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Autolysis and Autoaggregation in *Pseudomonas aeruginosa* Colony Morphology Mutants

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Two distinctive colony morphologies were noted in a collection of *Pseudomonas aeruginosa* transposon insertion mutants. One set of mutants formed wrinkled colonies of autoaggregating cells. Suppressor analysis of a subset of these mutants showed that this was due to the action of the regulator WspR and linked this regulator (and the chemosensory pathway to which it belongs) to genes that encode a putative fimbrial adhesin required for biofilm formation. WspR homologs, related in part by a shared GGDEF domain, regulate cell surface factors, including aggregative fimbriae and exopolysaccharides, in diverse bacteria. The second set of distinctive insertion mutants formed colonies that lysed at their center. Strains with the most pronounced lysis overproduced the *Pseudomonas* quinolone signal (PQS), an extracellular signal that interacts with quorum sensing. Autolysis was suppressed by mutation of genes required for PQS biosynthesis, and in one suppressed mutant, autolysis was restored by addition of synthetic PQS. The mechanism of autolysis may involve activation of the endogenous prophage and phage-related pyocins in the genome of strain PAO1. The fact that PQS levels correlated with autolysis suggests a fine balance in natural populations of *P. aeruginosa* between survival of the many and persistence of the few.

Pseudomonas aeruginosa, although readily defined taxonomically (69), shows no evidence of being constrained to a particular bacterial lifestyle (72). Indeed, this opportunistic pathogen proliferates within hosts as varied as plants, insects, nematodes, and mammals and interacts with amoebae and yeasts as well (13, 16, 31, 44, 58). The broad host range of *P. aeruginosa* can be used both to uncover the role of specific virulence factors and to characterize new regulatory systems controlling virulence (10, 12, 13, 16, 21, 44, 58). One study identified transposon insertion mutants of *P. aeruginosa* strain PAO1 that were less virulent in the fruit fly model host (16). Most such strains had an insertion in a chemotaxis-like regulatory locus (PA0408 to PA0417) required for surface spreading by twitching motility and controlling unknown factors required for efficient fly killing. Based only on their distinctive compact colony morphology, a large collection of these mutants was assembled for testing in the fly model.

In this same study (16), two even more striking colony morphologies were noted, and strains of each type were saved. One set of mutants formed wrinkled colonies and had additional properties described for autoaggregating cells of *Pseudomonas fluorescens* (59, 68) and *Salmonella enterica* serovar Typhimurium (61, 62, 83). The second set of mutants formed colonies with visible lysis, a characteristic noted in some of the earliest descriptions of *P. aeruginosa* isolates (5, 6, 26, 32), but for which the molecular basis has been elusive. Since autoaggregation and autolysis are likely to reflect fundamental aspects of the biology of *P. aeruginosa*, the genetic basis of the two mutant colony morphologies was explored. Autoaggregation, in the subset of strains analyzed, was found to require the GGDEF-type (28) response regulator WspR. Autolysis, in strains with the most pronounced phenotype, correlated with the level of the *Pseudomonas* quinolone signal (PQS), an extracellular signal (9, 49, 56) that interacts with the quorum sensing regulatory hierarchy.

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MATERIALS AND METHODS

Strains and culture conditions. *P. aeruginosa* strain PAO1 (from the laboratory of B. Iglewski) was grown with Luria-Bertani (LB) medium (with NaCl at 8 g/liter) at 37°C on 1.5% agar or in 5-ml cultures unless otherwise noted (all percentages indicate wt/vol). Medium ingredients (Difco) included brain heart infusion, nutrient broth, Bacto Agar, tryptone peptone, tryptic soy broth, and yeast extract. The wrinkled-colony phenotype of mutant cells was most evident after growth with the richest media. To observe swarming motility, cells were grown on 0.5% agar with 0.8% nutrient broth and 0.5% glucose (60), while for swimming motility, cells were grown in 0.3% agar with LB medium with NaCl at 8 g/liter and without the yeast extract. Freshly poured agar for motility tests was left at room temperature for 24 to 36 h before use. For PQS analysis, cells were grown with peptone tryptic soy broth (PTSB) (49).

Derivatives of plasmid pVSP61 carrying *wspR19* or *wspR9* (P. J. Goymer, S. G. Kahn, and P. B. Rainey, unpublished data) were transferred into *P. aeruginosa* by conjugation (as described below for transposon mutagenesis of the *wspF10* mutant). These plasmids were maintained using kanamycin at 500 μg/ml, and expression of the cloned genes from the plasmid *lac* promoter was constitutive in *P. aeruginosa* because of the absence of LacI. Plasmid pUCP18 (64) and derivatives were transferred into *P. aeruginosa* by transformation and maintained using carbenicillin at 200 μg/ml.

