, seemed to be redundant.

Activity, structure and composition of turbulent and laminar flow-generated biofilms

Table II shows the biofilm respiratory activity, mass, cellular density and the biochemical composition of

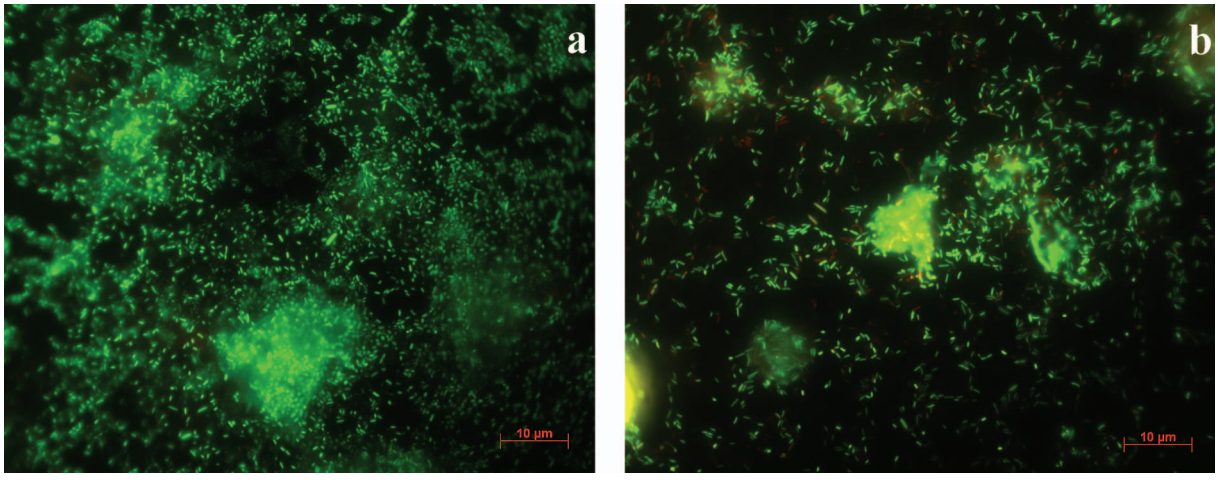

Figure 1. Epifluorescence photomicrographs of 7-d-old turbulent (a) and laminar (b) flow-generated biofilm cells. x1320; bar = 10 μm.

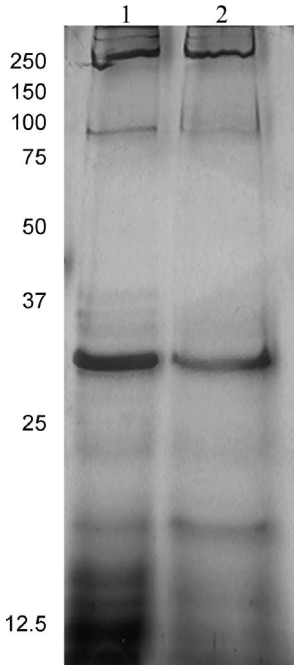

Figure 2. OMP profiles of *P. fluorescens* developed within turbulent (lane 1) and laminar (lane 2) flow-generated biofilms. Numbers on the left = molecular weights in kDa.

Table II. Characteristics of turbulent and laminar flow-generated biofilms of *P. fluorescens*.

Biofilm characteristics	Turbulent	Laminar
Respiratory activity ($mgO_2\ g^{-1}_{biofilm}\ min^{-1}$)	0.320 (±0.08)	0.068 (±0.01)
Biofilm mass ($mg_{biofilm}\ cm^{-2}$)	1.71 (±0.23)	0.717 (±0.19)
Log cellular density (cells cm^{-2})	14.6 (±0.09)	11.9 (±0.11)
Total proteins ($mg\ g^{-1}_{biofilm}$)	255 (±76.7)	71.3 (±25.1)
Matrix proteins ($mg\ g^{-1}_{biofilm}$)	37.4 (±6.73)	19.8 (±6.99)
Total polysaccharides ($mg\ g^{-1}_{biofilm}$)	136 (±33.2)	239 (±84.1)
Matrix polysaccharides ($mg\ g^{-1}_{biofilm}$)	115 (±28.0)	167 (±58.9)

Data are means of 5 experiments (95% confidence interval of are given in parenthesis).

biofilms formed under turbulent and laminar flow regimes.

