Influence of Quorum Sensing and Iron on Twitching Motility and Biofilm Formation in Pseudomonas aeruginosa

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Reducing iron (Fe) levels in a defined minimal medium reduced the growth yields of planktonic and biofilm Pseudomonas aeruginosa, though biofilm biomass was affected to the greatest extent and at FeCl3 concentrations where planktonic cell growth was not compromised. Highlighting this apparently greater need for Fe, biofilm growth yields were markedly reduced in a mutant unable to produce pyoverdine (and, so, deficient in pyoverdine-mediated Fe acquisition) at concentrations of FeCl3 that did not adversely affect biofilm yields of a pyoverdine-producing wild-type strain. Concomitant with the reduced biofilm yields at low Fe concentrations, P. aeruginosa showed enhanced twitching motility in Fe-deficient versus Fe-replete minimal media. A mutant deficient in low-Fe-stimulated twitching motility but normal as regards twitching motility on Fe-rich medium was isolated and shown to be disrupted in rhlI, whose product is responsible for synthesis of the N-butanoyl homoserine lactone (C4-HSL) quorum-sensing signal. In contrast to wild-type cells, which formed thin, flat, undeveloped biofilms in Fe-limited medium, the rhlI mutant formed substantially developed though not fully mature biofilms under Fe limitation. C4-HSL production increased markedly in Fe-limited versus Fe-rich P. aeruginosa cultures, and cell-free low-Fe culture supernatants restored the twitching motility of the rhlI mutant on Fe-limited minimal medium and stimulated the twitching motility of rhlI and wild-type P. aeruginosa on Fe-rich minimal medium. Still, addition of exogenous C4-HSL did not stimulate the twitching motility of either strain on Fe-replete medium, indicating that some Fe-regulated and RhlI/C4-HSL-dependent extracellular product(s) was responsible for the enhanced twitching motility (and reduced biofilm formation) seen in response to Fe limitation.

Pseudomonas aeruginosa is an opportunistic human pathogen that causes debilitating infections, particularly in patients with underlying diseases, such as cystic fibrosis (24, 50), where it can cause chronic infections characterized by the formation of biofilms (24, 25, 42, 50, 59, 77). These surface-associated communities of sessile bacteria embedded in a polysaccharide matrix are important features of many infectious diseases (25, 42, 45, 59), and their characteristic resistance to antimicrobials (antibiotics and biocides) and host immune responses (4, 20, 42, 45, 59) compromises infection control. Details of in vivo P. aeruginosa biofilm formation, structure, and properties are limited, with most of our understanding of these structures coming from the study of model biofilms formed in vitro (3, 15, 29, 30, 56, 80, 81, 84). In vitro biofilm development in P. aeruginosa is characterized by bacterial surface attachment, followed by microcolony formation by clonal expansion or motility-driven cell-to-cell aggregation and subsequent formation of a flat, uniform, confluent biofilm or heterogeneous, structured biofilms characterized by cell aggregates or “mushroom” structures separated by channels or spaces (38, 40).

A number of studies have linked quorum sensing (QS), cell-density control of gene expression involving acylhomoserine lactones (acyl-HSLs) (36, 69), and biofilm formation/development in P. aeruginosa (17, 26, 38, 64), although some studies indicate that QS has little or no role, with QS mutants being proficient in biofilm formation (27, 62, 67, 68). These discrepancies are generally linked to differences in biofilm model and/or culture conditions, and indeed, a recent study confirmed that the QS dependence of biofilm formation is nutritionally conditional (i.e., QS systems are needed for biofilm formation in some growth media [e.g., succinate] but not others [e.g., glucose or glutamate]) (73). Two primary acyl-HSL systems have been reported to occur in P. aeruginosa: las, involving the 3-oxo-dodecanoyl HSL product of the LasI synthase and the 3-oxo-dodecanoyl HSL-responsive DNA-binding regulator LasR, and rhl, involving the butanoyl HSL (C4-HSL) product of the RhlI synthase and the C4-HSL-responsive DNA-binding regulator RhlR (36, 69). In some studies, las but not rhl (17, 18, 64) or both las and rhl (18, 84) mutants are substantially altered as regards biofilm development, while in others, las mutants form nominally wild-type biofilms (27, 62, 84).

