Extracellular enzymes affect biofilm formation of mucoid *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa secretes a variety of hydrolases, many of which contribute to virulence or are thought to play a role in the nutrition of the bacterium. As most studies concerning extracellular enzymes have been performed on planktonic cultures of non-mucoid *P. aeruginosa* strains, knowledge of the potential role of these enzymes in biofilm formation in mucoid (alginate-producing) *P. aeruginosa* remains limited. Here we show that mucoid *P. aeruginosa* produces extracellular hydrolases during biofilm growth. Overexpression of the extracellular lipases LipA and LipC, the esterase EstA and the proteolytic elastase LasB from plasmids revealed that some of these hydrolases affected the composition and physicochemical properties of the extracellular polymeric substances (EPS). While no influence of LipA was observed, the overexpression of *estA* and *lasB* led to increased concentrations of extracellular rhamnolipids with enhanced levels of mono-rhamnolipids, elevated amounts of total carbohydrates and decreased alginate concentrations, resulting in increased EPS hydrophobicity and viscosity. Moreover, we observed an influence of the enzymes on cellular motility. Overexpression of *estA* resulted in a loss of twitching motility, although it enhanced the ability to swim and swarm. The *lasB*-overexpression strain showed an overall enhanced motility compared with the parent strain. Moreover, the EstA- and LasB-overproduction strains completely lost the ability to form 3D biofilms, whereas the overproduction of LipC increased cell aggregation and the heterogeneity of the biofilms formed. Overall, these findings indicate that directly or indirectly, the secreted enzymes EstA, LasB and LipC can influence the formation and architecture of mucoid *P. aeruginosa* biofilms as a result of changes in EPS composition and properties, as well as the motility of the cells.

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INTRODUCTION

Pseudomonas aeruginosa is a common environmental bacterium and represents an increasingly prevalent opportunistic human pathogen whose ecological success is based on a remarkable degree of genomic flexibility and phenotypic adaptation (Wehmhöner *et al.*, 2003). *P. aeruginosa* successfully colonizes a wide range of habitats, including natural soil and aquatic environments as well as artificial water systems (Botzenhart & Döring, 1993). It is

involved in acute and chronic diseases, for example in infections of surgery and burn patients, in urogenital tract and wound infections, chronic lung infections of cystic fibrosis patients, and nosocomial pneumonia in intubated and mechanically ventilated patients (Drenkard, 2003; Singh *et al.*, 2000).

The biofilm mode of life significantly contributes to the growth and persistence of *P. aeruginosa* under varying environmental conditions in nature, as well as in technical systems and the clinical setting. *P. aeruginosa* is now regarded as one of the medically most relevant biofilm-forming bacterial species. Therefore, it has become one of the best-studied model organisms for biofilm formation (McDougald *et al.*, 2008). Biofilm formation progresses through multiple developmental stages, beginning with reversible and then irreversible attachment of cells to a surface, followed by the formation of microcolonies, the maturation and differentiation of the biofilm with the expression of matrix polymers, and finally dispersal of cells

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Abbreviations: CLSM, confocal laser scanning microscopy; eDNA, extracellular DNA; EPS, extracellular polymeric substances.

A supplementary figure, showing gel electrophoretic analysis of PCR fragments amplified from *P. aeruginosa* SG81 genomic DNA, and a supplementary table, showing enzyme activities in EPS-containing cell-free supernatants from cultures of *P. aeruginosa* SG81 and its derivatives, are available with the online version of this paper.

from the biofilm (Stoodley *et al.*, 2002; Sauer *et al.*, 2004; McDougald *et al.*, 2008). It has been shown that flagella- and/or type IV pili-mediated motility is important in distinct steps during attachment and microcolony formation (O'Toole & Kolter, 1998; Sauer *et al.*, 2002; Klausen *et al.*, 2003a, b). Furthermore, extracellular polymeric substances (EPS), including proteins, polysaccharides such as alginate, Pel and Psl exopolysaccharides, and extracellular DNA (eDNA), can be involved in attachment processes during the initial stages of biofilm formation and/or are produced later during the formation of microcolonies and thereby influence the maturation of biofilms (Davey & O'Toole, 2000; Tielen *et al.*, 2005; Ryder *et al.*, 2007). Rhamnolipids produced and secreted by *P. aeruginosa* are composed of mono- or di-rhamnose linked to 3-hydroxy fatty acids of varying chain length. The most abundant rhamnolipid species of *P. aeruginosa* are L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate (mono-rhamnolipid) and L-rhamnosyl-L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate (di-rhamnolipid) (Rendell *et al.*, 1990; Maier & Soberón-Chávez, 2000). The biosynthesis and biochemistry of rhamnolipids have been extensively studied; however, their exact function is still incompletely understood. They are suspected to play a role in the biofilm structure by maintaining fluid channels and the detachment of cells from mature biofilm communities (Davey *et al.*, 2003; Espinosa-Urgel, 2003; Boles *et al.*, 2005). Moreover, they seem to play a role in the alteration of cell surface hydrophobicity (Zhang & Miller, 1994; Al-Tahhan *et al.*, 2000). Furthermore, rhamnolipids are required for swarming motility by acting as biosurfactants (Köhler *et al.*, 2000; Deziel *et al.*, 2003). Recently, a correlation between expression of the extracellular esterase EstA of *P. aeruginosa* and rhamnolipid production was described, indicating an important role for EstA in the biosynthesis of rhamnolipids (Wilhelm *et al.*, 2007a).

In the case of mucoid variants of *P. aeruginosa*, the EPS contain a high proportion of the exopolysaccharide alginate, which is a high-molecular-mass unbranched copolymer consisting of the (1→4)-linked uronic acid residues of β -D-mannuronate and α -L-guluronate (Jain & Ohman, 2004). In addition to this polysaccharide there is a significant fraction of proteins in the EPS of mucoid *P. aeruginosa* (Wingender *et al.*, 2001). Relatively little is known about the identity of extracellular proteins in biofilms, but at least some of them can be expected to be extracellular enzymes, which in general are ubiquitous in biofilms (Wingender & Jaeger, 2002). There are some indications that extracellular enzymes can accumulate in the biofilm matrix due to their interaction with exopolysaccharides (Wingender & Jaeger, 2002). Thus, lipase has been shown to bind to alginate, resulting in an increase in enzyme stability and the accumulation of lipase in the EPS matrix of mucoid *P. aeruginosa* (Wingender, 1990). Several functions have been attributed to extracellular enzymes, such as the degradation of biopolymers for nutrient acquisition by the bacteria or acting as virulence factors

during infection. *P. aeruginosa* is able to secrete a wide variety of degradative enzymes, some of which are well-known virulence factors (van Delden, 2004). Secreted enzymes have been identified as proteases (elastases LasA and LasB, alkaline protease, protease IV), lipolytic enzymes (lipases LipA and LipC, esterase EstA, phospholipases C), alkaline phosphatase and chitinase. Moreover, β -lactamases and other periplasmic enzymes have been identified, and are secreted via membrane vesicles into the extracellular space (Ciofu *et al.*, 2000). Several of the extracellular enzymes are known to be virulence factors, and alone or synergistically with others cause cell death, severe tissue damage and necrosis in the human host (Galloway, 1991; König *et al.*, 1996; van Delden, 2004). Proteolytic and lipolytic enzymes have been found in both environmental and clinical isolates of *P. aeruginosa* (Nicas & Iglewski, 1986), indicating a possible role for these enzymes in the survival of the bacteria under varying ecological conditions. Thus, the types of extracellular enzymes and especially the degree of expression differ between strains and seem to be strictly related to the colonized habitat (Hamood *et al.*, 1996; Rumbaugh *et al.*, 1999). Most studies concerning extracellular enzymes have been performed on planktonic cultures. A potential role for these enzymes in biofilm formation and their influence on the composition of the EPS have not been well studied. Since the biofilm mode of existence is an essential stage in the life cycle of *P. aeruginosa*, the objective of the present study was to examine the influence of selected lipolytic and proteolytic enzymes on the biofilm formation of mucoid *P. aeruginosa*, and the dependence of biofilm development on the expression of the enzymes.

METHODS

Bacterial strains, plasmids and growth conditions. Bacterial strains and plasmids are listed in Table 1. For conjugation experiments and determination of extracellular enzyme activities, bacterial strains were grown in Luria-Bertani (LB) broth and alginate-promoting medium (APM; Ohman & Chakrabarty, 1981) containing 100 mM Na-D-glucuronate, 100 mM KNO₃, 10 mM MgSO₄, 7.5 mM NaH₂PO₄ and 16.8 mM K₂HPO₄, at 180 r.p.m. and 36 °C. When required, antibiotics were added to the medium at the following concentrations: ampicillin, 100 µg ml⁻¹ (*Escherichia coli*); carbenicillin, 600 µg ml⁻¹ (*P. aeruginosa*); chloramphenicol, 50 µg ml⁻¹ (*E. coli*), 200 µg ml⁻¹ (*P. aeruginosa*); gentamicin 10 µg ml⁻¹ (*E. coli*), 30 µg ml⁻¹ (*P. aeruginosa*).

Construction of enzyme-overexpression strains. Strains were constructed via plasmid transfer by diparental mating using *E. coli* S17-1 as a donor strain (Simon *et al.*, 1983). For counter-selection of *E. coli* S17-1 donor cells from *P. aeruginosa* after conjugation, irgasan was used at a concentration of 25 µg ml⁻¹.

EPS isolation. For investigation of EPS composition and properties, *P. aeruginosa* strains were cultivated as agar-grown biofilms at 36 °C for 24 h on Pseudomonas Isolation Agar (PIA; Difco) in the form of confluent mucoid lawns, as described previously (Wingender *et al.*, 2001). The bacterial biomass was carefully scraped from the agar surface and suspended in 0.14 M NaCl. The bacteria were dispersed by vigorous stirring for 30 min at room temperature. The cells were

Table 1. Bacterial strains and plasmids used in this study

Tri^r, trimethoprim resistance; Str^r, streptomycin resistance; Amp^r, ampicillin resistance.