Comparison of *P. fluorescens* biofilms formed under different hydrodynamic conditions (Table II) showed that turbulent flow-generated biofilms were more metabolically active ($p < 0.05$), had about twice as much mass per cm^2 ($p < 0.05$) and a much higher cellular density ($p < 0.01$) than those formed under laminar flow. They also showed a higher total protein content ($p < 0.05$), which is probably related to community density effects, shown by the observed relevant cellular density of turbulent flow-generated biofilms and the associated statistical level of significance. The protein content distribution differed in biofilms formed under laminar and turbulent flow regimes; there was a considerably higher amount of non-matrix proteins within turbulent flow-generated biofilms ($p < 0.05$), while the amount of matrix proteins in turbulent and laminar flow-generated biofilms were statistically similar ($p > 0.1$). The total polysaccharide content was higher in biofilms formed under laminar flow for every biofilm constituent analysed ($p < 0.05$). This biofilm constituent was present in greater amounts in the matrix than in the cellular composition ($p < 0.05$).

Biofilm grown on stainless steel coupons under different flow regimes were inspected by direct visualisation (Figure 3). The structure of *P. fluorescens* biofilms depended on the flow conditions; biofilms formed under turbulent flow appeared more homogeneous and slimy while those formed under laminar flow were much more patchy.

Figure 4 displays SEM microphotographs representative of the several fields observed in each biofilm-covered metal surface. Although the morphology of the biofilm may have been altered by the dehydration process, the SEM results provided good comparative information demonstrating clear differences in the structure of turbulent and laminar flow-generated biofilms. Biofilms grown under turbulent and laminar flow look very different, emphasising that hydrodynamic conditions play an important role in biofilm architecture (Figure 4). It was also observed that biofilms formed on the stainless steel surfaces under laminar flow did not totally cover the surface (Figure 4b), corroborating the result obtained in Figure 3. SEM inspections also suggested the existence of a greater amount of cells in the biofilms formed under turbulent flow, as well as an almost non-existent biofilm matrix when compared with the biofilms formed under laminar flow.

Discussion

The characteristics and behaviour of biofilms have a significant impact on many industrial engineering systems, including the design of biofilm control procedures. It was already realised that flow regime plays an important role in biofilm characteristics and susceptibility to biocides and surfactants (Simões et al. 2003a; 2003b; 2005a). Biofilms, when exposed to antimicrobial agents, present specific survival strategies, i.e. expression of specific resistance genes, decreased growth rates, restricted diffusion of antimicrobial agents due to the presence of an EPS matrix, quorum sensing specific effects, existence of persister cells that explain their resistance

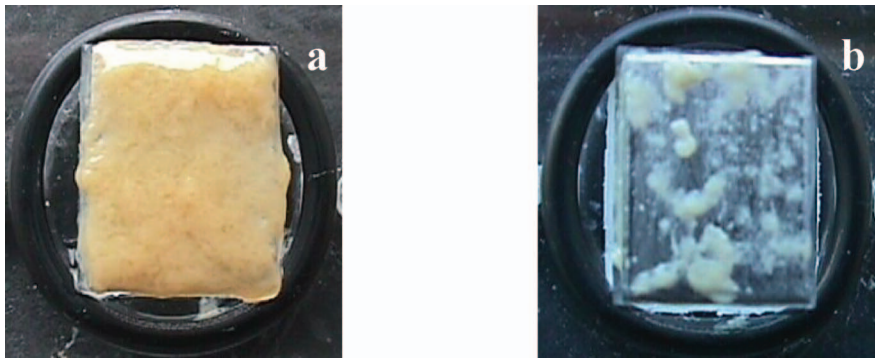

Figure 3. Photographs of coupons with wet biofilms formed on stainless steel slides under turbulent (a) and laminar flow (b).

