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Rhamnolipid Surfactant Production Affects Biofilm Architecture in *Pseudomonas aeruginosa* PAO1†

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In response to certain environmental signals, bacteria will differentiate from an independent free-living mode of growth and take up an interdependent surface-attached existence. These surface-attached microbial communities are known as biofilms. In flowing systems where nutrients are available, biofilms can develop into elaborate three-dimensional structures. The development of biofilm architecture, particularly the spatial arrangement of colonies within the matrix and the open areas surrounding the colonies, is thought to be fundamental to the function of these complex communities. Here we report a new role for rhamnolipid surfactants produced by the opportunistic pathogen *Pseudomonas aeruginosa* in the maintenance of biofilm architecture. Biofilms produced by mutants deficient in rhamnolipid synthesis do not maintain the noncolonized channels surrounding macrocolonies. We provide evidence that surfactants may be able to maintain open channels by affecting cell-cell interactions and the attachment of bacterial cells to surfaces. The induced synthesis of rhamnolipids during the later stages of biofilm development (when cell density is high) implies an active mechanism whereby the bacteria exploit intercellular interaction and communication to actively maintain these channels. We propose that the maintenance of biofilm architecture represents a previously unrecognized step in the development of these microbial communities.

Although bacteria are commonly viewed as solitary life forms, these organisms are more typically colonial creatures. In their natural settings, bacteria persist within microbial communities, where they exploit elaborate systems of intercellular interaction and communication to adjust to changing environmental parameters. Moreover, biofilm formation has also been linked to the emergence of a variety of opportunistic human pathogens (5). For example, organisms such as *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* form biofilms on implants and dead or living tissue, thereby contributing to a variety of persistent infections.

Thinking about bacterial populations as connected organisms capable of concerted multicellular activities has provided researchers with novel insights into microbial biology. The key to such multicellular behavior lies in the ability of each individual cell to sense and respond to information from nearby cells, and this behavior requires a certain population size or quorum of cells. The development of biofilms is a process that involves both a quorum of cells and multicellular behavior (6). Single-species biofilms are of particular interest due to their clinical importance and the monospecies biofilms formed by *P. aeruginosa* has become a prominent model for studying this aspect of microbial biology.

The complex structure of microbial biofilms has only recently been determined. Detailed analysis by scanning confocal laser microscopy has shown that biofilms of *P. aeruginosa* formed on solid surfaces and exposed to a continuous flow of fresh nutrients are open, highly hydrated structures consisting of cells embedded in an extracellular matrix filled with large void spaces (14). These void spaces, or channels, allow fluids to flow throughout the biofilm, resulting in the distribution of nutrients and oxygen. In addition, the channels between macrocolonies may also provide a means of removing metabolic end products.

It has been hypothesized that open-channel formation is not a stochastic process but instead represents an active process that creates a preferable structural design. For example, under some conditions *P. aeruginosa* mutants unable to make quorum-sensing molecules initiate biofilm formation but are unable to make normally structured biofilms (7). Other *P. aeruginosa* mutants, including those deficient in flagellum and pilus synthesis, as well as those with mutations in global regulators such as *gacA* and *crc*, have defects in the initial colonization of a surface (9, 22, 24, 27). Taken together, these data suggest that formation of the distinctive architecture of *P. aeruginosa* biofilms, including the macrocolonies surrounded by fluid-filled channels, is a regulated developmental process requiring distinct genetic surface structures and regulatory elements (23, 29, 30, 35). However, mechanisms by which the bacteria maintain these channels once they form have not been investigated. Our data indicate *P. aeruginosa* not only regulates development of its distinctive biofilm architecture but, once channels form, this organism utilizes rhamnolipid surfactants to actively maintain the void spaces surrounding macrocolonies. That is, we propose that rhamnolipids are not required for the formation of macrocolonies and channels but participate in the maintenance of channels once they are formed.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *P. aeruginosa* PAO1 was the wild-type strain used in the present study (13), and the *rhlA*::Tn*5* mutant strain was described previously (19). Plasmid pSMC21 (GFP^+ Ap^r Kn^r) constitutively expresses *gfp* and was used to label the strains for microscopy studies (1), and

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† For a commentary on this article, see page 699 in this issue.

plasmid pMH520 contains a *rhlA*::*gfp* transcriptional fusion (a gift from Matt Parsek, Northwestern University). All flow cell experiments were performed in EPRI medium as described previously (7), except that morpholinepropanesulfonic acid served as the buffer (2.2%) and the medium was supplemented with phosphate (0.0019% KH_2PO_4, 0.0063% K_2HPO_4). Dirhamnolipids were purified from the supernatant of *P. aeruginosa* as described previously (37) and were a gift of Thea Norman at Microbia, Inc. (Cambridge, Mass.).

Microtiter dish assay for biofilm formation and flow chamber experiments. For the biofilm formation assay, M63 medium (100 μl/well) containing arginine (0.05%) and $MgSO_4$ (100 μM) is inoculated (1:50) with an overnight L-broth-grown culture as described previously (25). The microtiter plates were then incubated at 37°C for the times indicated. The biofilm is macroscopically visualized by addition of 100 μl of a 0.1% solution of crystal violet to each well. Biofilm formation was quantified by the addition of 125 μl of 100% ethanol to each crystal violet-stained microtiter dish well; after 10 min 100 μl was transferred to a new microtiter dish, and the absorbance was determined with a plate reader at 550 nm.

Biofilms were also cultivated in flow chambers with channel dimensions of 5 by 1 by 30 mm. The flow system was assembled as described previously (4). The flow cell was inoculated from overnight L-broth-grown cultures diluted 10-fold in EPRI medium (final concentration, 10^8/ml). The medium flow was turned off prior to inoculation and for 1 h after inoculation. Thereafter, medium was pumped through the flow cell at a constant rate (1.8 ml/h) for the duration of the experiment. The flow was controlled with a PumpPro MPL (Watson-Marlow).

Microscopy and staining. A Leica DM IRB inverted microscope (Leica Microsystems, Wetzlar, Germany) equipped with a cooled charge-coupled device digital camera and a ×10 or ×63 PL Flotar objective lens was used for epifluorescence and phase-contrast microscopy analyses. With this instrument, digital images were captured and processed by using a G4 Macintosh computer with OpenLab software package (Improvision, Coventry, England). The images were processed for publication by using Photoshop software (Adobe, Mountain View, Calif.). Quantitative analysis of the flow cell-grown biofilms was performed with the COMSTAT image analysis software package (11, 12).

