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 elevated 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.

**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.
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 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-mannurionate and α-L-gulurionate (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-lactamas 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 & Igleswki, 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-glucoratone, 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

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype or phenotype</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td><strong>E. coli strain</strong></td>
<td></td>
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<tr>
<td>S17-1</td>
<td><em>thi pro hsdR</em>&lt;sup&gt;+&lt;/sup&gt; M&lt;sup&gt;+&lt;/sup&gt;, chromosomally integrated [RP4-2 Tc::Mu Km&lt;sup&gt;+&lt;/sup&gt;::Tn7, Tra&lt;sup&gt;+&lt;/sup&gt; Tri&lt;sup&gt;+&lt;/sup&gt; Str&lt;sup&gt;+&lt;/sup&gt;]</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td><strong>P. aeruginosa strains</strong></td>
<td></td>
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<tr>
<td>SG81</td>
<td>Mucoid biofilm isolate from technical water system</td>
<td>Grobe et al. (1995)</td>
</tr>
<tr>
<td>SG81MCS</td>
<td>Vector control pBBR1MCS</td>
<td>This study</td>
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<tr>
<td>SG81estA+</td>
<td>Overexpression of estA</td>
<td>This study</td>
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<tr>
<td>SG81lipC+</td>
<td>Overexpression of lipC</td>
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<tr>
<td>SG81lipA+</td>
<td>Overexpression of lipA</td>
<td>This study</td>
</tr>
<tr>
<td>SG81MMB</td>
<td>Vector control pMMB67EH</td>
<td>This study</td>
</tr>
<tr>
<td>SG81lasB+</td>
<td>Overexpression of lasB</td>
<td>This study</td>
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<tr>
<td><strong>Plasmids</strong></td>
<td></td>
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<tr>
<td>pBBR1MCS</td>
<td>lacZs Cm&lt;sup&gt;+&lt;/sup&gt; mob P&lt;sub&gt;Tac&lt;/sub&gt; P&lt;sub&gt;T7&lt;/sub&gt;</td>
<td>Kovach et al. (1994)</td>
</tr>
<tr>
<td>pBBX+</td>
<td>pBBR1MCS containing 3.3 kb XhoI fragment with estA under P&lt;sub&gt;Tac&lt;/sub&gt; control</td>
<td>Wilhelm et al. (2007a)</td>
</tr>
<tr>
<td>pBBLC</td>
<td>pBBLCAH AlpA (BamHI/PpuMI deletion), lipC/H under P&lt;sub&gt;Tac&lt;/sub&gt; control</td>
<td>Rosenau &amp; Jaeger (2003)</td>
</tr>
<tr>
<td>pBBL7</td>
<td>2.8 kb XmnI/Smal fragment with lipAI/H operon in pBBR1MCS under P&lt;sub&gt;Tac&lt;/sub&gt; control</td>
<td>Wilhelm et al. (2007b)</td>
</tr>
<tr>
<td>pMMB67EH</td>
<td>IncQ, lacI&lt;sup&gt;+&lt;/sup&gt;, P&lt;sub&gt;lac&lt;/sub&gt;, rmb, Amp&lt;sup&gt;+&lt;/sup&gt;, mob</td>
<td>Fürste et al. (1986)</td>
</tr>
<tr>
<td>pSAK5</td>
<td>2.7 kb EcoRI/HindIII fragment with lasB in pMMB67HE under P&lt;sub&gt;Tac&lt;/sub&gt; control</td>
<td>Kamath et al. (1998)</td>
</tr>
</tbody>
</table>