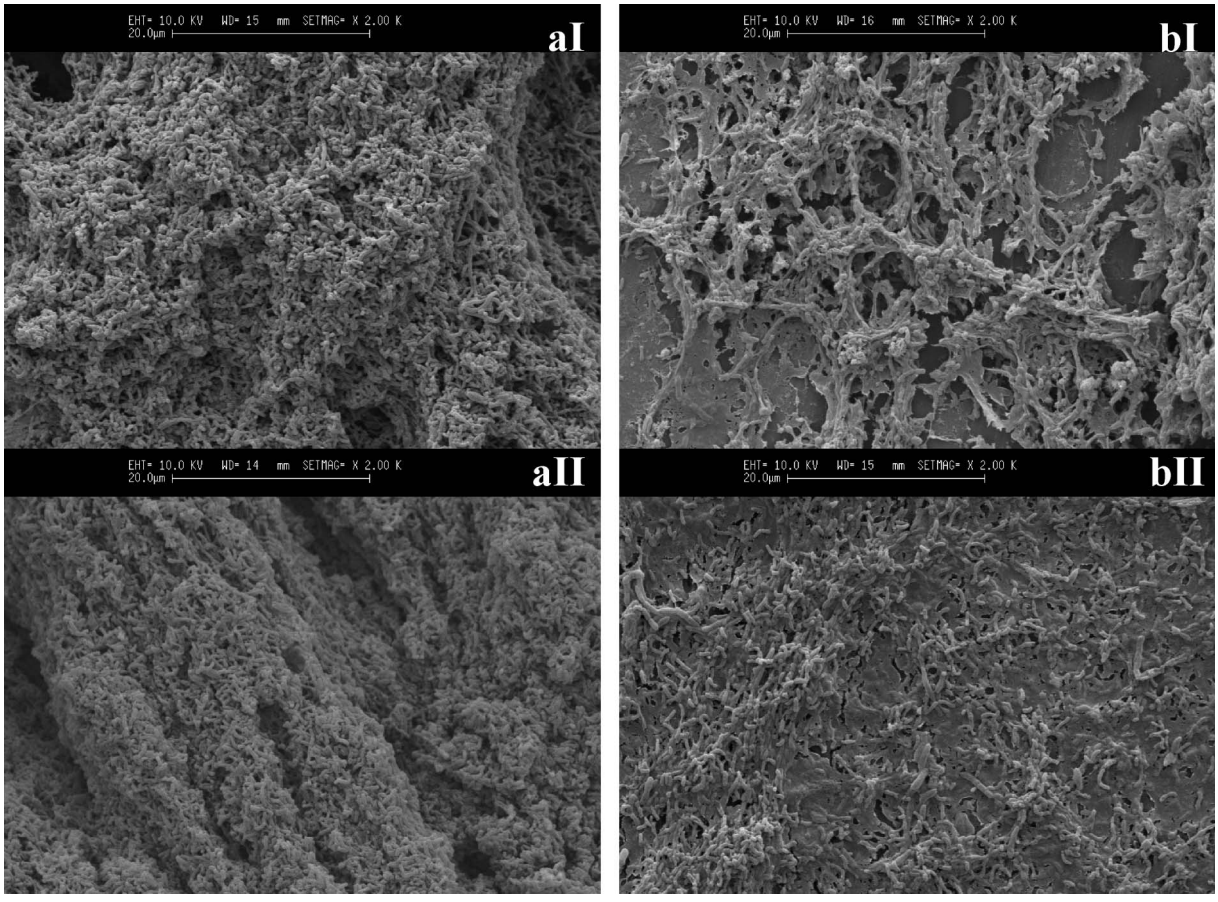

Figure 4. SEM micrographs of a 7-d-old *P. fluorescens* biofilm formed on stainless steel slides under turbulent (aI and aII) and laminar (bI and bII) flow. x 2000; bars = 20 μm.