Mixing experiments. Mixing experiments were performed by inoculating ~10^7 bacteria of both the wild type (carrying the pSMC21 *gfp*$^+$ plasmid) and the *rhlA* mutant into the same channel of the flow cell. Biofilms were allowed to form for 6 days, and then the cells were stained with 5-cyano-2,3-di-4-tolyl-tetrazolium chloride (CTC; Polysciences, Inc., Warrington, Pa.), a metabolic stain that renders metabolically active cells red. Cells expressing green fluorescent protein (GFP) quenched the red fluorescence and thus allowed the wild type and the *rhlA* mutants to be distinguished. Channels inoculated with the wild type alone or with the *rhlA* mutant alone were included in each experiment as controls. No difference in architecture or staining was observed when the *rhlA* mutant carried the GFP-expressing plasmid (data not shown).

Invasion experiments. The wild-type strain was allowed to form a biofilm in a flow cell for 6 days. The biofilm was stained with CTC for 1 h in the absence of flow, and then flow resumed for 2 h to remove the residual dye. This preformed, stained biofilm was "invaded" with ~10^7 wild-type cells carrying the pSMC21 *gfp*$^+$ plasmid, and the flow was stopped for 1 h. After the resumption of flow for an additional hour to remove the unattached bacteria, the biofilm was examined for red and green fluorescence, and the individual images were merged to form a composite of preformed biofilm cells and GFP-labeled invading cells.

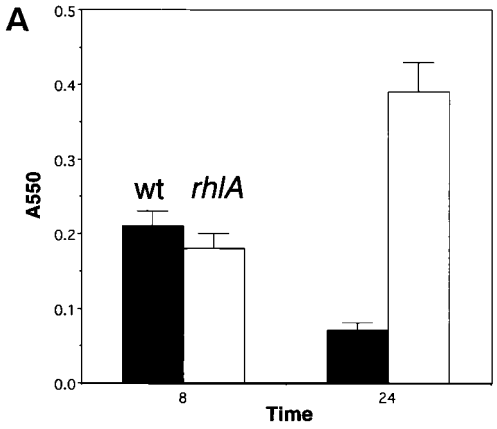

FIG. 1. A rhamnolipid mutant produces biofilms lacking characteristic architecture. (A) Biofilm formation by the wild type and *rhlA* mutant in microtiter dishes. The biofilm formation phenotype of the wild type (solid bars) and *rhlA* mutant (open bars) was quantitated over 24 h by using the microtiter dish assay, as reported elsewhere (26). The A_{550} value represents crystal violet-stained bacteria attached to the walls of the microtiter dish and is an indirect measure of the biofilm formed. The data represent two experiments, each performed in triplicate. (B) Flow cell architecture of mature biofilms. The wild-type and *rhlA*::Tn*5* strains carrying plasmid pSMC21, which constitutively expresses GFP, were inoculated into flow cells fed by minimal salts EPRI medium supplemented with glucose as the carbon and/or energy source. The top-down views shown here were acquired by epifluorescent microscopy by using the ×63 objective lens, which was used to monitor biofilm development in flow cells over 6 days. The large green macrocolonies are surrounded by dark areas, which are the sparsely colonized channel regions. At day 4, there is no difference between the wild type and the *rhlA* mutant, thus demonstrating that rhamnolipids are not required for the formation of macrocolonies. For the *rhlA* mutant, partial filling of the channels can be observed by day 5 and the channels are completely filled in this strain by day 6. The wild-type strain at day 6 displays the characteristic architecture of a mature *P. aeruginosa* biofilm. Scale bars are included and labeled on the figure. (C) Flow cell architecture of mature biofilms visualized under low magnification. This panel shows a day 6 biofilm obtained at low magnification (with a ×10 objective lens versus the ×63 objective lens used for the images in panel B). Scale bars are included and are labeled on the figure.

RESULTS

Rhamnolipids are required to maintain biofilm architecture. It was shown previously that the *lasR-lasI* quorum-sensing system in *P. aeruginosa* is involved in the later stages of biofilm differentiation, when cell density is sufficient to constitute a quorum (7). A biofilm produced by the *lasI* mutant is undifferentiated, i.e., the cells appear to grow as a continuous sheet on the surface lacking the characteristic water channels, indicating that *P. aeruginosa* has one (or more) systems to control the formation of macrocolony structures. Because *lasI* controls a whole suite of genes, the specific gene products contributing to this phenotype were unknown. One function controlled by the *lasI-lasR* system (indirectly via the *rhlI-rhlR* system) is rhamnolipid biosynthesis. Therefore, we investigated the biofilm formation phenotype of a mutant strain unable to synthesize rhamnolipids. The quorum-sensing-controlled *rhlA* gene codes for a rhamnosyltransferase whose only known function is in rhamnolipid synthesis. An *rhlA* mutant does not make rhamnolipids (20, 21).

To study the role rhamnolipids played in the formation of surface-attached communities, we monitored biofilm development of wild-type *P. aeruginosa* PAO1 and a *rhlA*-null mutant. Early biofilm formation was assayed in the microtiter dish assay. The wild-type strain forms a biofilm that peaks at 8 h and then declines at 24 h (Fig. 1A). Previous studies indicated that the *P. aeruginosa* biofilm formed in a 96-well microtiter dish declines after ca. 10 h of incubation, probably due to starvation in this batch culture system (25). In contrast, the biomass of the *rhlA* mutant biofilm continued to increase over this 24-h period to a final A_{550} value twofold greater than the maximum observed for the wild type. There was no discernible difference in the planktonic growth of these two strains. These data suggest