Growth curves were determined from experiments performed in triplicate: 35 ml of LB medium in a 300-ml Erlenmeyer flask with baffles was inoculated with 1 ml of a fresh 12-h culture and then incubated with shaking at 37°C for 3 days. The CFU in the cultures over time were determined by plating dilutions of 100-μl samples on LB agar.

Transposon mutagenesis. To provide a genetic background in the colony morphology mutants for a second round of mutagenesis with IS*phoA*/hah-Tc (16, 21), the transposable element was excised from the chromosome, as described

previously (45), by introduction of a plasmid expressing the Cre recombinase. Subsequent recombination between *loxP* sites flanking the transposable element left a chromosomal insertion of 189 bp and restored sensitivity to tetracycline (45). Insertional mutagenesis in the resulting strains was performed as in a previous study (16), but with the following modifications. Cultures of mating recipient cells were started with fresh single colonies of the *wspF* mutant grown at 23°C or of the *pqsL* mutant grown at 37°C. These cultures were incubated at 42°C without shaking for approximately 20 h. Colonies of cells in which a chromosomal insertion suppressed the mutant phenotype were identified after growth for 21 h at 37 and 30°C, respectively, for *wspF* mutants and *pqsL* mutants. The DNA sequence of the region flanking the insertion site was determined as before (16), and the identity of the mutated gene was determined by using data and annotations from the *Pseudomonas* Genome Project (http://www.pseudomonas.com).

PQS detection and quantitation. PQS analysis has been described previously (9, 56). Freshly grown colonies were used to inoculate 1-ml cultures. After growth with PTSB for 20 h at 37°C, the entire culture was extracted twice with three volumes of acidified ethyl acetate and with centrifugation at 10,000 × *g* (extraction of spent culture supernatants gives equivalent results). The ethyl acetate fractions from each extraction were pooled and analyzed by thin-layer chromatography (TLC). TLC plates (20 by 20 cm; Silica Gel 60 F_{254}; EM Science) were soaked in 5% KH_2PO_4 and activated at 100°C for 1 h. Sample extracts were separated on TLC plates using 17:2:1 methylene chloride-acetonitrile-dioxane as the solvent, conditions previously determined to be optimal for separation of PQS (56). TLC plates were then dried, illuminated with long-wave UV light, and photographed with a Polaroid 3000ISO camera and Polaroid 667 black-and-white film. PQS was quantitated by densitometry using ImageQuant software (Molecular Dynamics).

For autolysis restoration by extracellular complementation, spent culture supernatants were sterilized using Millipore filters (0.22-μm pore size), and sterility was confirmed by testing aliquots for growth. Synthetic PQS, synthesized previously by Jarad Milbank (56), was dissolved in 1:1 ethyl acetate-acetonitrile for addition to 6-mm-diameter paper disks (Becton Dickinson).

RESULTS

Autoaggregation in mutants of *P. aeruginosa* strain PAO1. Out of approximately 6,000 insertion mutants, previously generated using IS*phoA*/hah-Tc (16), six formed wrinkled colonies as shown in Fig. 1. DNA sequencing of the region flanking the insertion was successful for five of the six mutants, revealing three insertions in gene PA3703, one in gene PA0171, and one in gene PA2933 (gene numbers are from the *Pseudomonas* Genome Project). Different insertions in some of the same genes were also identified in wrinkled mutants obtained from transposon mutagenesis in separate studies: two insertions in PA3703, one in PA0171, and one in PA1121. DNA sequence analysis suggests that PA3703 encodes a CheB-like methylesterase and that PA2933 encodes an efflux protein of the major facilitator superfamily. The potential function of the remaining two genes is unknown. Gene PA3703, with five insertions, lies within a gene cluster predicted to encode a chemotaxis-like signal transduction cascade (72). This cluster is homologous to the *wsp* cluster from *P. fluorescens* that is also involved in autoaggregation and wrinkled-colony morphology (68; E. Bantinaki, A. J. Spiers, and P. B. Rainey, unpublished data). The *P. aeruginosa* homologs were consequently given the *wsp* designation, with PA3703 corresponding to *wspF* (Fig. 2), and the phenotype associated with one insertion, *wspF10*, was characterized in more detail.