Twitching, a form of surface motility mediated by type IV pili (46), is also implicated in biofilm development (41, 55), being involved in P. aeruginosa spreading over the surface of the substratum (in the initial stages of structured biofilm formation and in the formation of flat biofilms [41]) and in colonization of microcolony “stalks” to form the mushroom caps of...
We examine the impacts of Fe levels on biofilm formation. We find that twitching motility in P. aeruginosa and its derivatives, tetracycline (10 μg/ml) and chloramphenicol (50 μg/ml); to counterselect donor E. coli, putative rhlR deletion mutants were constructed to confirm the absence of mutations in the cloned DNA. Plasmid pCG001 was mobilized into P. aeruginosa K1120 from E. coli S17-1 by conjugation (78), and following selection of pCG001 transconjugants on tetracycline (50 μg/ml) and chloramphenicol (5 μg/ml) to counterselect donor E. coli, putative rhlR deletion strains were subsequently recovered on L agar supplemented with sucrose (10%).

**Materials and Methods**

**Bacterial strains, plasmids, and growth conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. Routine growth of P. aeruginosa and Escherichia coli was carried out in Luria broth (LB) as before (52), with antibiotic supplementation to maintain plasmids as needed (for pUC18, ampicillin (100 μg/ml); for mini-CTX-lacZ and its derivatives, tetracycline (10 μg/ml) for E. coli and 75 to 100 μg/ml for P. aeruginosa; for pK18mobSacB and its derivatives, kanamycin (50 μg/ml); for pFLP2, ampicillin (100 μg/ml) for E. coli) or carbenicillin (150 μg/ml for P. aeruginosa); and for pMMB207 and its derivatives, chloramphenicol [10 μg/ml for E. coli and 75 to 100 μg/ml for P. aeruginosa]. Fe-limited BM2 succinate and BM2 glucose minimal media have been described previously (61) and were supplemented with FeCl3 as and where indicated. Plasmid pGP003, carrying the rhlI gene, was constructed by amplification of rhlI from the chromosome of P. aeruginosa K1120 by PCR using primers rhlI-F (5′-GGATCCGGATCCCTGCAATGGACCGAC-3′; tandem HindIII sites underlined) and rhlI-R (5′-AAGCTTAAGCTTGATCAGCTG-3′; EcoRI site underlined). The upstream and downstream PCR products were subsequently cloned into pEX18Tc by cloning PCR-amplified 1-kb DNA fragments corresponding to the regions upstream and downstream of the rhlI sequences to be deleted. Sequences 5′ to the deletion were amplified using primers RhlRDown-For (5′-GATCCGATCCTCATCTGAAACG-3′; BamHI site underlined) and RhlRDown-Rev (5′-GATCCGGTATCATCTGAAACG-3′; BamHI site underlined) and RhlRDown-Rev (5′-GATCAGTGGATACGGAAGCAGTTC-3′; HindIII site underlined). The 50-μl PCR mixtures were formulated as described previously (79) (plus 10% [vol/vol] dimethyl sulfoxide for the downstream-arm PCR) and heated for 3 min at 95°C, followed by 29 cycles of 45 s at 95°C, 45 s at 64°C (upstream fragment) or 57°C (downstream fragment), and 2 min at 72°C, before finishing with a 10-min elongation at 72°C. The upstream and downstream PCR products were sequentially cloned into pEX18Tc to yield pCG001 and sequenced to confirm the absence of mutations in the cloned DNA. Plasmid pCG001 was mobilized into P. aeruginosa K1120 from E. coli S17-1 via conjugation (78), and following selection of pCG001 transconjugants on tetracycline (50 μg/ml) and chloramphenicol (5 μg/ml) to counterselect donor E. coli, putative rhlI deletion strains were subsequently recovered on L agar supplemented with sucrose (10%).

