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# Increase in Rhamnolipid Synthesis under Iron-Limiting Conditions Influences Surface Motility and Biofilm Formation in *Pseudomonas aeruginosa*▽†

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**Iron is an essential element for life but also serves as an environmental signal for biofilm development in the opportunistic human pathogen *Pseudomonas aeruginosa*. Under iron-limiting conditions, *P. aeruginosa* displays enhanced twitching motility and forms flat unstructured biofilms. In this study, we present evidence suggesting that iron-regulated production of the biosurfactant rhamnolipid is important to facilitate the formation of flat unstructured biofilms. We show that under iron limitation the timing of rhamnolipid expression is shifted to the initial stages of biofilm formation (versus later in biofilm development under iron-replete conditions) and results in increased bacterial surface motility. In support of this observation, an *rhlAB* mutant defective in biosurfactant production showed less surface motility under iron-restricted conditions and developed structured biofilms similar to those developed by the wild type under iron-replete conditions. These results highlight the importance of biosurfactant production in determining the mature structure of *P. aeruginosa* biofilms under iron-limiting conditions.**

The biofilm mode of bacterial growth is a surface-attached state in which cells are closely packed and encased in an extracellular polymeric matrix (10, 27). Biofilms are abundant in nature and are of clinical, environmental, and industrial importance. Biofilm development is known to follow a series of complex but discrete and tightly regulated steps (18, 27), including (i) microbial attachment to the surface, (ii) growth and aggregation of cells into microcolonies, (iii) maturation, and (iv) dissemination of progeny cells that can colonize new niches. Over the last decade, several key processes important for biofilm formation have been identified, including quorum sensing (12) and surface motility (28).

One of the best-studied model organisms for biofilm development is the bacterium *Pseudomonas aeruginosa* (10), a notorious opportunistic pathogen which causes many types of infections, including biofilm-associated chronic lung infections in individuals with cystic fibrosis (10, 24, 41). Like most organisms, *P. aeruginosa* requires iron for growth, as iron serves as a cofactor for enzymes that are involved in many basic cellular functions and metabolic pathways. Recent work has shown that at iron concentrations that are not limiting for growth, this metal serves as a signal for biofilm development (40). Iron limitation imposed, for example, by the mammalian iron chelator lactoferrin blocks the ability of *P. aeruginosa* biofilms to mature from thin layers of cells attached to a surface into large multicellular mushroom-like biofilm structures (40). By chelating iron, lactoferrin induces twitching motility (a specialized form of surface motility), which causes the cells to move across the surface instead of settling down to form structured communities (39, 40). In a recent paper, Berlutti et al. (5) provided further support for the role of iron in cell aggregation and biofilm formation. They reported that in the liquid phase, iron limitation induced motility and transition to the free-living (i.e., planktonic) mode of growth, while increased iron concentrations facilitated cell aggregation and biofilm formation. We recently demonstrated that iron limitation-induced twitching motility is regulated by quorum sensing (31). Quorum sensing allows bacteria to sense and respond to their population density via the production of small diffusible signal molecules. In *P. aeruginosa* and many other Gram-negative bacteria, these signal molecules are *N*-acyl homoserine lactones (acyl-HSLs), which have specific receptors (R proteins) (16, 30). *P. aeruginosa* possesses two acyl-HSL quorum-sensing systems, one for production of and response to *N*-3-oxo-dodecanoyl homoserine lactone ($3OC_{12}$-HSL) (LasR-LasI) and the other for production of and response to *N*-butanoyl homoserine lactone ($C_4$-HSL) (RhlR-RhlI) (35, 37). We have reported that an *rhlI* mutant unable to synthesize the $C_4$-HSL signal was impaired in iron limitation-induced twitching motility and formed structured biofilms under iron-limiting conditions (31).

The correlation between twitching motility, the RhlR-RhlI quorum-sensing system, and iron-regulated biofilm formation led us to hypothesize that rhamnolipids are involved in mediating this process. Rhamnolipids are surface-active amphi-

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TABLE 1. Strains and plasmids used in this study

| Strain or plasmid | Description[a] | Source or reference |
|---|---|---|
| Strains | | |
| *E. coli* strains | | |
| DH5α | F′ *endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1* Δ(*lacZYA-argF*)*U169 deoR* | 44 |
| SM10 λpir | *thi-1 thr leu tonA lacY supE recA*::RP4-2-Tc::Mu Km$^r$ λpir | 13 |
| *P. aeruginosa* strains | | |
| PAO1 | Wild type | 20 |
| PAO1 *rhlAB* | PAO1 with unmarked deletion in *rhlAB* | 6 |
| RT101 | PAO1 with an *rhlA-gfp* fusion inserted in *attB* | This study |
| RT102 | PAO1 with pKD-*rhlA* | This study |
| RT103 | PAO1 with pKD202 | This study |
| PAO1 *rhlI* | PAO1 IS*lacZ*/hah in *rhlI* | This study |
| PAO1 *pilA* | PAO1 IS*lacZ*/hah in *pilA* | This study |
| PAO1 *fliM* | PAO1 IS*lacZ*/hah in *fliM* | This study |
| Plasmids | | |
| pMS402 | Lux reporter vector | 15 |
| pKD-*rhlA* | pMS402 containing *rhlI* promoter | 15 |
| pKD202 | pMS402 containing *rhlA* promoter | 15 |
| pGP003 | pMMB207 containing *rhlI* | 31 |
| pME6032 | Tc$^r$ cloning vector | 19 |
| pRT2 | pME6032 carrying the *rhlAB* gene | This study |
| pMRP9-1 | Cb$^r$, constitutively expresses *gfp* | 12 |

[a] Cb$^r$, carbenicillin resistance; Tc$^r$, tetracycline resistance; Km$^r$, kanamycin resistance.

pathic molecules composed of a hydrophobic lipid and a hydrophilic sugar moiety and compose the main constituents of the biosurfactant produced by *P. aeruginosa* (reviewed in reference 42). The biosurfactant is required for a form of surface motility called swarming, where it functions as a wetting agent and reduces surface tension (8, 14). Furthermore, elements constituting the biosurfactant were recently shown to modulate the swarming behavior by acting as chemotactic-like stimuli (43). Rhamnolipids are also important in maintaining biofilm structure and inducing biofilm dispersion (6, 11, 29). Their synthesis requires the expression of the *rhlAB* operon, which is regulated by the RhlR-RhlI quorum-sensing system (14, 25, 32) and is also induced under iron-limiting conditions (14).

