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# Extracellular products as mediators of the formation and detachment of *Pseudomonas fluorescens* biofilms

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**Abstract**

*Pseudomonas fluorescens* B52 produces substantial biofilms at the air/liquid/solid interface of glass coverslips clamped vertically and partly submerged in liquid medium at 21°C. Biofilm formation was maximal ca. 20–50 h after inoculation of the liquid medium and, as indicated by environmental scanning electron microscopy (ESEM), contained large numbers of bacterial cells that were embedded within an extensive exopolymeric matrix. Incubation beyond 50 h led to reductions in biofilm which ESEM related primarily to losses of exopolymer. Both biofilm formation and the subsequent decline in exopolymer deposition was more rapid, and occurred to greater extents, when supernatants from two-day old cultures of B52 were used as the initial growth media. The addition of *N*-acyl-hexanoyl homoserine lactone to fresh growth medium had a similar effect upon biofilm formation as using spent culture medium. Homoserine lactones could not be demonstrated in spent culture supernatants by an *Agrobacterium tumefaciens* bioassay. An exopolysaccharide lyase was detected in spent culture media taken from dense biofilm cultures whose action was specifically directed towards biofilm exopolysaccharide. Results suggest that (i) cell-cell signals such as homoserine lactones are associated with the formation of *P. fluorescens* biofilms, (ii) the enzymic degradation of exopolymers has a specific role in the detachment of cells under starvation conditions, and (iii) whilst short chain ($C_6$) exogenous homoserines can trigger such responses in *P. fluorescens*, its own signal substance is likely to possess a longer ($>C_8$) fatty acyl chain. 

*Keywords:* Biofilm; Surface attachment; Surface detachment; Homoserine lactone

## 1. Introduction

In the vast majority of ecosystems, be they associated with industrial plants, the natural environment or animal and plant infections, microbial cells grow in association with surfaces. Surface-associated growth leads to the formation of biofilms, which are consortia of cells enveloped within exopolymer (EPS) matrices [1]. Attachment to surfaces is thought to initiate cascades of physiological change in the cells which lead, in part, to the overproduction of exopolymers [2,3]. These exopolymers not only immobilise the cells on the colonised surface, but also facilitate

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the spatial arrangement of different species within the biofilm. Such arrangements give the biofilm community metabolic and physiological capabilities which are not possible for the individual, unattached cells [4]. Notable amongst the unique properties of biofilms is their high level of resistance to a panoply of antimicrobial agents [5].

In order to colonise new surfaces and to avoid population density mediated starvation of attached bacteria, cells must be able to detach, and disperse, from mature biofilms and recolonise virgin surfaces. Such processess involve active detachment through enzymic cleavage of matrix polymers [6], or changes in the physiology of the attached cells [7]. There are suggestions that such mechanisms of detachment may be associated with the division cycle of individual cells of both Gram-negative [8] and Gram-positive [9] bacteria.

The ability of cells to transfer from biofilms to new surfaces is not only crucial for the long term survival of the organisms, but also has profound implications upon factory hygiene, water distribution and microbial disease and aetiology. Bacteria released from biofilms can lead to further colonisation of the manufacturing plant and contaminate contact surfaces and production lines, thereby affecting the quality and safety of the end product with potentially catastrophic consequences. In this respect, the role of *Pseudomonas* biofilms in industry has often been acknowledged [10]. Despite this awareness, the regulatory mechanisms that promote the attachment and detachment from surfaces and/or regulate the expression of the biofilm phenotype are as yet unknown. It has, however, been recently suggested that freely diffusible chemical signals in the form of *N*-acyl homoserine lactones (HSLs) play an important role in the development and maintenance of Gram-negative biofilms [11,12]. The data presented in this paper utilise a simple and quick method of assessing biofilm formation to demonstrate the influence of cellular exoproducts on the formation and dispersal of *Pseudomonas fluorescens* biofilms at wetted solid/air interfaces.

## 2. Materials and methods

### 2.1. Bacterial strains and growth conditions

*Pseudomonas fluorescens* B52 was used throughout, grown as batch cultures at 21°C with agitation (100 r.p.m.) in a minimal salts medium consisting of (g $l^{-1}$): $MgSO_4 \cdot 7H_2O$ (0.2), $K_2HPO_4$ (0.86), $NH_4Cl$ (0.65), *N*,*N*-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid (10.7), sodium pyruvate (11.0) and $CaCl_2$ (0.075). Where indicated, added HSL was 0.45 mg $l^{-1}$. Stock cultures were maintained as frozen glycerol (10% w/v) preparations at −70°C. Inocula consisted of washed end-of-log-phase cells to provide $10^7$ colony forming units $ml^{-1}$. *Agrobacterium tumefaciens* NT1 (pDC141E33), kindly provided by Dr S. Farrand, University of Illinois at Urbana-Champaign, IL, USA, contained a *lacZ* promoter for the presence of short chain ($\leq C_8$) HSLs. This strain was grown on nutrient agar, containing 50 μg $ml^{-1}$ kanamycin, at 28°C. Stock cultures of this organism were maintained as above.

