Pseudomonas aeruginosa extracellular products inhibit staphylococcal growth, and disrupt established biofilms produced by Staphylococcus epidermidis

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Multiple bacterial species often coexist as communities, and compete for environmental resources. Here, we describe how an opportunistic pathogen, Pseudomonas aeruginosa, uses extracellular products to interact with the nosocomial pathogen Staphylococcus epidermidis. S. epidermidis biofilms and planktonic cultures were challenged with P. aeruginosa supernatant cultures overnight. Results indicated that quorum-sensing-controlled factors from P. aeruginosa supernatant inhibited S. epidermidis growth in planktonic cultures. We also found that P. aeruginosa extracellular products, mainly polysaccharides, disrupted established S. epidermidis biofilms. Cellulase-treated P. aeruginosa supernatant, and supernatant from pelA, pslF and pelApslBCD mutants, which are deficient in polysaccharide biosynthesis, diminished the disruption of S. epidermidis biofilms. In contrast, S. epidermidis supernatant in overnight cultures had no effect on established P. aeruginosa biofilms and planktonic growth. These findings reveal that P. aeruginosa extracellular products are important microbial competition factors that overcome competition with S. epidermidis, and the results may provide clues for the development of a novel strategy for controlling S. epidermidis biofilms.

INTRODUCTION

Microbial species coexist in multicellular communities, and compete for common nutritional resources (Smith, 2002). In most ecological niches, surface-associated bacteria often form tightly packed exopolysaccharide-encased colonies, known as biofilms, to survive in hostile environments, and to occupy nutritional niches (Hall-Stoodley et al., 2004). Recent studies have shown that certain bacterial species secrete extracellular products that inhibit the settlement of potential competitors (Burgess et al., 1999). Bacteria produce natural products, such as secreted signalling molecules, biosurfactant and polysaccharide, that interfere with biofilm formation and cell-to-cell communication (Irie et al., 2005; Rasmussen & Givskov, 2006; Valle et al., 2006). Because biofilms are the most common mode of bacterial growth in nature, we hypothesize that there are many different methods used by bacteria to disrupt established biofilms formed by other bacterial species.

To test this hypothesis, we investigated the interaction between Pseudomonas aeruginosa and Staphylococcus epidermidis. P. aeruginosa is a ubiquitous Gram-negative bacterium that is frequently responsible for nosocomial and burn infections. P. aeruginosa is a model organism for the study of quorum-sensing extracellular virulence factors and biofilm formation (Costerton et al., 1995; Latifi et al., 1995; Passador et al., 1993). P. aeruginosa possesses two N-acyl-L-homoserine lactone (AHL)-dependent quorum-
sensing systems (Pesci et al., 1997) and a 2-heptyl-3-hydroxy-4(1H)-quinolone (PQS)-based quorum-sensing system (D’Argenio et al., 2002; Diggle et al., 2003; McKnight et al., 2000). *P. aeruginosa* quorum-sensing systems regulate biosurfactant rhamnolipid production, motility and the release of biofilm matrix materials (Allesen-Holm et al., 2006; Pesci et al., 1997; Shrouf et al., 2006). *P. aeruginosa* also produces two distinct extracellular polysaccharides (from the *pel* and *psl* genes) as structural components of the biofilm matrix (Friedman & Kolter, 2004a).

*S. epidermidis* is a Gram-positive, spherical, pathogenic bacterium responsible for nosocomial infections. It forms biofilms on the surfaces of indwelling medical devices (Rupp & Archer, 1994). *P. aeruginosa* and *S. epidermidis* commonly coexist in infected patients; for example, in the colonization of surfaces of indwelling medical devices and hydrogel contact lenses (Gilsdorf et al., 1989; Henriques et al., 2005). In the lungs of cystic fibrosis (CF) patients, *P. aeruginosa* is the predominant bacterium, but it can coexist with other species, including staphylococci (Govan & Deretic, 1996).