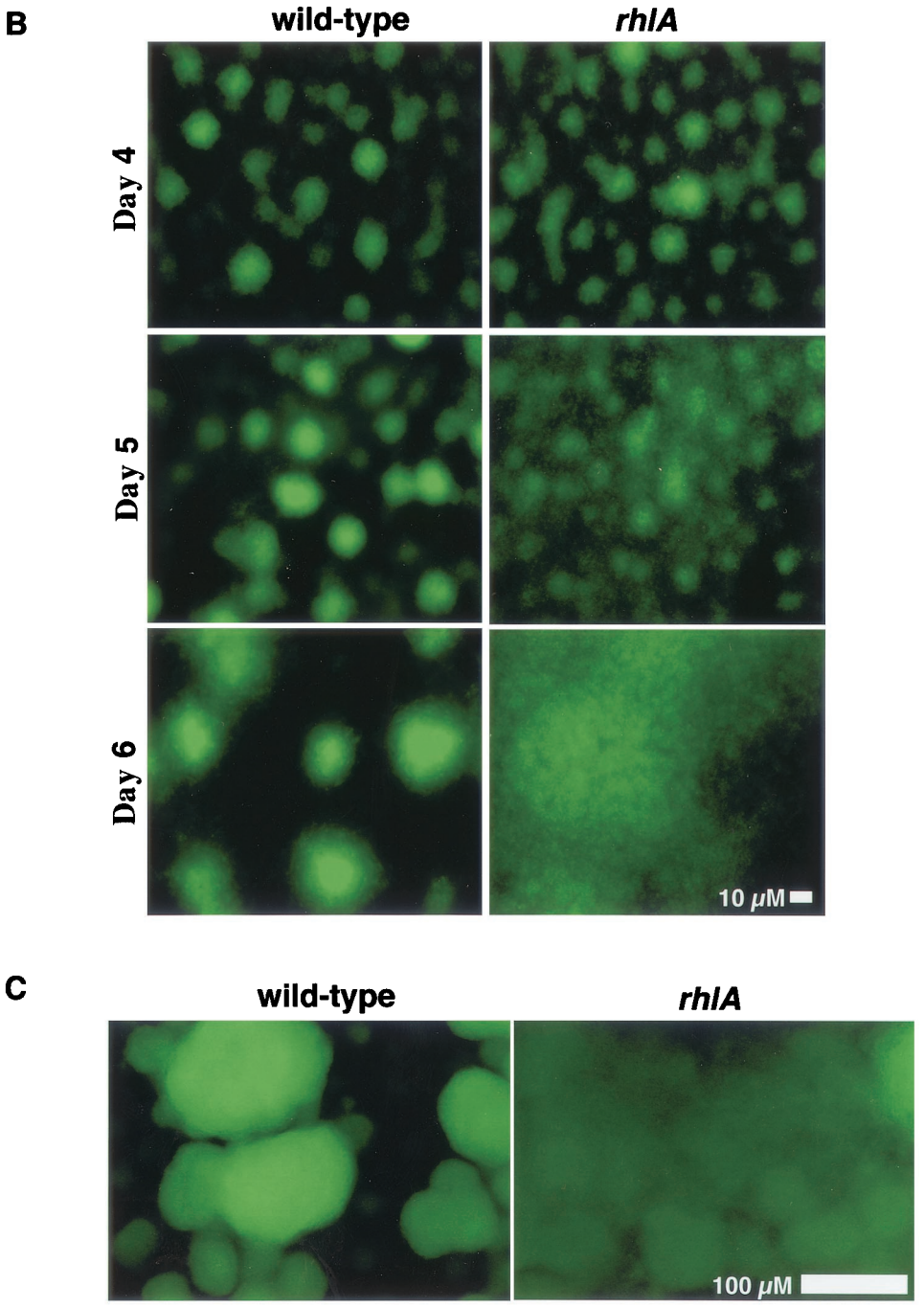

FIG. 1—*Continued.*

that the *rhlA* mutant is more proficient at early colonization than is the wild-type strain.

We followed further biofilm maturation over the course of 6 days by using a once-through flow cell system that provides a continuous supply of fresh nutrients to the biofilm. To monitor the bacteria by epifluorescence microscopy, the strains were transformed with plasmid pSMC21, which constitutively expresses the GFP. We had shown previously that this plasmid is maintained in the absence of antibiotic selection and confers no detectable metabolic load on the cells (1).

Macrocolony formation by the wild-type and *rhlA* strains appeared to be identical after 4 days of incubation (Fig. 1B). Both strains formed well-defined fluorescent (green) colonies separated by dark, fluid-filled channels. This observation indicates that the small advantage gained by the *rhlA* mutant in initial colonization in a microtiter dish assay (Fig. 1A) does not translate to an observable phenotype in the architecture of the 4-day-old flow cell-grown biofilm. By day 6, wild-type architecture was comprised of characteristic macrocolonies surrounded by open channels. The *rhlA* mutant, however, pro-

TABLE 1. Quantitative analysis of biofilm architecture

Strain	Mean ± SEM[a]			
	Thickness (μm)	Substratum coverage (%)	Roughness coefficient	Surface/volume ratio ($\mu m^2\ \mu m^{-3}$)
Wild type	15.2 ± 6.74	0.34 ± 0.09	1.28 ± 0.17	0.15 ± 0.06
rhlA::Tn*5* mutant	78.26 ± 20.4	0.87 ± 0.13	0.3 ± 0.27	0.05 ± 0.03

[a] Values are means of data from 12 *z*-series image stacks (three image stacks from two channels in two independent flow cell experiments at day 6).

duced a biofilm that was a thick, uniform mat of bacterial cells (Fig. 1B). Visualizing the biofilm at a lower magnification confirmed the difference in architecture (Fig. 1C). These data indicated that the *rhlA* mutant was able to form the channels surrounding the macrocolonies but was unable to maintain these channels.

Quantitative analysis of biofilm architecture. Visual inspection of biofilms produced by wild-type and the rhamnolipid mutant at day 6 indicated an altered biofilm structure for the mutant. To confirm this observation, we applied the COMSTAT image analysis program to perform a quantitative analysis of biofilm architecture (11, 12). As shown in Table 1, four variables (mean thickness, substratum coverage, roughness coefficient, and surface/volume ratio) were used to evaluate biofilm architecture. The mean thickness of the biofilm and the percent substratum coverage were much higher in the biofilms formed by the rhamnolipid mutant, a finding consistent with the images shown in Fig. 1. The roughness coefficient was much lower in the mutant, demonstrating that biofilms produced in the absence of rhamnolipids are much less heterogeneous. In addition, the surface/volume ratio was significantly lower in the biofilm produced by the mutant, indicating that a smaller fraction of the biomass is exposed to the nutrient flow. These data confirm the visual observation that the *rhlA* mutant produces a biofilm consisting of a thick, uniform mat of bacterial cells.