The *wspF10* mutant did not spread by twitching motility and formed wrinkled colonies that grew mostly vertically (Fig. 1). Cells of this mutant adhered so tightly to each other that the entire wrinkled colony was easily transferred with forceps from one agar surface to another, as was done to generate Fig. 1. In contrast, nonaggregative wild-type cells spread using type IV pili for twitching motility (65) and formed flatter colonies (Fig. 1). The *wspF10* mutant also failed to spread by either of two forms of flagellum-based motility (Fig. 3): swarming motility on the agar surface using self-generated biosurfactant or swimming within the agar (40, 60). Indeed, under conditions favoring swimming, the *wspF10* mutant grew predominately as a wrinkled mass protruding above the agar (Fig. 4), in stark contrast to the diffuse distribution of wild-type cells (Fig. 3).

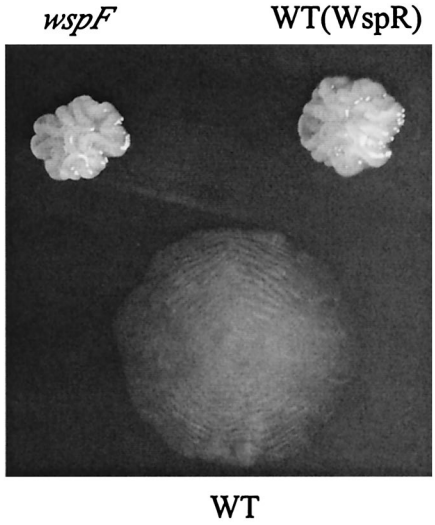

FIG. 1. Colony morphology of the wild-type *P. aeruginosa* strain PAO1 (WT), the *wspF10* insertion mutant, and strain PAO1 carrying a plasmid expressing the *P. fluorescens* constitutively active WspR19 [WT(WspR)]. Each strain was incubated separately on brain heart infusion agar, and after 3 days at 23°C, a colony of each of the two wrinkled mutants was transferred using forceps to the agar surface adjacent to a colony of strain PAO1. The WT colony had a diameter of approximately 0.3 cm.

Additional phenotypes of the *wspF10* mutant (not shown) suggested even closer parallels with properties of autoaggregating cells of *P. fluorescens* (59, 68) and *S. enterica* serovar Typhimurium (61, 62, 83). These phenotypes included increased bacterial lawn hydrophobicity, evident because *wspF10* mutant cells formed lawns with a dry appearance upon which water droplets persisted as tight beads, while wild-type cells formed lawns with a wet appearance upon which water droplets collapsed. In addition, the *wspF10* mutant grew in standing LB cultures primarily as a pellicle at 23°C and with diffuse turbidity at 42°C, indicating that autoaggregation was suppressed by higher growth temperatures. After prolonged incubation, autoaggregating cultures or colonies of the *wspF10* mutant were commonly overgrown by phenotypic revertants. A similar autoaggregation phenotype was described for spontaneous mutants of *P. aeruginosa* strain 57RP (17) and strain PAK (3), but the genes responsible were not identified.

WspR controls autoaggregation. Analysis of the wrinkled-colony phenotype of *P. fluorescens* implicated WspR (68), a protein related to the *Caulobacter crescentus* PleD global regulator controlling the transition between motile swarmer cell and nonmotile stalked cell (28). To test whether a similar regulatory system operated in *P. aeruginosa*, a constitutively active WspR from *P. fluorescens* (WspR19) (Goymer et al., unpublished) was expressed in strain PAO1. The resulting col-

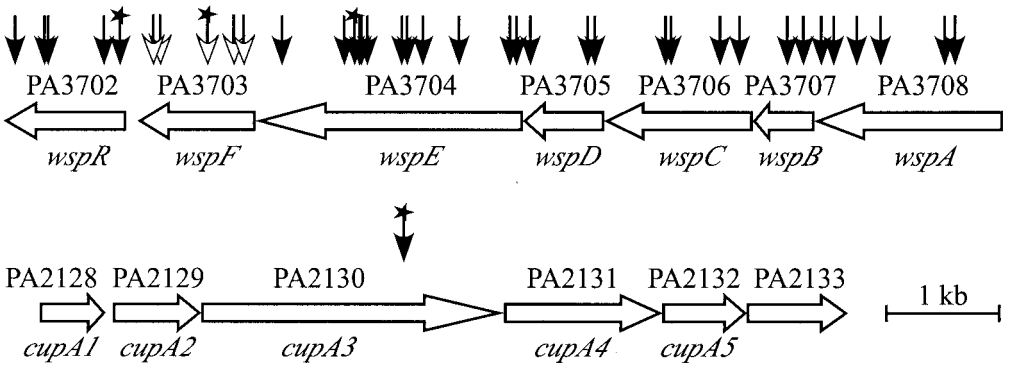

FIG. 2. *P. aeruginosa* genes in two chromosomal loci (distinguished by gene numbering) involved in autoaggregation and wrinkled-colony morphology. Vertical arrows indicate the sites of insertion of the IS*phoA*/hah-Tc transposable element. Unfilled arrowheads denote insertions, all in *wspF*, that confer autoaggregation. Stars indicate insertions in strains that were characterized in more detail. For suppressor analysis of the strain with the *wspF10* insertion, the transposon was excised from *wspF* (leaving a 189-bp insertion) and transposon mutagenesis of this strain was repeated: filled arrowheads denote insertions that suppress autoaggregation, either completely (insertions in the *wsp* genes) or partially (the insertion in *cupA3*). The one exception was the strain with the *wspE65* insertion, in which autoaggregation was suppressed at 37°C but not at 23°C.

