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Quorum Sensing Is Not Required for Twitching Motility in *Pseudomonas aeruginosa*

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It has been reported that mutations in the quorum-sensing genes *lasI* and *rhlI* in *Pseudomonas aeruginosa* result in, among many other things, loss of twitching motility (A. Glessner, R. S. Smith, B. H. Iglewski, and J. B. Robinson, J. Bacteriol. 181:1623-1629, 1999). We constructed knockouts of *lasI* and *rhlI* and the corresponding regulatory genes *lasR* and *rhlR* and found no effect on twitching motility. However, twitching-defective variants accumulated during culturing of *lasI* and *rhlI* mutants. Further analysis showed that the stable twitching-defective variants of *lasI* and *rhlI* mutants had arisen as a consequence of secondary mutations in *vfr* and *algR*, respectively, both of which encode key regulators affecting a variety of phenotypes, including twitching motility. In addition, when grown in shaking broth culture, *lasI* and *rhlI* mutants, but not the wild-type parent, also accumulated unstable variants that lacked both twitching motility and swimming motility and appeared to be identical in phenotype to the S1 and S2 variants that were recently reported to occur at high frequencies in *P. aeruginosa* strains grown as a biofilm or in static broth culture (E. Deziel, Y. Comeau, and R. Villemur, J. Bacteriol. 183:1195-1204, 2001). These results indicate that mutations in one regulatory system may create distortions that select during subsequent culturing for compensatory mutations in other regulatory genes within the cellular network. This problem may have compromised some past studies of regulatory hierarchies controlled by quorum sensing and of bacterial regulatory systems in general.

Pseudomonas aeruginosa is an opportunistic pathogen of many organisms, including plants, nematodes, insects, and animals (22, 30). In humans, it causes serious infections in those affected by cystic fibrosis, immunocompromised individuals such as those with AIDS or who are undergoing cancer chemotherapy, or burn patients who have breaches in their natural defenses (21, 39). This bacterium is very versatile and produces a wide range of virulence factors that are required for host colonization, including extracellular proteases, lipases, and toxins. Furthermore, *P. aeruginosa* possesses polar filaments called type IV pili (or fimbriae) that are involved in attachment and surface translocation by twitching motility and that also act as receptors for certain bacteriophages (2, 21). Twitching motility occurs in a wide variety of pathogenic bacteria, including *Neisseria gonorrhoeae* (40), and is mediated by pilus extension and retraction (24, 38). Twitching motility, at least in *P. aeruginosa*, is also required for the formation of biofilms (29).

Like many bacteria, *P. aeruginosa* has a quorum-sensing system by which it polls cell density and regulates the expression of many genes at high cell densities, including those encoding extracellular proteases, toxins, surfactants, and others (11, 14, 20, 50). Such systems usually consist of a transcriptional activator and a biosynthetic enzyme, the latter of which is responsible for producing a small diffusible molecule, termed an autoinducer (normally an acyl-homoserine lactone), that forms a complex with the former at high threshold levels to induce transcriptional activation of other genes (11, 14, 50). In *P. aeruginosa*, there are two well known quorum-sensing systems termed *las* and *rhl*. The *lasR* and *rhlR* genes encode transcriptional activators, and *lasI* and *rhlI* encode acyl-homoserine synthases that are responsible for the synthesis of *N*-(3-oxododecanoyl)-L-homoserine lactone and *N*-butyryl-L-homoserine lactone, respectively. LasR complexed with *N*-(3-oxododecanoyl)-L-homoserine lactone regulates transcription of *lasI*, *rhlR*, *lasA*, *lasB*, *apr*, and *toxA*, the latter four of which encode key virulence factors. RhlR complexed with *N*-butyryl-L-homoserine lactone regulates transcription of *rhlI*, *rhlA*, *lasB*, and *phzA*, which also encode key virulence factors (for recent reviews, see references 11, 14, and 50). Therefore, a hierarchy exists, with the *las* quorum-sensing system apparently situated above the *rhl* system.

However, it is becoming clear that the hierarchical quorum-sensing system is a great deal more complex than initially anticipated. Several additional regulatory proteins have been shown to exert control over the *las* or *rhl* system, including RpoS (19), GacA (31), RelA (41), and Vfr (1). There are also a number of conflicting reports in the literature, for example, that RpoS regulates *rhlI* (51), not vice versa, as previously reported (19). In fact, it has recently been shown that a secondary-site mutation in the *P. aeruginosa* wild-type strain used to construct a *lasR* knockout mutant accounts for at least some of this confusion (17).