In the present study, we show that quorum-sensing-controlled factors from *P. aeruginosa* supernatant can inhibit *S. epidermidis* growth in planktonic cultures. We also show that *P. aeruginosa* supernatant uses an extracellular-polysaccharide-dependent process to disrupt established *S. epidermidis* biofilms. The release of extracellular polysaccharide is independent of AHL- and PQS-based quorum-sensing systems.

**METHODS**

**Bacteria, and growth conditions.** *P. aeruginosa* PAO1 (strain PAO0001) was obtained from the Pseudomonas Genetic Stock Center (East Carolina University School of Medicine, Greenville, North Carolina, USA). The *lasH*III derivative was constructed by allelic displacement in PAO1, as described by Hentzer et al. (2003). The *pspA* mutant was constructed by D’Argenio et al. (2002) via transposon insertion in PAO1. The *rhlA* mutant was constructed by allelic displacement in PAO1, as described by Pamp & Tolker-Nielsen (2002). PA2233–PA2234, and *pelA* and *psl* mutants were from the *P. aeruginosa* PAO1 transposon mutant library (University of Washington, Seattle, Washington, USA) (Jacobs et al., 2003). *S. epidermidis* 1457 wild-type (WT) and the *agr* quorum-sensing mutant were kindly provided by Dr Yuan Lu (Li et al., 2004). Tryptic soy broth (TSB; Oxoid) containing 0.25% glucose was used as the culture medium for the production of biofilms by *S. epidermidis* in a static chamber system and in microtitre plates. Luria–Bertani (LB; Oxoid) broth was used as the culture medium for the production of *P. aeruginosa* biofilms and batch cultures were grown at 37°C, except when indicated otherwise. SYTO9 and propidium iodide (PI) (Live/Dead reagents; Molecular Probes) were used at a concentration of 1 μM for staining live (SYTO9, green signal) and dead (PI, red signal) bacteria in biofilms.

**Preparation of supernatants of *P. aeruginosa* and *S. epidermidis* strains.** Overnight cultures (~14 h) of *P. aeruginosa* in LB broth, or *S. epidermidis* in TSB containing 0.25% glucose, were centrifuged, and the crude supernatant was filtered (0.22 μm filter). Next, 10 μl filtered supernatant was plated on a LB or TSB agar plate to test possible contamination, and the remaining supernatant was stored at −20°C for further use.

**Cultivation of bacterial biofilms, and challenge by bacterial supernatants**

**Polystyrene microtitre plates.** Biofilm cultivation in polystyrene microtitre plates was carried out essentially as described by Christensen et al. (1985). Briefly, overnight cultures of *S. epidermidis* strains grown in TSB (containing 0.25% glucose) were diluted 1:200. The diluted cultures were transferred to wells of polystyrene microtitre plates (200 μl culture per well), and incubated at 37°C for 24 h. The wells were then washed gently three times with 200 μl sterile PBS, and a 100 μl volume of prepared *P. aeruginosa* supernatant was added to each well. LB broth was used instead of supernatant as a negative control in this assay. After 2 h incubation at 37°C, the wells were washed gently three times with 200 μl sterile PBS, air-dried, and stained with 2% crystal violet for 5 min. Then, the plate was rinsed under running tap water, air-dried, the crystal violet was resuspended in ethanol, and the OD490 was determined. For heat inactivation of the proteins in the *P. aeruginosa* supernatant, the supernatant was heated in a 100°C water bath for 20 min. To inactivate the polysaccharide, the supernatant was treated with cellulase (5 mg ml⁻¹; MP Biomedicals) at 37°C for 1 h, then the cellulase was inactivated by heat before it was added to the microtitre plates. Treatment of the supernatant with alginate lyase (1 mg ml⁻¹; Sigma) instead of cellulase was used for comparison.

