Biofouling
Vol. 24, No. 5, September 2008, 339–349

Taylor & Francis
Taylor & Francis Group

Antagonism between *Bacillus cereus* and *Pseudomonas fluorescens* in planktonic systems and in biofilms

Manuel Simões[a,b]*, Lúcia C. Simões[a], Maria O. Pereira[a] and Maria J. Vieira[a]

[a]*IBB-Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, Universidade do Minho, Campus de Gualtar, Braga, Portugal;* [b]*CITAB-Departamento de Fitotecnia e Engenharia Rural, Universidade de Trás-os-Montes e Alto Douro (UTAD), Edifício Ciências Agrárias, Apartado 1013, Vila Real, Portugal*

(*Received 24 April 2008; final version received 27 May 2008*)

In the environment, many microorganisms coexist in communities competing for resources, and they are often associated as biofilms. The investigation of bacterial ecology and interactions may help to improve understanding of the ability of biofilms to persist. In this study, the behaviour of *Bacillus cereus* and *Pseudomonas fluorescens* in the planktonic and sessile states was compared. Planktonic tests were performed with single and dual species cultures in growth medium with and without supplemental $FeCl_3$. *B. cereus* and *P. fluorescens* single cultures had equivalent growth behaviours. Also, when in co-culture under Fe-supplemented conditions, the bacteria coexisted and showed similar growth profiles. Under Fe limitation, 8 h after co-culture and over time, the number of viable *B. cereus* cells decreased compared with *P. fluorescens*. Spores were detected during the course of the experiment, but were not correlated with the decrease in the number of viable cells. This growth inhibitory effect was correlated with the release of metabolite molecules by *P. fluorescens* through Fe-dependent mechanisms. Biofilm studies were carried out with single and dual species using a continuous flow bioreactor rotating system with stainless steel (SS) substrata. Steady-state biofilms were exposed to a series of increasing shear stress forces. Analysis of the removal of dual species biofilms revealed that the outer layer was colonised mainly by *B. cereus*. This bacterium was able to grow in the outermost layers of the biofilm due to the inhibitory effect of *P. fluorescens* being decreased by the exposure of the cells to fresh culture medium. *B. cereus* also constituted the surface primary coloniser due to its favourable adhesion to SS. *P. fluorescens* was the main coloniser of the middle layers of the biofilm. Single and dual species biofilm removal data also revealed that *B. cereus* biofilms had the highest physical stability, followed by *P. fluorescens* biofilms. This study highlights the inadequacy of planktonic systems to mimic the behaviour of bacteria in biofilms and shows how the culturing system affects the action of antagonist metabolite molecules because dilution and consequent loss of activity occurred in continuously operating systems. Furthermore, the data demonstrate the biocontrol potential of *P. fluorescens* on the planktonic growth of *B. cereus* and the ability of the two species to coexist in a stratified biofilm structure.

Keywords: antagonist molecules; biofilm formation; biofilm control; dual-species biofilms; microbial interactions

Introduction

Microorganisms are found in a wide range of ecosystems as highly structured multi-species communities, termed biofilms (Stoodley et al. 2002; Palmer et al. 2007). The downside of microbial biofilms is associated with their involvement in major problems associated with industry, medicine and everyday life (Gilbert et al. 2002; Leriche et al. 2003; Hall-Stoodley et al. 2004; Shakeri et al. 2007). Bacterial adhesion to surfaces is a widespread phenomenon and is affected by many factors (Palmer et al. 2007; Simões et al. 2008a). To date, it has been documented that surface conditioning and the type of growth medium, growth temperature and pH, electrostatic and physical interactions between bacterial cell surfaces and substratum, cell–cell communication and signalling, are able to influence bacterial adhesion and the further development of biofilm (Davies et al. 1998; Doyle 2000; Gilbert et al. 2002; Palmer et al. 2007). Moreover, the colonisation of a surface by a bacterium can enhance the attachment of others to the same surface (Møller et al. 1998; Simões et al. 2007a). This gradual process can lead to functional bacterial consortia often possessing greater combined metabolic activity and resilience than that of each component species on its own (Møller et al. 1998). Understanding the mechanisms by which different species survive and interact in a biofilm should help to develop strategies for their elimination at source. Nevertheless, the physiology and metabolism of multispecies biofilm communities are immensely complex. The existence of multiple

*Corresponding author. Email: mjvsimoes@deb.uminho.pt

ISSN 0892-7014 print/ISSN 1029-2454 online

DOI: 10.1080/08927010802239154
http://www.informaworld.com

interactions or even the simple production of a metabolite can interfere with the development of a biofilm (Rasmussen et al. 2005; Simões et al. 2007a). For example, competition for substrate is considered to be one of the major evolutionary driving forces in bacteria (Banks and Bryers 1991). Numerous experimental data obtained in the laboratory show how different microorganisms may effectively outcompete others as a result of better utilisation of a given energy source (Banks and Bryers 1991; Christensen et al. 2002; Komlos et al. 2005).

Evidence of increased biofilm resistance to conventional antimicrobial treatments has led to several alternative control strategies. These include the use of interspecies interactions as biocontrol strategies (Gram et al. 1999; Mireles et al. 2001; Ammor et al. 2006), bacteriophages (Hughes et al. 1998; Tait et al. 2002; Sillankorva et al. 2004), enzymes (Meyer 2003; Olsen et al. 2007; Leroy et al. 2008), quorum-sensing inhibitors (Rasmussen et al. 2005) and other compounds that interfere with biofilm formation such as iron chelators (Singh et al. 2002; Banin et al. 2005). However, in order to develop strategies for preventing biofilm formation, it is necessary to better understand the mechanisms by which different species survive and interact within a biofilm. Therefore, this study was designed to evaluate the interactions between *Bacillus cereus* and *Pseudomonas fluorescens* in both planktonic and biofilm conditions. These bacteria are commonly found in industrial settings, causing numerous process and end product quality problems (Kolari et al. 2001; Lindsay et al. 2002; Simões et al. 2008b). They can represent a significant proportion of the contaminant biofilm microflora of constitutive dairy plants (Sharma and Anand 2002a; Dogan and Boor 2003; Kreske et al. 2006; Wijman et al. 2007).

The main objectives of this study were to evaluate the interactions between *B. cereus* and *P. fluorescens* as changes in bacterial counts in (1) a planktonic system with and without iron and (2) biofilms in a continuous bioreactor. Biofilm stratification and physical stability were also assessed on single and dual species biofilms after growth for 7 days. Furthermore, planktonic growth inhibition assays with and without iron were performed and the production of iron-chelating molecules was measured and correlated with the presence/absence of iron in the growth medium. Finally, the adhesion ability and the free energy of adhesion between the bacteria and stainless steel (SS) were assessed.

Materials and methods

Microorganism and culture conditions

P. fluorescens ATCC 13525^{T} and a *B. cereus* strain, isolated from a disinfectant solution and identified by 16S rRNA gene sequencing, were used throughout this study (Simões et al. 2007b). Bacterial growth conditions were 27 $\pm$ 2°C and pH 7, with glucose as the main carbon source. The bacteria were grown in independent chemostats, consisting of 0.5 l glass reactors (Quickfit, MAF4/41, England), with an air flow rate of 0.425 l min^{-1} and continuously fed with a sterile concentrated standard growth medium (glucose, 5 g l^{-1}; peptone, 2.5 g l^{-1}; and yeast extract, 1.25 g l^{-1}; prepared in 0.02 M phosphate buffer, pH 7) (Simões et al. 2007c). The continuous feeding, with the aid of a peristaltic pump (Ismatec Reglo, Germany) occurred at a rate of 10 ml h^{-1} (*P. fluorescens*) or 13 ml h^{-1} (*B. cereus*) of sterile medium.