### 2.2. Media and chemicals

Dehydrated culture media were obtained from Oxoid (Basingstoke, UK). Kanamycin, cetylpyridinium chloride, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and Coomassie Blue were obtained from Sigma (Poole, UK). All other reagents were of the purest available grade and were obtained from BDH (London, UK). *N*-Acyl-hexanoyl homoserine lactone was synthesised and kindly provided by Dr A. Eberhard, Ithaca College, New York, USA.

### 2.3. Attachment of microorganisms and development of biofilms

Attachment of bacterial cells was reproduced on sterile, nitric acid cleaned glass coverslips. These were clamped vertically in a radial arrangement in a carousel device and held across the air-liquid interface of cultures (55 ml) held in 500-ml beakers. Each carousel was constructed from teflon and could hold up to 16 glass coverslips. Coverslips were removed at regular intervals, fixed in cetylpyridinium chloride (10 mM, 2 min) and air dried for 24 h before staining

at room temperature with Coomassie Blue (0.1%, 2 min).

### 2.4. Imaging of colonised surface and quantitation of bacterial attachment

Digital images of the stained coverslips were obtained using a Bio-Rad GS-670 densitometer with a green filter in transmission mode at 64 μm resolution. The accumulation of biofilm was quantified using the Bio-Rad Molecular Analyst version 2.01 software by calculating total absorption readings for five replicate coverslips for each sample. The standard deviation for the mean absorption was ±0.003 of an optical density unit (ca. 5%). The accumulated biomass was calculated by reference to standard curves constructed for defined quantities of porcine mucin (50-μl aliquots) dried onto coverslips and stained. Linearity of response was demonstrated for the adsorbed material which could be related to accumulated biomass.

### 2.5. Environmental scanning electron microscopy (ESEM)

Samples were placed directly, without pre-treatment, onto the viewing stage of an environmental scanning electron microscope (Electroscan, Electroscan, MA, USA). Sample hydration was maintained whilst the micrographs were obtained. Sixteen consecutive images were taken across representative coverslips, through the air/liquid interface.

### 2.6. Generation of spent culture supernatant

Cells from three-day old cultures were removed by centrifugation (10 000 × *g*, 20 min) and the supernatant filter sterilised (0.22 μm).

### 2.7. Exopolymer extraction

Coverslips were removed from the carousel after 20 h growth and scraped to remove attached biomass. This was resuspended in saline (50 ml) and blended for 5 min before adding to 100 ml acetone (4°C). Following evaporation of the acetone (80°C), precipitated polymer was re-dissolved in distilled water and dialysed exhaustively against running tap water (96 h) and distilled water (24 h) before freeze drying. Exopolymers were extracted from the planktonic population by a similar process.

### 2.8. Polysaccharide lyase assay

Minimal salts medium was solidified with 1% agarose and supplemented with 0.1% (w/v) of the extracted, lyophilised exopolysaccharide. Wells were cut in the agarose and filled with 0.1 ml of cell-free overnight culture supernatants and incubated for 16 h. The plates were then flooded with 10% cetylpyridinium chloride (10 min), drained and counter stained with saturated congo red/10% Tween 80 in a 1:2 ratio. Lyase positive supernatants produced a clear zone against an opaque, orange background.

### 2.9. Homoserine lactone (HSL) assay

Soft agar containing X-gal at 40 μg $ml^{-1}$ and suspensions of the *A. tumefaciens* reporter strain were overlayed onto a base plate of minimal medium [13]. Once the overlay had solidified, 20-μl aliquots of cell-free culture supernatants were carefully pipetted onto the surface and the plates incubated at 28°C for 18 h. A diffuse blue zone spreading out from the test supernatant indicates a positive reaction and the presence of active *N*-acyl HSLs possessing a short fatty acid side chain ($\leq C_8$).

## 3. Results and discussion

Flask cultures of *P. fluorescens* were shown to produce substantial wall growth at the air/liquid interface which declined with progression into the stationary phase of growth. The progress of this was monitored by placing coverslips across the air/liquid interface and quantifying biomass spectrophotometrically.