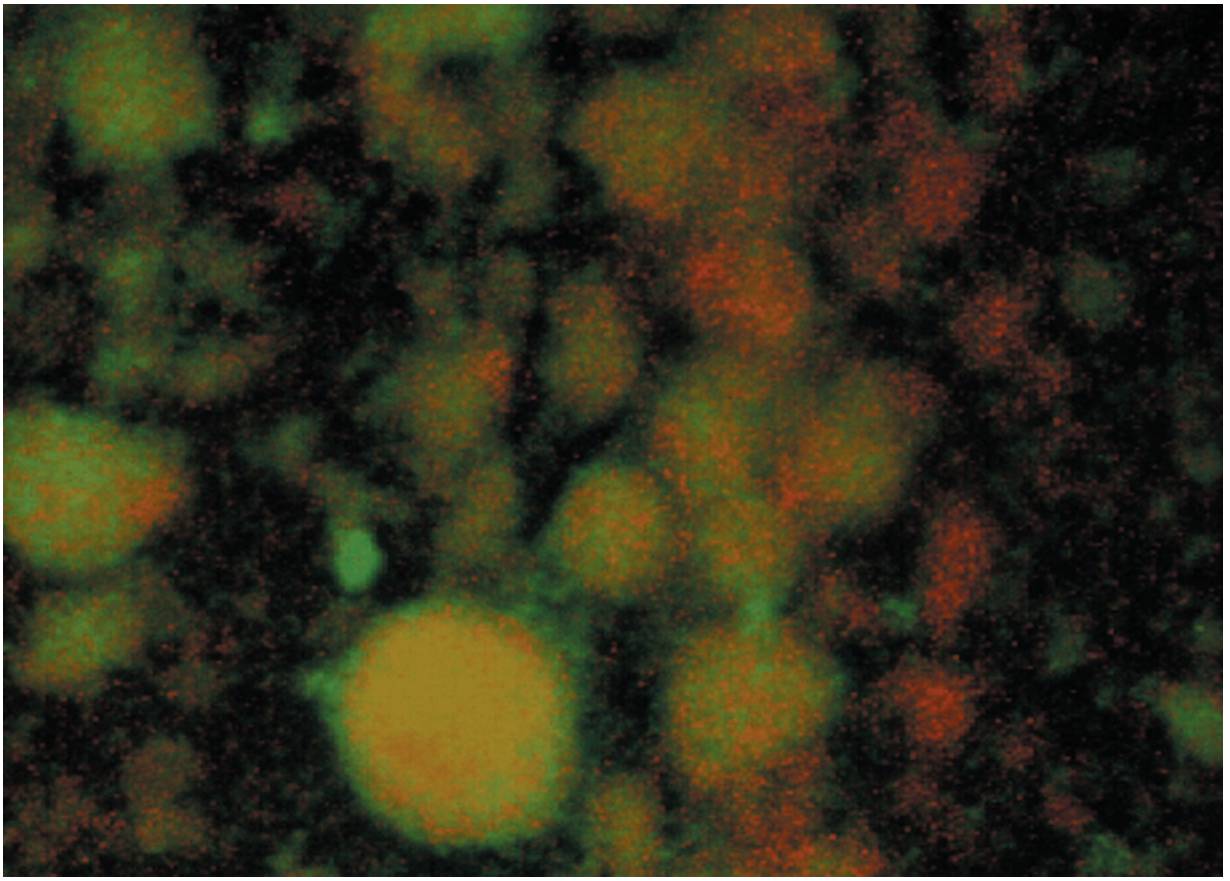

FIG. 2. Partial rescue of the *rhlA* mutant architecture phenotype by coculture with the wild-type strain. A mixing experiment was done to evaluate the ability of the wild type to rescue the phenotype of the *rhlA* mutant. The image is a top-down epifluorescent view of a 6-day-old biofilm formed in a flow cell where wild-type cells was mixed 50:50 with the *rhlA* mutant obtained with a ×10 objective lens. The *rhlA* mutants cells were visualized by CTC staining (red cells, see Materials and Methods), and the wild-type strain expresses the GFP from a plasmid (green cells). Regions of overlapping wild-type and mutant bacteria are yellow.