Planktonic growth of single and dual species

Three flasks, containing 200 ml of sterile concentrated standard growth medium, were inoculated with *B. cereus* and *P. fluorescens* from 0.5 l chemostats in the exponential phase of growth, to a cell density of 5×10^8 cells ml^{-1} (2.5×10^8 cells ml^{-1} of each species for co-culture experiments), and left to grow in an orbital shaker (120 rpm, 27°C) (Simões et al. 2007a). Bacterial growth was followed over time (48 h) by aseptically taking 0.5 ml samples from each flask and assessing the number of viable *B. cereus* and *P. fluorescens* cells by epifluorescence microscopy using the Live/Dead BacLight bacterial viability kit (Molecular Probes, L-7012, Leiden, The Netherlands), according to the procedure described by Simões et al. (2005b). Cell suspension samples were diluted such as to have 30–250 cells per microscopic field, and then microfiltered through a Nucleopore® (Whatman, Middlesex, UK) black polycarbonate membrane (pore size 0.22 μm), stained with 250 μl of SYTO 9^{TM} (component A) and 250 μl of propidium iodide (component B) from the Live/Dead kit, and left in the dark for 15 min. Dye solutions were prepared by dissolving 3 μl of component A in 1 ml of sterile-filtered (pore size 0.22 mm) distilled water and the same procedure was followed for component B. A Zeiss (AXIOSKOP; Zeiss, Göttingen, Germany) microscope, fitted with fluorescence illumination and a 100× oil immersion objective, was used to visualise the stained cells. The optical filter combination for optimal viewing of stained preparations consisted of a 480–500 nm excitation filter in combination with a 485 nm emission. Cell observations were recorded as micrographs using a microscope camera (AxioCam HRC, Carl Zeiss, Germany) and a programme path (AxioVision, Carl Zeiss Vision) involving image acquisition and processing. A programme path (Scan Pro 5, Sigma), involving object measurement and data output, was used to quantify the number of cells. Additional experiments were conducted as referred to above, but with the presence of 100 μM of $FeCl_3$ (Riedel-de-Haën,

Germany) in the growth medium. Spore numbers of *B. cereus* were assessed by surface plating (300 μl samples) after heat treatment of cell cultures (80°C, 5 min), according to Kolari et al. (2001). The plates of solid concentrated standard growth medium (13 g l^{-1} agar) were incubated at 27 $\pm$ 2°C for 72 h.

Assays of growth inhibition

B. cereus and *P. fluorescens* were grown in batch conditions for 2 days in 500 ml flasks (Schott, Germany) containing 200 ml of sterile concentrated standard growth medium. The suspensions were centrifuged (3777*g*, 5 min) and the supernatant filter-sterilised (0.2 μm, Whatman). Exponential phase bacteria (100 μl) from the chemostats referred to above, were spread on solid medium and air-dried for 30 min. The supernatant solutions were pipetted (10 μl) onto the lawns, as described by Kolari et al. (2001). The screening for inhibition halos was performed after 1 day at 27°C. Sterile medium was used for the negative control. Experiments were also conducted with supernatants from bacteria grown on standard medium supplemented with 100 μM of $FeCl_3$.

Production of iron-chelating molecules

The production of iron-chelating molecules was assayed on chrome azurol S (CAS) agar, based on the methodology described by Schwyn and Neilands (1987). In liquid medium, those molecules were detected by the CAS assay (Schwyn and Neilands 1987). Equal volumes of sterile-filtered culture supernatant and CAS assay solution were mixed and left for 30 min at room temperature (25 $\pm$ 2°C). The absorbance at 630 nm (BIO-TEK, Synergy HT, Vermont, USA) was measured against blanks of sterile medium and CAS assay solution. A negative value indicated the presence of iron-chelating molecules, such as siderophores.

Free energy of adhesion between bacteria and SS

The free energy of adhesion ($\Delta G_{\mathrm{Adhesion}}$) between the bacterial cells and SS surfaces was assessed according to the procedure described by Simões et al. (2007d, 2008a). To ascertain the bacterial surface properties, lawns of *B. cereus* and *P. fluorescens* were prepared as described by Busscher et al. (1984). SS surfaces were prepared for characterisation by immersion in a solution of commercial detergent (Sonasol Pril, Henkel Ibérica S. A.) in ultrapure water for 30 min. After rising with ultrapure water, the surfaces were dried at 65 $\pm$ 5°C for 3 h.

The contact angles of the bacteria and of the SS were determined by sessile drop contact angle measurements, using an apparatus (model OCA 15 Plus; DATAPHYSICS, Filderstadt, Germany) that allowed image acquisition and data analysis. The surface tension components of bacteria and SS were obtained by measuring the contact angles with three pure liquids. These measurements were carried out at 25 $\pm$ 2°C using water, formamide and α-bromonaphthalene (Sigma) as reference liquids. The surface tension components of the reference liquids were taken from the literature (Janczuk et al. 1993). Contact angle data were obtained from at least 25 determinations for each liquid and for each experiment. Afterwards, the hydrophobicity of bacteria and the SS surfaces was evaluated from contact angle measurements by the method of van Oss et al. (1987, 1988, 1989). With this method, the degree of hydrophobicity of a given material (1) is expressed as the free energy of interaction between two entities of that material immersed in water (w) – (ΔG_{iwi} mJ m^{-2}). ΔG_{iwi} was calculated from the surface tension components of the interacting entities, according to the equation

$$\Delta G_{\mathrm{iwi}} = -2\left(\sqrt{\gamma_{\mathrm{i}}^{\mathrm{LW}}} - \sqrt{\gamma_{\mathrm{w}}^{\mathrm{LW}}}\right)^2 + 4\left(\sqrt{\gamma_{\mathrm{i}}^{+}\gamma_{\mathrm{w}}^{-}} + \sqrt{\gamma_{\mathrm{i}}^{-}\gamma_{\mathrm{w}}^{+}} - \sqrt{\gamma_{\mathrm{i}}^{+}\gamma_{\mathrm{i}}^{-}} - \sqrt{\gamma_{\mathrm{w}}^{+}\gamma_{\mathrm{w}}^{-}}\right) \quad (1)$$

where γ^{LW} accounts for the Lifshitz-van der Waals component of the surface free energy and γ^{+} and γ^{-} are the electron acceptor and electron donor parameters, respectively, of the Lewis acid-base component (γ^{AB}), with $\gamma^{\mathrm{AB}} = 2\sqrt{\gamma^{+}\gamma^{-}}$.

The surface tension components were estimated by the simultaneous resolution of three equations of the type

$$(1+\cos\theta)\gamma_{\mathrm{i}}^{\mathrm{Tot}} = 2\left(\sqrt{\gamma_{\mathrm{s}}^{\mathrm{LW}}\gamma_{\mathrm{i}}^{\mathrm{LW}}} + \sqrt{\gamma_{\mathrm{s}}^{+}\gamma_{\mathrm{i}}^{-}} + \sqrt{\gamma_{\mathrm{s}}^{-}\gamma_{\mathrm{i}}^{+}}\right) \quad (2)$$

where θ is the contact angle and $\gamma^{\mathrm{Tot}} = \gamma^{\mathrm{LW}} + \gamma^{\mathrm{AB}}$.