Biomass accumulation on glass coverslips and their detachment was assessed for *P. fluorescens* as a function of time (Fig. 1). Substantial accretion was observed, maximal 20–50 h after inoculation which, with prolonged incubation, declined rapidly in extent. The detachment phenomenon was related to losses of exopolymer from the biofilm since environmental scanning electron microscope images indi-

cated thick biofilms at 20 h containing large numbers of bacterial cells enveloped within extensive exopolymer matrices (Fig. 2a). After 60 h growth, however, the biofilm had substantially reduced leaving clear images of single cells, denuded of EPS, associated with the surface (Fig. 2b). The rapid loss of biofilm from the coverslip and loss of EPS suggested an active process of detachment was occurring, possibly mediated by enzymic degradation.

Whilst several candidate exopolysaccharide lyase enzymes and biosurfactants have been previously identified and associated with microbial biofilms [6], their mode of regulation is unclear. Expression of such 'release agents' has been suggested to be regulated through environmental triggers, possibly associated with growth rate and/or nutrient starvation and stringency [14]. The simple salts growth medium employed in this study contains nutrients in excess for the growth of *P. fluorescens* B52. Cultures enter the stationary phase due to oxygen limitation. As such, planktonic cells can be cultured several times in the media without there being a change in stationary phase optical density or growth rate. Spent media from strain B52 could therefore be collected and filter sterilised to determine what effects, if any, this might have on de novo biofilm formation and detachment.

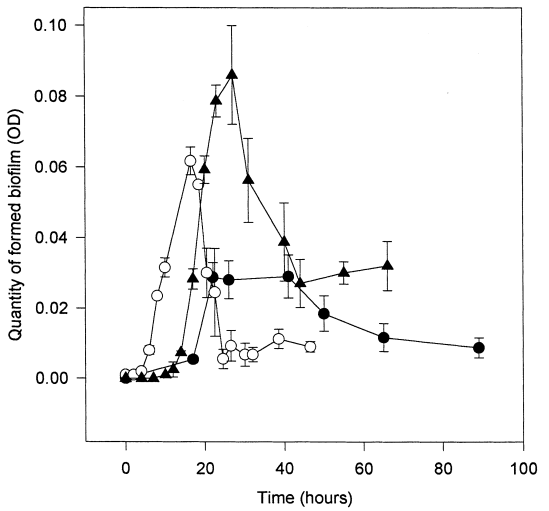


Fig. 1. Biofilm deposition by *P. fluorescens* B52 onto glass slides at the air/liquid interface. Cultures were grown in batch culture at 21°C either in fresh medium (●), filtered spent medium from B52 (○) or in fresh culture medium supplemented with *N*-acyl-hexanoyl homoserine lactone (▲).

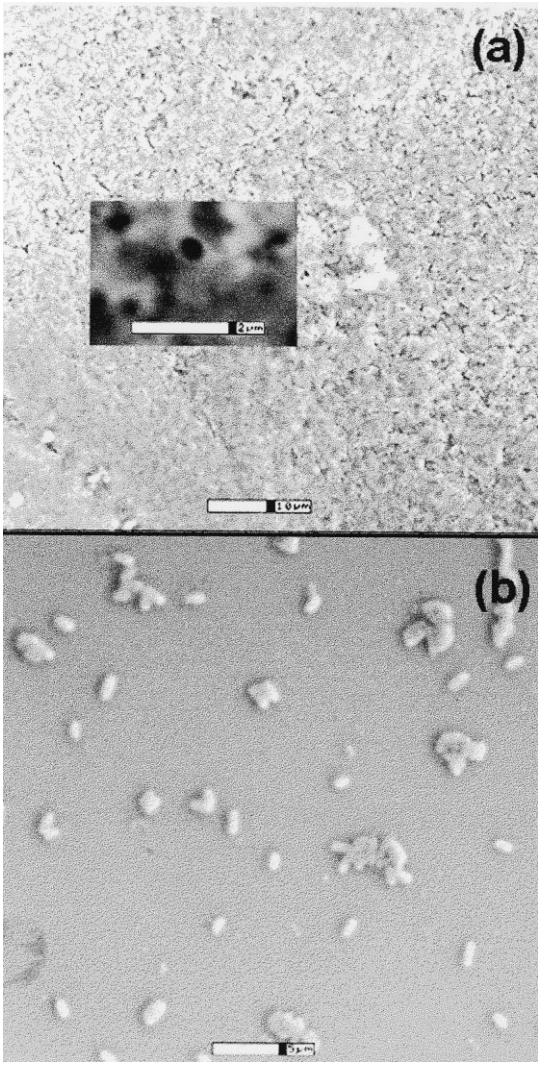


Fig. 2. Environmental scanning micrographs of *P. fluorescens* B52 biofilms formed on glass coverslips after (a) 20 h (bar = 10 μm) and (b) 60 h (bar = 5 μm) at 21°C. The insert to Fig. 2a represents the appearance of the covered surface at a higher magnification (bar = 2 μm).