TABLE 1. Bacterial strains and plasmids

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<tr>
<th>Species and plasmid</th>
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<tr>
<td><strong>E. coli</strong></td>
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<td>DH5α</td>
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<td>K2873</td>
<td>K1120 ΔrhlR</td>
<td>This study</td>
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<td>pUCP18::gfp; Ap(^{+}); Cb(^{+})</td>
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\(a\) Tc\(^{+}\), tetracycline resistant; Ap\(^{+}\), ampicillin resistant; Cm\(^{+}\), chloramphenicol resistant; Km\(^{+}\), kanamycin sensitive; Km\(^{\text{-}}\), kanamycin resistant; Cb\(^{+}\), carbenicillin resistant.
Chromosome were selected on L agar containing tetracycline (10 g/ml) and P. aeruginosa pGP001, mobilized into E. coli plate (Nunc Brand, Roskilde, Denmark). Wells were inoculated with 200 μl of P. aeruginosa (final absorbance at 600 nm [A600] of 0.05) in iron-limited BM2 succinate minimal media supplemented with the specified concentrations of FeCl3. Plates were incubated at 37°C for the specified times while shaking at 200 rpm. Biofilm mass was quantified by staining the biofilm-containing pegs on the lid with 1% (wt/vol) crystal violet solution for 20 min and then, after washing the unbound dye in water, eluting the bound dye in ethanol with gentle rocking. Both staining and eluting were performed in clean microtiter plates containing 200 μl of the respective solutions. An absorbance reading at 550 nm of the eluted dye on a plate reader spectrophotometer provided an indirect measure of the biofilm (mass) that was attached to each peg. Alternatively, the biomasses of 12 pegs from one concentration of FeCl3 were pooled for use in β-galactosidase assays. In both 1 h spectroscopy of 100 μl at room temperature. Confocal scanning laser microscopy with a MRC 1024 system (Bio-Rad) and an Axioskop (Zeiss) were used for three-dimensional images by using Volocity image analysis software (Improvision).

**Twitching assay.** Surface-associated twitching motility was assayed via sub-agar stab inoculations in various media based on a method described previously (71). Twitching media consisted of BM2 succinate, BM2 glucose, or L broth, each solidified with 1% (wt/vol) agar and, where indicated, supplemented with various concentrations of FeCl3. Colonies were transferred from solid BM2 medium and stab inoculated in the appropriate twitching medium. Twitching motility zones (occurring at the agar/petri plate interface) were allowed to develop for 40 (BM2 minimal media) or 24 (L broth) hours at 37°C, after which the agar was carefully removed, leaving the twitching zone attached to the petri plate. The twitching zones were then visualized by staining for 1 minute with 1% (wt/vol) crystal violet and their diameters measured. In some experiments, twitching plates were supplemented with C4-HSL (at 5 μM; Sigma) or cell-free culture supernatants from Fe-limited or Fe-rich cultures of wild-type and rhl P. aeruginosa. Culture supernatants were recovered following removal of cells by centrifugation, filtered sterile (Millipore GF Express Plus membrane; 0.22-μm pore size), and used at 1:10 dilution in the twitching medium. Where indicated, culture supernatants were heat (30 min at 95°C) and proteinase K (100 μg/ml for 1 h at 37°C) treated prior to their use in twitching plates.

**Construction of a PA4570-lacZ reporter fusion.** In order to permit ready assessment of iron limitation of P. aeruginosa during planktonic and biofilm growth, reporter strain K2589, in which a known iron limitation-inducible gene (PA4570) was fused to lacZ in the chromosome, was constructed. Initially, the 5′-upstream region of PA4570 was amplified via PCR using the conditions described above for cloning of rhl, with primers PA4570-F (5′-AACGCTTAACGCAATACCG GCC-3′; tandem HindIII sites underlined) and PA4570-R (5′-GGATCCGGATCCGCGT AGGCTGC-3′; tandem BamHI sites underlined), an annealing temperature of 67°C, and 30 cycles of amplification. The PA4570 promoter-containing product was cloned upstream of the promoterless lacZ gene of plasmid mini-CTX-loxZ and the resulting vector, pGp001, mobilized into P. aeruginosa K1120 via conjugation with pGP001-carrying E. coli SM10 (87). Transconjugants carrying pGp001 integrated into the chromosome were selected on L agar containing tetracycline (10 μg/ml) and imipenem (0.5 μg/ml) to counterselect donor E. coli. The mini-CTX plasmid backbone was subsequently excised from the chromosome by using the plasmid pFLP2-encoded Flp recombinase as described previously (28), leaving the PA4570-lacZ fusion behind.