onies were wrinkled and identical in appearance to those of the *wspF10* mutant (Fig. 1). Furthermore, expression of a dominant negative mutant WspR from *P. fluorescens* (WspR9) (Goymer et al., unpublished) had no apparent effect on colonies of strain PAO1 but completely suppressed the wrinkled phenotype of *wspF10* mutant colonies (data not shown). The latter colonies, however, were slightly smaller than those of the wild-type strain, suggesting that some autoaggregation remained.

WspF is mutated in five strains forming wrinkled colonies, including the *wspF10* mutant (Fig. 2). WspF is 30% identical (over 335 amino acids) to CheB, a methylesterase from *Escherichia coli*. In *E. coli*, CheB removes a methyl group from methyl-accepting chemotaxis proteins, reducing activity in the signal transduction cascade during chemotaxis (71). Assuming a similar function in *P. aeruginosa*, inactivation of WspF would result in hypermethylation of the methyl-accepting chemotaxis protein (WspA). This would cause constitutive activation of the Wsp chemosensory pathway and ultimately of the response regulator WspR, the predicted target of the Wsp phosphorelay (68). Since WspR controls the structural components necessary for the wrinkled-colony morphology (68; Goymer et al., unpublished), this would mimic the effect of expressing the heterologous constitutively active WspR.

To test this hypothesis, the transposon in the *wspF10* mutant was excised from the chromosome, leaving an insertion of 189 bp (see Materials and Methods) which maintained the wrinkled phenotype. A second round of transposon mutagenesis was then performed in this strain to identify genes required for the wrinkled-colony morphology. Approximately 60,000 insertion mutants were screened, yielding 95 suppressed double mutants. The insertion site was sequenced in those 39 suppressed mutants that were independently obtained, 38 of which had a wild-type colony morphology at 37°C. The one remaining mutant was only partly suppressed for autoaggregation. Of the 38 strains, 34 carried an insertion in the *wsp* genes (Fig. 2), and

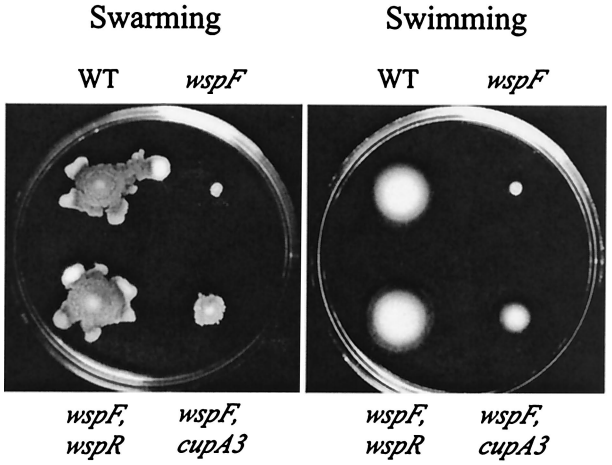

FIG. 3. Swarming motility (on the surface of 0.5% agar after 24 h at 37°C) and swimming motility (within 0.3% agar after 12 h at 37°C) of strain PAO1 (WT), the *wspF10* mutant, and strains in which autoaggregation caused by an insertion in *wspF* was suppressed by an insertion in either *wspR* or *cupA3* (Fig. 2).

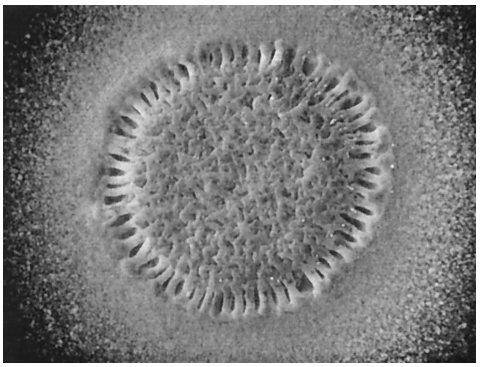

FIG. 4. Autoaggregation of the *wspF10* mutant growing in and above swimming-motility agar after 48 h at 23°C. The agar was inoculated using a toothpick with *wspF10* cells from a diffusely turbid culture grown at 42°C, a temperature that suppresses clumping (inoculation with cells grown at 23°C on an agar surface yielded equivalent results). Cells have dispersed to a maximum diameter of 0.5 cm; cells of strain PAO1 incubated in the same way disperse evenly over a zone approximately 4 cm in diameter.

all with the exception of three identical insertions in *wspR* were unique.