**The static-chamber system.** *S. epidermidis* biofilms were grown in coverglass cell culture chambers (Nunc), as described previously (Qin et al., 2007). Briefly, overnight cultures of *S. epidermidis* strains grown in TSB (containing 0.25% glucose) were diluted to OD600 0.01, inoculated into the wells of a chamber (1.5 ml per well), and incubated at 37°C for 24 h. Then, the chamber was washed gently three times with 1 ml sterile PBS, and 1 ml prepared *P. aeruginosa* supernatant was added to each well. After incubation at 37°C for 2 h, the chamber was washed gently three times with 1 ml sterile PBS, stained by using the Live/Dead reagents for 15 min, and observed under the microscope.

**Construction of the *pelA* complemented strain.** For genetic complementation of the *pelA* mutant, a 2.8 kb DNA fragment containing the entire *pelA* locus, with a unique overhanging EcoRI or XbaI cloning site, was PCR amplified by use of the primers *pelAF* (5'-GGATTCATGCGGTCGATCAGAGAA-3') and *pelAR* (5'-GCTG-CTAGAGCAGCGGCGAATGTTG-3'). The amplified fragment was cloned into the shuttle vector pUCP22 (Herrero et al., 1998), and electroporated into the *pelA* mutant, yielding the complemented strain.

**Construction of the *pelApsiBCD* mutant.** The allelic exchange vector pMPSL-KO was constructed in pEX18 Ap by amplifying a DNA fragment containing genes PA2232–PA2235 of the *psl* locus by using primers MPLUS (5'-CGGATCCACTGCGTTCCGACACCTGG-ACGACT-3') and MPSLND (5'-CGAAGCTTCGAGCTGCCGACCGCGGCGC-3'). The PCR product was ligated to the suicide vector pEX18 Ap via KpnI and HindIII restriction sites. PA2233–PA2234, and parts of PA2232 and PA2235, were replaced by a blunt-ended Sad fragment containing the gentamicin-resistance cassette from pPS858. The construct pMPSL-KO1 was then mobilized into the *pelA* strain to generate an allelic replacement, as described by Hoang et al. (1998).

**Measurement of the polysaccharide in the supernatant of *P. aeruginosa* strains.** The supernatant of *P. aeruginosa* strains was diluted, and adjusted to 1 ml sample volume with sterile MilliQ water. A 0.5 ml volume of 6% phenol was added, and allowed to...
react for 10 min. Next, 2.5 ml H2SO4 was added to the solution, and allowed to react for 20 min. The OD600 was then measured. ABT minimal medium was used as a background control. The amount of polysaccharide in the supernatant of P. aeruginosa strains was calculated based on a standard glucose concentration curve, in which different concentrations of glucose were prepared in the same way as the P. aeruginosa supernatants.

**Crude polysaccharide matrix isolation.** Isolation of crude polysaccharide matrix from the supernatant of overnight cultures of P. aeruginosa strains was performed using a method based on an ethanol-precipitation method (Friedman & Kolter, 2004a, b). An aliquot of 1 M NaOH was added to the supernatant, and the sample was vortexed every 2 min for 15 min. The sample was centrifuged at 39,000 r.p.m. (19,000 g) in an ultracentrifuge for 1 h at 4 °C. The supernatant was removed, and filtered through a 0.2 μm filter. The filtrate was neutralized with concentrated HCl, precipitated by the addition of ethanol to 70%, and stored at −20 °C overnight. The precipitate was collected by centrifugation at 9000 r.p.m. (4400 g) for 30 min at 4 °C. The pellet was washed with 70% ethanol, allowed to dry for 45 min, resuspended in water, and then lyophilized. The lyophilized material was resuspended in water, dialysed against water, and then lyophilized, weighed, and resuspended in sterile PBS for further use.