Recently, it was reported that the *las* and *rhl* quorum-sensing systems are required for twitching motility in *P. aeruginosa* (16). We have been studying the genetic control of the biogenesis and function of type IV pili in this bacterium for many

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years and, with others, have characterized a large number of genes, now totaling more than 35, that are involved in this process, including the genes encoding the main structural subunit (*pilA* or pilin), the leader peptidase (*pilD*), other proteins involved in pilus assembly and twitching motility (many of which are homologous to other proteins required for type II protein secretion and DNA uptake in *P. aeruginosa* and other bacteria), and at least three regulatory systems, including the two-component sensor regulator systems *pilSR* and *algR/fimS* and a complex chemosensory system (*pilGHIJK*, *chpABCDE*) (2). Here we show that twitching motility is unaffected by mutations in the genes encoding the *las* and *rhl* quorum-sensing systems but that such mutations can lead to specific secondary mutations in other key regulatory genes that do affect twitching motility and that apparently represent compensatory mutations induced by distortions in the regulatory architecture of the cell.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strain DH5α was used in all genetic manipulations and in the preparation of DNA sequencing templates, and *E. coli* S17-1 was used as the donor strain in bacterial conjugation (36). *P. aeruginosa* competent cells and transformations were prepared as described previously (23). *E. coli* and *P. aeruginosa* liquid cultures were maintained in Luria-Bertani (LB) broth (32), and solid medium was prepared by adding 1 to 1.5% Select agar (Gibco-BRL). Light microscopy was performed by using nutrient medium (4 g of Tryptone liter^{-1}, 2 g of yeast extract liter^{-1}, 2 g of NaCl liter^{-1}) solidified with 8 g of GelGro (ICN) liter^{-1} for greater optical clarity. The following antibiotic concentrations were used for selection of *E. coli*: 10 μg of tetracycline ml^{-1}, 100 μg of ampicillin ml^{-1}, 50 μg of kanamycin ml^{-1}, and 10 μg of gentamicin ml^{-1}. The concentrations of antibiotics used for selection of *P. aeruginosa* were 500 μg of carbenicillin ml^{-1}, 20 μg of rifampin ml^{-1}, 100 μg of gentamicin ml^{-1}, and 200 μg of tetracycline ml^{-1}. Sucrose (5%) was added to plates for *sacBR* counterselection during allelic exchange.

Isolation of genes and construction of isogenic mutants. Allelic-exchange mutations were constructed in the genes *lasR*, *lasI*, *rhlR*, *rhlI*, and *pheC* by using the sucrose selection system described previously (4, 33). All reference sequence data for these genes, as well as *algR* and *vfr*, and their local restriction site topography were taken from the *P. aeruginosa* genome database (http://pseudomonas.bit.uq.edu.au) (9). Comparison of amplimer sequences with the *P. aeruginosa* genome database provides information about the exact nature of the cloned gene sequences.

lasI, *rhlI*, *lasRI*, *rhlRI*, and *pheC* loci were amplified from chromosomal DNA by using flanking amplimers (designed against the 5′ upstream and 3′ downstream regions adjacent to the relevant coding regions), whose sequences are available on request. The *lasRI* amplicon was cloned into the *Xho*I-*Bam*HI sites of pOK12 (41), whereas the *rhlRI* and *pheC* amplicons were cloned into the *Not*I-*Xho*I sites of pOK12 (note that the *rhlRI* amplicon was cloned as a *Not*I-*Sal*I fragment due to an internal *Xho*I site). All plasmids were sequence verified. Single-knockout mutations in these genes were made by insertion of the gentamicin resistance cassette (Gm) from pX1918GT (34) or the tetracycline resistance cassette (Tc) from pSM-TET (25) into restriction sites within *lasI* (*Eco*RI), *lasR* (*Sal*I), *rhlI* (*Eco*RI), and *rhlR* (*Bam*HI). The disrupted genes were then isolated by digestion with *Spe*I and inserted into the corresponding site of the suicide vector pRIC380 (4). This vector carries the genes *sacBR*, which promote sensitivity to sucrose, and *oriT*, which enables conjugal transfer. The constructs were then used to transform *E. coli* donor strain S17-1 in preparation for mating with *P. aeruginosa*. Following conjugation, the transconjugants were plated on 5% sucrose medium containing tetracycline or gentamicin, as appropriate, to select for colonies in which the plasmid had been excised while leaving the homologously recombined mutated gene in the chromosome. Mutants were confirmed by Southern blot analysis.

The double mutations *lasI*::Gm *rhlI*::Tc and *lasR*::Gm *rhlR*::Tc were made by sequential antibiotic resistance insertions and allelic replacements by using the strategy outlined above. The quadruple mutation Δ*lasRI*::Gm Δ*rhlRI*::Tc was made by sequential replacement of *Sal*I-*Eco*RI and *Bam*HI-*Eco*RI fragments, which span *lasRI* and *rhlRI*, with gentamicin and tetracycline resistance cassettes, respectively. The Δ*lasRI*::Gm knockout also removes a small open reading frame termed *rsaL* between *lasR* and *lasI* (10).

The *pheC* knockout was made similarly by insertion of the tetracycline resistance cassette into its *Bam*HI site, and the *rhlI*::Gm *pheC*::Tc double mutation was made by subsequent insertion of the gentamicin cassette into the *Eco*RI site of *rhlI*.

PAK*lasI*::Tc and PAK*rhlI*::Tc were constructed by using allelic-exchange plasmids that differed from those used to construct the respective PAO1 mutants. Specifically, *lasI* and *rhlI* were amplified from chromosomal DNA by using flanking amplimers (sequences are available on request). The resulting amplicons were inserted as *Bam*HI-*Xba*I fragments into pOK12. Single-knockout mutations in these genes were made by insertion of the Tc-encoding sequence from pSM-TET (25) into *Eco*RI within *lasI* and into *Kpn*I within *rhlI*. The disrupted genes were then isolated by using *Spe*I and inserted into the corresponding site of the suicide vector pRIC380 (4). Isogenic knockout mutants of *P. aeruginosa* PAK were made as described previously. For complementation studies, pUCP*lasI* and pUCP*rhlI* were constructed by excising *lasI* and *rhlI* from the corresponding pOK12 plasmids as *Xho*I-*Xba*I fragments and inserting them into the *E. coli-P. aeruginosa* shuttle expression vector pUCPKS (44) (with *lac*).