Mutation of *wspR* completely suppressed colony wrinkledness and restored swarming and swimming motility (Fig. 3), consistent with WspR being the ultimate target of the Wsp phosphorelay and the proximate cause of the wrinkled phenotype. The same conclusion was reached recently based on the fact that a mutation in either *wspR* or the *wspABCDEF* operon eliminated wrinkledness in a *P. fluorescens* strain with a spontaneous mutation in *wspF* (A. J. Spiers, Z. Robinson, and P. B. Rainey, unpublished data). The strain with the *wspE65* insertion (Fig. 2) provided further evidence that autoaggregation was favored by lower growth temperatures. Of the double mutants forming colonies with a wild-type appearance at 37°C, only this strain formed wrinkled colonies at 23°C, suggesting that the *wspE65* insertion partly inactivated *wspE* and that this leaky mutation was sufficient to suppress the wrinkled phenotype only at higher growth temperatures.

Additional genes involved in autoaggregation. The one remaining mutant, in which autoaggregation at 37°C was only partly suppressed, carried an insertion in PA2130 (Fig. 2). Although growth curves indicated that this mutant grew at a wild-type rate in cultures shaken at 37°C, the mutant formed unwrinkled colonies on LB agar slightly smaller than those formed by the wild-type strain (not shown) and was partly impaired in swarming and swimming motility (Fig. 3). Excision of the transposon in this strain (again leaving a 189-bp insertion) restored the wrinkled-colony phenotype, suggesting that the transposon in PA2130 had a polar effect on expression of downstream genes. PA2130, recently designated *cupA3* in *P. aeruginosa* strain PAK, lies within a gene cluster (Fig. 2) that is predicted to encode a novel fimbrial adhesin (14, 75), and insertion mutations within this cluster prevent biofilm formation (75). In addition, at least two genes in this cluster, PA2128 and PA2129, are differentially regulated during growth as a biofilm (81), and PA2128 is related to the gene encoding the subunit of aggregative fimbriae which mediate biofilm formation and the wrinkled-colony phenotype in *Salmonella* (61, 62, 75, 83).

Autolysis in mutants of *P. aeruginosa* strain PAO1. Among the approximately 6,000 transposon insertion mutants previously generated (16), not only were the six that formed wrinkled colonies notable, but also three that formed colonies that lysed at their centers. In the areas of highest cell density in lawns of these three strains, plaque-like clearings developed, expanding and merging to form zones of confluent lysis (Fig. 5). The plaque-like clearings and the metallic, iridescent sheen associated with the lysed areas are both properties noted in early descriptions of *P. aeruginosa* isolates (5, 6, 26, 32) but absent in strain PAO1 (Fig. 5). In a recent collection of *P. aeruginosa* isolates from cystic fibrosis patients, 31% (59 of 191) had visible lysis (S. Miller, personal communication).

In the two insertion mutants with the most pronounced lysis, DNA sequencing revealed two different insertions in PA4190, here designated *pqsL*, and the phenotype associated with the *pqsL64* insertion was characterized in more detail (Fig. 5 and 6). The *pqsL* gene is predicted to encode a member of a family of monooxygenases in *P. aeruginosa* (72) that include products of *pobA* and *ubiH*, genes which encode hydroxylases involved in *p*-hydroxybenzoate catabolism and ubiquinone biosynthesis,