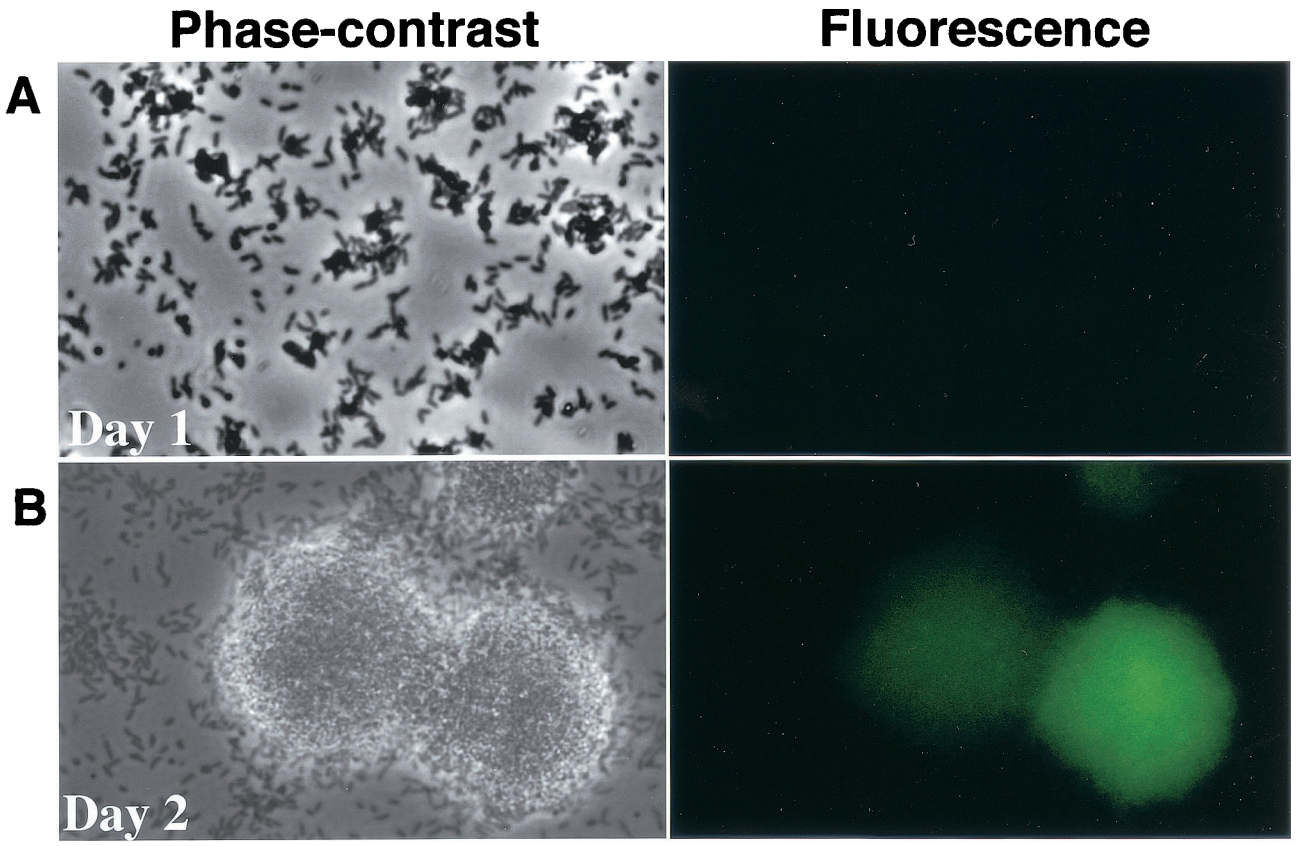

FIG. 3. Expression pattern of an *rhlA*::*gfp* transcriptional fusion during biofilm formation. The images are top-down views of individual cells and colonies at day 1 (A) and day 2 (B) for flow cell-grown bacteria (obtained with the ×63 objective lens). Both phase-contrast and fluorescent images of the same view are shown. Small clusters of bacteria produced no detectable fluorescence; however, larger colonies (high cell density) resulted in expression of the *rhlA*::*gfp* fusion.

Partial rescue of the *rhlA* biofilm architecture defect by the wild-type strain. If the defect in biofilm development is the result of a lack of rhamnolipid production, it follows that providing rhamnolipids should restore wild-type biofilm architecture. Rhamnolipids are likely produced by a certain subset of cells in response to specific environmental cues, resulting in surfactant production at a specific time and place, as has been reported elsewhere (8). Consistent with this idea, providing spent supernatants of *P. aeruginosa* cultures was insufficient to rescue the architecture defect of a *rhlA* mutant (data not shown). As an alternative approach to test the ability of rhamnolipids to rescue the phenotype of a *rhlA* mutant, we monitored biofilm development in a flow cell inoculated with a mixture of equal numbers of wild-type PAO1 (green cells) and the *rhlA*::Tn*5* mutant (red cells). As seen in Fig. 2, this mixing experiment resulted in a biofilm that consisted of macrocolonies made up in large part of a mixture of wild-type and *rhlA* mutant bacteria. Despite the presence of wild-type bacteria in many macrocolonies, the resulting biofilm is a mixture of typical wild-type architecture (comprised of macrocolonies surrounded by open areas) and dense compact areas where the channels were occupied by cells, a phenotype that is reminiscent of the *rhlA* mutant. These data indicate that the presence of the wild type could only partially rescue the biofilm architecture defect of the *rhlA* mutant.

Expression of *rhlA* during biofilm development. To evaluate where and when rhamnolipids are synthesized in the biofilm, we examined the expression of the *rhlA* gene during development. Expression of *rhlA* was determined indirectly by using plasmid p*rhlA*::*gfp*, which harbors a transcriptional fusion of the *rhlA* promoter region to the GFP. As shown in Fig. 3, on day 1, small microcolonies of 10 to 30 cells associated with the early stages in biofilm development can be seen by phase-contrast microscopy; however, no *rhlA-gfp*-dependent fluorescence was observed at this time point. However, by day 2, larger colonies comprised of thousands of fluorescent cells could be detected, indicating expression of the *rhlA* gene. Expression of this fusion was found to coincide with macrocolony formation and continued throughout biofilm development in areas where the cell density was high. The expression of *rhlA* only in macrocolonies is consistent with the observation that rhamnolipids are not required for the initiation of biofilm development. In control experiments, we could observe the fluorescence of individual batch grown planktonic, stationary-phase cells carrying the p*rhlA-gfp* plasmid (not shown). These data demonstrate that the digital camera used to obtain images