In this study, we test this hypothesis and demonstrate that rhamnolipid production is induced under iron-limiting conditions and that this promotes twitching motility. We found that increased expression of rhamnolipid synthesis genes during early biofilm development under iron-limiting conditions induces surface motility and results in formation of a thin flat biofilm. Furthermore, a mutant that is incapable of synthesizing rhamnolipids does not display twitching motility under iron-limiting conditions and thus forms structured biofilms under these conditions. These results highlight the importance of biosurfactant production in determining the architecture of mature *P. aeruginosa* biofilms under iron-limiting conditions.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and media.** The bacterial strains and plasmids used in this study are summarized in Table 1. *Escherichia coli* (DH5α and SM10 λpir) was used for the molecular biology work. The *gfp* vector pMRP9-1 was used to express green fluorescent protein (GFP) in *P. aeruginosa* (12). For growth curve and microtiter dish assays, we used BM2 medium as described previously (31). BM2 medium without the addition of iron served as an iron-limited medium, while BM2 medium supplemented with $FeCl_3$, as indicated, was used as an iron-replete medium. For flow cell biofilm experiments, we used either BM2 medium (with or without 50 μM $FeCl_3$) or 1% Bacto tryptic soy broth (TSB) (Becton Dickinson, Franklin Lakes, NJ), with or without lactoferrin (20 μg/ml) (3). LB medium (Becton Dickinson, Franklin Lakes, NJ) was used for growth of *E. coli* strains. All experiments were carried out at 37°C.

**Construction of strains and plasmids.** The *rhlAB*-complementing plasmid was constructed as follows. A fragment containing the *rhlAB* genes (bp 3893424 to 3890690) was amplified from *P. aeruginosa* PAO1 chromosomal DNA by PCR with the primers 5′-AAGGTACCGGTACCAGCGTTTCGACACCGG-3′ and 5′-AAGAATCCATTGGCCCGGGGTATGA-3′. The PCR product was digested with KpnI and EcoRI and cloned into KpnI-EcoRI-digested pME6032 (19). The resultant vector, pRT2, was introduced into *P. aeruginosa* by transformation. Transformants were selected on *Pseudomonas* isolation agar (Becton Dickinson) containing tetracycline (100 μg/ml). The *rhlI*, *pilA*, and *fliM* mutants were constructed using a method described in reference 36. In brief, we extracted DNAs from *rhlI*, *pilA*, and *fliM* mutants obtained from the comprehensive *P. aeruginosa* transposon mutant library at the University of Washington Genome Center (21). Using these DNAs, we transformed the PAO1 wild type with genomic DNA (40 to 80 μg). After transformation, colonies were selected on LB agar plates containing tetracycline (100 μg/ml). The mutation in each strain was confirmed by PCR.

**Growth curve and rhamnolipid gene expression assays.** To follow rhamnolipid biosynthetic gene expression at different iron concentrations, overnight cultures of the *P. aeruginosa* wild type carrying the *rhlA-lux* or *rhlI-lux* promoter fusion plasmid (15) were diluted in BM2 medium supplemented with various concentrations of $FeCl_3$ (0 to 50 μM). Growth was performed at 37°C with shaking. Growth was monitored by measuring the optical density of the samples at 595 nm, using an Ultrospec 2100 Pro spectrophotometer (Amersham-Pharmacia, Stockholm, Sweden). Luminescence measurements were carried out using a Synergy 2 plate reader (BioTek, VT).

**Surface motility assays.** Surface-associated twitching motility was assessed via subagar stab inoculation of Fe-deficient or Fe-replete (100 μM $FeCl_3$) BM2 glucose or L agar plates as described previously (31). In some experiments, the agar medium in twitching plates was supplemented with spent culture supernatant fluid (10% [vol/vol]) or purified rhamnolipidic biosurfactant (10 μg/ml). Preparation of cell-free supernatant (31) and rhamnolipid (43) has been described previously.

**Flow chamber biofilm experiments.** Biofilm development on a glass surface was assessed using flow cell biofilm reactors as described previously (2). To image biofilms, we used confocal scanning laser microscopy (CSLM). The CSLM was an MRC 1024 system with an Axioskop microscope (Bio-Rad, London, United Kingdom) or a Fluview FV1000 microscope (Olympus, Tokyo, Japan). Gener-

ally, we imaged GFP in strains containing pMRP9-1, which carries a constitutively expressed *gfp* gene (12). For GFP, the excitation and emission wavelengths were 488 and 522 nm (±35 nm), respectively, and for propidium iodide fluorescence, excitation was at 488 nm and emission wavelengths of >660 nm were collected with a 660LP filter. Three-dimensional images were constructed using Volocity image analysis software (Improvision, Coventry, United Kingdom). To determine surface motility during the initial stages of biofilm formation (4 to 8 h), we carried out time-lapse microscopy. Cells were tracked for 20-min intervals, with pictures taken every 2 min, and the surface motility distance was determined by using Imaris image analysis software (Bitplane, St. Paul, MN). To avoid any preinduction of quorum sensing-regulated genes due to inoculation with a high-density planktonic culture, the cells used for the time-lapse experiments were taken from mid-logarithmic-phase planktonic cultures and diluted to a final concentration of $10^7$ cells per ml prior to inoculation into the biofilm flow cell.

To follow rhamnolipid biosynthetic gene expression during biofilm development, we again used the *rhlA-gfp* fusion-containing strain. The biofilm was counterstained with propidium iodide as described previously (2). For quantifying *rhlA* gene expression in biofilms by using real-time PCR, the cells were grown in 10-cm silicon tubes under similar growth conditions. At each time point, three silicon tubes from each experimental condition were taken for RNA extraction.

**Real-time PCR analysis.** Real-time PCR analysis of the *rhlA* gene was performed essentially as described in reference 34. For monitoring of *rhlA* gene expression in biofilms, biofilms were grown in silicon tubes under constant flow in iron-replete and iron-restricted media, as stated above, and RNAs were extracted as previously described (1). Real-time PCR was performed with an ABI Prism 7900 sequence detection system (Applied Biosystems, Foster City, CA), using the primers *rhlA* forward (5′-GGCGCGAAAGTCTGTTGG-3′) and *rhlA* reverse (5′-CCAACGCGCTCGACATG-3′). Cycling parameters were 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Dissociation profiles of the amplified products were run to evaluate nonspecific amplification. Each PCR mixture contained 1′ Power SYBR green master mix (Applied Biosystems), 1 ng cDNA template, and 7.5 pmol of each primer in a 25-μl volume. Transcript levels were determined using the standard curve method. Standard curves were constructed with $10^{-4}$ to 10 pg of RNA-free genomic DNA purified from *P. aeruginosa* PAO1 (Genomic-tip kit; Qiagen, Valencia, CA). To more accurately compare transcript levels throughout growth, we utilized a two-step reaction and calibrated the amount of input cDNA before PCR. Thus, data are normalized to the total amount of cDNA.