**β-Galactosidase assay.** β-Galactosidase assays were performed according to the method of Miller (47) on log-phase P. aeruginosa cells grown in BM2 succinate without added FeCl3 supplementation. In the case of β-galactosidase assays of microtiter plate biofilm cells grown in the presence of various FeCl3 concentrations, pegs (12 for every different growth condition) with attached biofilms were detached from the 96-well-plate lid and vortexed with glass beads in 1 ml total of sterile phosphate-buffered saline (PBS). Following recovery of the biofilm-containing PBS and pelleting of biofilm biomass by centrifugation in a microcentrifuge, the pelleted cells were resuspended in 0.1 ml fresh PBS and assayed for β-galactosidase activity as described above with modifications. Limited cell recovery and, thus, minimal “signal” in this assay (which yielded substantial variation experiment to experiment), the A605 readings were normalized such that the highest A605 reading for any experiment was arbitrarily adjusted to 100 and all other A605 readings were adjusted proportionally. The corresponding planktonic cells from 12 wells were also recovered, pooled, and assayed for β-galactosidase activity by using the standard Miller assay.

**C4-HSL bioassay.** Relative levels of C4-HSL in cell-free culture supernatants of P. aeruginosa were determined using a bioassay approach described previously (58) with modifications. Filter-sterilized culture supernatants (50 μl) from P. aeruginosa grown in Fe-limited or Fe-rich minimal medium were prepared as described above and mixed with equal volumes of E. coli (pEPCP61.5) diluted to an A600 of 0.1 in L broth supplemented with 0.1 M isopropl-β-D-thiogalactopyranoside ( IPTG). After 6 h of incubation at 30°C, β-galactosidase assays (47) were carried out, and the values obtained were normalized per ml of supernatant from a culture with an A600 of 1.0. Plasmid pEPCP61.5 carries the C4-HSL-responsive gene rhlA fused to lacZ, so β-galactosidase production becomes an indirect measure of supernatant C4-HSL.

**Mini-Tn5-tet transposon mutagenesis.** P. aeruginosa strain K2589 was mutagenized with mini-Tn5-tet following mobilization of mini-Tn5-tet-carrying plasmid pUT from E. coli SM10 (Apir) as described previously (14). Approximately 5,000 mutants were screened for a loss of low-iron-enhanced twitching motility on iron-limited BM2 succinate twitching plates, and those showing reduced twitching motility relative to that of the K2589 parent were recovered. These were then examined on L agar and Fe-replete (100 μM FeCl3) BM2 succinate twitching plates to eliminate mutants with general defects in twitching motility (i.e., only those mutants showing reduced twitching motility on Fe-limited BM2 succinate were retained). The mini-Tn5-tet-disrupted genes from the twitching mutants were recovered following digestion of genomic DNA with PstI or NotI and shotgun cloning into pUC18. Transformants of E. coli DH5α carrying pUC18 with inserts of mini-Tn5-tet and flanking chromosomal DNA were selected on LB agar containing tetracycline (10 μg/ml), and the chromosomal DNA flanking the mini-Tn5-tet element was sequenced using primers mini-Tn5-Te-Right and mini-Tn5-Te-Left as before (79).

**RESULTS**

**Impact of Fe on biofilm formation.** Previous studies have suggested that Fe limitation compromises biofilm formation, though these generally relied on the use of metal chelators (e.g., lactoferrin) to impose Fe restriction (75, 76). Using a defined Fe-limited minimal medium and Fe supplementation, we sought to examine the specific impacts of various medium iron levels on biofilm formation. As seen in Fig. 1, biofilm yields of P. aeruginosa K1120 declined with declining Fe supplementation, with Fe concentrations below 8 μM markedly reducing biofilm yields. Planktonic cell growth also declined with declining Fe levels, though only at concentrations below 1 μM (Fig. 1), indicating that the Fe needs of biofilm cells are greater than those of planktonic cells. A mutant of P. aeruginosa unable to synthesize pyoverdine (K1203) was even more compromised as regards the impact of Fe limitation on biofilm formation, with Fe concentrations below 64 μM markedly reducing biofilm yields (Fig. 2). This highlights both the negative impact of Fe limitation on biofilm formation by P. aeruginosa.
pyoverdine-deficient mutants are less able to acquire Fe [61] and, so, more Fe limited) and the importance of pyoverdine as regards Fe acquisition in biofilm cells. To confirm that biofilm reduction was, indeed, a response to Fe limitation, a reporter \( \text{P. aeruginosa} \) strain carrying a chromosomal \( \text{lacZ} \) transcriptional fusion to a gene known to be induced in response to Fe limitation (PA4570) (54), K2589, was constructed. In planktonic cells, increasing \( \beta\)-galactosidase activity was clearly seen in \( \text{P. aeruginosa} \) K2589 as cell yields declined with declining Fe supplementation (Fig. 3), consistent with the known Fe limitation inducibility of PA4570. In biofilm K2589 cells, too, \( \beta\)-galactosidase activity increased in concert with reduced biofilm yields, confirming that biofilm cells were sensing Fe limitation and that this was likely responsible for biofilm reduction.