**Microscopy and image acquisition.** All microscope observations and image acquisitions were performed with a Zeiss LSM 510 confocal scanning laser microscope (CLSM; Carl Zeiss) equipped with detectors and filter sets for monitoring SYTO9, PI, 7-hydroxy-9H-1,3-dichloro-9,9-dimethylacridin-2-one (DDAO) and tetramethylrhodamine isothiocyanate (TRITC) fluorescence. Images were obtained using a ×63/1.4 objective or a ×40/1.3i objective. Simulated 3D images and sections were generated using the IMARIS software package (Bitplane).

**Influence of P. aeruginosa supernatant on S. epidermidis growth.** An overnight culture of S. epidermidis was diluted to OD600 0.05 in 50 ml fresh TSB (containing 0.25% glucose). A 1 ml volume of prepared supernatant of P. aeruginosa was added, and the culture was incubated at 37 °C with shaking. The OD600 was measured at 1 h intervals.

**Lysis of S. epidermidis by P. aeruginosa supernatant.** Lysis of S. epidermidis on agar in a Petri dish was performed by thoroughly swabbing a TSB agar plate with an overnight culture of P. aeruginosa was spotted onto the agar, air-dried and incubated at 37 °C for 24 h. Plates were imaged using an Alpha Innotech documentation system.

**Statistical analysis.** Two-tailed Student’s t tests were performed with a computer (Excel 2007), and P<0.05 was considered significant.

**RESULTS**

**P. aeruginosa supernatant inhibits S. epidermidis growth.**

We first investigated the interaction of P. aeruginosa and S. epidermidis in planktonic conditions, and on agar plates. As shown in Fig. 1(a, b), addition of the supernatant from P. aeruginosa PAO1 WT inhibited the growth of S. epidermidis 1457 WT and agr quorum-sensing mutant cultures, as compared with the control. In contrast, the supernatant from two P. aeruginosa PAO1 quorum-sensing mutants (lasIrhlI and pqsA) did not inhibit S. epidermidis growth. The supernatant from P. aeruginosa rhlA (deficient in rhamnolipid production) showed an inhibitory effect that was similar to that of P. aeruginosa PAO1. This indicates that the quorum-sensing-regulated biosurfactant does not inhibit S. epidermidis growth. For P. aeruginosa PAO1 WT and lasIrhlI mutant, addition of the supernatant from either S. epidermidis 1457 WT or agr mutant (quorum-sensing mutant) had no influence on growth of P. aeruginosa (Fig. 1c, d). On TSB agar plates, overnight cultures of P. aeruginosa PAO1 WT and the rhlA mutant strain lysed S. epidermidis 1457 cells effectively. However,
the diameter of the lysis zone was decreased by approximately 75 and 70%, respectively, when overnight cultures of lasIRhlI and pqsA mutants were used (Fig. 1e, *P*<0.05). In contrast, overnight cultures of *S. epidermidis* strains did not lyse *P. aeruginosa* cells (data not shown).

**P. aeruginosa** supernatant disrupts established *S. epidermidis* biofilms

To test the interaction of *P. aeruginosa* and *S. epidermidis* under biofilm conditions, we challenged established biofilms of *P. aeruginosa* PAO1 WT with supernatant from *S. epidermidis* 1457 WT, and vice versa. Biofilms were grown in microtitre plates in TSB for 24 h, and treated with supernatants for 2 h at 37 °C. As controls, we treated bacterial biofilms with fresh LB broth or TSB. At the end of the treatment period, the residual biofilm was stained with crystal violet, and the OD590 was measured. The results showed that the supernatant from *P. aeruginosa* PAO1 could efficiently remove >80% (*P*<0.05) of the *S. epidermidis* biofilm, while the supernatant from *S. epidermidis* 1457 had no effect on the *P. aeruginosa* PAO1 biofilm (Fig. 2).