## RESULTS

**Rhamnolipids induce twitching motility under conditions of iron limitation.** Our previous work suggested that an RhlI/RhlR-dependent iron-regulated cellular component promotes twitching motility in *P. aeruginosa* (31). There is also evidence suggesting that rhamnolipid production is repressed by high iron concentrations (14). To further characterize the iron regulation of rhamnolipid synthesis and its connection to the RhlI/RhlR quorum-sensing system, we used PAO1 wild-type strains carrying an *rhlA-lux* or *rhlI-lux* promoter fusion. We monitored *rhlA* and *rhlI* expression by measuring luminescence during growth. Iron limitation induced expression of the *rhlI* and *rhlA* genes, by approximately 3-fold, confirming that their expression is dependent on the iron concentration (Fig. 1A). The *rhlA* expression results were also confirmed by real-time PCR experiments (see Fig. S1 in the supplemental material). Consistent with the published data documenting RhlI/RhlR control of rhamnolipid expression, no *rhlA* gene expression was detected in the *rhlI* mutant strain carrying the *rhlA-lux* promoter fusion construct (see Fig. S2 in the supplemental material). Furthermore, examining *rhlI* and *rhlA* expression as a function of growth showed that the addition of iron did not affect the timing of expression in planktonic cells but rather decreased the level of expression of the *rhlI* and *rhlA* genes (Fig. 1B and C).

Based on our previous study (31) and the results presented

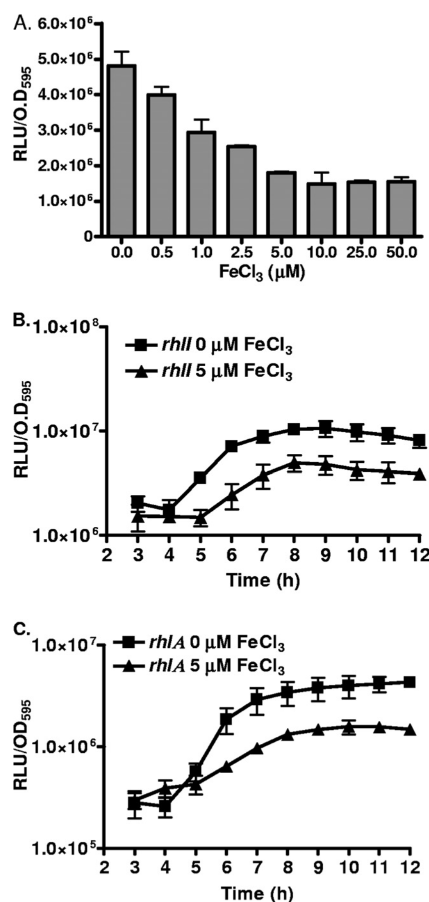


FIG. 1. Influence of iron on *rhlI-gfp* and *rhlA-gfp* expression in planktonic *P. aeruginosa* cultures. Wild-type *P. aeruginosa* PAO1 carrying an *rhlA-lux* (RT102) or *rhlI-lux* (RT103) promoter fusion was grown in BM2 medium, and expression of the *rhlA* and *rhlI* genes was assessed by measuring luminescence. (A) *rhlA-lux* measurements at different iron concentrations (results from measurements taken at 12 h are presented). Expression profiles for the *rhlI-lux* promoter fusion (B) and the *rhlA-lux* promoter fusion (C), grown with and without 5 μM iron, are shown. In all graphs, the promoter activity is given as relative luminescence units (RLU) per unit of growth (optical density at 595 nm). A promoterless *lux*-based vector control showed no luminescence (see Fig. S2 in the supplemental material).

above, we hypothesized that the *rhlI* mutant was not able to promote twitching motility under iron-limiting conditions because it cannot synthesize rhamnolipids. We reasoned that rhamnolipids might act to stimulate twitching motility because they are surfactants. To test this hypothesis, we examined the twitching motility of the *rhlI* mutant in the presence of purified rhamnolipids. In support of our hypothesis, the addition of rhamnolipids enhanced twitching motility in the *rhlI* mutant (Fig. 2A). These findings suggest that the general lack of twitching motility by the PAO1 parent strain on iron-replete medium (Fig. 2A) (31) results from a lack of rhamnolipid production. To confirm the rhamnolipid requirement for iron limitation-promoted twitching motility of wild-type *P. aeruginosa*, the twitching motility of an *rhlAB* deletion mutant (RT101) was examined. The mutant was deficient in iron limitation-promoted twitching motility, and a plasmid-borne copy of *rhlAB* restored twitching motility. These results are consis-