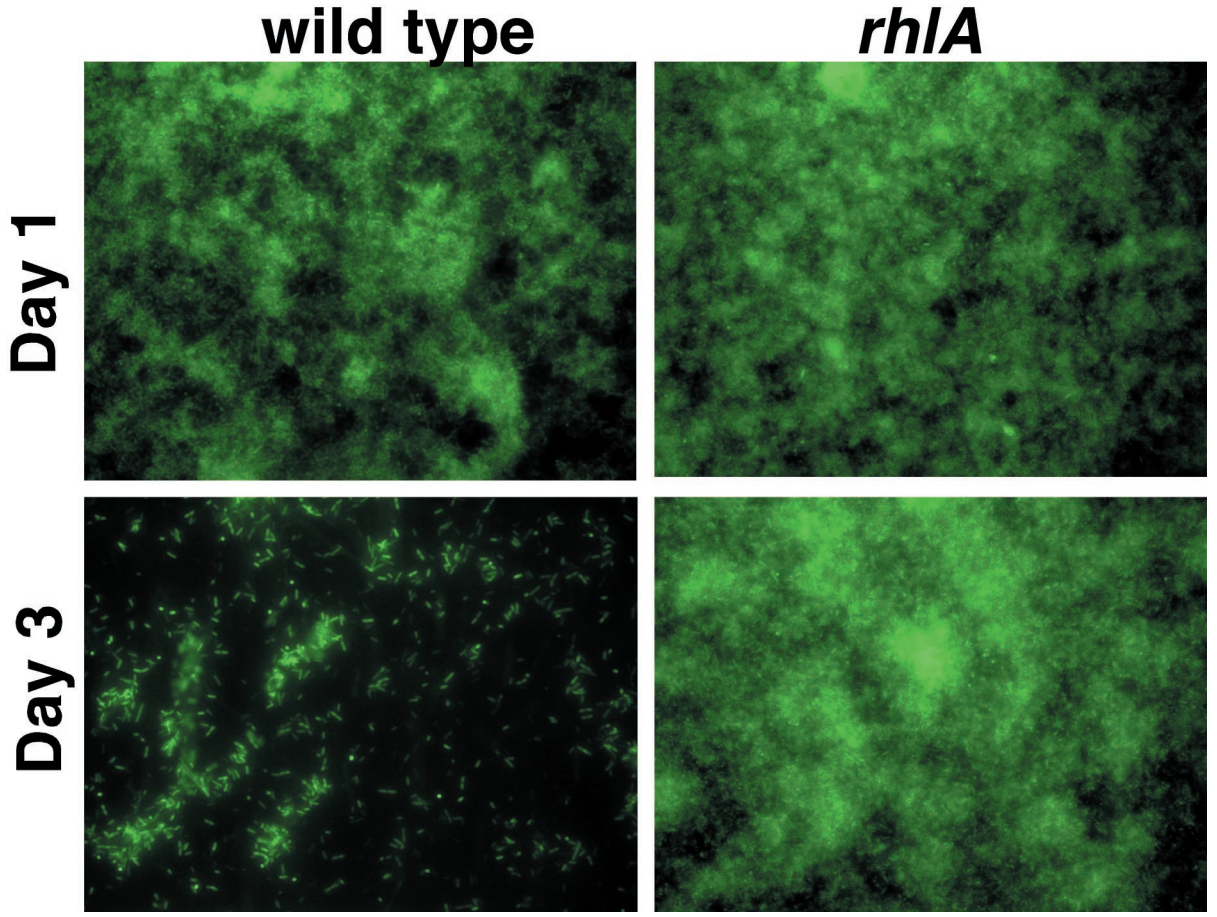

FIG. 4. Effect of rhamnolipid overexpression on biofilm formation. The images are fluorescent top-down views of flow cell-grown biofilms formed by wild type and the rhamnolipid mutant at days 1 and 3. PPGAS medium, a low-phosphate medium, induced the synthesis of high levels of rhamnolipids in the wild-type strain and resulted in a sparsely colonized surface. The *rhlA* mutant was unable to synthesize any rhamnolipids even in low-phosphate medium and made a dense biofilm by day 3.

was sufficiently sensitive to detect an individually fluorescing bacterium.

Overproduction of rhamnolipids inhibits biofilm development. Based on their surface-active properties, we predicted that rhamnolipids could affect the interactions of bacteria with surfaces and with each other. To address this hypothesis directly, we examined the effects of rhamnolipid overexpression on the interactions of bacteria with a surface. First, the effects of rhamnolipids on early biofilm formation were determined by growing bacteria under conditions leading to high-level expression of rhamnolipids. Rhamnolipids are typically synthesized during late-exponential or stationary phase of growth when the cell density is high, and this production is greatly enhanced by nutrient limitation (10). However, it has been shown previously that growth in a phosphate-limited complex medium induces the production of high levels of rhamnolipids (~100 μM in planktonic cultures) in the absence of high cell density (17). The formation of biofilms was observed in flow cells fed with phosphate-limited PPGAS medium (~0.03 mM inorganic phosphate, low-phosphate conditions). As shown in Fig. 4, when grown in low-phosphate, rhamnolipid-inducing conditions, wild-type cells started to attach at day 1, but by day 3 very few bacteria remained attached to the surface of the flow cell. In contrast, the *rhlA* mutant, which is unable to produce any rhamnolipids, formed a dense biofilm on the surface by day 3. Supplementing the PPGAS complex medium with 2.5 mM potassium phosphate (high-phosphate conditions) restored the ability of the wild-type strain to form a biofilm in the flow cell (data not shown). Therefore, expressing high levels of rhamnolipids can impede the formation of biofilms.

Rhamnolipids disrupt both cell-to-cell and cell-to-surface interactions. The data presented above indicated that production of rhamnolipids could help maintain the channels formed in a biofilm. How can rhamnolipids carry out such a function? As mentioned above, rhamnolipids are surfactants, or surface-active molecules, and a general property of surfactants involves their ability to alter the physical and/or chemical properties at interfaces (18). We hypothesized, therefore, that the rhamnolipid surfactants could influence *P. aeruginosa* cell-to-cell and/or cell-to-surface interactions.

To test these hypotheses, purified rhamnolipids were assessed for their effects on the initiation of biofilm development by using the previously described microtiter dish assay. Purified rhamnolipid (250 μM) added to bacteria at the time of inoculation into the microtiter dishes completely blocked biofilm