All knockout mutations were confirmed by Southern analysis with indicative restriction digests and relevant probes.

Wild-type and mutated *vfr* and *algR* alleles were PCR amplified from chromosomal DNA by using specific amplimers (sequences available on request). PCR products were inserted against *lac* as *Kpn*I-*Hin*dIII or *Bam*HI-*Hin*dIII fragments, respectively, into the *E. coli-P. aeruginosa* shuttle expression vector pUCPSK (44) for sequence analysis and complementation studies.

DNA sequencing. PCR-amplified genes were subcloned into pUCPSK (44) as described above. Plasmid DNA was isolated by alkaline lysis minipreps, and cloned inserts were sequenced (both strands) by using universal primers and internal primers with dye terminator chemistry on an ABI 377 DNA sequencer by the Australian Genome Research Facility, University of Queensland, Brisbane, Queensland, Australia. DNA sequences were compared with reference sequences from the *P. aeruginosa* genome (http://pseudomonas.bit.uq.edu.au).

Twitching motility assay. Twitching motility was assayed by the subsurface agar method described previously (3, 35). Briefly, the *P. aeruginosa* strain to be tested was stab inoculated through a 1% agar plate, and after overnight growth at 37°C, the zone of twitching motility elaborated at the interstitial surface between the agar and the petri dish was visualized by staining with Coomassie brilliant blue R250.

Mutation assays. Wild-type PAK, PAK*lasI*::Gm, and PAK*rhlI*::Tc were recovered from frozen stocks and streaked to single colonies on LB agar. To compare the frequencies of spontaneous twitching defects and streptomycin resistance among wild-type PAK and the PAK*lasI*::Tc and PAK*rhlI*::Tc mutants, dilutions of 2 ml of LB broth cultures (inoculated from a single colony and incubated for 24 h at 37°C in 10-ml polystyrene tubes) were diluted and spread plated on LB agar with or without 500 μg of streptomycin (Sigma) ml^{-1}. Twitching-defective colonies were identified after 24 h of incubation at 37°C by colony morphology and confirmed by twitching stab assay. Streptomycin-resistant colonies were scored on antibiotic-containing plates after 32 h of incubation at 37°C and patched onto fresh antibiotic-containing plates to confirm their phenotype.

Serial subcultures were performed by inoculating a fresh tube containing 2 ml of LB broth with 20 μl of a 10^{-4} dilution of the previous day's culture for 4 consecutive days. Broths were incubated at 37°C with agitation overnight, and dilutions were plated on LB agar plates. Each day for 4 days, at least 1,000 CFU from each experiment were scored for twitching motility by colony morphology and the phenotype of up to 50 twitching-defective colonies from each experiment was confirmed by twitching stab assay.

Competitive 1:1 growth assays were carried out by coinoculating fresh tubes containing 2 ml of LB broth with 20 μl of a 10^{-3} dilution of an overnight culture of each test strain. Similarly 1:100 or 100:1 competitive assays used 20 μl of a 10^{-3} dilution and 10^{-1} dilution of an overnight culture of each test strain or vice versa, respectively. Competitions were incubated overnight as described above, and the relative fitness of each strain was determined by plate counts using colony morphology to distinguish strains.

RESULTS AND DISCUSSION

Twitching motility is not controlled by quorum sensing. To investigate the relationship between quorum sensing and twitching motility, we constructed isogenic *lasI* and *rhlI* knockout mutants of *P. aeruginosa* strain PAK and *lasR*, *lasI*, *rhlR*,