Strain or plasmid	Relevant genotype or phenotype	Reference or source
<i>E. coli</i> strain		
S17-1	<i>thi pro hsdR</i> [−] M ⁺ , chromosomally integrated [RP4-2 Tc ^r ::Mu::Km ^r ::Tn7, Tra ⁺ Tri ^r Str ^r]	Simon <i>et al.</i> (1983)
<i>P. aeruginosa</i> strains		
SG81	Mucoid biofilm isolate from technical water system	Grobe <i>et al.</i> (1995)
SG81MCS	Vector control pBBR1MCS	This study
SG81estA+	Overexpression of <i>estA</i>	This study
SG81lipC+	Overexpression of <i>lipC</i>	This study
SG81lipA+	Overexpression of <i>lipA</i>	This study
SG81MMB	Vector control pMMB67EH	This study
SG81lasB+	Overexpression of <i>lasB</i>	This study
Plasmids		
pBBR1MCS	<i>lacZα</i> Cm ^r mob P _{lac} P _{T7}	Kovach <i>et al.</i> (1994)
pBBX+	pBBR1MCS containing 3.3 kb <i>Xho</i> I fragment with <i>estA</i> under P _{lac} control	Wilhelm <i>et al.</i> (2007a)
pBBLCH	pBBLCAH Δ <i>lipA</i> (<i>Bam</i> HI/ <i>Ppu</i> MI deletion), <i>lipC/H</i> under P _{lac} control	Rosenau & Jaeger (2003)
pBBL7	2.8 kb <i>Xmn</i> I/ <i>Sma</i> I fragment with <i>lipA/H</i> operon in pBBR1MCS under P _{lac} control	Wilhelm <i>et al.</i> (2007b)
pMMB67EH	IncQ, <i>lacI</i> ^q , P _{tac} , <i>rrnB</i> , Amp ^r , mob	Fürste <i>et al.</i> (1986)
pSAK5	2.7 kb <i>Eco</i> RI/ <i>Hind</i> III fragment with <i>lasB</i> in pMMB67HE under P _{tac} control	Kamath <i>et al.</i> (1998)

separated from the EPS by high-speed centrifugation of the suspension at 40 000 g for 1 h. Residual bacteria were removed by filtration of the supernatant through cellulose acetate membranes (0.2 µm pore-size) to yield a cell-free EPS solution. Alginate was purified from agar-grown biofilms of *P. aeruginosa* SG81, as described previously (Wingender *et al.*, 2001).

Determination of enzyme activities. Extracellular enzyme activities were determined in supernatants from overnight LB cultures and 24 h APM cultures after removal of cells by centrifugation (15 min, 10 000 g) and in cell-free EPS solutions prepared from agar-grown biofilms. Lipase activity was measured with *para*-nitrophenyl palmitate as substrate (Winkler & Stuckmann, 1979). An increase in A₄₁₀ of 1.0 unit per 15 min corresponds to a lipase activity of 48.3 nmol min^{−1} (ml solution)^{−1}. Esterase activity was determined with fluorescein diacetate as substrate (Küpfer *et al.*, 1999). An increase in A₄₉₀ of 1.0 unit per 60 min corresponds to an esterase activity of 0.282 nmol min^{−1} (ml solution)^{−1}. Protease activity was determined using azocasein as substrate (Prestidge *et al.*, 1971). One enzyme unit of extracellular protease corresponds to an increase in A₄₃₀ of 1.0 unit per 60 min (Obernesser *et al.*, 1981). Alkaline phosphatase activity was determined with *para*-nitrophenyl phosphate as substrate (Neu & Heppel, 1965). An increase in A₄₁₀ of 1.0 unit per 30 min corresponds to a phosphatase activity of 55.9 nmol min^{−1} (ml solution)^{−1}.

Analysis of EPS. Total carbohydrate and uronic acid (alginate) contents of the EPS solutions were determined by the phenol/sulfuric acid method (Dubois *et al.*, 1956) and the hydroxydiphenyl assay (Blumenkrantz & Asboe-Hansen, 1973), respectively, using purified alginate from *P. aeruginosa* SG81 as standard. Protein was determined with a modified Lowry assay (Frølund *et al.*, 1996), using BSA (fraction V, Sigma) as standard. Rhamnolipids were quantified by a modified orcinol method (Chandrasekaran & BeMiller, 1980), using L-rhamnose (Sigma) as standard. For this analysis, 600 µl EPS solution was extracted three times with 600 µl diethyl ether. The combined diethyl ether extracts were vacuum-concentrated to dryness by evaporation of the organic solvent. The rhamnolipids were redissolved in 100 µl deionized water, and 100 µl 1.6% (w/v) orcinol and 800 µl 60% (v/v) H₂SO₄ were added. After incubation at 80 °C

for 30 min and subsequently at room temperature for 10 min, the A₄₂₁ of the solution was measured. Rhamnolipid concentrations were calculated based on the assumption that 1 µg rhamnose corresponds to 2.5 µg rhamnolipid (Ochsner *et al.*, 1994).

Analysis of rhamnolipids by TLC. The identity of rhamnolipids was determined by a modified TLC method (Syldatk *et al.*, 1985), using purified mono- and di-rhamnolipids from *P. aeruginosa* PAO1 as standards. The extracted and dried rhamnolipids prepared as described above were dissolved in 15 µl chloroform and applied to TLC plates (silica gel HPTLC plates, 20 cm × 20 cm, Merck). The plates were developed for approximately 40 min, using a solvent system of chloroform/methanol/acetic acid in a ratio of 65:15:2 by volume. For visualization, a derivatization reagent (75 g orcinol, 4.2 ml concentrated sulphuric acid, 21 ml deionized water) was used.

Viscosity measurement. Specific viscosities of EPS solutions were determined at 25 °C in a Micro-Ubbelohde capillary viscometer (capillary diameter 0.32 mm). For the calculation of reduced viscosity, the specific viscosity was divided by the concentration of uronic acids (alginate) in the EPS solution.

Hydrophobicity of the EPS. The hydrophobicity of EPS solutions was determined according to a method for measuring the hydrophobicity of cell surfaces (Rosenberg *et al.*, 1980). A 1.2 ml volume of EPS solution in phosphate/urea/Mg (PUM) buffer, pH 7.5 (22.2 g K₂HPO₄ · 3H₂O, 7.26 g KH₂PO₄, 1.8 g urea, 0.2 g MgSO₄ · 6H₂O, 1 l deionized water), was mixed with 200 µl n-hexadecane with vigorous stirring for 2 min. After phase separation at room temperature for 15 min, the lower aqueous phase was removed and its OD₅₈₀ measured.

Determination of cell motility. Motility agar plate assays for determination of bacterial swimming, swarming and twitching motility were performed as described elsewhere (O'Toole & Kolter, 1998; Rashid & Kornberg, 2000).

Cultivation of biofilms in flow chambers. Biofilms were cultivated in two-channel flow cells on a borosilicate glass substratum, as

described previously (Tielen *et al.*, 2005). All experiments were performed at 30 °C at a flow rate of 20 ml h⁻¹, using a modified alginate-promoting medium (mAPM) (Tielen *et al.*, 2005). For inoculation, bacteria from a 24 h culture on PIA were suspended in mAPM to a density of approximately 10⁷ cells ml⁻¹, and each channel of the flow cell was inoculated with 5 ml cell suspension. After stopping the medium flow for 1 h, it was resumed at a rate of 20 ml h⁻¹ (83.3 cm h⁻¹), corresponding to a flow with a Reynolds number of 1.0. After 24, 48 and 72 h, biofilm cells were stained with SYTO 9 (Molecular Probes) by injecting 5 ml SYTO 9 solution (1.5 µl SYTO 9 added to 9 ml mAPM), and the biofilms were viewed by confocal laser scanning microscopy (CLSM).

CLSM and image analysis. Examinations of flow-cell biofilms were performed using an LSM 510 confocal laser scanning microscope (Zeiss). Images were obtained with a Zeiss LD Achroplan ×40/0.60 numerical aperture (NA) objective. 3D image stacks of 72 h-old flow-cell biofilms stained with SYTO 9 were recorded at an excitation wavelength of 488 nm by use of an argon laser in combination with an emission long pass filter LP 505 nm. Digital image acquisition and analysis of the CLSM optical thin sections were performed with the Zeiss LSM software (version 3.2).

Statistical analyses. The significance of the data was analysed using a two-sample Welch's *t* test and one-way analysis of variance (ANOVA; Fagerland & Sandvik, 2009). *P* < 0.05 was considered to be a significant difference.

RESULTS

Enzyme overexpression in mucoid *P. aeruginosa*

The mucoid environmental strain *P. aeruginosa* SG81, originally isolated from the biofilm of a technical water system (Grobe *et al.*, 1995), was used to investigate the influence of extracellular enzymes on biofilm composition, structure and properties. The enzymes considered in the present study were the lipolytic enzymes LipA (PA2862), LipC (PA4813) and EstA (PA5112), and the proteolytic enzyme LasB (PA3724). These enzymes were originally discovered and characterized in typically non-mucoid wild-type strains of *P. aeruginosa* (Jaeger *et al.*, 1996; Martínez *et al.*, 1999; Wilhelm *et al.*, 1999; Kamath *et al.*, 1998). Since the genetic background of *P. aeruginosa* SG81 was unknown, the identification of the genes encoding the enzymes was attempted by PCR amplification of the target genes from genomic DNA isolated from *P. aeruginosa* SG81. Using suitable primers with nucleotide sequences derived from the non-mucoid reference strain *P. aeruginosa* PAO1, PCR products of 1.0, 1.0, 2.0 and 1.5 kb were generated, which corresponded to the expected fragment lengths of *lipA*, *lipC*, *estA* and *lasB*, respectively (shown for *lipA*, *lipC* and *estA* in Supplementary Fig. S1).