When studying the interaction (free energy of adhesion ($\Delta G_{\mathrm{Adhesion}}$)) between substances i and I that are immersed or dissolved in water, the total interaction energy, $\Delta G_{\mathrm{iwI}}^{\mathrm{Tot}}$, can be expressed as

$$\Delta G_{\mathrm{iwI}}^{\mathrm{Tot}} = \gamma_{\mathrm{iI}}^{\mathrm{LW}} - \gamma_{\mathrm{iw}}^{\mathrm{LW}} - \gamma_{\mathrm{Iw}}^{\mathrm{LW}} + 2\left[\sqrt{\gamma_{\mathrm{w}}^{+}}\left(\sqrt{\gamma_{\mathrm{i}}^{-}} + \sqrt{\gamma_{I}^{-}} - \sqrt{\gamma_{\mathrm{w}}^{-}}\right) + \sqrt{\gamma_{\mathrm{w}}^{-}}\left(\sqrt{\gamma_{\mathrm{i}}^{+}} + \sqrt{\gamma_{I}^{+}} - \sqrt{\gamma_{\mathrm{w}}^{+}}\right) - \sqrt{\gamma_{i}^{+}\gamma_{I}^{-}} - \sqrt{\gamma_{i}^{-}\gamma_{I}^{+}}\right] \quad (3)$$

Thermodynamically, if $\Delta G_{iwI}^{Tot} < 0$ adhesion is favoured, whereas adhesion is not expected to occur if $\Delta G_{iwI}^{Tot} > 0$ mJ m^{-2}.

Adhesion assays

SS coupons (8 mm × 8 mm), prepared as indicated for the $\Delta G_{Adhesion}$ assessment, were inserted in the bottom of 24-well (15 mm diameter each well) microtiter plates (polystyrene, Orange Scientific, USA). Bacteria collected from the 0.5 l chemostats were centrifuged twice (3777*g*, 5 min) and resuspended in 0.2 M phosphate buffer to cell densities of 5×10^8 cells ml^{-1} or 2.5×10^8 cells l^{-1} of each bacterium, for dual species experiments. A volume of 2 ml (1 ml of each bacterium for dual species experiments) of each bacterial suspension was added to each well. Adhesion to SS was allowed to occur for 2 h at 25 ± 2°C, in a shaker at 150 rpm, according to the methods of Simões et al. (2007d). Negative controls were obtained from SS in phosphate buffer without bacteria. The experiments were performed in triplicates and repeated three times. At the end of the assay, each well was washed twice with phosphate buffer, to remove weakly adherent cells. Total counts (viable and non-viable cells) of adhered bacteria were assessed by Live/Dead bacterial viability kit following the procedure described above.

Single and dual species biofilm formation

Continuous cultures of each bacterium, grown in the 0.5 l glass chemostats as referred above, were used to inoculate (10 ml h^{-1} for *P. fluorescens* and 13 ml h^{-1} for *B. cereus*) continuously in a 3.5 l polymethyl methacrylate (Perspex) reactor, aerated at 0.243 l min^{-1}. This reactor was fed with diluted nutrient medium (glucose, 0.05 g l^{-1}; peptone, 0.025 g l^{-1}; and yeast extract, 0.0125 g l^{-1}; prepared in 0.02 M phosphate buffer, pH 7) (Azeredo and Oliveira 2000; Simões et al. 2005b). The flow rate was maintained at 1.7 l h^{-1}, so that it would support a cell density of 6×10^7 cells ml^{-1}. Single and dual biofilms were grown on ASI 316 SS cylinders, with a surface area of 34.6 cm^2 (diameter = 2.2 cm; length = 5 cm), inserted in the 3.5 l reactor and rotating at a constant low shear stress corresponding to a Reynolds number of agitation (Re_A) of 2400. Three SS cylinders were used in every experiment. Biofilms were allowed to grow for 7 days before further experimentation in order to obtain steady-state biofilms (Pereira et al. 2002).

In the case of dual species biofilms, two 0.5 l glass chemostats were used to grow *B. cereus* and *P. fluorescens* independently. The 3.5 l reactor was inoculated simultaneously with the two bacteria, and fed with the diluted nutrient medium at a flow rate two times higher (3.4 l h^{-1}) than used for single species biofilm formation, in order to obtain a cell density and residence time similar to the single species scenario.

Biofilm stratification and physical stability

Biofilm physical stability was assessed by determining the biomass loss due to exposure to increasing Re_A in a rotating system device, as described elsewhere (Simões et al. 2005b, 2008b). After biofilm formation for 7 days, the cylinders with biofilm were carefully removed from the 3.5 l reactor, accurately weighed and immersed in 170 ml vessels with 0.02 M phosphate buffer. Afterwards, the cylinders were consecutively subjected to serial shear stress forces corresponding to Re_A of 4000, 8100, 12,100, 16,100, for a period of 30 s each. The wet weight of the cylinders with biofilm attached was determined before and after each Re_A application. After exposure to shear stress and for dual biofilms, the proportion of *B. cereus* and *P. fluorescens* cells in the several layers (removed and the remaining) was quantified by direct cell counts. The residual biofilms were removed with an SS scraper according to the methodology of Simões et al. (2008a), and resuspended in 5 ml of phosphate buffer, for cell enumeration.

The experiments were repeated on three different occasions for every scenario tested with low variability for the results ($P > 0.05$). The wet mass of the biofilm removed from the surface of each cylinder, after each Re_A exposure, was expressed in terms of percentage biofilm removal, defined as the amount of biofilm adhered after exposure to the complete series of Re_A. The data are expressed as percentage of biofilm remaining, according to the following equations:

$$\text{Biofilm remaining}\,(\%) = (X_f - X_c)/(X_{biofilm} - X_c) \times 100 \quad (4)$$

$$\text{Biofilm removal}_i(\%) = (X_{biofilm} - X_i)/(X_{biofilm} - X_c) \times 100 \quad (5)$$

where $X_{biofilm}$ is the wet mass of the cylinder plus biofilm mass before exposure to the series of Re_A; X_c is the wet mass of the clean cylinder, ie without adhered biofilm; X_f is the wet mass of the cylinder plus biofilm mass after exposure to the entire series of Re_A; i–Re_A, ie 4000, 8100, 12,100 and 16,100; X_i is the wet mass of the cylinder plus biofilm mass after exposure to Re_A of 4000, 8100, 12,100 or 16,100.

Biofilm cell counts

Biofilm cells were obtained after extraction of extracellular polymeric substances, according to Frølund et al. (1996) in order to eliminate components (biofilm matrix) that would interfere with the staining technique (Simões et al. 2005b, 2007c). Afterwards, bacteria were stained with the Live/Dead BacLight bacterial viability kit as described for planktonic cell counts. Total cell counts were assessed by the amount of viable and non-viable bacteria. *B. cereus* and *P. fluorescens* were distinguished according to differences in cell size (Simões et al. 2007b, 2007c). *B. cereus* had cell sizes of 1.58 $\pm$ 0.09 μm (sessile) and 2.03 $\pm$ 0.13 μm (planktonic), whereas *P. fluorescens* had cell sizes of 0.583 $\pm$ 0.07 μm (sessile) and 1.16 $\pm$ 0.11 μm (planktonic).

The mean number of cells was determined from counts of a minimum of 20 microscopic fields, for each sample membrane.