TABLE 1. Plasmids, primers and *P. aeruginosa* strains used in this study

Strain or plasmid	Relevant characteristics[b]	Source or reference
Strains		
E. coli		
DH5α	*endA1 hsdR17*(r_K^- m_K^+) *glnV44 thi-1 recA1 gyrA relA1* Δ(*lacIZYA-argF*)*U169 deoR* [ϕ*80dlac*Δ(*lacZ*)*M15*]	Laboratory collection
S17-1	*thi pro hsdR recA chr*::*RP4-2*	36
P. aeruginosa		
PAK	Wild-type *P. aeruginosa* strain K	Laboratory collection[a]
PAO1	Wild-type *P. aeruginosa* strain ATCC 15692	Laboratory collection
PAK*pilA*::Tc	Previously referred to as AWK	45
PAK*lasI*::Tc	PAK with Tc cartridge inserted into unique *Eco*RI site of *lasI* (*vfr* wild type)	This study
PAK*lasI*-RT	PAK*lasI*::Tc reduced-twitching spontaneous mutant (VfrΔEQERS)	This study
PAK*lasI*-RT/T1	PAK*lasI*-RT normal-twitching spontaneous mutant (VfrΔEQERS, S194R)	This study
PAK*lasI*-RT/T2	PAK*lasI*-RT normal-twitching spontaneous mutant (VfrΔEQERS, D36A)	This study
PAK*rhlI*::Tc	PAK with Tc cartridge inserted into unique *Kpn*I site of *rhlI* (*algR* wild type)	This study
PAK*rhlI*-NT	PAK*rhlI*::Tc nontwitching spontaneous mutant (AlgR V241G)	This study
PAK*rhlI*-NT/T1	PAK*rhlI*-NT normal-twitching spontaneous mutant (*algR* wild type)	This study
PAK*rhlI*-NT/T2	PAK*rhlI*-NT normal-twitching spontaneous mutant (*algR* wild type)	This study
PAO1*lasI*::Gm	PAO1 with Gm cartridge inserted into unique *Eco*RI site of *lasI*	This study
PAO1*rhI*::Tc	PAO1 with Tc cartridge inserted into unique *Eco*RI site of *rhlI*	This study
PAO1*lasR*::Gm	PAO1 with Gm cartridge inserted into unique *Sal*I site of *lasR*	This study
PAO1*rhlR*::Tc	PAO1 with Tc cartridge inserted into unique *Bam*HI site of *rhlR*	This study
PAO1*pheC*::Tc	PAO1 with Tc cartridge inserted into unique *Bam*HI site of *pheC*	This study
PAO1*rhlI*::Gm *pheC*::Tc	PAO1*pheC*::Tc with Gm cartridge inserted into unique *Eco*RI site of *rhlI*	This study
PAO1*lasI*::Gm *rhlI*::Tc	PAO1*lasI*::Gm with Tc cartridge inserted into unique *Eco*RI site of *rhlI*	This study
PAO1*lasR*::Gm *rhlR*::Tc	PAO1*lasR*::Gm with Tc cartridge inserted into unique *Bam*HI site of *rhlR*	This study
PAO1Δ*lasRI*::Gm	PAO1 with Gm cartridge replacing *Pst*I fragment shared by *lasR* and *lasI*	This study
PAO1Δ*rhlRI*::Tc	PAO1 with Tc cartridge replacing *Bam*HI-*Eco*RI fragment shared by *rhlR* and *rhlI*	This study
PAO1Δ*lasRI*::Gm Δ*rhlRI*::Tc	PAO1Δ*lasRI*::Gm with Tc cartridge replacing *Bam*HI-*Eco*RI fragment shared by *rhlR* and *rhlI*	This study
Plasmids		
pSM-TET	Source of Tc cartridge	25
pX1918GT	Source of Gm cartridge	34
pRIC380	*P. aeruginosa* suicide vector	4
pOK12	*E. coli* Km^r cloning vector	43
pUCPSK	*P. aeruginosa-E. coli* shuttle vector	44
pSB209.5A	3-kb PCR fragment containing *lasR* and *lasI* cloned into *Xho*I/*Bam*HI sites of pOK12	This study
pSB222.7A	pSB209.5A with Gm cartridge inserted into unique *Eco*RI site of *lasI*	This study
pSB219.8A	pRIC380 carrying *lasI*::Gm on *Spe*I fragment from pSB222.7A	This study
pSB222.25C	pSB209.5A with Gm cartridge inserted into unique *Sal*I site of *las*R	This study
pSB219.9A	pRIC380 carrying *lasR*::Gm on *Spe*I fragment from pSB222.25C	This study
pSB222.10A	2.8-kb PCR fragment containing *rhlR* and *rhlI* cloned into *Not*I/*Xho*I sites of pOK12	This study
pSB224.8A	pSB222.10A with Tc cartridge inserted into unique *Eco*RI site of *rhlI*	This study
pSB224.12B	pRIC380 carrying *rhlI*::Tc on *Spe*I fragment from pSB224.8A	This study
pSB224.4A	pSB222.10A with Tc cartridge inserted into unique *Bam*HI site of *rhlR*	This study
pSB224.10A	pRIC380 carrying *rhlR*::Tc on *Spe*I fragment from pSB224.4A	This study
pSB222.13A	2.5-kb PCR fragment containing *pheC* cloned into *Not*I/*Xho*I sites of pOK12	This study
pSB224.6A	pSB222.13A with Tc cartridge inserted into unique *Bam*HI site of *pheC*	This study
pSB224.11A	pRIC380 carrying *pheC*::Tc on *Spe*I fragment from pSB224.6A	This study
pSB255.1A	pSB222.10A with Gm cartridge inserted into unique *Eco*RI site of *rhlI*	This study
pSB255.3A	pRIC380 carrying *rhlI*::Gm on *Spe*I fragment from pSB255.1A	This study
pSB222.4A	pSB209.5A with Gm cartridge inserted into *Sal*I/*EcoRI* sites encompassing *lasR* and *lasI*	This study
pSB219.7A	pRIC380 carrying Δ*lasRI*::Gm on *Spe*I fragment from pSB222.4A	This study
pSB236.4A	pSB222.10A with Tc cartridge inserted into *Bam*HI/*Eco*RI sites encompassing *rhlR* and *rhlI*	This study
pSB280.1H	pRIC380 carrying Δ*rhlRI*::Tc on *Spe*I fragment from pSB236.4A	This study
pAS001	0.95-kb PCR fragment containing *lasI* cloned into *Bam*HI-*Xba*I sites of pOK12	This study
pAS002	0.79-kb PCR fragment containing *rhlI* cloned into *Bam*HI-*Xba*I sites of pOK12	This study
pAS003	pAS001 with Tc cartridge inserted into unique *Eco*RI site of *lasI*	This study
pAS004	pAS002 with Tc cartridge inserted into unique *Kpn*I site of *rhl*	This study
pAS005	pRIC380 carrying *lasI*::Tc from pAS003 on *Spe*I fragment	This study
pAS006	pRIC380 carrying *rhI*::Tc from pAS004 on *Spe*I fragment	This study
pUCP*lasI*	*lasI* cloned from pAS001 as *Xho*I-*Xba*I fragment into pUCPKS with *lac*	This study
pUCP*rhlI*	*rhlI* cloned from pAS002 as *Xho*I-*Xba*I fragment into pUCPKS with *lac*	This study
pSB299.15A	*vfr* amplified from PAO1 wild type and cloned into pUCPSK against *lac*	This study
pSB299.16A	*vfr* amplified from PAK wild type and cloned into pUCPSK against *lac*	This study
pSB299.17A	*vfr* amplified from PAK*lasI*-RT and cloned into pUCPSK against *lac*	This study
pSB313.7A	*vfr* amplified from PAK*lasI* and cloned into pUCPSK against *lac*	This study
pSB402.20	*vfr* amplified from PAK*lasI*-RT/T1 and cloned into pUCPSK against *lac*	This study
pSB402.23	*vfr* amplified from PAK*lasI*-RT/T2 and cloned into pUCPSK against *lac*	This study
pSB400.2	*algR* amplified from PAK wild type and cloned into pUCPSK against *lac*	This study
pSB400.9	*algR* amplified from PAK*rhlI* and cloned into pUCPSK against *lac*	This study
pSB401.30	*algR* amplified from PAK*rhl*-NT and cloned into pUCPSK against *lac*	This study
pSB401.38	*algR* amplified from PAK*rhl*-NT/T1 and cloned into pUCPSK against *lac*	This study
pSB401.43	*algR* amplified from PAK*rhl*-NT/T2 and cloned into pUCPSK against *lac*	This study