To study the influence of extracellular enzymes on the biofilm formation of mucoid strains of *P. aeruginosa*, the genes of the four extracellular hydrolases were moderately overexpressed from plasmids in the mucoid strain *P. aeruginosa* SG81. Using the broad-host-range vector pBBR1MCS as a basis, appropriate vectors were constructed to overexpress *lipA*, *lipC* and *estA* *in trans*

(Wilhelm *et al.*, 2007a, b; Rosenau & Jaeger, 2003). Thereby, *lipA* and *lipC* were overexpressed together with their specific chaperone gene *lipH* in an operonic structure to ensure the proper folding of the enzymes (Rosenau *et al.*, 2004). The gene activities were controlled by the constitutive *lacZ* promoter. A *lasB*-overexpression system was constructed in the background of the broad-host-range vector pMMB67EH, with *lasB* under the control of the constitutive *tac* promoter (Kamath *et al.*, 1998). All vectors were transferred into the mucoid strain *P. aeruginosa* SG81 by conjugation, using *E. coli* S17-1 as donor.

Overexpression of target enzymes was first checked by determination of the enzyme activities in cell-free supernatants of overnight LB cultures (Supplementary Table S1). All enzyme-overexpressing strains revealed enhanced activities of the target enzymes compared with the parent strain *P. aeruginosa* SG81. Lipase activity normalized to cell density was increased 391-fold (*P* = 2.3 × 10⁻⁷) and 1.9-fold (*P* = 2.5 × 10⁻⁹) in the LipA- and LipC-overexpressing strains *P. aeruginosa* SG81lipA+ and *P. aeruginosa* SG81lipC+, respectively; esterase activity was enhanced 6.8-fold (*P* = 2.8 × 10⁻⁹) in the *estA*-overexpressing strain *P. aeruginosa* SG81estA+, and protease activity was enhanced 9.4-fold (*P* = 9 × 10⁻¹³) in the *lasB*-overexpressing strain *P. aeruginosa* SG81lasB+ (Supplementary Table S1).

The growth characteristics of all overexpression strains were investigated in liquid mAPM, which was also used for biofilm cultivation experiments (see below). Growth curves were monitored at 36 °C and 180 r.p.m. over 48 h (Table 2). The parent strain and both control vector strains showed identical growth characteristics. The exponential growth phase started after 13 h and ended after approximately 24 h, resulting in a maximal cell density corresponding to an OD₅₈₀ of 1.4. The growth rate of these strains was 0.4 h⁻¹. *P. aeruginosa* SG81estA+ showed a similar growth behaviour, with a growth rate of 0.48 h⁻¹ and a maximal cell density (OD₅₈₀) of 1.4 reached after 20 h. Both lipase-overproduction strains showed decreased growth rates of 0.12 h⁻¹ for *P. aeruginosa* SG81lipC+ and 0.28 h⁻¹ for *P. aeruginosa* SG81lipA+. However, after incubation for 24 h, both strains had a maximal cell density of OD₅₈₀ 1.5, similar to that of the parent strain. The elastase-overproducing strain *P. aeruginosa* SG81lasB+ showed a higher growth rate of 0.59 h⁻¹ and a prolonged exponential phase, resulting in a higher cell density (OD₅₈₀ 2.1) than that of the parent strain. This indicated that a higher level of LasB influences the physiology of *P. aeruginosa* and supports growth, possibly through enhanced nutrition of the bacterium.

Initial investigations of alginate production as the major EPS component of the mucoid *P. aeruginosa* strains showed that the strains produced alginate at different concentrations when grown in APM (Fig. 1). The LipA- and LipC-overproduction strains showed the same alginate content as the parent strain, whereas *P. aeruginosa* SG81estA+ showed a 2.7-fold reduced and *P. aeruginosa*

Table 2. Growth of *P. aeruginosa* SG81 and its derivative strains in mAPM cultures (36 °C, 180 r.p.m.)

Strain	Growth rate, μ (h ⁻¹)	Time point of maximal cell density (h)	Beginning of stationary phase (h)	Maximal cell density (OD ₅₈₀)
SG81	0.424	24	20	1.45
SG81MCS	0.414	24	20	1.40
SG81estA +	0.478	20	20	1.42
SG81lipC +	0.115	24	24	1.51
SG81lipA +	0.277	36	20	1.48
SG81MMB	0.380	24	20	1.42
SG81lasB +	0.594	36	20	2.08

SG81lasB+ an eightfold reduced alginate concentration compared with *P. aeruginosa* SG81.

Extracellular enzyme activities in agar-grown biofilms

The expression of extracellular enzymes was investigated in biofilms that had been grown for 24 h at 36 °C in the form of a confluent mucoid biomass on the surface of PIA medium. Previously, it had been shown that the carbohydrate fraction of the EPS from these biofilms consists predominantly of the exopolysaccharide alginate, but also contains significant levels of protein (Wingender *et al.*, 2001). In order to determine the specific activities of the extracellular enzymes of the mucoid *P. aeruginosa* strain SG81 compared with those of the enzyme-overexpressing strains, cell-free EPS solutions were prepared from the biofilms and assayed for enzyme activity.

The biofilms of *P. aeruginosa* SG81 revealed significant levels of extracellular lipase, esterase, protease and phosphatase activities (Table 3). All strains containing the enzyme-expressing plasmids showed higher activities of the target enzymes compared with the parent strain. The activities of the lipolytic enzymes LipA, LipC and EstA were significantly enhanced 445-fold ($P=2.2 \times 10^{-16}$), 2.7-fold ($P=9.3 \times 10^{-11}$) and 4.4-fold ($P=5.1 \times 10^{-7}$) in the respective strains; the proteolytic enzyme LasB revealed

an eightfold ($P=2.2 \times 10^{-16}$) increase in activity (Table 3). For comparison, the phosphatase activity was not increased in any of the strains, and decreased in the *lasB*-over-expressing strain (Table 3). The vector controls revealed only slight differences from the parent strain in their extracellular enzyme activities. This indicated that the introduction of the vector plasmids had no substantial influence on the expression and/or secretion of the enzymes. Thus, increased activities were observed for all enzymes in the agar-grown *P. aeruginosa* biofilms. The degree of overexpression varied depending on the type of enzyme, but was comparable with that observed in planktonic cultures grown in LB medium, as described above.

Effects of extracellular enzymes on EPS composition and properties

The influence of enhanced enzyme activities on EPS composition and properties was investigated by comparing the content of proteins, total carbohydrates, uronic acids and rhamnolipids of EPS solutions prepared from agar-grown biofilms (Table 4). In addition, EPS solutions were characterized by the determination of their viscosity and hydrophobicity (Fig. 2).

Overexpression of *lipA* and *lipC* did not result in significant changes in EPS composition, with the exception

Table 3. Enzyme activities in EPS solutions prepared from agar-grown biofilms of the mucoid strain *P. aeruginosa* SG81 and its derivative strains

Strain	Lipase [nmol min ⁻¹ (mg protein) ⁻¹]*	Esterase [nmol min ⁻¹ (mg protein) ⁻¹]*	Protease [enzyme units (mg protein) ⁻¹]*	Phosphatase [nmol min ⁻¹ (mg protein) ⁻¹]*
SG81	0.51 ± 0.03	0.048 ± 0.002	11.4 ± 0.1	42.10 ± 1.07
SG81MCS	0.55 ± 0.07	0.049 ± 0.004	9.2 ± 0.1	37.87 ± 0.50
SG81estA +	0.71 ± 0.07	0.209 ± 0.005	8.5 ± 0.1	32.45 ± 0.03
SG81lipC +	1.35 ± 0.12	0.050 ± 0.003	9.7 ± 0.2	39.05 ± 0.89
SG81lipA +	226.7 ± 22.7	0.065 ± 0.005	9.8 ± 0.1	39.78 ± 1.31
SG81MMB	0.58 ± 0.01	0.060 ± 0.001	9.1 ± 0.1	38.64 ± 0.17
SG81lasB +	0.79 ± 0.04	0.098 ± 0.004	87.8 ± 0.5	11.69 ± 0.25

*The results are expressed as the mean ± SD of four independent experiments.

Fig. 1. Alginate concentrations of EPS solutions from *P. aeruginosa* strains grown in APM (24 h, 36 °C). The results are expressed as mean values of two independent experiments performed in duplicate. Error bars, SD.

of a slightly increased extracellular protein level ($P=9.61 \times 10^{-5}$) in LipA-overproducing biofilms (Table 4). LipA and LipC overproduction also did not influence the viscosity and hydrophobicity of EPS (Fig. 2). Compared with the parent strain, the EstA-overproducing strain showed a 1.4-fold ($P=7.9 \times 10^{-10}$) increase in carbohydrate and a 1.3-fold ($P=6.4 \times 10^{-4}$) increase in rhamnolipid concentration. Moreover, the viscosity and hydrophobicity of the EPS solutions prepared from the biofilms were significantly enhanced in this strain (Fig. 2). Overexpression of the elastase gene *lasB* showed the strongest influence on both EPS composition and properties. The concentration of total carbohydrates was 1.5-fold enhanced ($P=2.2 \times 10^{-16}$), while the uronic acid

(alginate) content was 1.6-fold reduced, so that the uronic acid fraction of the extracellular carbohydrates was significantly ($P=1.3 \times 10^{-8}$) lower in the *lasB*-overexpressing strain compared with the other strains (Table 4). Reduced alginate concentrations were also observed for *P. aeruginosa* SG81estA+ and SG81lasB+ grown in APM liquid cultures, as described above (Fig. 1). In addition, in biofilms, the concentrations of rhamnolipids were significantly ($P=2.4 \times 10^{-12}$) enhanced in *P. aeruginosa* SG81lasB+ and were the highest of all the strains in the present study (Table 4). Furthermore, the hydrophobicity and viscosity of the EPS solutions of this strain were significantly higher than those of the parent strain *P. aeruginosa* SG81 (Fig. 2).