**Impact of Fe on twitching motility.** Reduced biofilm formation in response to Fe limitation has previously been attributed to enhanced twitching motility, though again, these studies were carried out using metal chelators (lactoferrin or desferrioxamine) to impose Fe restriction (75, 76), and studies of biofilm formation by other organisms (e.g., \( \text{Streptococcus mutans} \)) in response to Fe chelation have shown that lactoferrin can have effects on biofilm formation independent of Fe restriction (10). To directly examine the impact of Fe on twitching motility, we again used an Fe-limited minimal medium with or without Fe supplementation. While limited twitching motility of \( \text{P. aeruginosa} \) K1120 was observed in Fe-replete (100 \( \mu \text{M} \) Fe\( \text{Cl}_3 \)) medium, this increased markedly in Fe-limited (i.e., non-Fe-supplemented) medium (Fig. 4). Moreover, twitching motility remained limited at all levels of Fe supplementation above 4 \( \mu \text{M} \), with concentrations at or below 4 \( \mu \text{M} \) only promoting enhanced twitching motility (data not shown). The pyoverdine-deficient mutant K1203 showed increased (relative to that of K1120) twitching motility on Fe-limited medium (the twitching zone increased from 7.6 to 14 mm), presumably because it is less able to transport Fe and, so, is more Fe limited than its parent strain. The effect of Fe on twitching motility was not explainable by a negative impact of Fe on twitching and/or...
growth on twitching plates, insomuch as addition of 100 μM FeCl₃ to L agar twitching plates did not adversely affect twitching motility on this medium (data not shown), where P. aeruginosa displays substantial twitching motility (Fig. 5c). The enhanced twitching motility with decreasing Fe thus parallels the reduction in biofilm formation, consistent with low-Fe-stimulated twitching motility contributing to loss of biofilm formation.

**Low-Fe-stimulated twitching motility requires RhlI.** To gain insight into the mechanism of low-Fe-stimulated twitching motility and its apparently negative impact on biofilm formation, we sought to isolate mutants of P. aeruginosa specifically defective in the enhanced twitching motility seen in response to Fe limitation. Thus, P. aeruginosa K2589 was mutagenized using the mini-Tn5-tet transposon and mutants showing normal twitching motility on L agar with or without Fe supplementation, and following incubation at 37°C for 40 h, the twitching zones were stained (bottom) and their diameters measured (top). Error bars represent the standard errors of the means for a representative assay performed in triplicate. Bars under twitching zones (bottom) represent 10 mm. Similar results were observed using glucose minimal medium.

**FIG. 4. Influence of iron availability on twitching motility.** P. aeruginosa K1120 was inoculated onto succinate minimal agar with or without Fe supplementation, and following incubation at 37°C for 40 h, the twitching zones were stained (bottom) and their diameters measured (top). Error bars represent the standard errors of the means for a representative assay performed in triplicate. Bars under twitching zones (bottom) represent 10 mm. Similar results were observed using glucose minimal medium.

**FIG. 5. Involvement of rhlI in low-iron-stimulated twitching motility.** P. aeruginosa strains K2589 (RhlI⁺), K2590 (RhlI⁻), and K2590 harboring rhlI on low-copy-number plasmid pGP003, and K2590 with pMMB207 alone were inoculated onto iron-limited BM2 glucose (a), iron-replete (100 μM FeCl₃) BM2 glucose (b), and Lagar (c) twitching plates and twitching zones (diameter) measured after 24 (L agar) or 40 (minimal media) h of incubation at 37°C. Error bars represent the standard errors of the means for a representative assay performed at least in triplicate.