[a] Source: David Bradley, Memorial University of Newfoundland, St. John's, Newfoundland, Canada.
[b] Antibiotic resistance abbreviations: Tc, tetracycline; Gm, gentamicin; Km, kanamycin.

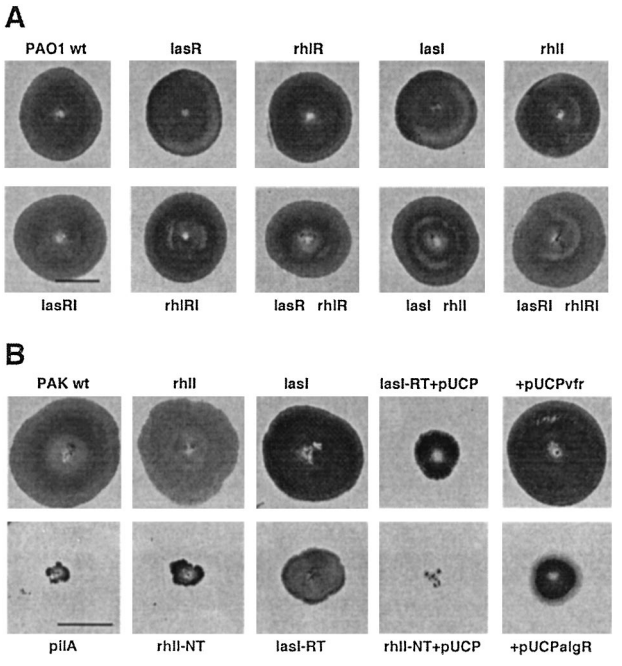

FIG. 1. Twitching motility phenotypes of quorum-sensing and other mutants. Assays were carried out by the subsurface agar stab method (3, 35). Twitching motility is observed as the large zone of Coomassie-stained cells radiating from the point of inoculation at the interstitial surface between the agar and the bottom of the petri dish. (A) *P. aeruginosa* PAO1 quorum-sensing mutants. (B) *P. aeruginosa* PAK quorum-sensing mutants, twitching-defective variants, and variants complemented with wild-type *vfr* and *algR* cloned on the multicopy vector pUCPSK (abbreviated pUCP) (44). Bar, 1 cm; wt, wild type.

and *rhlI* mutants of *P. aeruginosa* strain PAO1. However, we found no detectable differences in twitching motility between any of these mutants, including double (*lasI*::Gm *rhlI*::Tc, *lasR*::Gm *rhlR*::Tc, and Δ*lasRI*::Gm Δ*rhlRI*::Tc) and quadruple (Δ*lasRI*::Gm Δ*rhlRI*::Tc) knockouts, and the corresponding wild-type parents by the colonial twitching stab assay (Fig. 1) or by light microscopy (data not shown).

We examined several avenues to try to resolve the discrepancy between our observations and those reported by Glessner et al. (16). Those authors reported that the level of twitching motility in strain JP1 (PAO1*lasI*) was reduced to ~30% of the wild-type level and that strains PDO100 (PAO1*rhlI*) and JP2 (PAO1*rhlIlasI*) exhibited complete loss of twitching motility. These strains have been widely used to study quorum sensing in *P. aeruginosa* (11). We considered the possibility that our quorum-sensing mutants were somehow defective; however, the integrity of all of the mutants was confirmed by Southern analysis and the elastase- and rhamnolipid-deficient phenotypes of our *lasI* and *rhlI* mutants, respectively, were consistent with those reported in the literature (8, 15, 17) (data not shown).