Analysis of the extracellular rhamnolipids by TLC revealed that *estA*- and *lasB*-overexpression strains showed an altered composition of extracellular rhamnolipids. Both strains produced both mono- and di-rhamnolipids, while the parent strain and the other enzyme-overexpression strains produced di-rhamnolipid alone (Fig. 3). In addition, a higher amount of di-rhamnolipid in the *estA*-overexpressing strain was detected by TLC compared with the other strains, confirming the higher level of rhamnolipids determined by the photometric orcinol assay (Table 4). The results indicate that elastase and esterase influence the EPS composition, which leads to changed physicochemical properties of the EPS.

Influence of enzyme overexpression on cellular motility

Since flagellum- and/or type IV pili-mediated swimming, swarming and twitching motility can affect biofilm formation, the cellular motility of the enzyme-overexpressing strains was investigated in comparison with the parent strain, using agar plate assays (Fig. 4). *lipA* overexpression had no influence on all three types of motility, while

Table 4. Biochemical analysis of EPS isolated from agar-grown biofilms of the mucoid parent strain *P. aeruginosa* SG81 and its derivative strains

Strain	Analyte			
	Protein* [mg (10 ⁹ cells) ⁻¹]	Carbohydrate* [mg (10 ⁹ cells) ⁻¹]	Uronic acids* [mg (10 ⁹ cells) ⁻¹]	Rhamnolipids† [μg (10 ⁹ cells) ⁻¹]
SG81	0.16 ± 0.05	0.30 ± 0.1	0.35 ± 0.09	14.94 ± 2.4
SG81MCS	0.17 ± 0.02	0.30 ± 0.06	0.37 ± 0.06	15.33 ± 0.04
SG81estA +	0.18 ± 0.03	0.43 ± 0.1	0.36 ± 0.08	18.90 ± 2.0
SG81lipC +	0.13 ± 0.01	0.32 ± 0.1	0.35 ± 0.08	13.77 ± 1.0
SG81lipA +	0.21 ± 0.05	0.34 ± 0.1	0.39 ± 0.07	16.12 ± 1.5
SG81MMB	0.18 ± 0.01	0.28 ± 0.02	0.30 ± 0.03	11.58 ± 0.07
SG81lasB +	0.21 ± 0.09	0.44 ± 0.1	0.22 ± 0.04	28.49 ± 4.3

*The results are expressed as the mean ± SD of four independent experiments in triplicate.

†The results are expressed as the mean ± SD of two independent experiments in triplicate.

Fig. 2. Hydrophobicity and viscosity of EPS solutions from *P. aeruginosa* strains. The hydrophobicity was measured as the OD₅₈₀ of EPS solutions after treatment with n-hexadecane (light-grey bars; results expressed as mean values of three independent experiments). The reduced viscosities of EPS solutions were determined using a capillary viscometer (dark-grey bars; results expressed as mean values of four independent experiments). The significance of the data was calculated by *t* test analyses: * $P=2.8 \times 10^{-10}$, ** $P=5.25 \times 10^{-8}$, † $P=8.9 \times 10^{-11}$, †† $P=1.7 \times 10^{-6}$. Error bars, SD.

overexpression of *lipC* abolished twitching motility, although swimming and swarming remained unaffected. The overexpression of *estA* resulted in a loss of twitching motility, whereas swimming and swarming motility was stronger than that of the parent strain *P. aeruginosa* SG81. The overexpression of *lasB* led to more pronounced swimming, swarming and twitching motility (Fig. 4).

Fig. 3. TLC of rhamnolipid diethyl ether extracts of the EPS of the enzyme-overexpression strains and the parent strain *P. aeruginosa* SG81. Rhamnolipids were extracted from 40 µg biofilm mass and stained after TLC with an orcinol reagent.

Fig. 4. Swimming (a), swarming (b) and twitching (c) motility of mucoid strain *P. aeruginosa* SG81 and its enzyme-overexpressing derivatives. The motility of the *P. aeruginosa* strains was analysed on agar plates containing different agar concentrations to investigate swimming (0.3% agar, incubation at 36 °C for 24 h), swarming (0.5% agar, incubation at 36 °C for 48 h) and twitching motility (3 mm thick LB agar, incubation at 36 °C for 24 h), and subsequently at room temperature for 24 h. Shown are representative images from three independent experiments. (a, b) Top view of agar plates showing hazy zones around the inoculation point in the middle of the plates; (c) photomicrographs of colony rims at $\times 10$ magnification.

Influence of enzyme overexpression on biofilm architecture

Biofilms were grown in flow cells for up to 72 h and analysed by CLSM (Figs 5 and 6). The mucoid parent strain formed a heterogeneous biofilm characterized by the presence of large microcolonies of different height and diameter separated by regions with lower surface coverage. The same type of biofilms were produced by the vector control strains. Among the enzyme-overexpressing strains, only the LipA-overproducing strain *P. aeruginosa* SG81lipA+ revealed no significant differences from the parent strain, while overexpression of the other enzymes influenced the biofilm architecture. In contrast to the parent strain, cells overproducing LipC were more heterogeneously distributed within the biofilm, which was predominantly composed of thicker microcolonies of varying diameter consisting of densely packed cells with large void spaces between the microcolonies. Overproduction of EstA resulted in premature biofilm dissolution. After 48 h of cultivation, biofilms resembled those of the LipC-overproducing strain (Fig. 5), but after 72 h the surface was only colonized by single cells evenly distributed over the glass surface (Fig. 6). Overproduction of LasB had the most pronounced effect on biofilms. Over the observation period of 72 h, the surface was only

Fig. 5. CLSM images of 48 h-old biofilms of mucoid *P. aeruginosa* at $\times 200$ magnification. The biofilms were grown in a continuous-culture flow-cell system on glass surfaces at 30 °C. Biofilm cells were stained with SYTO 9. Shown are optical sections in the middle of the biofilms. Bars, 50 μ m.

sparsely and unevenly colonized in the form of single cells and bacterial accumulations of only a few cells (Fig. 6).

DISCUSSION

In this study, the effect of extracellular enzyme over-expression in biofilms of a mucoid strain of *P. aeruginosa* was studied, using two different biofilm model systems: (i) bacteria grown on agar surfaces, representing static unsaturated biofilms; and (ii) bacteria growing on glass

surfaces under continuous flow conditions, representing submerged biofilms. Agar-grown biofilms were used to study the influence of enzyme activities on the composition and properties of EPS, because this type of biofilm provides sufficient amounts of biomass for EPS analysis (Wingender *et al.*, 2001). This growth model has been used for the investigation of *P. aeruginosa* by several groups to address different scientific questions (e.g. Steinberger *et al.*, 2002; Borriello *et al.*, 2006; Kim *et al.*, 2009). It represents an unsaturated biofilm growing at the solid–air interface, which can also be found for example in soil environments,

Fig. 6. CLSM images of 72 h-old biofilms of mucoid *P. aeruginosa* at $\times 200$ magnification. The biofilms were grown in a continuous-culture flow-cell system on glass surfaces at 30 °C. Biofilm cells were stained with SYTO 9. Shown are top and side views of the biofilm covering an area of 240 μ m $\times$ 240 μ m. Bars, 50 μ m.

on plants, or on surfaces of mucous membranes or teeth (Auerbach *et al.*, 2000; Steinberger *et al.*, 2002; Chang *et al.*, 2007). Flow-chamber experiments were performed to investigate the effects of enzyme activities on biofilm development and architecture on glass surfaces in transparent flow cells, since they allowed the non-destructive microscopic analysis of biofilms by CLSM. This type of experimental setup has been used in various studies to analyse the architecture of *P. aeruginosa* biofilms (e.g. Hentzer *et al.*, 2001; Davey *et al.*, 2003; Sauer *et al.*, 2002). One of the main differences between these two biofilm models is nutrient availability. While the agar-biofilm model represents a static culture in which nutrients are depleted during growth (Steinberger *et al.*, 2002), the flow chamber is a continuous system. Nutrients are constantly supplied and metabolic end-products can be removed. The combination of both biofilm growth models is a strategy often used to obtain complementary information about different structural and functional aspects of biofilms (e.g. Mah *et al.*, 2003; Werner *et al.*, 2004; Waite *et al.*, 2005). The same relationships in enzyme activities between the mutants and the parent strain were found even in APM liquid cultures and PIA-grown biofilms. Also, the alginate concentrations were comparable in both systems, indicating that the observed phenotypic properties of our mutants are stable, independently of the growth conditions used in the present study. However, for other factors it is well known that there are significant differences in gene expression and therefore in the phenotypic properties of *P. aeruginosa* growing planktonically compared with the sessile biofilm mode of growth (Sauer *et al.*, 2002; Waite *et al.*, 2005).

Biochemical analysis of EPS from agar-grown biofilms confirmed that the mucoid environmental strain *P. aeruginosa* SG81 produced high amounts of uronic acids, which have previously been identified as alginate (Grobe *et al.*, 1995; Wingender *et al.*, 2001). In addition, the EPS contained significant amounts of proteins. The identification of lipase, esterase and protease activities in the EPS of the biofilms confirmed that at least some of the proteins were extracellular enzymes. These observations indicate that mucoid *P. aeruginosa* grown in unsaturated biofilms expresses and secretes lipolytic and proteolytic enzymes, which then accumulate in the EPS matrix of these biofilms.

EPS such as polysaccharides, eDNA and structural proteins determine the integrity of biofilms. They are involved in the adhesion of biofilms to surfaces, mediate the mechanical stability of biofilms (Körstgens *et al.*, 2001; Flemming & Wingender, 2003) and determine biofilm architecture (Wingender *et al.*, 1999). Less is known about the influence of extracellular enzymes on biofilm formation and architecture. The first evidence for the influence of the esterase EstA on the biofilm structure of non-mucoid *P. aeruginosa* PAO1 was reported by Wilhelm *et al.* (2007a).