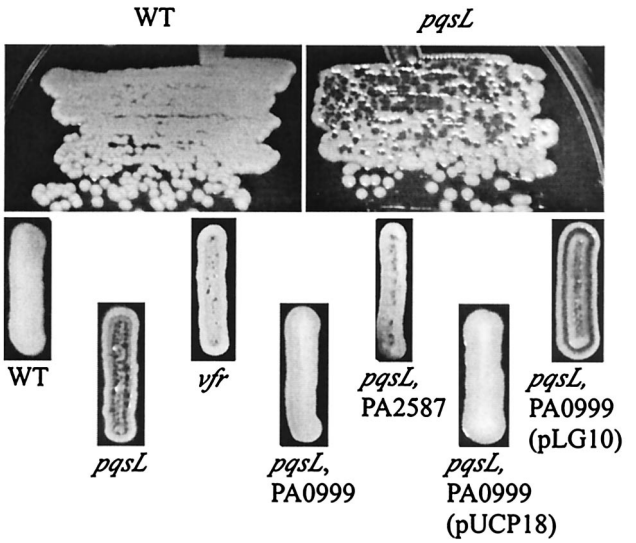

FIG. 5. Autolysis visible as plaque-like clearings (darker zones) in areas of dense growth on LB agar after 18 h at 37°C (top row) or in toothpick-generated streaks after 18 h at 37°C followed by 6 days at 23°C (bottom row). Visible autolysis is absent in *P. aeruginosa* strain PAO1 (WT), in contrast to the mutant with the *pqsL64* insertion or an insertion in *vfr*. In double-mutant strains, autolysis caused by an insertion in *pqsL* is suppressed by an insertion in PA0999 or PA2587. The transposon was excised from the chromosome in the former mutant, leaving an insertion of 189 bp and resulting in an insertion of 63 amino acids in the protein encoded by PA0999. Autolysis in this strain was restored by complementation with plasmid pLG10 (22) carrying PA0996 to PA1000, but the vector pUCP18 had no effect.

respectively. PA4190 is also related to three quorum-sensing-controlled genes (80): PA2587 (*qsc105*), PA3328 (*qsc125*), and PA4217 (*qsc132*), of which the third gene encodes the hydroxylase PhzS involved in phenazine biosynthesis (48). The one remaining mutant had less pronounced lysis and carried an insertion in the gene encoding Vfr (Fig. 5). Vfr is a homolog of the *E. coli* cyclic AMP receptor protein CRP (4, 78) and acts at the top of the quorum sensing regulatory hierarchy (1, 4). An insertion in PA4190 was also identified in a mutant with visible lysis obtained from transposon mutagenesis in a separate study, but no more strains with this phenotype were found by screening an additional 3,000 transposon insertion mutants.

Colonies of the *pqsL64* mutant had a particularly distinctive morphology after prolonged incubation. As the band of peripheral cells spread outward from the central lysed area, the center of the band itself developed plaque-like holes which coalesced, forming concentric zones of lysis as this process repeated (Fig. 7). The same pattern of lysis was observed for *pqsL64* mutant colonies incubated in the dark, indicating a different mechanism of cell death than that by which visible light kills *E. coli ubiH* (*visB*) mutant cells (51). In liquid culture, the *pqsL64* mutant grew like the wild-type strain for the first 24 h, reaching a density of approximately 10^{10} CFU/ml, and then both strains lost viability over time. However, by 72 h, the cultures of the *pqsL64* mutant had lost an average of four times more CFU than those of the wild-type strain, with CFU dropping by a factor of 48 versus 12, respectively. No phenotypic revertants were noted. The relative subtlety of this mutant phenotype suggested that high cell density, or another condi-

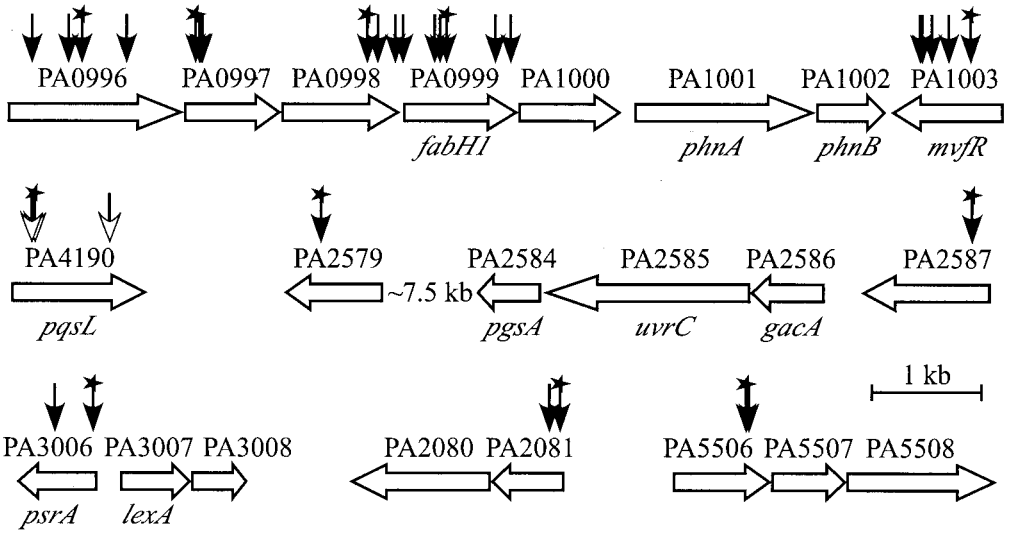

FIG. 6. *P. aeruginosa* genes in six chromosomal loci (distinguished by gene numbering) involved in autolysis. Vertical arrows indicate the sites of insertion of the IS*phoA*/hah-Tc transposable element. Unfilled arrowheads denote insertions, all in *pqsL*, that confer autolysis. Stars indicate insertions in strains that were characterized in more detail. For suppressor analysis of the strain with the *pqsL64* insertion, the transposon was excised from *pqsL* (leaving a 189-bp insertion) and transposon mutagenesis was repeated: filled arrowheads denote insertions that suppress autolysis, either completely (insertions in PA0996 to PA1003) or partially.