(Brown & Gilbert, 1993; Costerton et al. 1995; Lewis, 2001; Pereira & Vieira, 2001; Stewart, 2003; Xavier et al. 2005). Other physiological changes are induced by environmental conditions (Stoodley et al. 1999a; Pereira et al. 2002b; Purevdorj et al. 2002). The present work shows that entrapped biofilm cells and biofilms formed under different flow regimes (turbulent/laminar) express distinct physiological characteristics, providing new biofilm data (mainly quantitative, and complementing previous reference studies related to the effect of hydrodynamics on the morphological characteristics of biofilms (thickness, surface coverage, density), as well as on their growth kinetics and mass transfer events, based essentially on microscopic and structure associated observations (Vieira et al. 1993; Bishop et al. 1997; Stoodley et al. 1999a; Pereira et al. 2002b; Wäsche et al. 2002). The highly spatial distributions of bacterial populations, structure and activity was demonstrated in a pioneer study by Zhang and Bishop (1994), showing the

non-uniform distribution of biofilm properties. The present study provides new and reliable comparative data between biofilms formed under different flow regimes. The biofilm and cellular parameters studied here are relevant in a biofilm control context by the application of chemical or mechanical stresses (Simões et al. 2005a; 2005b; 2006).

In biofilms, metabolic activity may reach an extent where biofilm bacteria are still viable, even if they do not show signs of viability such as the capability to grow in a solid medium. This cellular condition therefore induces loss of culturability although the cells remain metabolically active and structurally intact. This viable but non-culturable state is the main reason for the low detection rate of biofilms by routine culture (Fux et al. 2005). Consequently, when biofilms are an issue, the assessment of respiratory activity due to oxygen uptake rate may be more accurate than the traditional method of colony formation on agar media to assess the viability of bacteria within biofilms (Simões et al. 2005c). Also, the non-aggressive EPS extraction procedure used (Frølund et al. 1996) allowed the separation of cells from matrix material, providing reliable comparative data. Cells within biofilms formed under laminar flow were more active than those in the turbulent situation (Table I). This distinct cellular metabolic activity is possibly a consequence of a cascade of physical and biochemical mechanisms. Vieira et al. (1993) found that mass transfer limitations existed to a greater extent in biofilms formed under laminar flow than under turbulent conditions. Other authors (Horn & Hempel, 1997; Stoodley et al. 1997; Wäsche et al. 2002) also demonstrated the correlation between nutrient mass transfer in biofilms and flow velocities. Consequently, the higher oxygen rate and substrate transport from the fluid to the biofilm (mass transfer effects), should favour microbial metabolism and cell replication. Additionally, studies analysing the electron transport system, have shown that high shear stress can stimulate the catabolic activity of biofilms (Liu & Tay, 2001). However, the differences found in the overall biofilm metabolic activity is most likely related to the higher cellular density found within turbulent flow-generated biofilms (14.6 log cells cm^{-2} *vs* 11.9 log cells cm^{-2} for laminar biofilms) (Table II).

The differences in the amounts of cellular proteins and polysaccharides in turbulent and laminar flow-generated biofilm cells (Table I) was arguably related to the stress imposed by the flow conditions on the resultant bacterial physiology (Figure 1). For instance, laminar flow-generated biofilm cells had a larger cell size and also a higher content of total proteins and polysaccharides. However, such biochemical differences do not account for OMP expression changes (Figure 2). Cells within turbulent and laminar flow-generated biofilms expressed a similar major OMP. It is interesting to note that the molecular weights of the OMP expressed by both turbulent and laminar flow-generated biofilm cells were similar to those of the well-characterised OMP, OprF (32 ± 3 kDa) and OprH or OprL (21 ± 2 kDa), which are apparently implicated in the process of biofilm formation and development (Kragelund et al. 1996; Seyer et al. 2005). In the context of biofilm control, the OMPs seem not to be the main cellular property involved in the recalcitrance and resistance of turbulent flow-generated biofilms, when compared with the laminar flow-generated biofilms. Previous studies (Simões et al. 2003a; 2003b; 2005a) revealed that the latter were more easily inactivated with biocides and surfactants than turbulent biofilms, an event attributed to the distinct biofilm physiology (Simões et al. 2003a; 2003b). Comparing the OMP profiles of biofilm cells (Figure 2) with previous reports concerning planktonic *P. fluorescens* grown under similar operational conditions (Simões et al. 2006), it was found, as expected, that the OMP profiles of cells in different states differed significantly in the expression of major OMPs.