Statistical analysis

The data were analysed using the statistical programme Statistical Package for the Social Sciences, version 14.0. The mean and standard deviations within samples were calculated for all cases. All data were analysed by the application of the non-parametric Wilcoxon test (confidence level $\geq$ 95%).

Results

Single and dual species planktonic tests

B. cereus and *P. fluorescens* single cultures in standard (Figure 1a) and Fe-supplemented medium (Figure 1b) had similar growth profiles and rates ($P > 0.05$). For these conditions, *B. cereus* spores were detected at residual numbers compared to vegetative cells, and at similar amounts in both standard and Fe-supplemented medium (Figure 2). In the dual species system grown in standard medium (Figure 1c), *B. cereus* and *P. fluorescens* co-existed for approximately 8 h, but after this period, there was a sharp decrease in the number of vegetative cells of *B. cereus*, as their growth was repressed by *P. fluorescens*. The proportions of the two species were therefore significantly affected, with the density of viable cells of *B. cereus* after 48 h being *ca.* 90% lower than that of *P. fluorescens*. The reduction in the number of *B. cereus* cells induced spore formation to some extent, although this was not correlated with a decrease in viable cells (Figure 2). Spores were detected at 10 h with their numbers increasing over time at smaller levels than the decrease in viable cells ($P < 0.05$). Moreover, the number of spores was significantly higher than in single cultures ($P < 0.05$).

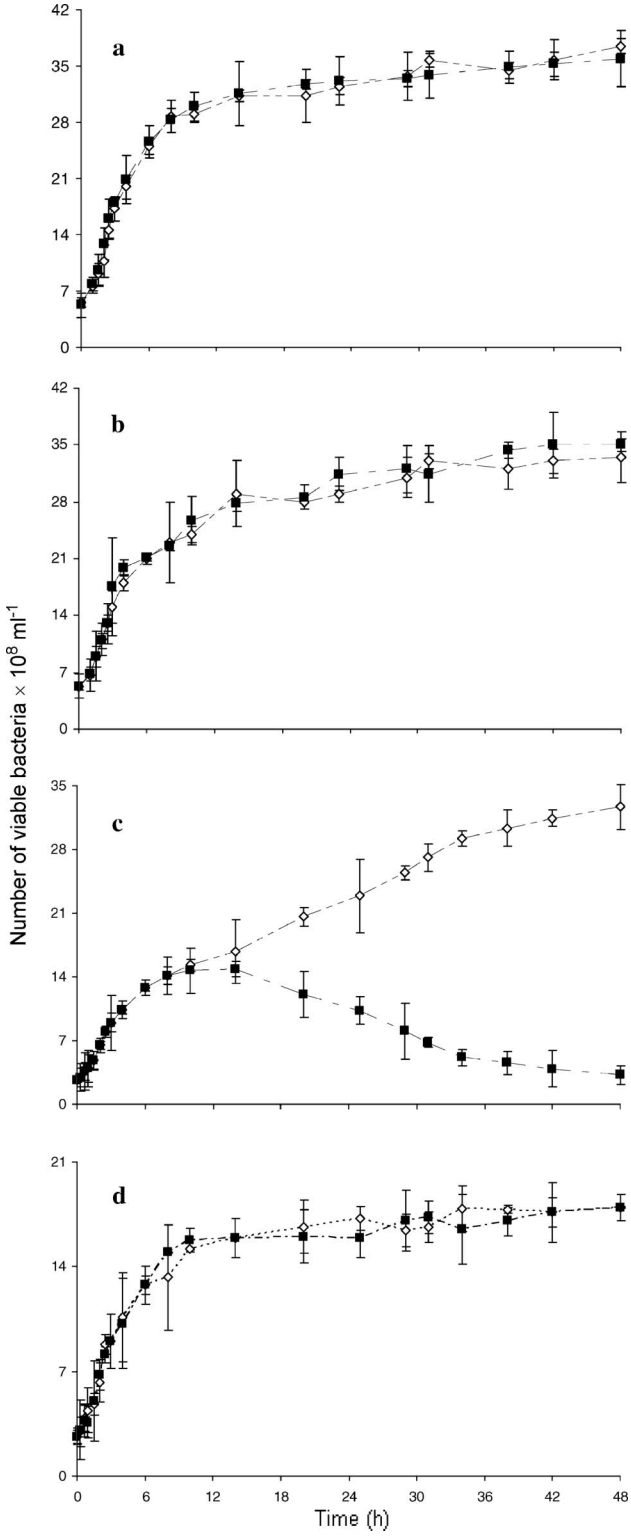

Figure 1. Planktonic growth of *B. cereus* (■), and *P. fluorescens* (◇) as single cultures in standard growth medium not supplemented (a) and supplemented with $FeCl_3$ (b). Dual cultures of *B. cereus* (■) and *P. fluorescens* (◇) in conventional (c) and $FeCl_3$ supplemented growth medium (d). The means $\pm$ SDs for at least three replicates are illustrated.

The antimicrobial activity of *P. fluorescens* on *B. cereus* was quenched when the medium was supplemented with 100 μM of $FeCl_3$ (Figure 1d). In this condition, both species grew with similar growth profiles and cell numbers ($P > 0.05$). Spore formation was also detected (Figure 2) in similar amounts to those detected for co-culture in Fe-limited conditions ($P > 0.05$), indicating that spore formation is dependent on the presence or absence of *P. fluorescens*, but not of iron.

To explore the nature of the antagonism observed, extracts from broth cultures of *B. cereus* and *P. fluorescens* in standard growth medium with or without iron, were analysed for antimicrobial effects. Only cell-free supernatants of *P. fluorescens* grown in standard growth medium caused inhibition of the growth of *B. cereus*, whereas the inverse was not found. This showed that growth inhibition of *B. cereus* by *P. fluorescens* was due to metabolites secreted under iron starvation. In fact, no zones of inhibition were observed when the medium was supplemented with iron.

Tests with CAS agar revealed the production of Fe-chelating molecules only by *P. fluorescens*. Moreover, Figure 3 shows that the quantity of Fe-chelating substances produced by *P. fluorescens* increased over time (the most negative values indicate higher concentrations of Fe-chelating molecules), when the bacteria were in the standard medium. This effect was also observed in co-culture suspensions grown in standard medium (results not shown). Under Fe-supplemented conditions, the concentration of Fe-chelating molecules was negligible and did not vary ($P > 0.05$). This suggests that the antagonistic activity of *P. fluorescens* against *B. cereus* is related to the presence of Fe-chelating molecules.

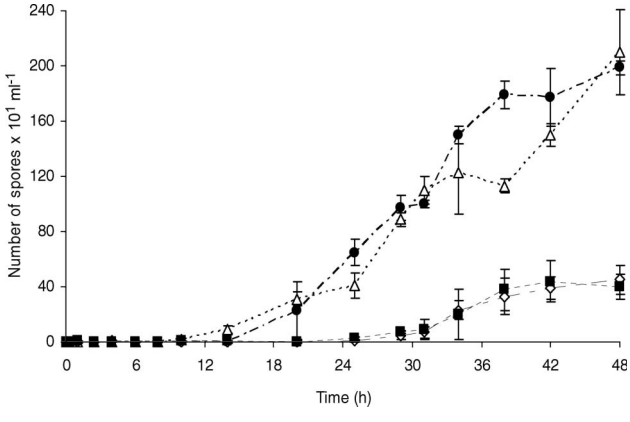

Figure 2. Spore counts from pure cultures of *B. cereus* in standard growth medium not supplemented (■) and supplemented with $FeCl_3$ (◇) and from a bacterial co-culture in conventional (△) and $FeCl_3$ supplemented growth medium (●). The means ±SDs for at least three replicates are illustrated.