The PDO100 and JP2 strains that were studied by Glessner et al. (16) contain an ~5-kb deletion that knocks out not only *rhlI* but also the adjacent *pheC* gene (8). PheC is homologous to FliY of *E. coli*, which has been implicated in cell density sensing and appears to be a periplasmic binding protein predicted to feed into chemosensory systems (26), raising the possibility that loss of *pheC* was responsible for the twitching motility defects observed in these strains. We therefore constructed an isogenic *pheC* knockout, as well as an *rhlI*::Gm *pheC*::Tc double mutant, but again found no effect on twitching motility.

Twitching-defective variants spontaneously arise among *lasI* and *rhlI* mutants. During the course of these experiments, however, we noticed that spontaneous defects in twitching motility, similar to those reported by Glessner et al. (16), commonly appeared in the PAK*lasI* and PAK*rhlI* quorum-sensing mutants. Specifically, nontwitching variants of PAK*rhlI* arose frequently during the allelic-exchange procedure and reduced-twitching variants of PAK*lasI* arose frequently after recovery from frozen stock culture. We isolated the stable twitching-defective mutants from these PAK*lasI* and PAK*rhlI* cultures. These mutants were nontwitching in the case of the PAK*rhlI* strain and showed reduced twitching in the case of the PAK*lasI* strain (hereinafter referred to as PAK*rhlI*-NT and PAK*lasI*-RT, respectively) (Fig. 1), phenotypes that were indistinguishable from those described for PDO100 and JP1, respectively (16) (see above). We confirmed that PAK*rhlI*-NT and PAK*lasI*-RT were bona fide variants of the PAK*rhlI*::Tc and PAK*lasI*::Tc strains, respectively, by Southern analysis (data not shown). Furthermore, we were unable to complement the twitching phenotypes of PAK*rhlI*-NT and PAK*lasI*-RT with cloned wild-type *rhlI* and *lasI*, respectively (data not shown). Therefore, the likeliest explanation was the occurrence of a spontaneous secondary-site mutation within a gene required for normal twitching motility.

Careful examination of the phenotypes of the PAK*rhlI*-NT and PAK*lasI*-RT strains and comparison of these with the known twitching-defective mutants in our collection (on the basis of such indices as the nature of the twitching zones, the micromorphology of the colony edge under twitching conditions, the susceptibility of these strains to pilus-specific bacteriophage, and the level of expression of the structural subunit PilA and its subcellular location [data not shown]) indicated that the phenotype of the PAK*rhlI*-NT strain was similar to that of *algR* mutants and the phenotype of the *lasI*-RT strain was similar to that of *vfr* mutants. AlgR is an atypical regulator that was first identified as being required for alginate production in mucoid strains of *P. aeruginosa* (12) and later shown to be also required for twitching motility, along with its cognate sensor FimS (49). Vfr is a homolog of the *E. coli* catabolite repressor protein CRP (6, 18, 48) and has been shown to regulate exotoxin A production, *regA* transcription, and quorum sensing, apparently via control of *lasR* transcription (1, 47, 48). We have recently identified Vfr as being required for normal twitching motility by screening of a transposon library of twitching-defective mutants (5). In agreement with the phenotypic similarities between these mutants, we found that twitching motility could be restored to PAK*rhlI*-NT and PAK*lasI*-RT by complementation with wild-type *algR* and wild-type *vfr*, respectively (Fig. 1). Conversely, PAK*rhlI*-NT and PAK*lasI*-RT were not complemented by wild-type *vfr* and *algR*, respectively.

Mutations in *algR* and *vfr* cause the twitching defect in PAK*rhlI*-NT and PAK*lasI*-RT, respectively. We then isolated and sequenced the *algR* gene from wild-type strain PAK and

TABLE 2. Proportions of bacterial populations exhibiting twitching defects after four consecutive subcultures[a]

Strain	Expt 1	Expt 2	Expt 3	Expt 4	Expt 5	Expt 6
PAK	0	0	0	0	0	0
lasI	0.05[b,c]	0.55[c,d]	0.24[c]	0	0.02[b,c]	0.23[d]
rhlI	0.63[c,d]	0.67[c,d]	0	0.07[d]	0.12[c]	0

[a] Proportion is based on ≈1,000 colonies examined for each experiment.
[b] Variants with twitching defect similar to *vfr* null mutant identified; *vfr* harbors EQERS deletion.
[c] S1-type variants identified.
[d] S2-type variants identified.

the PAK*lasI*::Tc, PAK*lasI*-RT, PAK*rhlI*::Tc, and PAK*rhlI*-NT mutant strains. The *algR* sequence was identical to that of the wild type in all of the strains except PAK*rhlI*-NT, which had a GGGGT→GGGGG transversion causing a V241G substitution. Homopolymeric runs of nucleotides are common sites for mutations. We also found that PAK*rhlI*-NT mutants spontaneously reverted to a normal-twitching phenotype at low frequencies when cultured on solid medium. Reversion was characterized by the emergence of a kidney-shaped flare of twitching cells at the edge of an otherwise smooth, nontwitching colony and was typically observed in approximately 1% of PAK*rhlI*-NT colonies after 3 or 4 days of incubation. The *algR* alleles from two such revertants were also sequenced, and both were found to have recovered the wild-type *algR* sequence (data not shown).