tion required for the pronounced lysis during surface growth, was not completely reproduced by growth in liquid.

Autolysis is suppressed by mutations that reduce the level of PQS. The colony phenotype of the *pqsL64* mutant suggested that autolysis would be as amenable to genetic analysis as autoaggregation. The transposon in the *pqsL64* mutant was excised, with the remaining 189-bp chromosomal insertion preserving the phenotype, and transposon mutagenesis of this strain was repeated. Out of approximately 20,000 insertion mutants, 37 strains formed colonies in which visible lysis was either reduced or absent, and these strains had a wild-type growth rate based on colony size (400 strains that had delayed lysis because of slower growth were not analyzed further). The insertion site was sequenced in those 30 suppressed mutants that were independently obtained: 22 strains with no apparent lysis (typified by one with an insertion in PA0999 in Fig. 5) had a unique insertion in a single chromosomal region (Fig. 6). The remaining eight strains with reduced lysis (typified by one with an insertion in PA2587 in Fig. 5) had unique insertions more dispersed around the chromosome (Fig. 6).

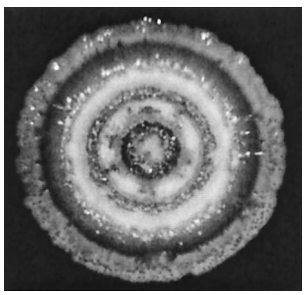

FIG. 7. Concentric zones of lysis (darker bands) formed during growth of the *pqsL64* mutant on LB agar. The agar was inoculated with 20 μl of 10 mM $MgSO_4$ containing cells of the *pqsL64* mutant from six small colonies from LB agar. After 36 h at 30°C, the plate was sealed with Parafilm, inverted, and incubated for 25 days at 23°C. The colony has grown to a diameter of 3.5 cm, and the plaque-like holes in the outermost band of cells have not yet coalesced. No lysis was visible in colonies of wild-type cells generated in the same way (as also seen in Fig. 5).

The 22 strains with autolysis completely suppressed carried insertions in PA0996, PA0997, PA0998, PA0999, and PA1003 (Fig. 6). PA1003, designated *mvfR* in *P. aeruginosa* strain PA14 (10), encodes a transcriptional regulator required for production of multiple virulence factors, including pyocyanin, elastase, and cyanide. PA1003 is consequently required for full virulence in diverse model hosts (10, 21). Recently, both *mvfR* (10) and the operon including PA0996 to PA0999 (22) were found to be involved in biosynthesis of PQS, an extracellular signal in the quorum sensing hierarchy (9, 49, 56). Measurement of the amount of PQS made by the strains in this study revealed that the *pqsL64* mutant produced approximately four times as much PQS as the wild-type strain, and all the suppressor mutations reduced this amount (Fig. 8 and 9). Those strains in which autolysis was completely suppressed made no detectable PQS (Fig. 8). In a mutant with a nonpolar suppressor mutation in PA0999, autolysis was restored by complementation with a plasmid containing PA0996 to PA1000 (22), while the vector alone had no effect (Fig. 5).

Autolysis in the PQS-overproducing *pqsL64* mutant occurred in the areas of highest cell density during surface growth (Fig. 5), consistent with a causal role for PQS, an extracellular compound whose production peaks in late stationary phase (49). Supporting this connection, lysis of the partially suppressed PA2587 mutant was triggered by addition of synthetic PQS (Fig. 10). Further implicating PQS, DNA sequence analysis of one mutant with partially suppressed autolysis suggested that the affected gene, PA2579 (Fig. 6), encodes tryptophan 2,3-dioxygenase. This is the first enzyme in the kynurenine pathway from tryptophan to anthranilate (53), which is a precursor of PQS (9). Partial suppression of autolysis by mutation