We further studied disruption of the *S. epidermidis* 1457 biofilm by *P. aeruginosa* supernatant in a static-chamber cultivation system (Fig. 3). An established biofilm (24 h) of *S. epidermidis* 1457 in the static-chamber system was treated with supernatant from *P. aeruginosa* PAO1 WT for 0, 20, 40, 60, 90 and 120 min at 37 °C. After washing, the cells in the biofilm were stained with SYTO9 and PI, and observed by CLSM. As the treatment time increased, the *P. aeruginosa* supernatant was able to reduce the multilayered *S. epidermidis* biofilms down to a single layer on the surface of the static chamber, but almost all of the residual biofilm cells were living (no red PI staining was observed in the images). Detached *S. epidermidis* cells were collected and stained with Live/Dead reagent. Results indicated that most of the bacteria were alive after detachment from the chamber surface (data not shown).

**Disruption of *S. epidermidis* biofilms by *P. aeruginosa* supernatant is mainly due to secreted polysaccharides**

The composition of *P. aeruginosa* supernatant is very complex, so we were interested in learning which components were involved in the disruption of *S. epidermidis* biofilms. Thus, we compared the biofilm-disruption abilities of supernatants from isogenic mutants of *P. aeruginosa* PAO1 in microtitre plates. The mutants were: the lasIRhlI double mutant, which is deficient in AHL-based quorum-sensing; the pqsA mutant, which is deficient in PQS-based quorum-sensing; the rhlA mutant, which is deficient in biosynthesis of biosurfactant rhamnolipid; the pelA mutant, which is deficient in biosynthesis of cellulase-susceptible polysaccharide; and the psf mutant, which is deficient in biosynthesis of another polysaccharide. The polysaccharide mutants were chosen because recently Valle *et al.* (2006) have reported that a soluble capsular polysaccharide derived from *Escherichia coli* strains prevents biofilm formation by a wide range of Gram-positive and Gram-negative bacteria. Therefore, we hypothesized that the extracellular polysaccharide produced by *P. aeruginosa* may have a similar function to that of the *E. coli* polysaccharide. Our results showed that the supernatants of *P. aeruginosa* PAO1 and the *rhlA* mutant substantially disrupted the biofilm formed by *S. epidermidis* 1457 in microtitre plates, and the supernatants of *P. aeruginosa* PAO1 quorum-sensing mutants (lasIRhlI and pqsA) showed only slightly less ability to disrupt the biofilm (Fig. 4a). Notably, among the mutants, the supernatant of the *pelA* strain had significantly decreased ability to remove the *S. epidermidis* biofilm (Fig. 4a, *P*<0.05). The supernatant of another polysaccharide-biosynthesis-deficient strain, the *pslF* mutant, also showed reduced ability for removing the *S. epidermidis* biofilm, producing results that were similar to those of the *pelA* mutant in our assay (*P*<0.05). The supernatant of the *pel* and *psl* double mutant (*pelApslBCD*) showed virtually no ability to remove the *S. epidermidis* biofilm (*P*<0.05). Interestingly, the supernatants from the *pelA* and the *pslF* mutants showed similar ability to *P. aeruginosa* PAO1 WT strain in inhibiting *S. epidermidis* growth in planktonic conditions (Fig. 4b). These data also indicate that the extracellular polysaccharide secreted by *P. aeruginosa* is not involved in inhibition of *S. epidermidis* growth.

In addition, similar results to those obtained using microtitre plates were obtained using the static-chamber system (Fig. 5). The control *S. epidermidis* culture (treated with LB broth) formed compact microcolonial biofilm structures (Fig. 5a), while treatment with the supernatants of *P. aeruginosa* PAO1 and the *rhlA* mutant completely

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**Fig. 2.** Established ageing biofilms (24 h-old) of *P. aeruginosa* PAO1 or *S. epidermidis* 1457 on microtitre plates treated with *S. epidermidis* 1457 or *P. aeruginosa* PAO1 supernatant for 2 h at 37 °C. The values are means of three separate assays, and the bars indicate so. *P*<0.05 (vs medium control) in Student's *t* test.