Adhesion and free energy of adhesion between bacteria and SS

The free energy of adhesion between bacteria and SS was ascertained by thermodynamic interaction between cell-adhesion and the surface. *B. cereus*, which had $\Delta G_{Adhesion} = -2.23 \pm 0.11$ mJ m^{-2}, showed favourable adhesion to SS compared with *P. fluorescens* ($\Delta G_{Adhesion} = 11.8 \pm 1.3$ mJ m^{-2}).

Adhesion assays demonstrated that in single species experiments, $8.8 \times 10^5 \pm 3.1 \times 10^4$ cells cm^{-2} of *B. cereus* and $1.1 \times 10^5 \pm 1.8 \times 10^4$ cells cm^{-2} of *P. fluorescens* adhered to SS. In the dual species system, the adhered cell numbers were $7.8 \times 10^5 \pm 4.3 \times 10^4$ cells cm^{-2} of *B. cereus* and $2.5 \times 10^5 \pm 5.1 \times 10^4$ cells cm^{-2} of *P. fluorescens*. This result, combined with the $\Delta G_{Adhesion}$ data provides strong evidence that *B. cereus* is the primary surface coloniser.

Biofilm removal due to mechanical stress – dual species biofilm stratification

B. cereus and *P. fluorescens*, individually and in co-culture, colonised the SS surfaces to a significant extent, a phenomenon easily seen by visual inspection (Figure 4). Dual biofilms were composed of log values of 13.9 (±0.1) and 13.6 (±0.09) cells cm^{-2} of *B. cereus* and *P. fluorescens* ($P < 0.05$), respectively, whereas single species biofilms had cell densities of 13.0 (±0.21) of *B. cereus* and 14.0 (±0.11) cells cm^{-2} of *P. fluorescens*.

The detachment of bacterial cells from biofilms is a critical factor in their control processes. In this study, the physical stability of biofilms was assessed by exposure to different shear stresses, corresponding to increasing Re_A. Biofilms subjected to mechanical

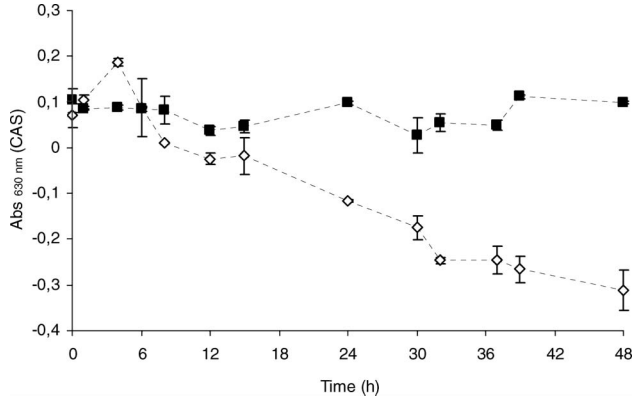

Figure 3. Indication of iron-chelating molecule production (CAS reaction with negative values) by *P. fluorescens* in conventional (◇) and $FeCl_3$ supplemented growth medium (■). The means ± SDs for at least three replicates are illustrated.

treatments were hardly removed by a Re_A of 4000. This Re_A only removed 13%, 14% and 24% of *B. cereus, P. fluorescens* and dual biofilms, respectively (Figure 5a). However, when the Re_A was raised from 4000 to 8100, additional biofilm detachment was observed. The highest increase in single and dual species biofilm removal occurred following exposure to a Re_A of 8100. For the other Re_A, single species biofilm removal was similar ($P > 0.05$), whereas the removal of dual biofilms was dependent on the shear stress applied ($P < 0.05$).

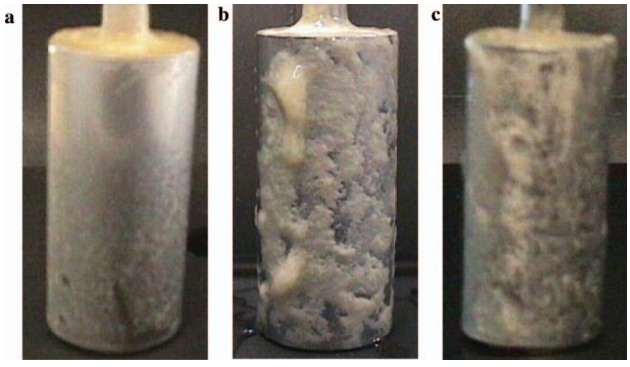

Figure 4. Stainless steel cylinders covered with biofilms after growth for 7 days of *B. cereus* (a), *P. fluorescens* (b), and dual species (c).

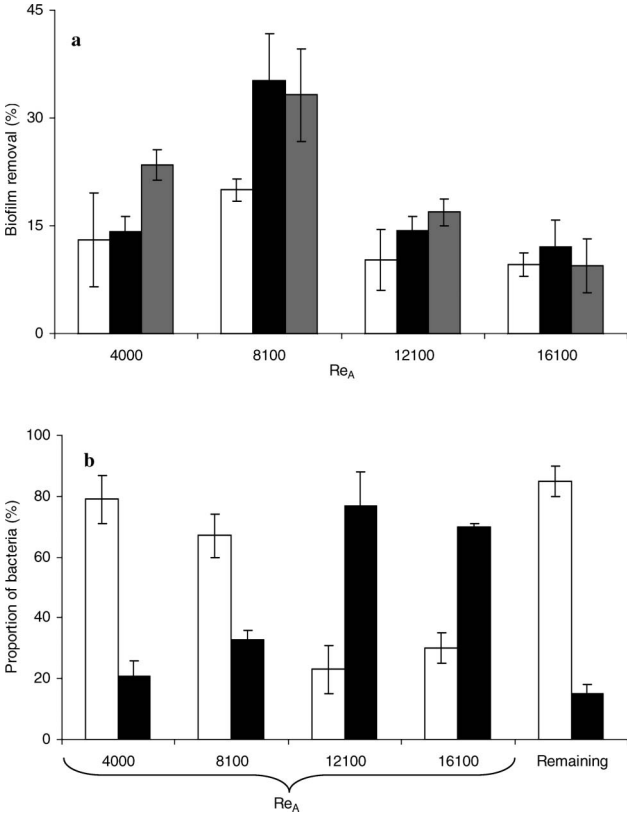

Figure 5. Percentage removal of *B. cereus* (□), *P. fluorescens* (■), and dual species (▣) biofilm after exposure to a series of Re_A (a). Proportion of *B. cereus* (□) and *P. fluorescens* (■) composing the several removed layers and the remaining adhered layer of a dual species biofilm exposed to a series of Re_A (b). The means ±SDs for at least three replicates are illustrated.

The method used allowed the removal of biofilm layers (Azeredo and Oliveira 2000), which was dependent on the mechanical stability of the biofilm and the shear stress force applied. However, it is expected that if biofilms were only exposed to the highest Re_A, removal would be similar to the total removal achieved by the increasing series of Re_A. When the effects of the series of Re_A shear stresses on biofilm removal were tested, the results were similar to those for biofilms exposed to a Re_A of 16,100 ($P > 0.05$). Similar removal results were also found for single species biofilms of *B. cereus* and *P. fluorescens* exposed to a Re_A of 4000 and for *P. fluorescens* single biofilms and dual biofilms exposed to Re_A of 8100 and 12,100 ($P > 0.05$). Figure 5a also shows that the series of Re_A did not give rise to total removal. Residual biofilm was still observed after exposure to the series of Re_A, with 47% of *B. cereus*, 24% of *P. fluorescens* and 17% of the mass of the dual biofilm remaining adhered to the SS surface. This result demonstrates that biofilms of *B. cereus* had a higher physical stability than those of *P. fluorescens* or the dual biofilms.