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 A<sub>490</sub> of 1.0 unit per 15 min corresponds to a lipase activity of 48.3 nmol min<sup>−1</sup> (ml solution)<sup>−1</sup>. Esterase activity was determined with fluorescein diacetate as substrate (Küpper et al., 1999). An increase in A<sub>380</sub> of 1.0 unit per 60 min corresponds to an esterase activity of 0.282 nmol min<sup>−1</sup> (ml solution)<sup>−1</sup>. Protease activity was determined using azocasein as substrate (Prestidge et al., 1971). One enzyme unit of extracellular protease corresponds to an increase in A<sub>540</sub> 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 A<sub>410</sub> of 1.0 unit per 30 min corresponds to a phosphatase activity of 55.9 nmol min<sup>−1</sup> (ml solution)<sup>−1</sup>. For 30 min and subsequently at room temperature for 10 min, the A<sub>421</sub> 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* PA01 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 x 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, 600 ml solution was extracted three times with 600 ml deionized water, and 100 ml 60 % (v/v) H<sub>2</sub>S<sub>4</sub>O<sub>7</sub>) 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 K<sub>2</sub>HPO<sub>4</sub>, 3H<sub>2</sub>O, 7.26 g KH<sub>2</sub>PO<sub>4</sub>, 1.8 g urea, 0.2 g MgSO<sub>4</sub>, 6H<sub>2</sub>O, 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 OD<sub>580</sub> 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 x40/0.60 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 PA01, 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 19-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=2.8×10⁻⁸) 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
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 strain, 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-overexpressing 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

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

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

*The results are expressed as the mean ± SD of four independent experiments.
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-overexpressing strain showed a 1.4-fold ($P=7.9 \times 10^{-10}$) increase in carbohydrate and a 1.3-fold ($P=6.4 \times 10^{-8}$) 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 ($P=1.3 \times 10^{-5}$) 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-overexpression 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-overexpression 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

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Analyte</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Protein* [mg (10^9 cells)^{-1}]</td>
</tr>
<tr>
<td>-------------------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td>SG81</td>
<td>0.16 ± 0.05</td>
</tr>
<tr>
<td>SG81MCS</td>
<td>0.17 ± 0.02</td>
</tr>
<tr>
<td>SG81estA+</td>
<td>0.18 ± 0.03</td>
</tr>
<tr>
<td>SG81lipC+</td>
<td>0.13 ± 0.01</td>
</tr>
<tr>
<td>SG81lipA+</td>
<td>0.21 ± 0.05</td>
</tr>
<tr>
<td>SG81MBM</td>
<td>0.18 ± 0.01</td>
</tr>
<tr>
<td>SG81lasB+</td>
<td>0.21 ± 0.09</td>
</tr>
</tbody>
</table>

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

**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 \textit{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 predominately 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

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**Fig. 2.** Hydrophobicity and viscosity of EPS solutions from \textit{P. aeruginosa} strains. The hydrophobicity was measured as the OD$_{580}$ 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: $^{*}P=2.8\times10^{-10}$, $^{**}P=5.25\times10^{-8}$, $^{t}P=8.9\times10^{-11}$, $^{tt}P=1.7\times10^{-6}$. Error bars, SD.

**Fig. 3.** TLC of rhamnolipid diethyl ether extracts of the EPS of the enzyme-overexpression strains and the parent strain \textit{P. aeruginosa} SG81. Rhamnolipids were extracted from 40\,$\mu$g biofilm mass and stained after TLC with an orcinol reagent.

**Fig. 4.** Swimming (a), swarming (b) and twitching (c) motility of mucoid strain \textit{P. aeruginosa} SG81 and its enzyme-overexpressing derivatives. The motility of the \textit{P. aeruginosa} strains was analysed on agar plates containing different agar concentrations to investigate swimming (0.3\% agar; incubation at 36\,$^\circ\text{C}$ for 24 h), swarming (0.5\% agar; incubation at 36\,$^\circ\text{C}$ for 48 h) and twitching motility (3\,mm thick LB agar; incubation at 36\,$^\circ\text{C}$ 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 $\times10$ magnification.