One strategy for investigating the physiological functions of enzymes is to compare the phenotypic properties of

protein-overexpression strains with those of the unmodified parent strains. For example, this technique has been applied to study the functions of LasB and EstA in the non-mucoid wild-type *P. aeruginosa* PAO1 (Kamath *et al.*, 1998; Wilhelm *et al.* 2007a). Although the construction of gene-knockout mutants is a common strategy to investigate the function of enzymes and their role in the physiological processes of the cell, the phenotypic characterization of overexpression strains is also an accepted strategy to investigate specific questions (e.g. Liu & Golden, 2002; Wiriyathanawudhiwong *et al.*, 2009). In some cases, interesting phenotypes are only observable in overproducing strains. For example, the role of the universal stress protein UspA in *E. coli* was recognized only by the use of overexpression strains (Nyström & Neidhardt, 1996). Even in *P. aeruginosa*, the overproduction of the extracellular polysaccharide alginate yields a specific phenotype with respect to virulence and biofilm-forming ability (Govan & Deretic, 1996; Hentzer *et al.*, 2001).

In the present study, strains overexpressing the genes *lipA*, *lipC*, *estA* and *lasB* were generated and compared with the mucoid parent strain *P. aeruginosa* SG81. In both liquid cultures and agar-grown biofilms, higher levels of the respective enzyme activities were observed in the enzyme-overexpression strains compared with the parent strain. Changing levels of enzyme expression have also been reported for different *P. aeruginosa* strains isolated from different habitats (Hamood *et al.*, 1996; Rumbaugh *et al.*, 1999). However, the relative levels of enzyme activities varied depending on the enzyme. Although both lipases were overexpressed via the plasmid pBBR1MCS controlled by the constitutive promoter of the *lacZ* gene, the *lipA*-overexpressing strain *P. aeruginosa* SG81*lipA*⁺ showed a 170-fold higher lipase activity than the *lipC*-overexpressing strain, indicating that (i) the two lipases exhibit a different ability to cleave the used test substrate *para*-nitrophenyl palmitate, (ii) their post-translational processing is controlled through different regulatory systems, or (iii) LipC is secreted via a different pathway from LipA that differs in secretion efficiency.

Since the composition of the EPS can influence biofilm architecture (e.g. Hentzer *et al.*, 2001; Nivens *et al.*, 2001; Davey *et al.*, 2003; Tielen *et al.*, 2005; Ma *et al.*, 2006), we investigated the concentrations of proteins, uronic acids, rhamnolipids and total carbohydrates in the EPS of the overexpression strains. All strains revealed significant changes in EPS composition. The LipA-overproducing strain *P. aeruginosa* SG81*lipA*⁺ showed an increased protein content, which is probably due to a higher amount of the extracellular LipA protein. Also, in the non-mucoid background of *P. aeruginosa* PAO1, the overproduction of LipA from pBBL7 reveals an increased lipase content of 150 mg (1 LB culture)⁻¹ (Rosenau & Jaeger, 2003). The strains *P. aeruginosa* SG81*estA*⁺ and *P. aeruginosa* SG81*lasB*⁺ showed an enhanced total carbohydrate concentration, which is likely due to the higher content of rhamnolipids (Table 4). Furthermore, the LasB-over-

producing strain showed a decreased concentration of uronic acids. This may explain the decrease in biofilm-formation ability in flow-chamber experiments. It is known that alginate plays a role in the adhesion of *P. aeruginosa* cells and in the stability of biofilms. For example, mucoid *P. aeruginosa* strains are able to attach up to 1000-fold better to lung epithelia (Gacesa, 1998); however, it has been shown that adhesion to abiotic surfaces such as glass is decreased for alginate-overproducing strains (Gómez-Suárez *et al.*, 2002). However, adherence to surfaces depends significantly on the physical properties of the surface, which are mainly determined by the conditioning film formed by media components (Pringle & Fletcher, 1986).

Mohr *et al.* (1990) reported a correlation between the expression of *lasB* and that of *algD*, which encodes the key enzyme GDP-mannose dehydrogenase of alginate biosynthesis. An active *algD* promoter leads to a loss of *lasB* expression. A contrary effect can be proposed in the case of high *lasB* expression. *algD* is regulated through the global transcriptional regulator AlgR (Deretic *et al.*, 1987). AlgR, together with the sensor kinase AlgQ, upregulates the biosynthesis of siderophores, depending on environmental stimuli (Deretic & Konyecsni, 1989; Ledgham *et al.*, 2003). This accords with our results, since decreased pigment concentrations were found in the *lasB*-overexpression strain (data not shown), indicating an inactive two-component system. Furthermore, it has been shown that AlgR/AlgQ downregulates the LasR/RhlR quorum-sensing systems of *P. aeruginosa* (Ledgham *et al.*, 2003), which has a negative influence on the expression of *lasB* and other virulence factors, e.g. rhamnolipids (Pesci & Iglewski, 1999; Wingender & Jaeger, 2002). Accordingly, overexpression of *lasB* possibly supplies negative feedback to the AlgR/AlgQ system, with decreased *algD* expression as a consequence. This would result in decreased alginate and siderophore concentrations and an increased amount of rhamnolipids upregulated via quorum sensing, which fits exactly with our observations.

The physicochemical properties of the EPS can also affect biofilm stability (Körstgens *et al.*, 2001). An increased hydrophobicity and, moreover, an enhanced viscosity of the EPS were observed for the *estA*- and the *lasB*-overexpressing strains. An enhanced hydrophobicity of the EPS may be an indication of the presence of a higher content of amphiphilic substances such as rhamnolipids and lipopolysaccharides (LPS), which corresponds to the observations for the two strains. Kadurugamuwa & Beveridge (1995) described the release of membrane vesicles from the outer membrane during the normal growth of *P. aeruginosa*. Furthermore, it has been reported that rhamnolipids induce the removal of LPS from the outer membrane of *P. aeruginosa* (Al-Tahhan *et al.*, 2000). Both components may increase the viscosity of EPS solutions by the formation of micelles and/or take part in interactions with other EPS components. Other studies have demonstrated that tensoactive rhamnolipids can

actively maintain open water channels around *P. aeruginosa* microcolonies (Rendell *et al.*, 1990; Maier & Soberón-Chávez, 2000; Davey *et al.*, 2003). Moreover, rhamnolipids are essential for the swarming motility of non-mucoid *P. aeruginosa* strains, which primarily depends on the motion of flagella and type IV pili (Köhler *et al.*, 2000). Strikingly, *P. aeruginosa* SG81estA+ and *P. aeruginosa* SG81lasB+ showed a noticeable increase in swarming motility (Fig. 4). Also, swimming motility was slightly enhanced, while twitching motility was reduced for *P. aeruginosa* SG81estA+. In a recent study, Wilhelm *et al.* (2007a) demonstrated that a deletion in the *estA* gene in the *P. aeruginosa* PAO1 background leads to reduced production of rhamnolipids and consequently a decreased ability to swarm. In agreement with the present study, in an *EstA*-overproduction strain they found an increased amount of rhamnolipids and an enhanced swarming motility. In the mucoid background of *P. aeruginosa* SG81, the overexpression of *estA* led to only a marginal increase in the rhamnolipid concentration (Table 4). However, TLC analysis showed a detectable amount of mono-rhamnolipid in the EPS of the *estA*-overexpression strain, while the wild-type produced only di-rhamnolipid (Fig. 2), which is the same observation as that of Wilhelm *et al.* (2007a) for the non-mucoid *P. aeruginosa* PAO1.

Microscopic images of flow-chamber biofilms demonstrated the multiplication of progeny cells of the mucoid parent strain *P. aeruginosa* SG81, resulting after 72 h of growth in a highly organized biofilm consisting of microcolonies with water-filled channels in between (Fig. 6). The biofilms formed by non-mucoid strains of *P. aeruginosa* under flow-through conditions have been found to be heterogeneous, with mushroom-shaped microcolonies, in studies in which glucose was used as the carbon source, but flat, uniform and densely packed in studies in which citrate was used as the carbon source (e.g. Klausen *et al.*, 2003a), indicating that *P. aeruginosa* biofilm development depends on the carbon source. Other studies of mature biofilms of mucoid *P. aeruginosa* in different experimental systems have also reported a highly structured biofilm architecture based on microcolony formation during biofilm development (Hentzer *et al.*, 2001; Nivens *et al.*, 2001; Matz *et al.*, 2004), also with gluconate as a carbon source (Tielen *et al.*, 2005). Furthermore, Borriello and co-workers have reported anaerobic areas in the depths of biofilms (Borriello *et al.*, 2004). The use of KNO₃ as a nitrogen source in the mAPM used in this study permitted the growth of *P. aeruginosa* also under anaerobic conditions via denitrification, so that there could be growth even after prolonged culture.

The decreased biofilm structure observed for the *estA*-overexpression strain *P. aeruginosa* SG81estA+ seemed to be caused by the enhanced swarming motility, which was possibly influenced by the altered rhamnolipid production. Another hint that biofilm formation is influenced by the content of rhamnolipids was the late dispersal (after 48 h; Figs 5 and 6) of the normally formed biofilm at the onset of the experiment. Since the production of rhamnolipids is

regulated by quorum sensing (Ochsner & Reiser, 1995; Pearson *et al.*, 1997), it does not start until the population reaches an appropriate cell density.