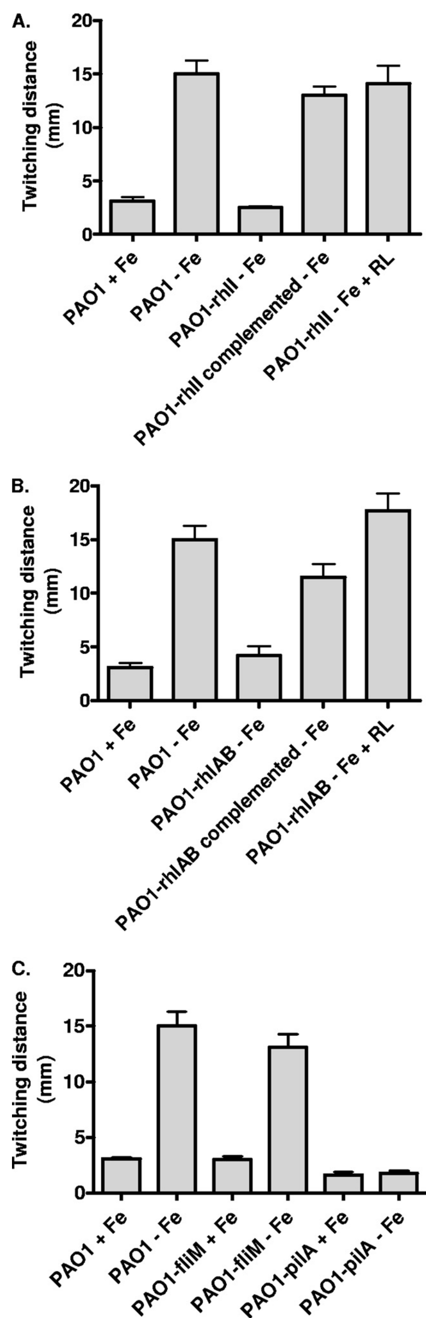


FIG. 2. Influence of rhamnolipids on twitching motility in *P. aeruginosa*. (A) Wild-type *P. aeruginosa* PAO1, its *rhlI* mutant (PAO1 *rhlI*), and the complemented *rhlI* mutant (PAO1 *rhlI* carrying the plasmid pGP003) were inoculated onto iron-replete (100 μM $FeCl_3$) or iron-limited BM2 glucose medium, with (+ RL) or without purified rhamnolipids (10 μg/ml), as indicated. (B) *P. aeruginosa* PAO1, its *rhlAB* mutant (PAO1 *rhlAB*), and the *rhlAB* mutant carrying the *rhlAB*-complementing vector (pRT2) were inoculated onto iron-replete or iron-limited BM2 glucose medium, with (+ RL) or without rhamnolipids, as indicated. (C) *P. aeruginosa* PAO1, a flagellum mutant (PAO1 *fliM*), and a type IV pilus mutant (PAO1 *pilA*) were inoculated onto iron-replete (100 μM $FeCl_3$) or iron-limited BM2 glucose medium. In all cases, plates were incubated at 37°C for 40 h, the twitching zones were stained, and their diameters were measured. Results shown represent the means ± standard deviations for a representative assay (16 individual twitching zones were measured per strain and/or growth condition on 2 separate plates) performed in duplicate.

tent with rhamnolipids being required for iron limitation-promoted twitching. Interestingly, the *rhlAB* mutant was not compromised for twitching on a rich medium (L broth) (data not shown), indicating that the twitching defect, and thus the rhamnolipid requirement for twitching, was growth medium dependent.

Type IV pili and flagella have been implicated in surface motility (type IV pili in twitching motility and flagella in swarming motility). To assess whether iron-induced twitching motility also involves flagella, we examined surface motility of a flagellum mutant (PAO1 *fliM*). In contrast to a PilA mutant (PAO1 *pilA*), which was defective in twitching motility, the FliM mutant (PAO1 *fliM*) was proficient in twitching motility, indicating that flagella are indeed not required for iron-induced twitching motility (Fig. 2C).

**A rhamnolipid-deficient mutant forms structured biofilms under iron-limited conditions.** The experiments described above provided evidence that the RhlI/RhlR-dependent iron-regulated component that induces twitching motility under iron-limiting conditions is rhamnolipid. An increase in twitching motility has been suggested to impair biofilm formation under low-iron conditions. To begin to analyze the role of rhamnolipids in iron-dependent biofilm development of *P. aeruginosa*, we examined biofilm formation in a biosurfactant-deficient *rhlAB* mutant (RT101) under iron-limiting conditions. It was shown previously that rhamnolipids are important in maintaining biofilm architecture only during the late stages of biofilm development under iron-sufficient conditions—no difference in biofilm development was observed between the wild type and the rhamnolipid mutant during the early stages of development (11). Our results support these findings, inasmuch as both the wild type and the *rhlAB* mutant formed mushroom-like structures (Fig. 3). However, under iron-limiting conditions, the wild-type strain formed a thin uniform layer, while the *rhlAB* mutant formed structured biofilms reminiscent of those formed by the wild-type strain under iron-sufficient conditions (Fig. 3). Complementation of the *rhlAB* mutation (using the *rhlAB*-carrying vector pRT2) restored the wild-type phenotype of flat biofilms under iron-limiting conditions (Fig. 3). This result suggested that rhamnolipid production affects biofilm formation under iron-limiting conditions.

**Iron limitation induces surface motility during early biofilm development.** It was previously proposed that increased twitching motility may account for the impaired biofilm formation of *P. aeruginosa* observed under low-iron conditions (40). Based on our results, we speculated that induction of rhamnolipid biosynthesis under iron-limiting conditions promotes twitching motility, which in turn produces a thin, flat, uniform biofilm. To determine whether rhamnolipid-dependent twitching motility is important in early biofilm formation, we carried out time-lapse experiments with the wild type and the *rhlAB* strain. Our time-lapse results showed that under iron-limiting conditions a higher percentage of the wild-type cells (Table 2) were motile (>2-μm movement) than the percentage of motile *rhlAB* mutant cells (Table 2) (68% versus 23%). Upon examination of the highly motile population (>5-μm movement), the difference was even more dramatic (22% of motile cells of the wild type versus 3% of the mutant cells). Furthermore, the observed motility of the *rhlAB* mutant under iron-limiting conditions was similar to that of the wild-type strain under iron-

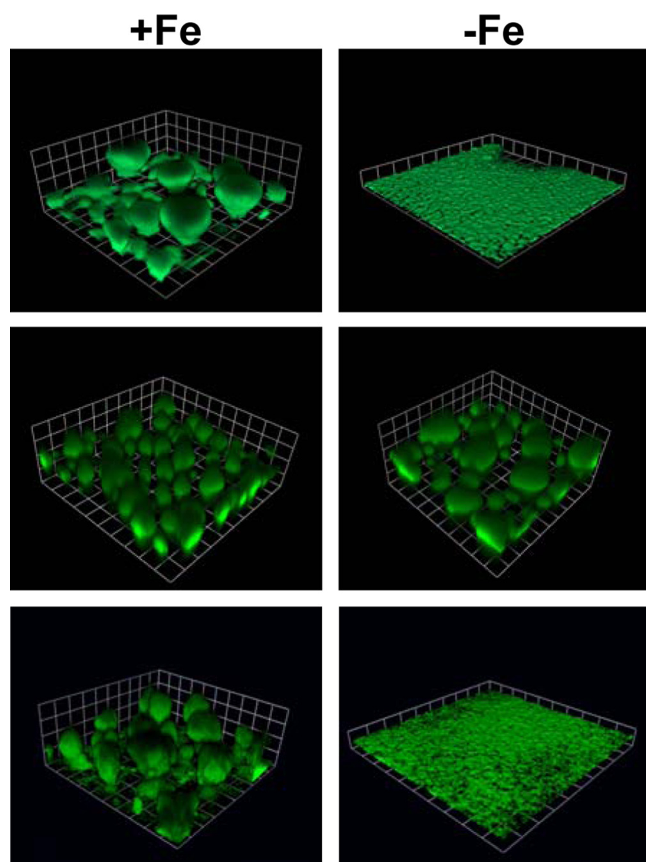


FIG. 3. Impact of *rhlAB* mutation on *P. aeruginosa* biofilm formation under iron-replete and iron-restricted conditions. Biofilms of wild-type *P. aeruginosa* PAO1 (top), the *rhlAB* mutant (PAO1 *rhlAB*) (middle), and the complemented *rhlAB* mutant (bottom), grown in flow cells for 4 days in iron-restricted or iron-replete medium as described in Materials and Methods, are shown. The three-dimensional (3-D) images presented were reconstructed from CSLM scans of 4-day-old biofilms. Each square is 25 μm on a side.