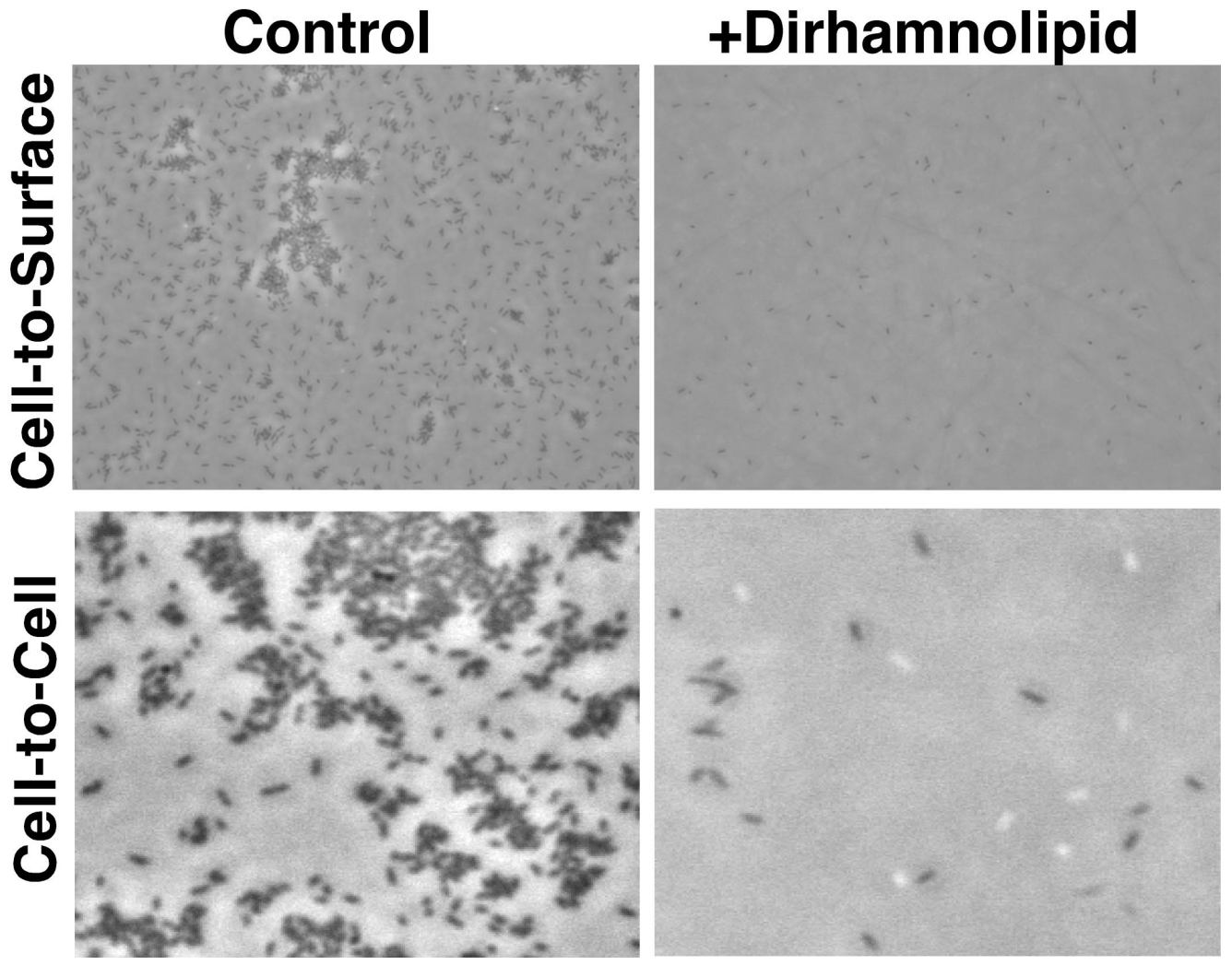

FIG. 5. Effect of purified rhamnolipids on cell-surface and cell-cell interactions. (Upper panels) Cell-surface interactions. The ability to form a biofilm on polyvinylchloride plastic in the presence or absence of rhamnolipids was examined. The rhamnolipid was added at the time of inoculation, and the biofilm formation was examined after 8 h of incubation. The addition of 250 μM rhamnolipid completely blocked biofilm formation (right panel). (Lower panels) Cell-cell interactions. The effect of rhamnolipids on rafts or pellicles of wild-type *P. aeruginosa* cells was examined. The disruption of cell-to-cell interactions caused by adding rhamnolipids was captured by phase-contrast microscopy immediately after addition of the compound (right panel). The addition of the same volume of water had no effect on biofilm formation or disruption of the rafts (left panels).

formation (Fig. 5). Partial inhibition of biofilm formation was observed at lower concentrations of rhamnolipid (not shown).

We also examined the effect of purified rhamnolipids on cell-to-cell interactions. *P. aeruginosa* typically forms a pellicle or raft of cells on the surface of a liquid culture. As shown in Fig. 5B, the addition of 100 μM dirhamnolipid disrupted cell-to-cell interactions, and this disruption occurred immediately after the addition of the compound. Taken together, these data demonstrate that rhamnolipids can disrupt both cell-to-cell and cell-to-surface interactions. Interestingly, neither purified rhamnolipid nor rhamnolipid-containing spent supernatants of *P. aeruginosa* cultures had any discernible effect on a preformed 6-day-old biofilm (not shown). These data suggest that rhamnolipids can only interfere with earlier stages of biofilm development.

Invasion of a preformed biofilm. Based on the evidence that biofilm channel structure is maintained over time, we predicted that few planktonic cells invading a preformed biofilm would be capable of attaching to this community. We performed an invasion experiment by introducing GFP-tagged *P. aeruginosa* cells ($\sim 10^7$ cells) into a flow cell containing a preformed mature 5-day-old biofilm, and the tagged cells were allowed to attach for 1 h under static conditions (Fig. 6a and c). After this period of attachment, flow was resumed for an additional hour to remove unattached bacteria before microscopic examination at low and high magnifications. As shown in Fig. 6b and d, very few of the invading GFP-tagged cells attached to the biofilm. These data are consistent with the hypothesis that preformed biofilms have the means, including but not necessarily limited to the production of rhamnolipids, to control the attachment of planktonic bacteria.

DISCUSSION

Our studies show that in situ production of rhamnolipid surfactants by *P. aeruginosa* cells affects biofilm architecture but that the loss of rhamnolipid production does not appear to