The disrupted gene in K2590 was subsequently identified as rhlI, encoding the C4-HSL synthase (43), which affects a number of genes in P. aeruginosa whose expression is cell density dependent (i.e., regulated by QS) (69). Introduction of the cloned rhlI gene into K2590 restored enhanced twitching motility on Fe-limited minimal medium (Fig. 5a) but showed the same twitching motility as the K2589 parent on Fe-replete minimal medium (Fig. 5b) and L agar (Fig. 5c), indicating that it did not have a general defect in twitching motility. The disrupted gene in K2590 was subsequently identified as rhlI, encoding the C4-HSL synthase (43), which affects a number of genes in P. aeruginosa whose expression is cell density dependent (i.e., regulated by QS) (69). Introduction of the cloned rhlI gene into K2590 restored enhanced twitching motility on Fe-limited minimal medium (Fig. 5a), consistent with the rhlI mutation being responsible for the low-Fe-stimulated twitching motility defect of this mutant. In agreement with this, rhlI mutants obtained from two different transposon mutant libraries (33, 44) (Table 1) also showed the same twitching motility defect on Fe-limited medium (data not shown).

**Impact of RhlI on low-Fe-abrogated biofilm formation.** If the enhanced twitching motility seen in P. aeruginosa in response to growth in Fe-limited medium is responsible for reduced biofilm formation, loss of this low-Fe-stimulated twitching motility in the rhlI mutant should largely restore biofilm formation. With a flow cell biofilm reactor, RhlI⁺ strain K2589 was shown to form well-developed three-dimensional mushroom-like biofilms during growth under Fe-replete conditions (Fig. 6A), with Fe limitation yielding flat, undeveloped biofilms (Fig. 6B), as reported previously (6). In the rhlI mutant strain K2590, structured biofilm formation was largely restored during growth in Fe-limited medium (Fig. 6D), though mushroom “caps” were generally lacking, and this was also true of K2590 grown in Fe-rich medium (Fig. 6C). Introduction of the cloned rhlI gene on plasmid pGP003 into K2590 yielded wild-type biofilms in both media (Fig. 6E and F). Addition of exogenous C4-HSL to K2590 also restored the wild-type biofilm phenotype in low-Fe medium (Fig. 6G). Thus, while an rhlI mutant is somewhat defective as regards mature biofilm formation under either growth condition, biofilm formation by the mutant is not negatively affected by Fe limitation as it is in the RhlI⁺ parent strain.

**Impact of C4-HSL on twitching motility.** The impact of an rhlI mutation on twitching motility and biofilm formation in Fe-limited medium might simply be explained by loss of C4-HSL production, which may be needed for expression of one or more genes involved in twitching motility. Initially, cell-free spent culture supernatants from RhlI⁺ P. aeruginosa K1120 grown in Fe-limited minimal medium (as a crude source of C4-HSL) were tested for their abilities to restore the twitching
motility of the *rhl* mutant K2590 on Fe-limited medium. As seen in Fig. 7A, the Fe-limited culture supernatant of *P. aeruginosa* K1120 promoted substantial twitching motility of K2590 on Fe-limited medium and, indeed, enhanced the twitching motility of K1120 on this medium as well (Fig. 7A). In contrast, cell-free supernatants from Fe-replete (10 \( \mu \)M FeCl\(_3\)) cultures of *P. aeruginosa* K1120 did not promote the twitching motility of K2590 on Fe-limited medium (Fig. 7A). This concentration of Fe was chosen to represent Fe-replete conditions in this study, because unlike supernatants from 100 \( \mu \)M FeCl\(_3\) cultures, which adversely affected the twitching motility of K1120 on Fe-limited plates (likely owing to Fe contamination of the twitching plates), supernatants from 10 \( \mu \)M FeCl\(_3\) cultures did not (Fig. 7A). Thus, the absence of an effect of the Fe-replete K1120 supernatant on K2590 twitching on Fe-limited plates could not attributed to excess Fe (from the culture supernatant) introduced into the Fe-limited twitching medium interfering with any twitching motility-promoting component that might have been present in the supernatant. As expected, supernatants prepared from Fe-limited K2590 did not promote the twitching motility of K2590 (or any strain tested) on Fe-limited medium (Fig. 7A). These data were consistent with C4-HSL being produced under Fe-limited but not Fe-replete conditions and, so, specifically stimulating twitching motility in the former instance. Using a bioassay involving *E. coli* carrying a C4-HSL-responsive lacZ reporter fusion to measure C4-HSL in spent culture supernatants of *P. aeruginosa* K1120, we confirmed that \( \beta \)-galactosidase activity (and, so, C4-HSL levels) increased markedly in Fe-limited versus Fe-replete minimal media (Fig. 8). Moreover, C4-HSL supplementation (5 \( \mu \)M) of Fe-limited twitching medium stimulated the twitching motility of K2590 (Fig. 9). Enhanced C4-HSL production by *P. aeruginosa* under Fe-limiting conditions is consistent with previous reports of low-Fe stimulation of *rhlR* expression (21, 34) and suggests that the observed low-Fe stimulation of twitching motility results from the C4-HSL/RhlR-promoted expression of a gene(s) required for twitching. Consistent with this, a \( \Delta rhlR\) mutant (K2873) was also defective in low-Fe-stimulated twitching motility (Fig. 7A) and this was not reversed upon C4-HSL supplementation (Fig. 9).