sufficient conditions (Table 2) (26% versus 23%, respectively). These results are consistent with the formation of comparable biofilm structures by the *rhlAB* mutant under iron-limiting conditions (Fig. 3). This suggests that rhamnolipids may be induced specially under low-iron conditions, where their influence on motility promotes flat biofilm formation, while their low level under iron-replete conditions (or absence in the *rhlAB* mutant) compromises surface motility and thus allows for more-structured biofilm formation.

**Rhamnolipid expression in biofilms is induced under low-iron conditions.** Lequette and Greenberg (22) previously reported on the timing of rhamnolipid synthesis gene expression in developing biofilms. We utilized a similar experimental approach and followed GFP expression from an *rhlA-gfp* fusion in wild-type *P. aeruginosa* biofilms grown under iron-sufficient and iron-limited conditions. Similar to the results reported by Lequette and Greenberg (22), microcolonies formed during the first 3 days of growth under iron-sufficient conditions, but the fluorescence of cells in the microcolonies remained low (Fig. 4A to C), consistent with minimal *rhlAB* transcription and thus rhamnolipid synthesis. After an additional 1 to 2 days of growth, the typical mushroom-like structures of mature biofilms were observed (as revealed by propidium iodide staining) (Fig. 4E). The stalk regions of these structures were brightly fluorescent, but the caps showed only low levels of GFP fluorescence (Fig. 4E), indicating significant *rhlAB* expression by bacteria in the stalks only. In contrast, under iron-limiting conditions, the cells formed a flat biofilm, GFP fluorescence was evident throughout the biofilm during the first day of biofilm formation (Fig. 4A), and GFP fluorescence increased in the following days (Fig. 4B to E). To quantify these results, we also followed *rhlA* expression during biofilm development by using real-time PCR. In agreement with the above data, *rhlA* expression in iron-replete medium was induced only on days 3 and 4 (Fig. 5). The results presented in Fig. 5 clearly show that in iron-depleted medium *rhlA* expression was induced (approximately 7- to 10-fold) early in the initial stages of biofilm development (i.e., at 24 to 72 h) compared to that under iron-replete conditions. Taken together, these results demonstrate that under iron-limiting conditions, the timing of rhamnolipid production shifts and rhamnolipids are synthesized earlier during the initial stages of biofilm development.

## DISCUSSION

Iron is an important environmental signal for *P. aeruginosa* biofilm development. Previous studies have suggested that under iron-limiting conditions *P. aeruginosa* twitching motility is induced and that, as a result, cells continue to move on the surface and normal biofilm formation is impaired (31, 40). The results of the present study provide evidence that iron limitation-inducible production of rhamnolipids during early stages of biofilm formation enhances twitching motility and that, as a result, flat unstructured biofilms form.

Surface motility is known to be important in biofilm formation. The role of rhamnolipids in surface motility, and most notably swarming motility, is well documented (8, 14, 43). Thus, it seems that the abilities of rhamnolipids to act as wetting agents and to reduce surface tension most likely underpin the enhancement of twitching motility under iron-limiting conditions. Our finding that rhamnolipids are important for twitching motility is in agreement with the study of Pamp and Tolker-Nielsen (29).

Rhamnolipids also play a critical role in determining biofilm architecture. Davey et al. (11) reported that mutants defective

TABLE 2. Influence of iron on surface motility of wild-type *P. aeruginosa* and an *rhlAB* mutant in biofilms

| Strain | Presence of iron | % of cells showing motility[a] | | |
|---|---|---|---|---|
| | | 0–2 μm | 2–5 μm | >5 μm |
| Wild type | −Fe | 32 | 46 | 22 |
| | +Fe | 74 | 22 | 4 |
| *rhlAB* mutant | −Fe | 77 | 20 | 3 |

[a] Time-lapse experiments were conducted on 4- to 8-h-old biofilm cultures grown in flow cells in order to allow single-cell tracking. The cells were tracked over 20-min intervals, during which an image was taken every 2 min. The results are presented as percentages of cells that moved a certain distance from the total cells tracked. The data presented are averages obtained from eight different time-lapse experiments for each sample, and in each time-lapse experiment, >150 cells were tracked.