The composition of the several layers of dual species biofilms was evaluated in terms of the number of *B. cereus* and *P. fluorescens* cells, as shown in Figure 5b. There was a higher proportion of *B. cereus* cells in the outer biofilm layers, corresponding to biofilm removal by a Re_A of 4000 and 8100 (70–80% of the removed population), whereas *P. fluorescens* colonised to a greater a extent, the layers removed by the application of a Re_A of 12,100 and 16,100 (70–80%). The inner layer, ie the biofilm remaining on the surface after the series of Re_A, was predominantly colonised by *B. cereus* (85%).

Similar results in terms of single and dual species biofilm formation, composition and behaviour were detected when biofilms were developed with diluted nutrient medium with and without a supplement of 100 μM $FeCl_3$ (results not shown).

Discussion

Interactions between microorganisms are well recognised phenomena, and are believed to be important for the selection of a specific microflora in a given ecological niche (Gram et al. 1999; Komlos et al. 2005; Hansen et al. 2007). Microbial diversity leads to

a variety of complex relationships, involving both inter- and intra-species interactions (Simões et al. 2007a). This study highlights factors regulating interactions between *B. cereus* and *P. fluorescens* in both planktonic and biofilm modes and demonstrates the biocontrol potential of *P. fluorescens* on the planktonic growth of *B. cereus*. In batch conditions, it is expected that a microorganism will out-compete all others if it holds a higher substrate utilisation efficiency, leading to an inauspicious environment for the less adapted. In this study, both bacteria showed similar growth behaviour when grown in single cultures or in co-cultures in Fe-supplemented medium. Under standard growth (iron limited) conditions, *P. fluorescens* released metabolites with antimicrobial activity on the vegetative cells of *B. cereus*. The decrease in the number of viable vegetative cells was not correlated with sporulation. The antimicrobial effect detected was apparently related to iron metabolism and the production of Fe-chelating molecules. No effects on the planktonic growth of *B. cereus* were detected when the medium was Fe-supplemented. Moreover, the appearance of inhibitory activity in spent supernatants from *P. fluorescens* standard growth medium coincided with a strong CAS reaction, indicating the presence of Fe-chelating molecules such as siderophores. However, the data do not allow the specific implication of siderophores in the active mechanism or a conclusion on the exact nature of the inhibitory molecules. In a previous study, Gram et al. (1999) also reported the inhibitory effects of uncharacterised molecules of a strain of *P. fluorescens* against the fish pathogenic bacterium *Vibrio anguillarum* released under Fe-limited conditions. Some strains of *P. fluorescens* are known to have biocontrol activity on many microorganisms due to antibiotics and/or siderophores (Dufy and Défago 1999; Cornelis and Matthijs 2002). To the authors' knowledge, this is the first study indicating the biocontrol effect of *P. fluorescens* on planktonic growth of *B. cereus*.

Dual biofilms were colonised to a greater extent by *B. cereus* (13.9 *vs.* 13.6 log cells cm^{-2} of *P. fluorescens*). Although cell density differences were statistically significant, it seems unlikely that they are biologically and ecologically relevant. Biofilm cells were present in a higher number than those found in industrial environments (Elvers et al. 1998; Sharma and Anand 2002b), but at similar levels to those formed by *P. fluorescens* under turbulent flow using flow cell reactors (Simões et al. 2007c). The high biofilm cell counts reported were apparently related to the characteristics of the experimental system used. This bioreactor system and the operating conditions were optimised to improve the potential of bacteria to form biofilms (Azeredo and Oliveira 2000; Simões et al. 2005a, 2008a). Single and dual biofilms were characterised in terms of physical stability in response to external mechanical stress conditions (Simões et al. 2005a). According to Stoodley et al. (1999), the biofilm matrix develops an inherent internal tension, which is in equilibrium with the shear stress under which the biofilm is formed. Thus, the removal of a well-established biofilm requires the forces that maintain its integrity to be overcome (Körstgens et al. 2001). Detachment of biofilms formed on the bioreactor rotating system occurred in layers, where the increase in the shear stress may progressively thin the biofilm, with mechanical failure and total detachment being the ultimate effects expected (Azeredo and Oliveira 2000). This detachment mechanism differs from that described for flowing systems, such as flow cells, where detachment of single cells and clusters are the main events (Stoodley et al. 2001). Experiments on biofilm removal showed that biofilms were hardly removed with a Re_A of 4000. When the Re_A was raised from 4000 to 8100, significant additional detachment was observed for the biofilms studied. Nevertheless, a significant biofilm layer still remained on the surface, even when the highest Re_A was applied. A previous report (Melo and Vieira, 1999) on the removal of *P. fluorescens* biofilm by increasing liquid flow velocity also demonstrated a very low removal efficiency when the flow was increased about 2.86 (40% biofilm removal) or 1.54 (0% biofilm removal) times compared with the flow conditions under which the biofilms were developed. Garret et al. (2008) found that about 93% of the population of 12 h-adhered *P. fluorescens* cells were removed when exposed to shear stress by a water flow for 960 s. The authors, furthermore, demonstrated that the older the biofilm, the more resistant the biomass was to removal. This study, in addition to other aspects (Chen et al. 2005), shows the effects of species type and association on the mechanical stability of biofilms.

The amount of biofilm remaining adhered to the SS surfaces indicated that dual biofilms had the lowest physical stability and biofilms of *B. cereus* were more stable. The apparent thinner and more compact biofilm structure of *B. cereus*, in comparison with *P. fluorescens*, could account for the differences in physical stability found between the single biofilms (Simões et al. 2007b). Moreover, other phenotypic characteristics of *B. cereus* and *P. fluorescens* biofilms are important in maintaining the physical stability of the biofilm (Simões et al. 2007b, 2007c). The presence of a high number of *P. fluorescens* cells in the inner layers of the dual biofilms could account for the lower physical stability compared with single species biofilms.

Comparisons between planktonic and biofilm data show that the behaviour of planktonic dual species did

not predict which microorganism would prevail in the biofilm system. Antagonist molecules were a probable factor in differential biofilm removal by a Re_A of 12,100 and 16,100. *P. fluorescens* dominated the biofilm layers that were removed (70–80%), which suggests that *B. cereus* antagonist molecules operate only in closed systems, such as batch conditions and biofilm inner layers. Conversely, in a continuously operating biofilm open system, all substances would be diluted at residual concentrations, thus reaching sub-effective concentrations. Also, the antagonism of such substances seems to indicate that efficacy is restricted to bacteria in close proximity, analogous to what has been suggested for oral biofilms, where most metabolic signals communicate only microdistances (Egland et al. 2004). Moreover, low levels of spore formation were detected in single biofilms of *B. cereus* and in dual species biofilms (results not shown). Ryu and Beuchat (2005) also found that spores were only present in residual number in biofilms compared with vegetative cells.