**Low-Fe-promoted C4-HSL production does not explain low-Fe-stimulated twitching motility.** If the Fe-regulated production of C4-HSL could explain the enhanced twitching motility of Fe-limited *P. aeruginosa* K1120 and the general lack of twitching motility for Fe-replete K1120, addition of exogenous C4-HSL would promote twitching of this strain on Fe-replete
medium. Exogenous C4-HSL failed, however, to promote the twitching motility of K1120 and, indeed, the rhlI and rhlR mutant strains on Fe-replete medium (Fig. 9). Interestingly, however, spent culture supernatants from Fe-limited (but not Fe-replete) K1120 strongly stimulated the twitching motilities of all three strains on Fe-replete medium (Fig. 7B) and, unlike Fe-replete) K1120 strongly stimulated the twitching motility on Fe-limited medium. While an earlier report suggested that Fe limitation adversely affects twitching motility (75, 76), our observation here and elsewhere (6) that a defect in siderophore (pyoverdine)-mediated Fe acquisition compromises biofilm formation seems also to be linked to enhanced twitching, presumably because such mutants are more Fe limited than their wild-type counterparts.

While these results indicate that limited Fe negatively affects biofilm formation by P. aeruginosa, other studies clearly show that elevated levels of Fe also compromise biofilm formation (51, 82) and, indeed, promote biofilm dispersal (51). The suggestion in one instance that low Fe thus favors P. aeruginosa biofilm formation (82), however, is incorrect and misleading, inasmuch as the Fe supplementation that negatively affected biofilm formation in these studies was typically >50 μM (51, 82) (a modest effect was seen at 10 μM, and concentrations below this were not tested [82]). In our studies, Fe levels below 8 μM were needed to effect Fe limitation and the attendant negative impact on biofilm formation, so clearly, Fe limitation and Fe excess both adversely affect biofilm formation in P. aeruginosa. Indeed, we also noted a reduction in biofilm formation, usually at levels of FeCl3 supplementation above 32 to 64 μM (G. Patriquin, unpublished results).

A significant finding of these studies is an RhlIR/C4-HSL requirement for the twitching motility seen in Fe-limited minimal medium. While an earlier report suggested that rhlI mutants were compromised for twitching on a rich medium (23), the authors of this study could not complement this defect with the cloned rhlI gene. A subsequent study revealed that rhlI mutants were not, in fact, altered with respect to their abilities to migrate via twitching motility on rich medium (a finding also

**DISCUSSION**

Apparent Fe influences on P. aeruginosa biofilm formation (6) and twitching motility (75, 76) have been reported previously, although given the general use of rich media in these studies (supplemented with metal chelators to effect Fe limitation) and the known medium/environmental dependence of these processes (73), we sought to assess the impact of this metal on biofilm formation and twitching motility directly by using a minimal medium with/without Fe supplementation. Our observation that Fe limitation compromises biofilm formation is in agreement with previous studies of P. aeruginosa (6) and, interestingly, contrasts with studies of biofilms in another organism, Staphylococcus aureus, where Fe limitation appears to stimulate biofilm formation (35). The negative impact of Fe limitation on biofilm formation coincides with its stimulation of twitching motility, again in agreement with previous studies showing that Fe-binding lactoferrin also promotes the twitching motility of P. aeruginosa (75, 76). The observation here and elsewhere (6) that a defect in siderophore (pyoverdine)-mediated Fe acquisition compromises biofilm formation seems also to be linked to enhanced twitching, presumably because such mutants are more Fe limited than their wild-type counterparts.