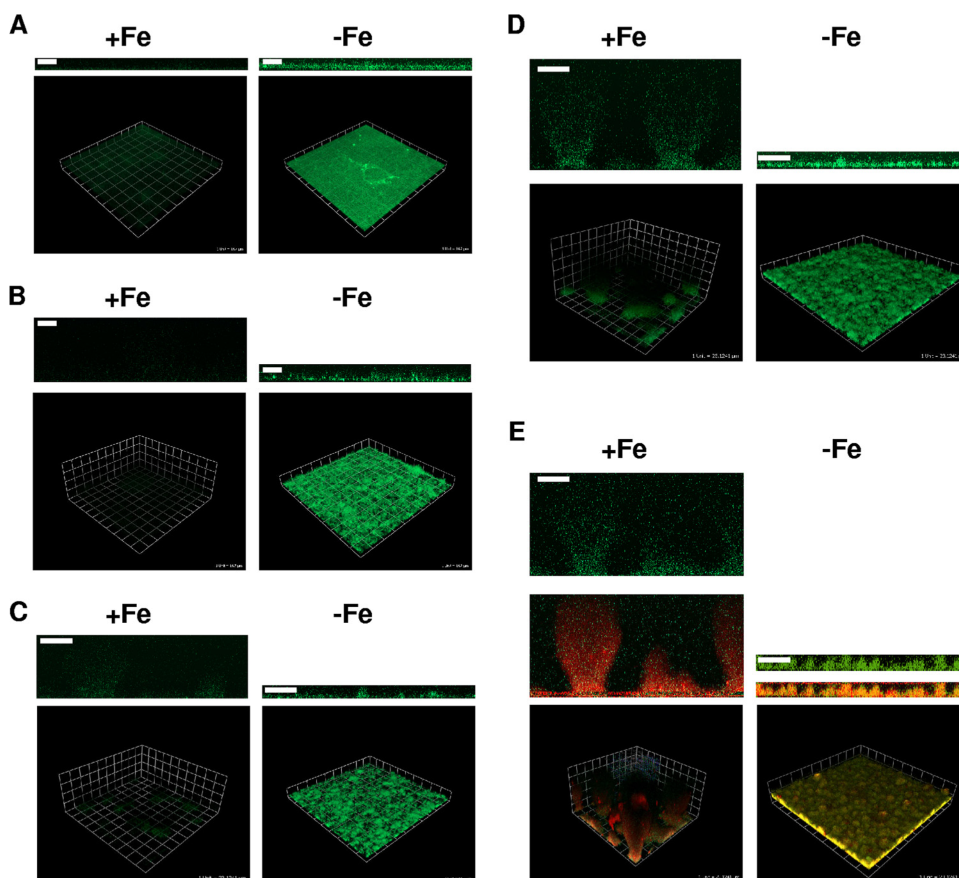


FIG. 4. Influence of iron on *rhlA-gfp* expression in *P. aeruginosa* biofilms. GFP fluorescence was followed over a 5-day period (A to E) in wild-type *P. aeruginosa* carrying an *rhlA-gfp* promoter fusion (RT101) and grown in flow cells under iron-restricted (−Fe) or iron-replete (+Fe) conditions. Images of 3-D reconstructions (bottom images) and horizontal sections near the center of the biofilms (top images) are shown for all time points. (A) 1 day; (B) 2 days; (C) 3 days; (D) 4 days; (E) 5 days. On day 5 (E), the biofilm was stained with propidium iodide to visualize the entire biofilm structure. A horizontal view of the green channel alone is shown (top), and a horizontal section and 3-D reconstruction of the merged red and green channels are also presented (middle and bottom). For the 3-D images, the squares for panels A and B are 14 μm on a side, and those for panels C to E are 24 μm on a side. Bars, 10 μm (A and B) and 35 μm (C to E).

in rhamnolipid production are unable to maintain water channels. These mutants exhibited normal microcolony formation in the initial stages of biofilm development (until day 4), but then the water channels around the biofilm were colonized and the biofilm matured into a thick flat biofilm. This result correlates with the study by Lequette and Greenberg (22), who showed that expression of rhamnolipid biosynthetic genes within a biofilm is strongly enhanced during the later stages of biofilm formation (after 3 days) and is localized to the stalk region of the mushroom-like structure, in agreement with our results. Thus, it seems that the timing of induction of rhamnolipid gene expression is crucial for development of structured biofilms. Our rhamnolipid biofilm gene expression studies support this hypothesis, as iron limitation caused early induction of rhamnolipid biosynthetic gene expression and resulted in more surface motility and a flat biofilm phenotype. Because it has been shown that overproduction of rhamnolipids results in biofilm dispersal, we believe that although rhamnolipids are needed for maturation of structured biofilms, rhamnolipid production must be tightly regulated, even in late-stage biofilm development (6). We speculate that under iron-sufficient conditions, wild-type *P. aeruginosa* produces small amounts of rhamnolipids, sufficient to maintain water channels, during late-stage biofilm development. Under iron-limiting conditions, rhamnolipid production occurs earlier in biofilm development and in amounts that enhance twitching motility, yielding a flat biofilm phenotype.

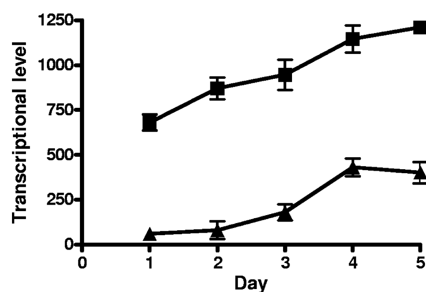


FIG. 5. Quantification of *rhlA* expression in *P. aeruginosa* biofilms. *rhlA* expression was monitored over a 5-day period in *P. aeruginosa* biofilms grown under iron-restricted (solid squares) or iron-replete (solid triangles) conditions. The level of *rhlA* was measured by real-time PCR as described in Materials and Methods. Transcript levels are given in picograms, normalized to a genomic DNA standard.

The role of biosurfactants in biofilm formation has also been reported by Pamp and Tolker-Nielsen (29), who showed that rhamnolipid-deficient mutants were defective in migration-dependent development of the characteristic mushroom-shaped biofilm structures. Using mixed-species biofilm experiments, they elegantly demonstrated that wild-type and *rhlA* and *pilA* mutant (defective in twitching motility) strains formed distinct subpopulations within the biofilm, related to their abilities to produce rhamnolipids. In their experimental system, the *rhlA* mutant developed mushroom caps of reduced size on top of mushroom stalks formed by the *pilA* mutant (in mixed cultures). It is important that in contrast to the Davey et al. study (11) and our current work, the *rhlAB* mutant used by Pamp and Tolker-Nielson did not form microcolonies in the initial stages of biofilm development but rather developed a flat biofilm phenotype when grown in monocultures. This difference in the biofilm phenotype was most likely due to differences in strain backgrounds and growth conditions used in these different studies, as suggested by Pamp and Tolker-Nielson in their study (29). We did attempt to address the possibility of secondary mutations in our strains. Beatson et al. previously demonstrated that quorum-sensing mutants that showed twitching motility defects contained secondary mutations in the *algR* and *vfr* genes which were responsible for the twitching motility defect (4). We sequenced our wild-type, *rhlAB*, and *rhlI* strains and did not see any mutations in these genes (data not shown).

Rhamnolipid expression is known to be regulated by the RhlI/RhlR quorum-sensing system and to be induced in the late stages of logarithmic growth (25). It has also been shown that rhamnolipid synthesis genes and biosurfactant production are induced under conditions of iron limitation (14, 17). Our *rhlA-gfp* promoter fusion experiments with planktonic cells support these findings and demonstrate that while the timing of expression did not change under conditions of iron limitation versus iron sufficiency, the levels of expression did, increasing approximately 3- to 4-fold in cells grown under iron limitation. In the biofilm mode of growth, however, both the timing and level of rhamnolipid biosynthetic gene expression were altered, with the *rhlAB* genes expressed earlier and at higher levels under iron-limiting conditions. This increase was sufficient to dramatically enhance surface motility (Fig. 2 and Table 2). How does iron influence *rhlAB* expression? There is growing evidence suggesting a link between the quorum-sensing and iron regulons (7, 9, 23, 26). Our *rhlI-lux* expression experiments and previous work showed that expression of *rhlI* and, subsequently, the amount of $C_4$-HSL were increased under conditions of iron limitation (31). This increase in the $C_4$-HSL signal will result in enhanced expression of genes regulated by RhlR, such as the *rhlAB* operon.