An agreement between biofilm spatial distribution, adhesion assays and free energy of adhesion data was found. The strength of interaction between the bacterial cells and SS was assessed through the determination of the free energy of adhesion and confirmed by adhesion assays. This result is in accordance with previous studies, proposing that *Bacillus* species are one of the microorganisms primarily responsible for formation of slimy deposits (Blanco et al. 1996; Kolari et al. 2001). Moreover, for the system used here, the favourable interaction between *B. cereus* and SS surfaces seems to play a more significant role on the inner layer species composition of the biofilm than the potential antagonist molecules produced by *P. fluorescens*. The results indicate that, in dual *P. fluorescens* and *B. cereus* biofilms, the latter species is the primary coloniser of SS. However, thermodynamically based calculations and adhesion tests only provide a partial explanation for the events occurring in the biofilm formation process. For example, *P. fluorescens* develops biofilms on SS surfaces with a higher cellular density and mass than those formed by *B. cereus* (Simões et al. 2007b), although the former bacterium has a lower ability to adhere to SS than *B. cereus*.

In conclusion, this study shows significant differences in the behaviour and composition of planktonic and sessile dual species communities of *B. cereus* and *P. fluorescens*. Planktonic mixed growth of *P. fluorescens* and *B. cereus* was regulated by iron availability. Under Fe-deficiency, *P. fluorescens* inhibited the vegetative growth of *B. cereus*. This inhibition was apparently related to the production of Fe-chelating molecules. Dual biofilms were colonised to a higher extent by *B. cereus*, the primary surface coloniser, which attached to SS more effectively than *P. fluorescens*. These biofilms had a stratified structure with a middle layer composed mostly of *P. fluorescens*, surrounded by two layers where *B. cereus* was the predominant species. The prevalence of *B. cereus* in the outermost layer of dual biofilms seems to be due to the constant supply of fresh medium, which is needed for the bioreactor system. Such a constant flow minimises the concentration of inhibitory factors produced by *P. fluorescens* within the bioreactor. Furthermore, the two species biofilms had decreased physical stability relative to that of the single species biofilms. Further studies are being carried out to characterise the exact chemical nature of the *B. cereus* antagonist molecule produced by *P. fluorescens*.

Acknowledgement

The authors acknowledge the financial support provided by the Portuguese Foundation for Science and Technology (Project Biomode-PTDC/BIO/73550/2006, SFRH/BD/31661/2006-L. C. Simões; Ciência 2007-M. Simões).

References

Ammor S, Tauveron G, Dufour E, Chevalier I. 2006. Antibacterial activity of lactic acid bacteria against spoilage and pathogenic bacteria isolated from the same meat small-scale facility 1-screening and characterization of the antibacterial compounds. Food Control. 17:454–461.

Azeredo J, Oliveira R. 2000. The role of exopolymers produced by *Sphingomonas paucimobilis* in biofilm formation and composition. Biofouling. 16:17–27.

Banin E, Vasil ML, Greenberg PE. 2005. Iron and *Pseudomonas aeruginosa* biofilm formation. Proc Natl Acad Sci USA. 102:11076–11081.

Banks MK, Bryers JD. 1991. Bacterial species dominance within a binary culture biofilm. Appl Environ Microbiol. 57:1974–1979.

Blanco MA, Negro C, Gaspar I, Tijero J. 1996. Slime problems in the paper and board industry. Appl Microbiol Biotechnol. 46:203–208.

Busscher HJ, Weerkamp AH, van der Mei HC, Pelt AWJ, de Jong HP, Arends J. 1984. Measurements of the surface free energy of bacterial cell surfaces and its relevance for adhesion. Appl Environ Microbiol. 48:980–983.

Chen MJ, Zhang Z, Bott TR. 2005. Effects of operating conditions on the adhesive strength of *Pseudomonas fluorescens* biofilms in tubes. Colloids Surf B: Biointerfaces. 43:61–71.

Christensen BB, Haagensen JAJ, Heydorn A, Molin S. 2002. Metabolic commensalism and competition in a two-species microbial consortium. Appl Environ Microbiol. 68:2495–2502.

Cornelis P, Matthijs S. 2002. Diversity of siderophores-mediated iron uptake system in fluorescent pseudomonads: not only pyoverdines. Environ Microbiol. 4:787–798.

Davies DG, Parsek MR, Pearson JP, Iglewski BH, Costerton JW, Greenberg PE. 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science. 280:295–298.

Dogan B, Boor KJ. 2003. Genetic diversity and spoilage potential among *Pseudomonas* spp. isolated from fluid milk products and dairy processing plants. Appl Environ Microbiol. 69:130–138.

Doyle RJ. 2000. Contribution of the hydrophobic effect to microbial adhesion. Microbes Infect. 2:391–400.

Dufy BK, Défago G. 1999. Environmental factors modulating antibiotic and siderophores biosynthesis by *Pseudomonas fluorescens* biocontrol strains. Appl Environ Microbiol. 65:2429–2438.

Egland PG, Palmer RJ, Kolenbrander PE. 2004. Interspecies communication in *Streptococcus gordonii-Veillonella atypica* biofilms: signalling in flow conditions requires juxtaposition. Proc Natl Acad Sci USA. 101:16917–16922.

Elvers KT, Leeming K, Moore CP, Lappin-Scott HM. 1998. Bacterial-fungal biofilms in flowing water photo-processing tanks. J Appl Microbiol. 84:607–618.

Frølund B, Palmgren R, Keiding A, Nielsen PH. 1996. Extraction of extracellular polymers from activated sludge using a cation exchange resin. Water Res. 30:1749–1758.

Garret TR, Bhakoo M, Zhang Z. 2008. Characterisation of bacterial adhesion and removal in a flow chamber by micromanipulation. Biotechnol Lett. 30:427–433.

Gilbert P, Maira-Litran T, McBain AJ, Rickard AH, Whyte FW. 2002. The physiology and collective recalcitrance of microbial biofilm communities. Adv Microb Physiol. 46:202–256.

Gram L, Melchiorsen J, Spanggaard B, Huber I, Nielsen TF. 1999. Inhibition of *Vibrio anguillarum* by *Pseudomonas fluorescens* AH2, a possible probiotic treatment of fish. Appl Environ Microbiol. 65:969–973.

Janczuk B, Chibowski E, Bruque JM, Kerkeb ML, Gonzalez-Caballero FJ. 1993. On the consistency of surface free energy components as calculated from contact angle of different liquids: an application to the cholesterol surface. J Colloid Interface Sci. 159:421–428.

Hall-Stoodley L, Costerton JW, Stoodley P. 2004. Bacterial biofilms: from the natural environment to infectious diseases. Nature Rev Microbiol. 2:95–108.

Hansen SK, Rainey PB, Haagensen JAJ, Molin S. 2007. Evolution of species interactions in a biofilm community. Nature. 445:533–536.

Hughes KA, Sutherland IW, Jones MV. 1998. Biofilm susceptibility to bacteriophage attack: the role of phage-borne polysaccharide depolymerase. Microbiology. 144:3039–3047.

Kolari M, Nuutinen J, Salkinoja-Salonen MS. 2001. Mechanisms of biofilm formation in paper machine by *Bacillus* species: the role of *Deinococcus geothermalis*. J Ind Microbiol Biotechnol. 27:343–351.

Körstgens V, Flemming H-C, Wingender J, Borchard W. 2001. Uniaxial compression measurement device for investigation of the mechanical stability of biofilms. J Microbiol Meth. 46:9–17.