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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 overexpression 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,
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 (Nystrom & 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 pBRR1MCS controlled by the constitutive promoter of the lacZ gene, the lipA-overexpressing strain P. aeruginosa SG81lipA+ 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 SG81lipA+ 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 (Rosena & Jaeger, 2003). The strains P. aeruginosa SG81estA+ and P. aeruginosa SG81lasB+ 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 \textit{P. aeruginosa} cells and in the stability of biofilms. For example, mucoid \textit{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 \textit{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 \textit{et al.} (1990) reported a correlation between the expression of \textit{lasB} and that of \textit{algD}, which encodes the key enzyme GDP-mannose dehydrogenase of alginate biosynthesis. An active \textit{algD} promoter leads to a loss of \textit{lasB} expression. A contrary effect can be proposed in the case of high \textit{lasB} expression. \textit{algD} is regulated through the global transcriptional regulator AlgR (Deretic \textit{et al.}, 1987). AlgR, together with the sensor kinase AlgQ, upregulates the biosynthesis of siderophores, depending on environmental stimuli (Deretic & Konyecsni, 1989; Ledgham \textit{et al.}, 2003). This accords with our results, since decreased pigmentation concentrations were found in the \textit{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 \textit{P. aeruginosa} (Ledgham \textit{et al.}, 2003), which has a negative influence on the expression of \textit{lasB} and other virulence factors, e.g. rhamnolipids (Pesci & Iglewski, 1999; Wingender & Jaeger, 2002). Accordingly, overexpression of \textit{lasB} possibly supplies negative feedback to the AlgR/AlgQ system, with decreased \textit{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 \textit{et al.}, 2001). An increased hydrophobicity and, moreover, an enhanced viscosity of the EPS were observed for the \textit{esta-} and the \textit{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 \textit{P. aeruginosa}. Furthermore, it has been reported that rhamnolipids induce the removal of LPS from the outer membrane of \textit{P. aeruginosa} (Al-Tahhan \textit{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 \textit{P. aeruginosa} microcolonies (Rendell \textit{et al.}, 1990; Maier & Soberón-Chávez, 2000; Davey \textit{et al.}, 2003). Moreover, rhamnolipids are essential for the swarming motility of non-mucoid \textit{P. aeruginosa} strains, which primarily depends on the motion of flagella and type IV pili (Köhler \textit{et al.}, 2000). Strikingly, \textit{P. aeruginosa} SG81estA+ and \textit{P. aeruginosa} SG81lasB+ showed a noticeable increase in swarming motility (Fig. 4). Also, swimming motility was slightly enhanced, while twitching motility was reduced for \textit{P. aeruginosa} SG81estA+. In a recent study, Wilhelm \textit{et al.} (2007a) demonstrated that a deletion in the \textit{esta} gene in the \textit{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 \textit{EstA-} overproduction strain they found an increased amount of rhamnolipids and an enhanced swarming motility. In the mucoid background of \textit{P. aeruginosa} SG81, the overexpression of \textit{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 \textit{esta}-overexpression strain, while the wild-type produced only di-rhamnolipid (Fig. 2), which is the same observation as that of Wilhelm \textit{et al.} (2007a) for the non-mucoid \textit{P. aeruginosa} PAO1.

Microscopic images of flow-chamber biofilms demonstrated the multiplication of progeny cells of the mucoid parent strain \textit{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 \textit{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 \textit{et al.}, 2003a), indicating that \textit{P. aeruginosa} biofilm development depends on the carbon source. Other studies of mature biofilms of mucoid \textit{P. aeruginosa} in different experimental systems have also reported a highly structured biofilm architecture based on microcolony formation during biofilm development (Hentzer \textit{et al.}, 2001; Nivens \textit{et al.}, 2001; Matz \textit{et al.}, 2004), also with gluconate as a carbon source (Tiene \textit{et al.}, 2005). Furthermore, Borriello and co-workers have reported anaerobic areas in the depths of biofilms (Borriello \textit{et al.}, 2004). The use of KNO3 as a nitrogen source in the mAMP used in this study permitted the growth of \textit{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 \textit{esta-}overexpression strain \textit{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 SG81lasB+ 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 swelling conditions (Overhage et al., 2008), indicating a potential role for LasB in the highly complex swelling 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 swelling 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 SG81lasB+ 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 staphylolysin elastase LasD (Folders et al., 2000). LasD in turn is necessary for the proteolytic degradation of the prepeptite 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 SG81lasB+ 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 PA01 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 SG81lipC+ 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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Extracellular enzymes and biofilm formation


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