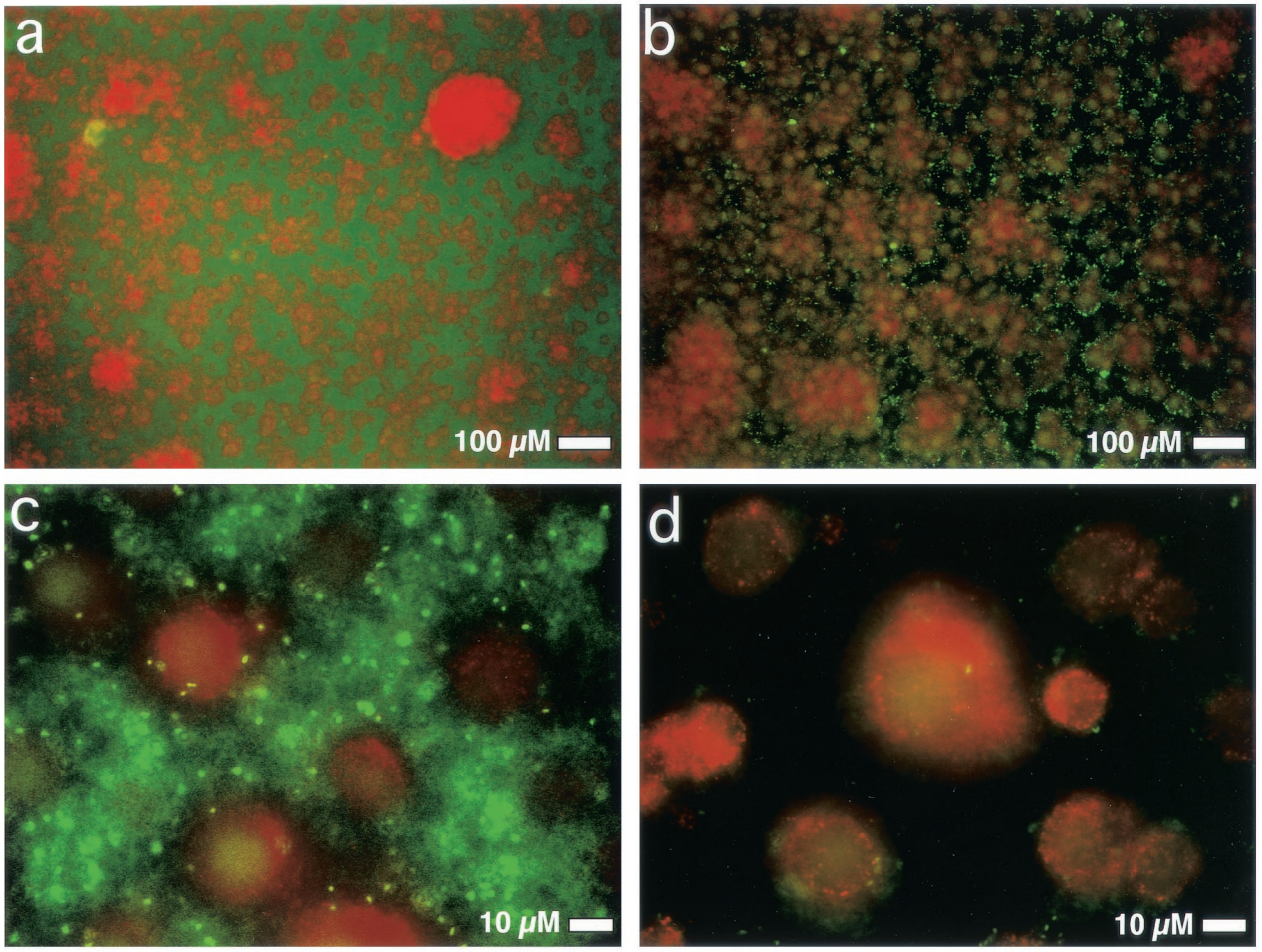

FIG. 6. Biofilm invasion assay. A biofilm of non-GFP-labeled wild-type bacteria was allowed to form for 5 days in a flow cell and then stained with CTC. (a and c) The flow was turned off, ca. 10^7 GFP-labeled wild-type *P. aeruginosa* cells were injected into the flow cell, and the flow chamber was incubated without flow for 1 h. (b and d) Flow was resumed for 1 h to remove planktonic bacteria, and the biofilm was examined by fluorescence microscopy. A merged composite image of the fluorescence micrographs is shown. The images in panels a and b were captured by using a ×63 objective lens, and the images in panels c and d were captured by using a ×10 objective lens. Scale bars are included in the images.

affect the initial development of macrocolonies and open channels. However, the *rhlA* mutant is incapable of maintaining open channels, eventually forming a relatively homogeneous layer of cells as thick as, or thicker than, the wild-type biofilm (Fig. 1). Such a role for surface-active compounds in the structuring of microbial populations at interfaces is not unprecedented in the microbial world. Surfactants are known to play a role in the emergence of aerial hyphae in both fungi and the filamentous bacterium *Streptomyces coelicolor* (31, 36) and in the formation of hydrophobic air channels in fruiting bodies of fungi (15). In addition, a recent study of fruiting body formation in the bacterium *Bacillus subtilis* showed that a mutant (*sfp*) deficient in surfactin production formed atypical surface-associated pellicle (biofilm) structures (2).

How does the production of rhamnolipids maintain the open channels formed during biofilm development? At this point, the answer to this question is not clear. As has been reported, high cell density planktonic growth induces the synthesis of quorum-sensing-dependent rhamnolipid production (3, 21, 28), and similar density-dependent transcription of the RhlI-RhlR system and *rhlA* is also seen in biofilms (8) (Fig. 3). We propose that surfactant production may inhibit planktonic cells from attaching to the preformed biofilm. This contention is supported by the "invasion assay" shown in Fig. 6. Alternatively, rhamnolipids may cause the detachment of cells or microcolonies from the biofilm, preventing the accumulation of new biomass in the channels. These models are not mutually exclusive. Finally, our data indicate that exposing a preformed biofilm to rhamnolipids has no significant effect on established biofilm structure, suggesting that the production of surfactants by preformed macrocolonies would not necessarily cause wholesale detachment of the biofilm.

The ability to block colonization of a preformed biofilm is likely due in large part to the well-known properties of surfactants at interfaces. Surfactants have the potential to affect both cell-to-cell and cell-to-surface interactions (18), and our data support the hypothesis that rhamnolipids produced by *P. aeruginosa* do affect these interface interactions. We showed that purified rhamnolipids, the only characterized surfactants produced by *P. aeruginosa*, are capable of disrupting interac-

tions between cells and each other and a surface. Hence, rhamnolipids are able to modulate cell-to-surface and cell-to-cell interactions. The ability of surfactants to disrupt bacterial attachment has been observed for the surfactant produced by *Lactobacillus* spp., known as surlactin, which blocked initial surface adhesin of uropathogenic *Enterococcus faecalis*, *Escherichia coli*, and *Staphylococcus epidermidis*, as well as two yeast strains (32–34). The surfactant produced by *B. subtilis*, called surfactin, also inhibits biofilm formation by *Salmonella enterica*, *E. coli*, and *Proteus mirabilis* (16). Studies in our laboratory indicate that purified *P. aeruginosa* rhamnolipids can block initial adherence by fluorescent pseudomonads and *E. coli* (M. E. Davey and G. A. O'Toole, unpublished data), suggesting that surfactants may have a broad and relatively nonspecific ability to interfere with cell-to-cell and cell-to-surface interactions. Therefore, *P. aeruginosa* rhamnolipid may prevent colonization not only of "self" but also of other planktonic microbes attempting to take up residence within the channels of its biofilm.

The findings presented here indicate that open-channel maintenance is not a stochastic process but represents an active process by the bacterial community that stabilizes a preferable biofilm architecture. Recent studies in *P. aeruginosa* suggest a role for a number of genes in the formation of biofilm architecture, indicating that a distinct set of genetic factors is required for the development of biofilm architecture in *P. aeruginosa*. We suggest that the production of rhamnolipid surfactants is one mechanism employed by *P. aeruginosa* for the active maintenance of biofilm architecture. We believe that other, as-yet-unidentified mechanisms may also contribute to the maintenance of architecture. For example, a decrease or cessation of growth in large macrocolonies may slow or stop the filling of channels between these structures. Finally, we propose that the maintenance of biofilm architecture is a previously undescribed stage in the development of *P. aeruginosa* biofilms.

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