In contrast to *estA* overexpression, *lasB* overexpression led to a complete lack of biofilm formation. Only a few small microcolonies were formed by *P. aeruginosa* SG81*lasB* + over the incubation time of 72 h. Moreover, we observed an increase in swimming, swarming and twitching motility compared with the parent strain (Fig. 4). In contrast, no significant difference was observed between the biofilms of a mucoid *P. aeruginosa* strain and those of an *aprA* deletion mutant, indicating that AprA does not have an important function in the development and/or maintenance of the 3D structure of mucoid *P. aeruginosa* biofilms (Sarkisova *et al.*, 2005). Recently, transcriptome analysis of *P. aeruginosa* has shown a 4.9-fold increase in *lasB* expression under swarming conditions (Overhage *et al.*, 2008), indicating a potential role for LasB in the highly complex swarming process. Similarly, in *Bacillus subtilis* it has been shown that swarming motility is enhanced by the expression of extracellular protease activity (Connelly *et al.*, 2004). Our results indicated a strong influence of LasB on swarming as well as on swimming motility. Flagella- and/or type IV pili-mediated motility can affect biofilm formation both in the first steps of adhesion (O'Toole & Kolter, 1998; Klausen *et al.*, 2003a) and during the maturation of the biofilm (Klausen *et al.*, 2003b). Furthermore, Sauer *et al.* (2002) reported the dispersal of free-living cells from voids inside microcolonies of *P. aeruginosa* biofilms. Another explanation for the absence of biofilm in SG81*lasB* + could be the degradation or modification of extracellular proteins by the activity of the proteolytic LasB. Proteome analysis has shown that extracellular proteins undergo a substantial post-translational degradation during biofilm maturation (Nouwens *et al.*, 2003). LasB has been identified as one of the enzymes involved. For example, it has been shown that the extracellular chitin-binding protein CbpD is cleaved by the proteolytic activity of LasB to the staphylolytic elastase LasD (Foldes *et al.*, 2000). LasD in turn is necessary for the proteolytic degradation of the propeptide of LasA, which acts as an inhibitor for LasA (Park & Galloway, 1998; Kessler *et al.*, 1998). Similar post-translational processing cascades of extracellular proteins have also been found in other organisms (Vu & Werb, 2000; Kearns *et al.*, 2002; Klauck *et al.*, 2005). However, no decrease in protein concentration was observed, although reduced phosphatase activity was observed in the LasB-overproducing strain (Table 3). The enhanced protein concentration of SG81*lasB* + may be explained by the enhanced production of the LasB protein. Similar observations were made in the LipA-overexpression strain, as discussed above. At least some of the extracellular proteins of *P. aeruginosa* could function as structural components, for example as lectin-like proteins within the biofilms. In *P. aeruginosa*, the galactose-specific lectin LecA and fucose-specific lectin LecB have been implicated in biofilm formation (Tielker *et al.*, 2005; Diggle *et al.*, 2006). Degradation or

even modification of such structural proteins could diminish biofilm stability and subsequently affect biofilm architecture.

The overproduction of the lipase LipA did not result in significant differences, while the overproduction of the other lipase, LipC, resulted in a heterogeneously distributed biofilm. The microcolonies were conspicuously thicker compared with the parent strain and consisted of densely packed cells. These types of microcolonies have been reported to develop as a result of clonal growth in combination with reduced cellular motility (Chiang & Burrows, 2003). Moreover, it has been reported that formation of microcolonies in the non-mucoid strain *P. aeruginosa* PAO1 is prevented because cells migrate from the initially formed microcolonies and spread over the substratum by means of twitching motility (Klausen *et al.*, 2003a, b). In accordance with this, the LipC-overproducing strain *P. aeruginosa* SG81*lipC* + lacked twitching motility in agar plate assays (Fig. 4). The reason why the overexpression of *lipC* influences the production or even the functionality of type IV pili-mediated twitching motility is so far unknown.

Overall, these results clearly indicate that extracellular enzymes can affect EPS composition and properties, thereby influencing biofilm development. These data provide strong evidence that extracellular enzymes, in addition to their roles in virulence and nutrition, play a key role in the differentiation of microbial biofilms.

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REFERENCES

- Al-Tahhan, R. A., Sandrin, T. R., Bodour, A. A. & Maier, R. M. (2000). Rhamnolipid-induced removal of lipopolysaccharide from *Pseudomonas aeruginosa*: effect on cell surface properties and interaction with hydrophobic substrates. *Appl Environ Microbiol* **66**, 3262–3268.
- Auerbach, I. D., Sorensen, C., Hansma, H. G. & Holden, P. A. (2000). Physical morphology and surface properties of unsaturated *Pseudomonas putida* biofilms. *J Bacteriol* **182**, 3809–3815.
- Blumenkrantz, N. & Asboe-Hansen, G. (1973). New method for quantitative determination of uronic acids. *Anal Biochem* **54**, 484–489.
- Boles, B. R., Thoendel, M. & Singh, P. K. (2005). Rhamnolipids mediate detachment of *Pseudomonas aeruginosa* from biofilms. *Mol Microbiol* **57**, 1210–1223.
- Borriello, G., Werner, E., Roe, F., Kim, A. M., Ehrlich, G. D. & Stewart, P. S. (2004). Oxygen limitation contributes to antibiotic tolerance of *Pseudomonas aeruginosa* in biofilms. *Antimicrob Agents Chemother* **48**, 2659–2664.
- Borriello, G., Richards, L., Ehrlich, G. D. & Stewart, P. S. (2006). Arginine or nitrate enhances antibiotic susceptibility of *Pseudomonas aeruginosa* in biofilms. *Antimicrob Agents Chemother* **50**, 382–384.

- Botzenhart, K. & Döring, G. (1993). Ecology and epidemiology of *Pseudomonas aeruginosa*. In *Pseudomonas aeruginosa as an Opportunistic Pathogen*, pp. 1–18. Edited by M. Campa, M. Bendinelli & H. Friedman. New York: Plenum Press.
- Chandrasekaran, E. V. & BeMiller, J. N. (1980). Constituent analysis of glycosaminoglycans. In *Methods in Carbohydrate Chemistry*, VIII, pp. 89–96. Edited by R. L. Whistler & J. N. BeMiller. New York: Academic Press.
- Chang, W. S., van de Mortel, M., Nielsen, L., Nino de Guzman, G., Li, X. & Halverson, L. J. (2007). Alginate production by *Pseudomonas putida* creates a hydrated microenvironment and contributes to biofilm architecture and stress tolerance under water-limiting conditions. *J Bacteriol* **189**, 8290–8299.
- Chiang, P. & Burrows, L. L. (2003). Biofilm formation by hyperpilated mutants of *Pseudomonas aeruginosa*. *J Bacteriol* **185**, 2374–2378.
- Ciofu, O., Beveridge, T. J., Kadurugamuwa, J., Walther-Rasmussen, J. & Høiby, N. (2000). Chromosomal beta-lactamase is packaged into membrane vesicles and secreted from *Pseudomonas aeruginosa*. *J Antimicrob Chemother* **45**, 9–13.
- Connelly, M. B., Young, G. M. & Sloma, A. (2004). Extracellular proteolytic activity plays a central role in swarming motility in *Bacillus subtilis*. *J Bacteriol* **186**, 4159–4167.
- Davey, M. E. & O'Toole, G. A. (2000). Microbial biofilms: from ecology to molecular genetics. *Microbiol Mol Biol Rev* **64**, 847–867.
- Davey, M. E., Caiazza, N. C. & O'Toole, G. A. (2003). Rhamnolipid surfactant production affects biofilm architecture in *Pseudomonas aeruginosa* PAO1. *J Bacteriol* **185**, 1027–1036.
- Deretic, V. & Konyecsni, W. M. (1989). Control of mucoidy in *Pseudomonas aeruginosa*: transcriptional regulation of *algR* and identification of the second regulatory gene, *algQ*. *J Bacteriol* **171**, 3680–3688.
- Deretic, V., Gill, J. F. & Chakrabarty, A. M. (1987). *Pseudomonas aeruginosa* infection in cystic fibrosis: nucleotide sequence and transcriptional regulation of the *algD* gene. *Nucleic Acids Res* **15**, 4567–4581.
- Deziel, E., Lepine, F., Milot, S. & Villemur, R. (2003). *rhlA* is required for the production of a novel biosurfactant promoting swarming motility in *Pseudomonas aeruginosa*: 3-(3-hydroxyalkanoyloxy)alkanoic acids (HAAs), the precursors of rhamnolipids. *Microbiology* **149**, 2005–2013.
- Diggle, S. P., Stacey, R. E., Dodd, C., Cámara, M., Williams, P. & Winzer, K. (2006). The galactophilic lectin, LecA, contributes to biofilm development in *Pseudomonas aeruginosa*. *Environ Microbiol* **8**, 1095–1104.
- Drenkard, E. (2003). Antimicrobial resistance of *Pseudomonas aeruginosa* biofilms. *Microbes Infect* **5**, 1213–1219.
- Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Anal Chem* **28**, 350–356.
- Espinosa-Urgel, M. (2003). Resident parking only: rhamnolipids maintain fluid channels in biofilms. *J Bacteriol* **185**, 699–700.
- Fagerland, M. W. & Sandvik, L. (2009). Performance of five two-sample location tests for skewed distributions with unequal variances. *Contemp Clin Trials* **30**, 490–496.
- Flemming, H.-C. & Wingender, J. (2003). The crucial role of extracellular polymeric substances in biofilms. In *Wastewater Treatment: an Interdisciplinary Approach*, pp. 178–210. Edited by S. Wuerzt, P. L. Bishop & P. A. Wilderer. London: IWA Publishing.
- Folders, J., Tommassen, J., van Loon, L. C. & Bitter, W. (2000). Identification of a chitin-binding protein secreted by *Pseudomonas aeruginosa*. *J Bacteriol* **182**, 1257–1263.
- Frølund, B., Palmgren, R., Keiding, K. & Nielsen, P. H. (1996). Extraction of extracellular polymers from activated sludge using a cation exchange resin. *Water Res* **30**, 1749–1758.
- Fürste, J. P., Pansegrau, W., Frank, R., Blocker, H., Scholz, P., Bagdasarian, M. & Lanka, E. (1986). Molecular cloning of the plasmid RP4 primase region in a multi-host-range *tacP* expression vector. *Gene* **48**, 119–131.
- Gacesa, P. (1998). Bacterial alginate biosynthesis – recent progress and future prospects. *Microbiology* **144**, 1133–1143.
- Galloway, D. R. (1991). *Pseudomonas aeruginosa* elastase and elastolysis revisited: recent developments. *Mol Microbiol* **5**, 2315–2321.
- Gómez-Suárez, C., Pasma, J., van der Borden, A. J., Wingender, J., Flemming, H. C., Busscher, H. J. & van der Mei, H. C. (2002). Influence of extracellular polymeric substances on deposition and redeposition of *Pseudomonas aeruginosa* to surfaces. *Microbiology* **148**, 1161–1169.
- Govan, J. R. & Deretic, V. (1996). Microbial pathogenesis in cystic fibrosis: Mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. *Microbiol Rev* **60**, 539–574.
- Grobe, S., Wingender, J. & Trüper, H. G. (1995). Characterization of mucoid *Pseudomonas aeruginosa* strains isolated from technical water systems. *J Appl Bacteriol* **79**, 94–102.
- Hamood, A. N., Griswold, J. A. & Duhan, C. M. (1996). Production of extracellular virulence factors by *Pseudomonas aeruginosa* isolates obtained from tracheal, urinary tract, and wound infections. *J Surg Res* **61**, 425–432.
- Hentzer, M., Teitzel, G. M., Balzer, G. J., Heydorn, A., Molin, S., Givskov, M. & Parsek, M. R. (2001). Alginate overproduction affects *Pseudomonas aeruginosa* biofilm structure and function. *J Bacteriol* **183**, 5395–5401.
- Jaeger, K.-E., Schneidinger, B., Liebeton, L., Haas, D., Reetz, M. T., Philippou, S., Gerritse, G., Ransac, S. & Dijkstra, B. W. (1996). Lipase of *Pseudomonas aeruginosa*: molecular biology and biotechnological application. In *Molecular Biology of Pseudomonads*, pp. 319–330. Edited by T. Nakazawa, K. Furukawa, D. Haas & S. Silver. Washington, DC: American Society for Microbiology.
- Jain, S. & Ohman, D. E. (2004). Alginate biosynthesis. In *Pseudomonas*, vol. 3, pp. 53–83. Edited by J.-L. Ramos. New York: Kluwer Academic/Plenum Publishers.
- Kadurugamuwa, J. L. & Beveridge, T. J. (1995). Virulence factors are released from *Pseudomonas aeruginosa* in association with membrane vesicles during normal growth and exposure to gentamicin: a novel mechanism of enzyme secretion. *J Bacteriol* **177**, 3998–4008.
- Kamath, S., Kapatral, V. & Chakrabarty, A. M. (1998). Cellular function of elastase in *Pseudomonas aeruginosa*: role in the cleavage of nucleoside diphosphate kinase and in alginate synthesis. *Mol Microbiol* **30**, 933–941.
- Kearns, D. B., Bonner, P. J., Smith, D. R. & Shinkets, L. J. (2002). An extracellular matrix-associated zinc metalloprotease is required for dilauroyl phosphatidylethanolamine chemotactic excitation in *Myxococcus xanthus*. *J Bacteriol* **184**, 1678–1684.
- Kessler, E., Safrin, M., Gustin, J. K. & Ohman, D. E. (1998). Elastase and the LasA protease of *Pseudomonas aeruginosa* are secreted with their propeptides. *J Biol Chem* **273**, 30225–30231.
- Kim, J., Hahn, J. S., Franklin, M. J., Stewart, P. S. & Yoon, J. (2009). Tolerance of dormant and active cells in *Pseudomonas aeruginosa* PAO1 biofilm to antimicrobial agents. *J Antimicrob Chemother* **63**, 129–135.
- Klauck, E., Becker, G. & Hengge, R. (2005). Proteolyse als Regulationsprinzip in Prokaryoten. *BIOspektrum (Heidelb)* **2**, 166–168.