■, Medium control; □, supernatant.
reduced these compact structures to single-cell layers (Fig. 5b, e). The supernatant of the *P. aeruginosa* PAO1 quorum-sensing mutants (*lasIrhlI* and *pqsA*) showed reduced ability (Fig. 5c, d) to inhibit the biofilm structure. Interestingly, treatment with supernatant of the *pelA* or the *pslF* mutant had little effect on the surface microcolonies, and this was especially so for the *pelApslBCD* double mutant (Fig. 5f–h).

To investigate whether cellulase-susceptible polysaccharide from the *P. aeruginosa* PAO1 WT supernatant was able to disrupt *S. epidermidis* biofilms, we used cellulase-pretreated *P. aeruginosa* PAO1 supernatant to treat an established *S. epidermidis* biofilm. We found that cellulase-pretreated PAO1 supernatant had virtually no ability to remove *S. epidermidis* biofilms (Fig. 6, *P* < 0.05). As a control, alginate-lyase pretreatment of the *P. aeruginosa* PAO1 supernatant had no effect on its ability to disrupt biofilms. Heating the supernatant did not destroy the inhibitory ability either. A complementation plasmid carrying the *pelA* gene (*pelA+pUCP22::pelA*) was able to restore the biofilm-removing ability of the supernatant from the *pelA* mutant, whereas the control plasmid (*pelA+pUCP22*) was not able to restore the activity (Fig. 6).

We also measured the amount of polysaccharide in the supernatant from *P. aeruginosa* PAO1 WT and its isogenic mutants. The results showed that the amount of polysaccharide was similar in supernatants from *P. aeruginosa* PAO1 and the quorum-sensing mutants *lasIrhlI* and *pqsA* (0.8–0.9 mg ml$^{-1}$). In comparison, the amount of polysaccharide was significantly reduced in the supernatants from *P. aeruginosa* *pelA* (~0.3 mg ml$^{-1}$) and *pslF* (0.5 mg ml$^{-1}$) mutants, and reduced even further in that of the *pelApslBCD* mutant (only ~0.06 mg ml$^{-1}$). The *pelA* complemented strain (*pelA+pUCP22::pelA*) recovered polysaccharide levels similar to that produced by PAO1, but the control plasmid strain (*pelA+pUCP22*) produced results that were similar to those of the *pelA* mutant (Fig. 7).

Finally, to confirm that *P. aeruginosa* extracellular polysaccharide is involved in disrupting established *S. epidermidis* biofilms, we isolated the crude polysaccharide.

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**Fig. 3.** Established biofilms (24 h) of *S. epidermidis* 1457 WT in the static chamber were treated with supernatant of *P. aeruginosa* PAO1 WT for 0 (a), 20 (b), 40 (c), 60 (d), 90 (e) and 120 min (f) at 37 °C. The cells in biofilms were stained with SYTO9 (green) and PI (red), and observed by using CLSM. Bars, 30 μm. Each image represents a typical image obtained from three independent experiments.

**Fig. 4.** (a) Established ageing biofilms (24 h-old) of *S. epidermidis* 1457 on microtitre plates were treated with supernatant of *P. aeruginosa* PAO1 wild-type and isogenic mutant strains for 2 h at 37 °C. The cells in biofilms were stained with crystal violet and measured at OD$_{590}$. The values are means of three separate assays, and the bars indicate sd. *P* < 0.05 (vs PAO1) in Student’s t test. ◆, LB control; ■, PAO1; ▲, *pelA*; ●, *pslF*. (b) Effect of supernatants of *P. aeruginosa* cultures on growth of *S. epidermidis* 1457 WT in planktonic culture. Each curve represents a typical curve obtained from three independent experiments.
matrices from the supernatant of \textit{P. aeruginosa} strains, and assessed their effects on the biofilms by measuring the OD$_{590}$. Compared with the PBS control, the matrices from \textit{P. aeruginosa} PAO1 and quorum-sensing mutants showed marked disruption of the biofilms in microtitre plates (Fig. 8). In contrast, the matrices isolated from \textit{P. aeruginosa} \textit{pelA}, \textit{pslF} and \textit{pelApslBCD} mutants had significantly less ability to disrupt the biofilms (\(P<0.05\)).