Quorum sensing-regulated surface motility and biofilm formation are not regulated only by changes in iron concentration. In fact, Shrout et al. (38) demonstrated that quorum sensing-regulated biofilm formation is nutritionally conditional and that biofilm formation by quorum-sensing mutants can vary dramatically depending on the available carbon source. Their study also showed that quorum sensing exerted this nutritionally conditional control of biofilm development through regulation of swarming motility (38). Similar to our findings with iron, they demonstrated that when a certain carbon source promoted surface motility, the result was a flat and unstructured biofilm. When the mutants were provided with a carbon source that did not promote motility, they formed structured biofilms (38). Thus, it seems that several nutritional conditions (e.g., carbon sources and iron) can influence biofilm formation. This raises a question regarding the interplay between these nutritional signals. Since *P. aeruginosa* is an opportunistic pathogen which readily encounters iron limitation in the host, this suggests that iron concentration may act as an important signal that determines the ability of *P. aeruginosa* to form biofilms *in vivo*.

In conclusion, the present study provides some explanation for the observed impact of iron availability on biofilm formation. Still, this is certainly not the only influence that iron has on biofilm physiology. It has been shown, for example, that less DNA, a major matrix component in *P. aeruginosa* biofilms, is released into biofilms grown with high versus low levels of iron (33). Furthermore, the impact of iron on quorum sensing probably provides additional effects, as cell-cell communication is known to be important in coordinating biofilm formation.

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## REFERENCES

1. **Bagge, N., M. Schuster, M. Hentzer, O. Ciofu, M. Givskov, E. P. Greenberg, and N. Hoiby.** 2004. *Pseudomonas aeruginosa* biofilms exposed to imipenem exhibit changes in global gene expression and beta-lactamase and alginate production. Antimicrob. Agents Chemother. **48:**1175–1187.
2. **Banin, E., K. M. Brady, and E. P. Greenberg.** 2006. Chelator-induced dispersal and killing of *Pseudomonas aeruginosa* cells in a biofilm. Appl. Environ. Microbiol. **72:**2064–2069.
3. **Banin, E., M. L. Vasil, and E. P. Greenberg.** 2005. Iron and *Pseudomonas aeruginosa* biofilm formation. Proc. Natl. Acad. Sci. U. S. A. **102:**11076–11081.
4. **Beatson, S. A., C. B. Whitchurch, A. B. Semmler, and J. S. Mattick.** 2002. Quorum sensing is not required for twitching motility in Pseudomonas aeruginosa. J. Bacteriol. **184:**3598–3604.
5. **Berlutti, F., C. Morea, A. Battistoni, S. Sarli, P. Cipriani, F. Superti, M. G. Ammendolia, and P. Valenti.** 2005. Iron availability influences aggregation, biofilm, adhesion and invasion of *Pseudomonas aeruginosa* and *Burkholderia cenocepacia*. Int. J. Immunopathol. Pharmacol. **18:**661–670.
6. **Boles, B. R., M. Thoendel, and P. K. Singh.** 2005. Rhamnolipids mediate detachment of *Pseudomonas aeruginosa* from biofilms. Mol. Microbiol. **57:**1210–1223.
7. **Bollinger, N., D. J. Hassett, B. H. Iglewski, J. W. Costerton, and T. R. McDermott.** 2001. Gene expression in *Pseudomonas aeruginosa*: evidence of iron override effects on quorum sensing and biofilm-specific gene regulation. J. Bacteriol. **183:**1990–1996.
8. **Caiazza, N. C., R. M. Shanks, and G. A. O'Toole.** 2005. Rhamnolipids modulate swarming motility patterns of *Pseudomonas aeruginosa*. J. Bacteriol. **187:**7351–7361.
9. **Cornelis, P., and S. Aendekerk.** 2004. A new regulator linking quorum sensing and iron uptake in *Pseudomonas aeruginosa*. Microbiology **150:**752–756.
10. **Costerton, J. W., P. S. Stewart, and E. P. Greenberg.** 1999. Bacterial biofilms: a common cause of persistent infections. Science **284:**1318–1322.
11. **Davey, M. E., N. C. Caiazza, and G. A. O'Toole.** 2003. Rhamnolipid surfactant production affects biofilm architecture in *Pseudomonas aeruginosa* PAO1. J. Bacteriol. **185:**1027–1036.
12. **Davies, D. G., M. R. Parsek, J. P. Pearson, B. H. Iglewski, J. W. Costerton, and E. P. Greenberg.** 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science **280:**295–298.
13. **de Lorenzo, V., and K. N. Timmis.** 1994. Analysis and construction of stable phenotypes in gram-negative bacteria with Tn5- and Tn10-derived minitransposons. Methods Enzymol. **235:**386–405.
14. **Déziel, E., F. Lepine, S. Milot, and R. Villemur.** 2003. *rhlA* is required for the production of a novel biosurfactant promoting swarming motility in *Pseudomonas aeruginosa*: 3-(3-hydroxyalkanoyloxy)alkanoic acids (HAAs), the precursors of rhamnolipids. Microbiology **149:**2005–2013.