- Klausen, M., Heydorn, A., Ragas, P., Lamberthsen, L., Aes-Jorgensen, A., Moli, S. & Tolker-Nielsen, T. (2003a). Biofilm formation by *Pseudomonas aeruginosa* wild type, flagella and type IV pili mutants. *Mol Microbiol* **48**, 1511–1524.
- Klausen, M., Aes-Jorgensen, A., Molin, S. & Tolker-Nielsen, T. (2003b). Involvement of bacterial migration in the development of complex multicellular structures in *Pseudomonas aeruginosa* biofilms. *Mol Microbiol* **50**, 61–68.
- Köhler, T., Curty, L. K., Barja, F., van Delden, C. & Pechere, J. C. (2000). Swarming of *Pseudomonas aeruginosa* is dependent on cell-to-cell signaling and requires flagella and pili. *J Bacteriol* **182**, 5990–5996.
- König, B., Jaeger, K. E., Sage, A. E., Vasil, M. L. & König, W. (1996). Role of *Pseudomonas aeruginosa* lipase in inflammatory mediator release from human inflammatory effector cells (platelets, granulocytes, and monocytes). *Infect Immun* **64**, 3252–3258.
- Körstgens, V., Flemming, H.-C., Wingender, J. & Borchard, W. (2001). Influence of calcium ions on the mechanical properties of a model biofilm of mucoid *Pseudomonas aeruginosa*. *Water Sci Technol* **43**, 49–57.
- Kovach, M. E., Phillips, R. W., Elzer, P. H., Roop, R. M., II & Peterson, K. M. (1994). pBBR1MCS: a broad-host-range cloning vector. *Biotechniques* **16**, 800–802.
- Küpper, H., Wingender, J., Jaeger, K.-E. & Flemming, H.-C. (1999). Eignung von Fluoresceinsternen zur Bestimmung der Esterase-Aktivitäten von Wasserbakterien Tagungsband. *Jahrestagung der Fachgruppe Wasserchemie in der Gesellschaft Deutscher Chemiker, Regensburg 1999*, 208–211.
- Ledgham, F., Soscia, C., Chakrabarty, A., Lazdunski, A. & Foglino, M. (2003). Global regulation in *Pseudomonas aeruginosa*: the regulatory protein AlgR2 (AlgQ) acts as a modulator of quorum sensing. *Res Microbiol* **154**, 207–213.
- Liu, D. & Golden, J. W. (2002). *hetL* overexpression stimulates heterocyst formation in *Anabaena* sp. strain PCC7120. *J Bacteriol* **184**, 6873–6881.
- Ma, L., Jackson, K. D., Landry, R. M., Parsek, M. R. & Wozniak, D. J. (2006). Analysis of *Pseudomonas aeruginosa* conditional *psl* variants reveals roles for the *psl* polysaccharide in adhesion and maintaining biofilm structure postattachment. *J Bacteriol* **188**, 8213–8221.
- Mah, T. F., Pitts, B., Pellock, B., Walker, G. C., Stewart, P. S. & O'Toole, G. A. (2003). A genetic basis for *Pseudomonas aeruginosa* biofilm antibiotic resistance. *Nature* **426**, 306–310.
- Maier, R. M. & Soberón-Chávez, G. (2000). *Pseudomonas aeruginosa* rhamnolipids: biosynthesis and potential applications. *Appl Microbiol Biotechnol* **54**, 625–633.
- Martínez, A., Ostrovsky, P. & Nunn, D. N. (1999). LipC, a second lipase of *Pseudomonas aeruginosa*, is LipB and Xcp dependent and is transcriptionally regulated by pilus biogenesis components. *Mol Microbiol* **34**, 317–326.
- Matz, C., Bergfeld, T., Rice, S. A. & Kjelleberg, S. (2004). Microcolonies, quorum sensing and cytotoxicity determine the survival of *Pseudomonas aeruginosa* biofilms exposed to protozoan grazing. *Environ Microbiol* **6**, 218–226.
- McDougald, D., Klebensberger, J., Tolker-Nielsen, T., Webb, J. S., Conibear, T., Rice, S. A., Kirov, S. M., Matz, C. & Kjelleberg, S. (2008). *Pseudomonas aeruginosa*: a model for biofilm formation. In *Pseudomonas. Model Organism, Pathogen, Cell Factory*, pp. 215–253. Edited by B. H. A. Rehm. Weinheim: Wiley-VCH Verlag.
- Mohr, C. D., Rust, L., Albus, A. M., Iglewski, B. H. & Deretic, V. (1990). Expression patterns of genes encoding elastase and controlling mucoidy – co-ordinate regulation of two virulence factors in *Pseudomonas aeruginosa* from cystic fibrosis. *Mol Microbiol* **4**, 2103–2110.
- Neu, H. C. & Heppel, I. A. (1965). The release of enzymes from *Escherichia coli* by osmotic shock and during the formation of spheroplasts. *J Biol Chem* **240**, 3685–3692.
- Nicas, T. I. & Iglewski, B. H. (1986). Production of elastase and other exoproducts by environmental isolates of *Pseudomonas aeruginosa*. *J Clin Microbiol* **23**, 967–969.
- Nivens, D. E., Ohman, D. E., Williams, J. & Franklin, M. J. (2001). Role of alginate and its O-acetylation in formation of *Pseudomonas aeruginosa* microcolonies and biofilms. *J Bacteriol* **183**, 1047–1057.
- Nouwens, A. S., Beatson, S. A., Whitchurch, C. B., Walsh, B. J., Schweizer, H. P., Mattick, J. S. & Cordwell, S. J. (2003). Proteome analysis of extracellular proteins regulated by the *las* and *rhl* quorum sensing systems in *Pseudomonas aeruginosa* PAO1. *Microbiology* **149**, 1311–1322.
- Nyström, T. & Neidhardt, F. C. (1996). Effects of overproducing the universal stress protein, UspA, in *Escherichia coli* K-12. *J Bacteriol* **178**, 927–930.
- Obernesser, H.-J., Döring, G. & Botzenhart, K. (1981). Extrazelluläre Toxine von *Pseudomonas aeruginosa*. I. Reinigung und Charakterisierung zweier Exoproteasen. *Zentralbl Bakteriol A* **A249**, 76–88.
- Ochsner, U. A. & Reiser, J. (1995). Autoinducer-mediated regulation of rhamnolipid biosurfactant synthesis in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **92**, 6424–6428.
- Ochsner, U. A., Fiechter, A. & Reiser, J. (1994). Isolation, characterization, and expression in *Escherichia coli* of the *Pseudomonas aeruginosa* *rhlAB* genes encoding a rhamnosyltransferase involved in rhamnolipid biosurfactant synthesis. *J Biol Chem* **269**, 19787–19795.
- Ohman, D. E. & Chakrabarty, A. M. (1981). Genetic mapping of chromosomal determinants for the production of the exopolysaccharide alginate in a *Pseudomonas aeruginosa* cystic fibrosis isolate. *Infect Immun* **33**, 142–148.
- O'Toole, G. A. & Kolter, R. (1998). Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Mol Microbiol* **30**, 295–304.
- Overhage, J., Bains, M., Brazas, M. D. & Hancock, R. E. (2008). Swarming of *Pseudomonas aeruginosa* is a complex adaptation leading to increased production of virulence factors and antibiotic resistance. *J Bacteriol* **190**, 2671–2679.
- Park, S. & Galloway, D. R. (1998). *Pseudomonas aeruginosa* LasD processes the inactive LasA precursor to the active protease form. *Arch Biochem Biophys* **357**, 8–12.
- Pearson, J. P., Pesci, E. C. & Iglewski, B. H. (1997). Roles of *Pseudomonas aeruginosa* *las* and *rhl* quorum-sensing systems in control of elastase and rhamnolipid biosynthesis genes. *J Bacteriol* **179**, 5756–5767.
- Pesci, E. C. & Iglewski, B. H. (1999). Quorum sensing in *Pseudomonas aeruginosa*. In *Cell–Cell Signaling in Bacteria*, pp. 147–155. Edited by G. M. Dunny & S. C. Winans. Washington, DC: American Society for Microbiology.
- Prestidge, L., Gage, V. & Spizizien, J. (1971). Protease activities during the course of sporulation in *Bacillus subtilis*. *J Bacteriol* **107**, 815–823.
- Pringle, J. H. & Fletcher, M. M. (1986). Influence of substratum hydration and adsorbed macromolecules on bacterial attachment to surfaces. *Appl Environ Microbiol* **51**, 1321–1325.
- Rashid, M. H. & Kornberg, A. (2000). Inorganic phosphate is needed for swimming, swarming, and twitching motilities of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **97**, 4885–4890.
- Rendell, N. B., Taylor, G. W., Somerville, M., Todd, H., Wilson, R. & Cole, P. J. (1990). Characterisation of *Pseudomonas* rhamnolipids. *Biochim Biophys Acta* **1045**, 189–193.