**DISCUSSION**

Bacteria grown in biofilms have greatly enhanced tolerance to stress, antimicrobial agents, and host immunological defences (Hall-Stoodley \textit{et al.}, 2004). Biofilm-related infections pose serious health problems for hospital patients with indwelling medical devices (Donlan, 2001). Thus, developing effective anti-biofilm strategies for the treatment of biofilm-related infections is critical. However, once established, biofilms are extremely difficult to eradicate. Current first-line antibiotics are ineffective at eradicating biofilms (Hall-Stoodley \textit{et al.}, 2004). 2-Heptyl-4-hydroxyquinoline N-oxide from \textit{P. aeruginosa} cultures has been reported to have anti-staphylococcal activity (Machan \textit{et al.}, 1992). Our results suggest that the production of these anti-staphylococcal compounds is under the control of quorum sensing, because \textit{P. aeruginosa} \textit{lasIrhlI} and \textit{pqsA} mutants showed marked reduction in the production of anti-staphylococcal compounds in plank-

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**Fig. 5.** Established biofilms (24 h) of \textit{S. epidermidis} 1457 WT in the static-chamber cultivation system were treated with LB medium control (a), the supernatant of \textit{P. aeruginosa} PAO1 WT (b), and mutants \textit{lasIrhlI} (c), \textit{pqsA} (d), \textit{rhlA} (e), \textit{pelA} (f), \textit{pslF} (g) and \textit{pelApslBCD} (h) for 2 h at 37 °C. The cells in biofilms were stained with SYTO9 (green) and PI (red), and observed using CLSM. Bars, 30 μm. Each image represents a typical image obtained from three independent experiments.

**Fig. 6.** Established ageing biofilms (24 h-old) of \textit{S. epidermidis} 1457 on microtitre plates were treated with differently pretreated supernatants of \textit{P. aeruginosa} PAO1, \textit{pelA} complementation, and plasmid-control strains. Before being added to \textit{S. epidermidis} biofilms, \textit{P. aeruginosa} PAO1 supernatants were treated with cellulase (5 mg ml$^{-1}$) at 37 °C for 1 h and heat-inactivated at 100 °C for 20 min and cooled, or treated with alginate lyase (10 mg ml$^{-1}$) at 37 °C for 1 h and heat-inactivated at 100 °C for 20 min and cooled. All the treatments lasted for 2 h at 37 °C. The cells in biofilms were stained with crystal violet and measured using OD$_{590}$. The values are means of three separate assays, and the bars indicate sd. *\(P<0.05\) (vs PAO1 boiled treatment) in Student’s \(t\) test.

**Fig. 7.** Measurement of the amount of polysaccharide in the supernatant from \textit{P. aeruginosa} PAO1 and mutants. The values are means of three separate assays, and the bars indicate sd. *\(P<0.05\) (vs PAO1) in Student’s \(t\) test.
The lytic activity of LasA protease against S. epidermidis is notable staphylolytic activity is LasA protease (Kessler et al., 2005). Another extracellular protein secreted by P. aeruginosa that has significant antibiotic activity against Gram-positive bacteria is LasA protease (Kessler et al., 2005, Machan et al., 1992). Antimicrobial quinolones can be packaged into extracellular membrane vesicles (MV) to cause direct lysis of S. epidermidis (Mashburn & Whiteley, 2005). Another extracellular protein secreted by P. aeruginosa that has notable staphyloytic activity is LasA protease (Kessler et al., 1993), and its expression is under the control of a lasIhlI quorum-sensing system (Nouwens et al., 2003).