We also isolated and sequenced the *vfr* gene from wild-type PAK and the PAK*rhlI*::Tc, PAK*rhlI*-NT, PAK*lasI*::Tc, and PAK*lasI*-RT mutant strains. The *vfr* sequence was identical to that of the wild type in all of the strains except PAK*lasI*-RT, which had a very precise 15-nucleotide in-frame microdeletion in the second half of an imperfect tandem repeat situated in the region of Vfr that closely matches the CRP cyclic AMP-binding domain (46), the net result of which is the loss of five residues (EQERS). Interestingly, and perhaps importantly, this allele (VfrΔEQERS) is able to restore elastolytic activity but not twitching motility when supplied in *trans* to *vfr*-null mutants (5).

The PAK*lasI*-RT (VfrΔEQERS) mutants were also found to revert spontaneously to a normal-twitching phenotype at low frequencies. *vfr* alleles from two such revertants were cloned and sequenced. Both *vfr* sequences contained the EQERS deletion, but each sequence contained a different point mutation elsewhere in *vfr* that caused an amino acid substitution (S194R and D36A). These *vfr* alleles were also able to complement *vfr* knockout mutants for twitching motility (data not shown)—therefore, these mutations appear to overcome the effect of the EQERS deletion. Interestingly, the revertant carrying the D36A mutation did not recover twitching motility completely and the corresponding *vfr* allele could only complement *vfr* knockout mutants to ~75% of wild-type twitching motility. This indicates that the effect of the EQERS deletion is only partly overcome by the D36A mutation. Analysis of specific compensatory mutations in the *vfr* alleles of further *lasI*-RT revertants should yield significant insights into the structure and function of Vfr itself.

Twitching-defective variants predominate in liquid culture of quorum mutants. We were interested in further characterizing the emergence of the spontaneous mutations among *lasI* and *rhlI* mutants. Our findings suggested either that these secondary mutations were occurring at high frequencies as a result of a hypermutator phenotype (due to down-regulation of systems that are involved in DNA repair or that protect cells from oxidative damage) or that such mutations had some selective advantage in this background. However, the frequency at which twitching defects arose among *lasI* and *rhlI* cells plated on solid medium was found to be similar to that in the wild type, between 10^{-4} and 10^{-6} in three independent trials (data not shown), which is consistent with the normal rate of spontaneous mutation given that a large number of genes are known to be required for twitching motility (representing approximately 0.1% of the genome). In addition, there was no significant difference in the frequency of spontaneous streptomycin resistance mutations in these strains (data not shown), which frequency was consistent with previous studies (27).

When serial subcultures in liquid medium (with shaking) were carried out, both PAK*rhlI*::Tc and PAK*lasI*::Tc cultures, but not wild-type PAK cultures, developed twitching defects (as measured by plating of dilutions after every subculture) that appeared to predominate as subculturing continued (Table 2). These experiments were repeated several times. The majority of these twitching-defective variants exhibited phenotypes that appeared to be identical to those of the S1 and S2 variants previously described by Deziel et al. in an environmental isolate of *P. aeruginosa* (strain 57RP) (13). Our S1- and S2-like mutants were defective in both twitching motility and swimming motility and formed a pellicle at the air-broth interface when cultured as a static broth but rapidly reverted to a normal twitching-swimming state, apparently by phase variation (data not shown), as had been previously observed by Deziel et al. (13). We did not find such variants among the wild-type cultures, suggesting that these as-yet-unidentified phenotypic variants, which were observed otherwise only when *P. aeruginosa* cells were grown as a biofilm or in static broth culture, also have a selective growth advantage in quorum-sensing mutants when they are grown in a shaken broth culture. Perhaps importantly, Deziel et al. noted that, apart from strain 57RP, S variants also arose in *P. aeruginosa* strains that demonstrated a long lag phase before growth on hexadecane (13). It has been reported that *rhl* mutants grow poorly in hexadecane (28). Taken together, these observations add support to the idea that the appearance of S variants may be quorum sensing related.

Interestingly, Deziel et al. also observed that S variants show decreased elastase and increased pyocyanin production (13). However, it was difficult to measure the pyocyanin phenotypes of our S variants due to the fact that strain PAK only produces very low levels of pyocyanin and because of the rapidity of reversion of such variants to a wild-type phenotype. Further-

more, as noted earlier, our *lasI* and *rhlI* mutants demonstrated elastase and rhamnolipid deficiencies that were consistent with published reports (8, 15, 17). This precluded the measurement of quorum-sensing-related phenotypes in the S variants of the *rhlI* and *lasI* mutants. Nevertheless, the range of phenotypes affected in S variants suggests that the apparent phase variation occurs within or affects the expression of a gene encoding a global regulator. However, neither our S1 nor our S2 variants could be complemented by pUCP*vfr* or pUCP*algR* and the *vfr* and *algR* alleles from representative S variants were identical in sequence to the wild type (data not shown), indicating that *vfr* and *algR* are not mutated in these S variants.