Biomass accumulation was more rapid (10–20 h) and occurred to a greater extent when the cell-free supernatant from a two-day old culture of B52 was used as the growth medium (Fig. 1). Similarly, detachment of strain B52 occurred at a much faster rate (20–30 h) in spent medium compared to that in fresh media.

Of interest was the observation that biofilm formation in strain B52 was accelerated when cells were grown in spent culture medium. This could suggest the presence of a cell-density-dependent signal substance, present within stationary phase cultures, which promotes attachment to glass and biofilm formation. It is becoming increasingly recognised that many, if not all Gram-negative bacteria produce small diffusible signals, termed autoinducers, in the form of HSLs [15], which enable the bacterial population to monitor its own cell density and which can trigger a coordinated and unified response at the population level [16,17]. HSL production occurs constitutively at a low basal level but is upregulated when the cell density, and hence HSL level, reaches a critical threshold concentration. Because the HSL is believed to be freely diffusible across cell membranes [18], induction in one cell leads directly to induction in others. This creates a positive feedback loop that can generate a large and rapid response of the population to a small stimulus. For such sensing to occur it is assumed that the cell is capable of HSL uptake and possesses a positive transcriptional activator which responds to the signal stimulating transcription of target genetic regulons [12]. *P. fluorescens* strain B52 could not be demonstrated to produce HSLs (results not shown), as indicated by an *Agrobacterium tumefaciens* indicator strain [19]. Since several *Pseudomonas* spp., including *P. fluorescens* and related species, have previously been shown to produce HSLs, however [20], the likelihood is that an HSL is produced but was not recognised by the indicator system employed. Indeed, the *A. tumefaciens* indicator strain is only noted to be sensitive for HSLs possessing a fatty acid chain length of eight or less carbon residues. It is highly likely, therefore, that strain B52 produces a longer chain HSL.

Addition of *N*-hexanoyl-L-homoserine lactone (HHL) to fresh medium accelerated and increased biofilm formation in strain B52 in a similar manner to that following growth in spent media (Fig. 1). Biomass reduction, however, was unaffected by the addition of HHL. This would imply that attachment and biofilm formation in *P. fluorescens* are influenced by the presence of HSLs, even by one that it cannot make itself (HHL), and that the organism produces such signalling substances in a cell-density dependent manner.

Spent cell-free supernatant (20 h culture) from B52 planktonic (unattached) and biofilm cultures were assayed for polysaccharide lyase activity using exopolysaccharide derived from both cultures as the test substrate. Lyase activity was detected in both supernatants. Whilst lyase activity detected in the planktonic supernatants was, however, only active against planktonic-derived EPS, lyase activity in biofilm supernatants was active against both biofilm and planktonic EPS (Table 1). Not only does this imply the production of at least two polysaccharide lyase enzymes by biofilm cultures, but it would also suggest that the biofilm EPS is of a different composition to the planktonic material. Demonstration of such biofilm-specific activity would support the earlier view that the rapid detachment of cells from the glass substratum is brought about by the action of a specific EPS lyase. Moreover, since lyase activity was not detected in earlier (10 h) culture supernatants, this would indicate induction to occur under starvation conditions.

Although suspended in a nutrient replete macroenvironment, starvation conditions are likely to occur in a dense, mature biofilm where nutrient and oxygen gradients will form from the nutrient-rich biofilm periphery to the nutrient-deprived biofilm core. As biofilms mature and EPS deposition increases, then the magnitude of nutrient and gaseous gradients will increase and the net growth rate of the community will become reduced, possibly with the onset of dormancy and the triggering of stringent response genes. Thus, in order to obtain nutrients for continued growth and division, cells in the depth of the biofilm must possess some mechanism by

Table 1
Exopolysaccharide lyase activity in *P. fluorescens* B52 biofilm and planktonic culture

| Supernatant | Exopolymer source | |
|---|---|---|
| | Biofilm | Planktonic |
| 10 h planktonic | − | − |
| 10 h biofilm | − | − |
| 20 h planktonic | − | + |
| 20 h biofilm | + | + |

Cell free supernatants from biofilm and planktonic culture at different stages of biofilm deposition and dispersal were assayed for the presence of lyase activity against exopolysaccharides extracted from both modes of growth.

which they can be released. In this respect, changes in environmental parameters, such as oxygen availability, associated with growth at the biofilm/substratum interface may stimulate the production and liberation of lyase enzymes [7].

Thus, the results presented in this paper suggest quorum sensing systems to be involved in promoting cell attachment and biofilm formation in *P. fluorescens,* but that these do not involve short chain HSLs. By contrast, removal of EPS, which plays a part in cell detachment and decline of the biofilm under starvation conditions, does not appear to be under the control of HSLs. Rather, enzymic degradation of EPS is thought to play a part in cell detachment and dispersal.

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