15. **Duan, K., and M. G. Surette.** 2007. Environmental regulation of *Pseudomonas aeruginosa* PAO1 Las and Rhl quorum-sensing systems. J. Bacteriol. **189:**4827–4836.
16. **Fuqua, C., S. C. Winans, and E. P. Greenberg.** 1996. Census and consensus in bacterial ecosystems: the LuxR-LuxI family of quorum-sensing transcriptional regulators. Annu. Rev. Microbiol. **50:**727–751.
17. **Guerra-Santos, L., O. Kappeli, and A. Fiechter.** 1984. *Pseudomonas aeruginosa* biosurfactant production in continuous culture with glucose as carbon source. Appl. Environ. Microbiol. **48:**301–305.
18. **Hall-Stoodley, L., J. W. Costerton, and P. Stoodley.** 2004. Bacterial biofilms: from the natural environment to infectious diseases. Nat. Rev. Microbiol. **2:**95–108.
19. **Heeb, S., C. Blumer, and D. Haas.** 2002. Regulatory RNA as mediator in GacA/RsmA-dependent global control of exoproduct formation in *Pseudomonas fluorescens* CHA0. J. Bacteriol. **184:**1046–1056.
20. **Holloway, B. W., V. Krishnapillai, and A. F. Morgan.** 1979. Chromosomal genetics of *Pseudomonas*. Microbiol. Rev. **43:**73–102.
21. **Jacobs, M. A., A. Alwood, I. Thaipisuttikul, D. Spencer, E. Haugen, S. Ernst, O. Will, R. Kaul, C. Raymond, R. Levy, L. Chun-Rong, D. Guenthner, D. Bovee, M. V. Olson, and C. Manoil.** 2003. Comprehensive transposon mutant library of *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. U. S. A. **100:**14339–14344.
22. **Lequette, Y., and E. P. Greenberg.** 2005. Timing and localization of rhamnolipid synthesis gene expression in Pseudomonas aeruginosa biofilms. J. Bacteriol. **187:**37–44.
23. **Lequette, Y., J. H. Lee, F. Ledgham, A. Lazdunski, and E. P. Greenberg.** 2006. A distinct QscR regulon in the *Pseudomonas aeruginosa* quorum-sensing circuit. J. Bacteriol. **188:**3365–3370.
24. **Lyczak, J. B., C. L. Cannon, and G. B. Pier.** 2000. Establishment of *Pseudomonas aeruginosa* infection: lessons from a versatile opportunist. Microbes Infect. **2:**1051–1060.
25. **Ochsner, U. A., and J. Reiser.** 1995. Autoinducer-mediated regulation of rhamnolipid biosurfactant synthesis in *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. U. S. A. **92:**6424–6428.
26. **Oglesby, A. G., J. M. Farrow III, J. H. Lee, A. P. Tomaras, E. P. Greenberg, E. C. Pesci, and M. L. Vasil.** 2008. The influence of iron on *Pseudomonas aeruginosa* physiology: a regulatory link between iron and quorum sensing. J. Biol. Chem. **283:**15558–15567.
27. **O'Toole, G., H. B. Kaplan, and R. Kolter.** 2000. Biofilm formation as microbial development. Annu. Rev. Microbiol. **54:**49–79.
28. **O'Toole, G. A., and R. Kolter.** 1998. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. Mol. Microbiol. **30:**295–304.
29. **Pamp, S. J., and T. Tolker-Nielsen.** 2007. Multiple roles of biosurfactants in structural biofilm development by *Pseudomonas aeruginosa*. J. Bacteriol. **189:**2531–2539.
30. **Parsek, M. R., and E. P. Greenberg.** 2000. Acyl-homoserine lactone quorum sensing in gram-negative bacteria: a signaling mechanism involved in associations with higher organisms. Proc. Natl. Acad. Sci. U. S. A. **97:**8789–8793.
31. **Patriquin, G. M., E. Banin, C. Gilmour, R. Tuchman, E. P. Greenberg, and K. Poole.** 2008. Influence of quorum sensing and iron on twitching motility and biofilm formation in *Pseudomonas aeruginosa*. J. Bacteriol. **190:**662–671.
32. **Pearson, J. P., E. C. Pesci, and B. H. Iglewski.** 1997. Roles of *Pseudomonas aeruginosa las* and *rhl* quorum-sensing systems in control of elastase and rhamnolipid biosynthesis genes. J. Bacteriol. **179:**5756–5767.
33. **Qin, Z., Y. Ou, L. Yang, Y. Zhu, T. Tolker-Nielsen, S. Molin, and D. Qu.** 2007. Role of autolysin-mediated DNA release in biofilm formation of *Staphylococcus epidermidis*. Microbiology **153:**2083–2092.
34. **Sandoz, K. M., S. M. Mitzimberg, and M. Schuster.** 2007. Social cheating in *Pseudomonas aeruginosa* quorum sensing. Proc. Natl. Acad. Sci. U. S. A. **104:**15876–15881.
35. **Schuster, M., and E. P. Greenberg.** 2006. A network of networks: quorum-sensing gene regulation in *Pseudomonas aeruginosa*. Int. J. Med. Microbiol. **296:**73–81.
36. **Schuster, M., A. C. Hawkins, C. S. Harwood, and E. P. Greenberg.** 2004. The *Pseudomonas aeruginosa* RpoS regulon and its relationship to quorum sensing. Mol. Microbiol. **51:**973–985.
37. **Schuster, M., C. P. Lostroh, T. Ogi, and E. P. Greenberg.** 2003. Identification, timing, and signal specificity of *Pseudomonas aeruginosa* quorum-controlled genes: a transcriptome analysis. J. Bacteriol. **185:**2066–2079.
38. **Shrout, J. D., D. L. Chopp, C. L. Just, M. Hentzer, M. Givskov, and M. R. Parsek.** 2006. The impact of quorum sensing and swarming motility on *Pseudomonas aeruginosa* biofilm formation is nutritionally conditional. Mol. Microbiol. **62:**1264–1277.
39. **Singh, P. K.** 2004. Iron sequestration by human lactoferrin stimulates *P. aeruginosa* surface motility and blocks biofilm formation. Biometals **17:**267–270.
40. **Singh, P. K., M. R. Parsek, E. P. Greenberg, and M. J. Welsh.** 2002. A component of innate immunity prevents bacterial biofilm development. Nature **417:**552–555.
41. **Singh, P. K., A. L. Schaefer, M. R. Parsek, T. O. Moninger, M. J. Welsh, and E. P. Greenberg.** 2000. Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. Nature **407:**762–764.
42. **Soberon-Chavez, G., F. Lepine, and E. Déziel.** 2005. Production of rhamnolipids by *Pseudomonas aeruginosa*. Appl. Microbiol. Biotechnol. **68:**718–725.
43. **Tremblay, J., A. P. Richardson, F. Lepine, and E. Déziel.** 2007. Self-produced extracellular stimuli modulate the *Pseudomonas aeruginosa* swarming motility behaviour. Environ. Microbiol. **9:**2622–2630.
44. **Woodcock, D. M., P. J. Crowther, J. Doherty, S. Jefferson, E. DeCruz, M. Noyer-Weidner, S. S. Smith, M. Z. Michael, and M. W. Graham.** 1989. Quantitative evaluation of *Escherichia coli* host strains for tolerance to cytosine methylation in plasmid and phage recombinants. Nucleic Acids Res. **17:**3469–3478.