Interestingly, all of the nontwitching variants isolated from serial broth subcultures of our PAK*rhlI* mutants were found to resemble either S1- or S2-type variants (Table 2) but not the original PAK*rhlI*-NT strains. Therefore, we have yet to identify the precise conditions (if any exist) that selected for the *algR* mutation in *rhlI* mutants that occurred at high frequencies during the allelic-exchange procedure. In contrast, after four consecutive subcultures, two of six PAK*lasI* cultures contained reduced-twitching mutants that resembled the original PAK-*lasI*-RT variant and were subsequently complemented with pUCP*vfr*. Significantly, these independent isolates both contained precisely the same mutation in *vfr* as previously observed in PAK*lasI*-RT, suggesting that in an agitated liquid culture, there is a selective pressure for this particular mutation in *P. aeruginosa lasI* mutants.

To detect whether there may be a selective advantage of the VfrΔEQERS mutation, we performed a number of direct competitions. Unlike the S1 and S2 mutants, which always outcompete the parent strain (or the wild type), PAK*lasI*-RT mutants harboring the VfrΔEQERS mutation do not appear to have a significant growth advantage over normal-twitching PAK*lasI* mutants in overnight 1:1 or 1:100 competitive assays (data not shown). Therefore, it is possible that the selective advantage of this mutation is more subtle than any that can be detected by a simple competitive assay. Consistent with this hypothesis are our findings that *vfr* mutations generally arise after three or four consecutive subcultures and do not numerically predominate, whereas S1 or S2 variants usually arise after one or two subcultures and often become the most predominant type after four subcultures (Table 2). We found no difference in the overall unselected rate of mutation (as measured by the frequency of streptomycin resistance development), suggesting that a generally higher incidence of mutation in the PAK*lasI* mutant could not be the cause of these VfrΔEQERS mutations. However, we cannot rule out the possibility that a specific type of mutation (one that favors deletion of the 15-nucleotide sequence within *vfr* but has no effect on the genes for streptomycin sensitivity) occurs more often in *lasI* mutants.

Quorum-sensing mutants and spontaneous mutation of regulatory genes. The frequent occurrence of nontwitching variants of PAK*rhlI*::Tc that harbor mutations in *algR* and that appear to be phenotypically similar to that described by Glessner et al. (16) indicates the possibility of a specific compensatory regulatory mutation occurring in *algR* in the *rhlI* background. Similarly, the fact that three independent twitching-defective variants of *lasI* mutants all contained the same precise mutation in *vfr* and are phenotypically similar to that reported for *lasI* mutants by Glessner et al. (16) suggests that this mutation has a specific advantage in the *lasI* background, probably as a compensatory mechanism by which to relieve stresses on the regulatory circuitry imposed by the lack of the *lasI* quorum-sensing signal. Interestingly, the twitching defects reported in the *lasI* mutant JP1 and the *rhlI* mutant PDO100 were not reported to be complemented by wild-type *lasI* or *rhlI* provided in *trans* (16). Furthermore, our findings that S1 and S2 phase variants that affect a variety of phenotypes appear commonly in quorum-sensing mutants but not wild-type cells grown in normal broth culture also suggests that spontaneous mutation is selected for by distortions in the quorum-sensing regulatory system. Taken together, these results suggest that mutations in the *lasI* and *rhlI* quorum-sensing systems create conditions that can select for secondary mutations in other regulatory genes, presumably because the latter relieve regulatory and/or physiological stresses on the cells caused by the primary mutations. These secondary mutations may themselves also cause new stresses and selective pressures that may induce flow-on genetic changes during subculturing of such mutants, many of which have been grown and studied in (different) laboratories for many years and that may have gone unnoticed in the past. Indeed, if it had not been for the fact that twitching motility is an easily scored phenotype in *P. aeruginosa* colonies, this problem would not have been recognized at all, and there may well be other genes that are also subject to selection pressure in such regulatory mutants but whose incidence is hidden. These unanticipated changes may also account for much of the confusion and contradictory results in the literature. This has been demonstrated recently by a study showing that a pre-existing mutation in a wild-type strain used to create a defined *lasR* mutant is the reason behind contradictory reports of the role of *lasR* in the control of rhamnolipid production (17). In the light of this finding, it appears that other reports of starvation selection for the elastase and rhamnolipid phenotypes in *lasR* mutants (42) and the related study showing *dksA* inhibition of quorum sensing (7) might have been similarly affected by this unexpected mutation.

At the minimum, our findings indicate that reports of the phenotypic effects of mutations in the *las* and *rhl* quorum-sensing systems in *P. aeruginosa* (and probably other bacteria) need to be confirmed by complementation of the mutants, as certain phenotypic characteristics might be due to secondary mutations in regulators such as AlgR and Vfr and, possibly, other global regulatory proteins. Clearly, any quorum-sensing mutant exhibiting a twitching motility defect harbors a secondary-site mutation and should be appropriately checked. The fact that the *vfr* mutations involve a precise deletion in a tandem repeat that affects some signaling pathways but not others suggests that this may be an important site for phase variation, as does the incidence of mutations in a poly-G tract in *algR*. Similar influences may be at work during the evolution of *P. aeruginosa* communities in the cystic fibrosis lung, where it is known that there are mutations in genes affecting transcriptional regulation and that there are different ratios of lactones than those observed in vitro (37). More broadly, it is clear that caution is required when investigating regulatory mutations, as such mutations may cause stresses in the cell that create conditions for the emergence of compensating mutations in other genes.

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