The analysis of the overall biofilm activity, structure and composition revealed that different hydrodynamic conditions promoted the formation of biofilms with distinct morphological structure (Figures 3 and 4), respiratory activity, cellular density, mass amount and total and extracellular biochemical composition (Table II). It must be emphasised that the quantitative comparative analysis reached a relevant statistical level of significance ($p < 0.05$), showing physiological differences between turbulent and laminar flow-generated biofilms. Other authors (Liu & Tay, 2001; Purevdorj et al. 2002) documented that physical phenomena rather than biological effects were responsible for the relationship between hydrodynamic shear and biofilm structure. In the present study, contrary to existing assumptions, it was demonstrated that high shear stress promoted the formation of higher cell density biofilms with lower amounts of matrix polysaccharides than low shear stress conditions (Table II). It has been proposed (Lazarova et al. 1994; Liu & Tay, 2001; Pereira et al. 2002b) that high detachment forces can induce the biofilms to secrete more EPS, which in turn would result in a balanced biofilm structure under given hydrodynamic conditions. Furthermore, based on CLSM analysis, Pereira et al. (2002b) proposed that laminar flow-generated biofilms had more cells than in the turbulent situation. In the present study, using epifluorescence microscopy and DAPI, for direct cell counts, it was found that turbulent flow-generated biofilms had a much higher cell density ($p < 0.01$) than in the laminar situation (Table II),

a result in accordance with a previous report (Stoodley et al. 1999a). Turbulent conditions contributed to the adhesion of a higher cell number and the consequent higher amount of mass, rather than the production of an extensive extracellular polymeric matrix (Table II), a fact probably related to the decreased cellular (single-cell) metabolic activity (Table I). Furthermore, the main reasons for the distinct structure, physiological composition and metabolic characteristics of turbulent and laminar flow-generated biofilms are strongly related to the different transport rates of oxygen, nutrients and cells from the fluid to the biofilm, the effect of flow conditions on the structural plasticity of biofilms (mass transfer limitations) and cellular induced reactions, acting as single or concomitant factors (Vieira et al. 1993; Liu & Tay, 2001; Hall-Stoodley & Stoodley, 2002; Pereira et al. 2002b). The selected SEM microphotographs also illustrate the significant morphological differences found in turbulent and laminar flow-generated biofilms. Currently, CLSM in combination with fluorescent probes is applied as a powerful tool for structural studies of biofilms (Neu et al. 2004; Garny et al. 2006). The concept of biofilms as complex three-dimensional structures with channel networks through which liquid flow occurs originated from studies using CLSM (Pereira et al. 2002b; Neu et al. 2004). However, SEM images are also useful for describing biofilm morphotypes and allowing the estimation of the dimensions of structures (Horath et al. 2006).

The ability of biofilms to adapt their physiology and morphology to different environmental conditions, found in this work, may help to explain their tenacious nature and recalcitrance to control as found in previous studies (Simões et al. 2003a; 2003b; 2005a). In conclusion, quantitative characterisation has demonstrated that cells within turbulent flow-generated biofilms were less metabolically active, had a reduced size and smaller amounts of proteins and polysaccharides in their composition and expressed similar OMPs when compared with laminar flow-generated biofilm cells. The analyses of overall biofilm activity, structure and composition revealed that distinct flow regimes lead to the formation of physiologically distinct biofilms. Biofilms formed under turbulent flow were more metabolically active due to the higher cellular density, had a higher amount of mass, total proteins, similar amounts of matrix proteins and a smaller amount of total and matrix polysaccharides when compared with biofilms formed under laminar flow. This detailed characterisation of biofilm physiological responses to hydrodynamic stress may represent a useful tool in many industrial engineering based processes, where the flow conditions can be controlled.

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