- Rosenau, F. & Jaeger, K.-E. (2003). Overexpression and secretion of biocatalysts in *Pseudomonas*. In *Enzyme Functionality: Design, Engineering, and Screening*, pp. 617–631. Edited by A. Sevensden. New York: Marcel Dekker.
- Rosenau, F., Tommassen, J. & Jaeger, K.-E. (2004). Lipase-specific foldases. *ChemBioChem* 5, 152–161.
- Rosenberg, M., Gutnick, D. & Rosenberg, E. (1980). Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. *FEMS Microbiol Lett* 9, 29–33.
- Rumbaugh, K. P., Griswold, J. A. & Hamood, A. N. (1999). *Pseudomonas aeruginosa* strains obtained from patients with tracheal, urinary tract and wound infection: variations in virulence factors and virulence genes. *J Hosp Infect* 43, 211–218.
- Ryder, C., Byrd, M. & Wozniak, D. J. (2007). Role of polysaccharides in *Pseudomonas aeruginosa* biofilm development. *Curr Opin Microbiol* 10, 644–648.
- Sarkisova, S., Patrauchan, M. A., Berglund, D., Nivens, D. E. & Franklin, M. J. (2005). Calcium-induced virulence factors associated with the extracellular matrix of mucoid *Pseudomonas aeruginosa* biofilms. *J Bacteriol* 187, 4327–4337.
- Sauer, K., Camper, A. K., Ehrlich, G. D., Costerton, J. W. & Davies, D. G. (2002). *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. *J Bacteriol* 184, 1140–1154.
- Sauer, K., Cullen, M. C., Rickard, A. H., Zeef, L. A., Davies, D. G. & Gilbert, P. (2004). Characterization of nutrient-induced dispersion in *Pseudomonas aeruginosa* PAO1 biofilm. *J Bacteriol* 186, 7312–7326.
- Simon, R., Priefer, U. & Pühler, A. (1983). A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram negative bacteria. *Biol/Technology* 1, 784–791.
- Singh, P. K., Schaefer, A. L., Parsek, M. R., Moninger, T. O., Welsh, M. J. & Greenberg, E. P. (2000). Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. *Nature* 407, 762–764.
- Steinberger, R. E., Allen, A. R., Hansa, H. G. & Holden, P. A. (2002). Elongation correlates with nutrient deprivation in *Pseudomonas aeruginosa* unsaturated biofilms. *Microb Ecol* 43, 416–423.
- Stoodley, P., Sauer, K., Davies, D. G. & Costerton, J. W. (2002). Biofilms as complex differentiated communities. *Annu Rev Microbiol* 56, 187–209.
- Syldatk, C., Lang, S., Wagner, F., Wray, V. & Witte, L. (1985). Chemical and physical characterization of four interfacial-active rhamnolipids from *Pseudomonas* spec. DSM 2874 grown on n-alkanes. *Z Naturforsch [C]* 40, 51–60.
- Tielen, P., Strathmann, M., Jaeger, K.-E., Flemming, H.-C. & Wingender, J. (2005). Alginate acetylation influences initial surface colonization by mucoid *Pseudomonas aeruginosa*. *Microbiol Res* 160, 165–176.
- Tielker, D., Hacker, S., Loris, R., Strathmann, M., Wingender, J., Wilhelm, S., Rosenau, F. & Jaeger, K.-E. (2005). *Pseudomonas aeruginosa* lectin LecB is located in the outer membrane and is involved in biofilm formation. *Microbiology* 151, 1313–1323.
- van Delden, C. (2004). Virulence factors in *Pseudomonas aeruginosa*. In *Pseudomonas*, vol. 2, pp. 3–45. Edited by J.-L. Ramos. New York: Kluwer Academic/Plenum Publishers.
- Vu, T. H. & Werb, Z. (2000). Matrix metalloproteinases: effectors of development and normal physiology. *Genes Dev* 14, 2123–2133.
- Waite, R. D., Papakonstantinou, A., Littler, E. & Curtis, M. A. (2005). Transcriptome analysis of *Pseudomonas aeruginosa* growth: comparison of gene expression in planktonic cultures and developing and mature biofilms. *J Bacteriol* 187, 6571–6576.
- Wehmhöner, D., Häussler, S., Tümmler, B., Jänsch, L., Bredenbruch, F., Wehland, J. & Steinmetz, I. (2003). Inter- and intracolon diversity of the *Pseudomonas aeruginosa* proteome manifests within the secretome. *J Bacteriol* 185, 5807–5814.
- Werner, E., Roe, F., Bugnicourt, A., Franklin, M. J., Heydorn, A., Molin, S., Pitts, B. & Stewart, P. S. (2004). Stratified growth in *Pseudomonas aeruginosa* biofilms. *Appl Environ Microbiol* 70, 6188–6196.
- Wilhelm, S., Tommassen, J. & Jaeger, K.-E. (1999). A novel lipolytic enzyme located in the outer membrane of *Pseudomonas aeruginosa*. *J Bacteriol* 181, 6977–6986.
- Wilhelm, S., Gdynia, A., Tielen, P., Rosenau, F. & Jaeger, K.-E. (2007a). The autotransporter esterase EstA of *Pseudomonas aeruginosa* is required for rhamnolipid production, cell motility, and biofilm formation. *J Bacteriol* 189, 6695–6703.
- Wilhelm, S., Rosenau, F., Becker, S., Buest, S., Hausmann, S., Kolmar, H. & Jaeger, K.-E. (2007b). Functional cell-surface display of a lipase-specific chaperone. *ChemBioChem* 8, 55–60.
- Wingender, J. (1990). Interactions of alginate with exoenzymes. In *Pseudomonas Infection and Alginates. Biochemistry, Genetics and Pathology*, pp. 160–180. Edited by P. Gacesa & N. J. Russell. London: Chapman & Hall.
- Wingender, J. & Jaeger, K.-E. (2002). Extracellular enzymes in biofilms. In *Encyclopedia of Environmental Microbiology*, vol. 3, pp. 1207–1223. Edited by G. Bitton. New York: Wiley.
- Wingender, J., Neu, T. & Flemming, H.-C. (1999). What are bacterial extracellular polymeric substances? In *Microbial Extracellular Polymeric Substances*, pp. 1–15. Edited by J. Wingender, T. Neu & H.-C. Flemming. Berlin, Heidelberg, New York: Springer-Verlag.
- Wingender, J., Strathmann, M., Rode, A., Leis, A. & Flemming, H.-C. (2001). Isolation and biochemical characterization of extracellular polymeric substances from *Pseudomonas aeruginosa*. *Methods Enzymol* 336, 302–314.
- Winkler, U. K. & Stuckmann, M. (1979). Glycogen, hyaluronate, and some other polysaccharides greatly enhance the formation of exolipase by *Serratia marcescens*. *J Bacteriol* 138, 663–670.
- Wiriathanawudhiwong, N., Ohtsu, I., Li, Z. D., Mori, H. & Takagi, H. (2009). The outer membrane TolC is involved in cysteine tolerance and overproduction in *Escherichia coli*. *Appl Microbiol Biotechnol* 81, 903–913.
- Zhang, Y. & Miller, R. M. (1994). Effect of a *Pseudomonas* rhamnolipid biosurfactant on cell hydrophobicity and biodegradation of octadecane. *Appl Environ Microbiol* 60, 2101–2106.

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