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**FIG. 8.** Bioassay of C4-HSL production by P. aeruginosa during growth under Fe-limited and Fe-replete conditions. Aliquots (500 μl) of cell-free spent culture supernatants of P. aeruginosa K1120 (RhlI+) and K2590 (RhlI−) grown overnight in Fe-limited (−Fe/K1120 and −Fe/K2590) or Fe-replete (100 μM FeCl3 (+Fe/K1120 and +Fe/K2590) glucose minimal medium were incubated with E. coli/H11002 and K2590 (RhlI+) for two independent experiments performed in duplicate and are normalized to 1 ml of supernatant from a culture with an A600 of 1.

**FIG. 9.** Influence of exogenous C4-HSL on twitching motility. P. aeruginosa K1120, H2590 (RhlI−), and K2784 (RhlR+) were inoculated onto Fe-limited (−Fe) or Fe-replete (100 μM FeCl3 (+Fe) glucose minimal agar without (−HSL) or supplemented with (+HSL) 5 μM C4-HSL. Following incubation at 37°C for 40 h, the twitching zones were stained and their diameters measured. Results shown represent the means ± standard deviations for a representative assay (16 individual twitching zones measured per strain and/or growth condition on two separate plates) performed in duplicate.
confirmed here), although twitching motility-defective variants of this mutant did accumulate during culturing, as a consequence of secondary mutations in algR (8). This suggested that secondary algR mutations may well explain the earlier report of a twitching defect in the rhl mutant. The observation that the RhlIR requirement for twitching is thus nutritionally conditional is in line with earlier observations that the QS dependence of other processes is also dependent on the growth medium/environmental conditions. In one report, for example, a negative impact of rhl and las mutations on biofilm formation and swarming was seen during growth on succinate but not on glucose or glutamate (73).

A link between Fe and QS is now well established, with lasIR (11, 21, 37) and rhlIR (12, 21, 34) expression both reportedly enhanced by Fe limitation and/or repressed by Fe supplementation. One explanation, then, for the general lack of twitching on Fe-replete minimal medium and the enhanced twitching on Fe-limited medium is the increased rhlIR expression and concomitant C4-HSL production observed in Fe-limited medium, with the latter upregulating a gene(s) required for twitching. Still, the failure of exogenous C4-HSL to stimulate twitching of Rhl” _P. aeruginosa_ under Fe-replete conditions argues that, while RhlIR/C4-HSL are clearly required for low-Fe-stimulated twitching (rhlIR mutants are twitching defective on Fe-limited minimal medium, and C4-HSL restores twitching of an rhl mutant), other Fe-regulated factors are also involved. Additional screening of our mini-Tn5tet mutant library for mutants defective in low-Fe-promoted twitching (to identify possible Fe- and RhlIR-regulated genes that might be responsible for this twitching phenomenon) yielded a mutant, K2592, showing a more modest reduction (from ca. 8 mm to 5 mm) in twitching motility on Fe-limited medium than its K2589 parent. The twitching motility of K2592 was unaltered relative to that of K2589 on Fe-replete medium or L agar (data not shown).

This mutant was disrupted in the mexI gene of the mexHI-oprD multidrug efflux operon (2, 70). Still, given previous observations that mutants defective in this efflux system show markedly reduced synthesis of C4-HSL (1, 2), it is likely that the twitching defect seen in K2592 on Fe-limited medium again relates to defects in C4-HSL production.

The observation that spent culture supernatants from Fe-limited but not Fe-replete RhlIR” _P. aeruginosa_ are able to promote twitching of Rhl” _P. aeruginosa_ and Rhl” _P. aeruginosa_ under Fe-replete conditions is consistent with some soluble, extracellular factor(s) which is low Fe inducible (and RhlIR dependent; supernatants from an rhl mutant do not promote twitching) positively influencing twitching. Rhamnolipids are extracellular glycolipids implicated in another form of surface motility, swarming (19), and interestingly, expression of the rhlA rhamnolipid biosynthetic gene is both RhlIR dependent (13, 53) and Fe regulated (Fe limitation yielded an 80-fold increase in rhlAB expression in M9 medium but not TSB [54], while Fe supplementation of a glucose-nutrient broth strongly reduced rhlA expression [19]). Moreover, rhamnolipid production by _P. aeruginosa_ is typically observed in Fe-limited media (19), making rhamnolipids a possible candidate for the twitching motility-promoting component of Fe-limited _P. aeruginosa_ culture supernatants. The observation that heat and protease treatment did not adversely affect the twitching motility-promoting activities of these supernatants is consistent with this, though the role, if any, for rhamnolipids in twitching motility remains to be seen.

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