Moreover, the anti-staphylococcal compounds controlled by P. aeruginosa quorum-sensing systems (especially the pqs system) may be partially involved in disrupting S. epidermidis biofilms, although our results showed that this contribution is very limited (Figs 4 and 5). Interestingly, MVs have also been found in the matrix of P. aeruginosa biofilms (Schooling & Beveridge, 2006), but it remains unknown whether such MVs are effective against S. epidermidis cells embedded in a biofilm structure. Likewise, the same question exists regarding the staphyloytic activity of LasA protease against S. epidermidis biofilm cells. In most cases, the cell density is always low when a bacterium starts to invade niches occupied by other species. Thus, the organism is unable to activate its quorum-sensing systems and use quorum-sensing-regulated products to compete with other species. This may explain events that occur in some CF patients: in the early stage of CF, P. aeruginosa inhabits lung tissues, along with other species, including staphylococci, and P. aeruginosa eventually becomes the only or the prominent pathogen within these patients as their disease progresses (Govan & Deretic, 1996).

In this study, we reported that P. aeruginosa might employ quorum-sensing-independent extracellular products (mainly polysaccharides) to disrupt established S. epidermidis biofilms. Two operons have been found to be involved in the biosynthesis of polysaccharide in P. aeruginosa: pel (Friedman & Kolter, 2004b) and psl (Jackson et al., 2004). The pel operon contains seven adjacent genes that are responsible for the production of a glucose-rich matrix material required for the formation of biofilms by P. aeruginosa PA14 strain (Friedman & Kolter, 2004b). The exopolysaccharide encoded by the psl locus is essential for P. aeruginosa PA01 biofilm formation, because a disruption of the first two genes of the psl cluster (PA2231 and PA2232) severely compromises biofilm initiation (Jackson et al., 2004). Interestingly, our results indicated that the polysaccharides produced by the pel and psl systems were able to disrupt established S. epidermidis biofilms. This type of disruption activity by the polysaccharides has also been found in Staphylococcus aureus biofilms (Z. Qin & others, unpublished data).

We hypothesize that the disruption of staphylococcal biofilms by P. aeruginosa polysaccharide is independent of a bactericidal effect, because the supernatants from P. aeruginosa pelA and pslF mutants were able to inhibit S. epidermidis growth in planktonic conditions, as did the supernatant from the PA01 WT strain (Fig. 4). Moreover, the S. epidermidis cells detached from the biofilms after P. aeruginosa supernatant treatment have been shown to be mostly alive (Z. Qin & others, unpublished data). Our data also indicate that the biosynthesis of polysaccharide by pel and psl is independent of the quorum-sensing systems, because the production of polysaccharide in P. aeruginosa quorum-sensing mutants (lasIhlI and pqsA) was comparable to that of the PA01 WT strain (Fig. 7). More recently, it has been reported that P. aeruginosa can produce an organic compound: cis-2-decenoic acid, which is capable of inducing the dispersion of established biofilms and inhibiting biofilm development by many Gram-positive and Gram-negative bacteria (Davies & Marques, 2009). However, it remains unknown whether the regulation of cis-2-decenoic acid biosynthesis is associated with pel, psl or quorum-sensing systems.

Interestingly, the major structural component of the staphylococcal biofilm matrix is polysaccharide intercellular adhesin (Heilmann et al., 1996). Therefore, it is of interest to know how polysaccharide derived from P. aeruginosa can interact with the staphylococcal biofilm matrix components (e.g. polysaccharide intercellular adhesin) to disrupt them. Although the polysaccharide encoded by pel in P. aeruginosa is cellulase susceptible, chemical analysis has revealed that it is not cellulose (Friedman & Kolter, 2004b). These findings warrant further investigation into the chemical structure of these polysaccharides produced by P. aeruginosa. Such studies may provide more...
information about the mechanisms of biofilm removal by *P. aeruginosa*. Despite these unresolved questions, extracellular polysaccharides secreted by *P. aeruginosa* could represent a promising strategy to be used against staphylococcal biofilms in future applications.

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**REFERENCES**


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