Komlos J, Cunningham AB, Camper AK, Sharp RR. 2005. Interaction of *Klebsiella oxytoca* and *Burkholderia cepacia* in dual-species batch cultures and biofilms as a function of growth rate and substrate concentration. Microb Ecol. 49:114–125.

Kreske AC, Ryu JH, Pettigrew CA, Beuchat LR. 2006. Lethality of chlorine, chlorine dioxide, and a commercial produce sanitizer to *B. cereus* and *Pseudomonas* in a liquid detergent, on stainless steel, and in biofilm. J Food Prot. 69:2621–2634.

Leriche V, Briandet R, Carpentier B. 2003. Ecology of mixed biofilms subjected daily to a chlorinated alkaline solution: spatial distribution of bacterial species suggests a protective effect of one species to another. Environ Microbiol. 5:64–71.

Leroy C, Delbarre C, Ghillebaert F, Compere C, Combes D. 2008. Effects of commercial enzymes on the adhesion of a marine biofilm-forming bacterium. Biofouling. 24:11–22.

Lindsay D, Brözel VS, Mostert JF, von Holy A. 2002. Differential efficacy of a chlorine dioxide-containing sanitizer against single and binary biofilms of a dairy-associated *Bacillus cereus* and a *Pseudomonas fluorescens* isolate. J Appl Microbiol. 92:352–361.

Melo LF, Vieira MJ. 1999. Physical stability and biological activity of biofilms under turbulent flow and low substrate concentration. Bioprocess Eng. 20:363–368.

Meyer B. 2003. Approaches to prevention, removal and killing of biofilms. Int Biodeterior Biodegr. 51:249–253.

Mireles JR, Toguchi A, Harshey RM. 2001. *Salmonella enterica* serovar Typhimurium swarming mutants with altered biofilm-forming abilities: surfactin inhibits biofilm formation. J Bacteriol. 183:5848–5854.

Møller S, Steinberg C, Andersen JB, Christensen BB, Ramos JL, Givskov M, Molin S. 1998. *In situ* gene expression in mixed-culture biofilms: evidence of metabolic interactions between community members. Appl Environ Microbiol. 64:721–732.

Olsen SM, Pedersen LT, Laursen MH, Kiil S, Dam-Johansen K. 2007. Enzyme-based antifouling coatings: a review. Biofouling. 23:369–383.

Palmer J, Flint S, Brooks J. 2007. Bacterial cell attachment, the beginning of a biofilm. J Ind Microbiol Biotechnol. 34:577–588.

Pereira MO, Kuehn M, Wuertz S, Neu T, Melo L. 2002. Effect of flow regime on the architecture of a *Pseudomonas fluorescens* biofilm. Biotechnol Bioeng. 78:164–171.

Rasmussen TB, Skindersoe ME, Bjarnsholt T, Phipps RK, Christensen KB, Jensen PO, Anderson JB, Koch B, Larsen TO, Hentzer M, et al. 2005. Identity and effects of quorum-sensing inhibitors produced by *Penicilium* species. Microbiology. 151:1325–1340.

Ryu J-H, Beuchat LR. 2005. Biofilm formation and sporulation by *Bacillus cereus* on a stainless steel surface and subsequent resistance of vegetative cells and spores to chlorine, chlorine dioxide, and a peroxyacetic acid-based sanitizer. J Food Prot. 68:2614–2622.

Schwyn B, Neilands JB. 1987. Universal chemical assay for the detection and determination of siderophores. Anal Biochem. 160:47–56.

Shakeri S, Kermanshahi RK, Moghaddam MM, Emtiazi G. 2007. Assessment of biofilm cell removal and killing and biocide efficacy using the microtiter plate test. Biofouling. 23:79–86.

Sharma M, Anand SK. 2002a. Characterization of constitutive microflora of biofilms in dairy processing lines. Food Control. 19:627–636.

Sharma M, Anand SK. 2002b. Biofilms evaluation as an essential component of HACCP for food/dairy processing industry – a case. Food Control. 13:469–477.

Sillankorva S, Oliveira DR, Vieira MJ, Sutherland IW, Azeredo J. 2004. Bacteriophage ΦS1 infection of *Pseudomonas fluorescens* planktonic cells *versus* biofilms. Biofouling. 20:133–138.

Simões M, Pereira MO, Vieira MJ. 2005a. Effect of mechanical stress on biofilms challenged by different chemicals. Water Res. 39:5142–5152.

Simões M, Pereira MO, Vieira MJ. 2005b. Validation of respirometry as a short-term method to assess the efficacy of biocides. Biofouling. 217–223.

Simões LC, Simões M, Vieira MJ. 2007a. Biofilm interactions between distinct bacterial genera isolated from drinking water. Appl Environ Microbiol. 73:6192–6200.

Simões M, Pereira MO, Vieira MJ. 2007b. Influence of biofilm composition on the resistance to detachment. Water Sci Technol. 55:473–480.

Simões LC, Simões M, Oliveira R, Vieira MJ. 2007d. Potential of the adhesion of bacteria isolated from drinking water to materials. J Basic Microbiol. 2:174–183.

Simões M, Simões LC, Pereira MO, Vieira MJ. 2008b. Sodium dodecyl sulfate allows the persistence and recovery of biofilms of *Pseudomonas fluorescens* formed under different hydrodynamic conditions. Biofouling. 24:35–44.

Simões M, Pereira MO, Sillankorva S, Azeredo J, Vieira MJ. 2007c. The effect of hydrodynamic conditions on the phenotype of *Pseudomonas fluorescens* biofilms. Biofouling. 23:249–258.

Simões M, Simões LC, Cleto S, Pereira MO, Vieira MJ. 2008a. The effects of a biocide and a surfactant on the detachment of *Pseudomonas fluorescens* from glass surfaces. Int J Food Microbiol. 121:335–341.

Singh PK, Parsek MR, Greenberg PE, Welsh MJ. 2002. A component of innate immunity prevents bacterial biofilm development. Nature. 417:552–555.

Stoodley P, Lewandowski Z, Boyle JD, Lappin-Scott HM. 1999. Structural deformation of bacterial biofilms caused by short-term fluctuations in fluid shear: an in situ investigation of biofilm rheology. Biotechnol Bioeng. 65:83–92.

Stoodley P, Sauer K, Davies DG, Costerton JW. 2002. Biofilms as complex differentiated communities. Annu Rev Microbiol. 56:187–209.

Stoodley P, Wilson S, Hall-Stoodley L, Boyle JD, Lappin-Scott, Costerton JW. 2001. Growth and detachment of cell clusters from mature mixed-species biofilms. Appl Environ Microbiol. 67:5608–5613.

Tait K, Skillman LC, Sutherland IW. 2002. The efficacy of bacteriophage as a method of biofilm eradication. Biofouling. 18:305–311.

van Oss CJ, Chaudhury MK, Good RJ. 1987. Monopolar surfaces. Adv Colloid Interfaces Sci. 28:35–64.

van Oss CJ, Good RJ, Chaudhury MK. 1988. Additive and nonadditive surface tension components and the interpretation of contact angles. Langmuir. 4:884–891.

van Oss CJ, Ju L, Chaudhury MK, Good RJ. 1989. Estimation of the polar parameters of the surface tension of liquids by contact angle measurements on gels. J Colloid Interfaces Sci. 128:313–319.

Wijman JG, de Leeuw PP, Moezelaar R, Zwietering MH, Abee T. 2007. Air–liquid interface biofilms of *Bacillus cereus*: formation, sporulation, and dispersion. Appl Environ Microbiol. 73:1481–1488.