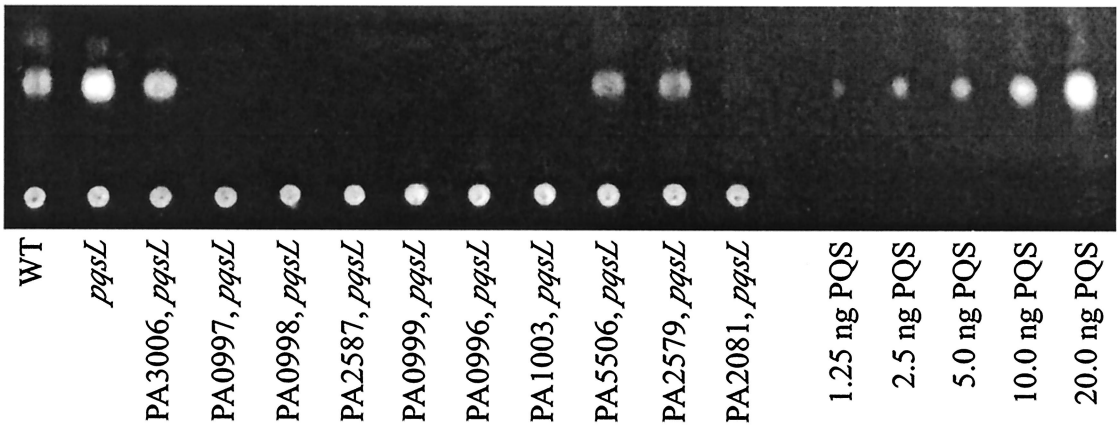

FIG. 8. TLC analysis of PQS produced by *P. aeruginosa* strain PAO1 (WT), the *pqsL64* mutant, and derivatives of the latter strain in which autolysis is suppressed (Fig. 6). Extracts of 1-ml overnight cultures were analyzed by TLC using conditions previously optimized for separation and visualization of PQS (see Materials and Methods). Various quantities of synthetic PQS (56) were analyzed for comparison. Doubling the amount of sample extract in the TLC analysis yielded equivalent results.

of *psrA* (41) (Fig. 6), encoding an activator for expression of the stationary-phase sigma factor RpoS, also implicates stationary-phase processes.

Although PQS may be required for the full autolysis observed for the *pqsL64* mutant, it appears not to be the sole factor involved. In the partially suppressed mutants (with lysis still evident), PQS levels are equivalent to that in the wild-type strain or are not detectable, as in the PA2587 mutant (Fig. 8). *P. aeruginosa* is known to make multiple quinolones in addition to PQS (8, 42), and some that also require PA0996 to PA0999 for biosynthesis may account for the residual lysis. Indeed, lysis of the partially suppressed PA2587 mutant could be triggered with spent culture supernatant from the wild-type strain, but not with supernatant from a mutant carrying an insertion in PA0999 (Fig. 10).

DISCUSSION

Intracellular signaling and autoaggregation. The wrinkled-colony phenotype has been analyzed for cells of *P. fluorescens* (59, 68), *S. enterica* serovar Typhimurium (61, 62, 83), and now *P. aeruginosa*. These studies have uncovered the role in autoaggregation played by regulatory proteins that are related to *C. crescentus* PleD (28), in part because of a shared GGDEF domain (23, 28). The genetic basis of autoaggregation, which was revealed using wrinkled colonies of mutant cells, is likely to apply to multicellular behavior of wild-type bacteria as well. Autoaggregation of the plague bacterium *Yersinia pestis*, for instance, requires the GGDEF-containing protein HmsT (35). Clumping cells of this organism block food intake of both nematode worms (15) and fleas (30). In the latter case, this results in increased transmission by the flea vector (30). For *P.*

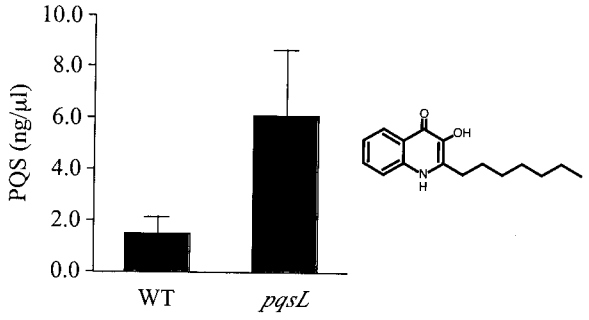

FIG. 9. PQS in cultures of *P. aeruginosa* strain PAO1 (WT) and the *pqsL64* mutant. PQS was separated by TLC as described in the legend for Fig. 8 and quantitated by densitometry. Relative PQS concentration was determined by comparing spots associated with sample extracts with those generated by known amounts of PQS. Data are the averages (error bars, standard deviations) of three independent experiments. PQS (2-heptyl-3-hydroxy-4-quinolone) is shown on the right.

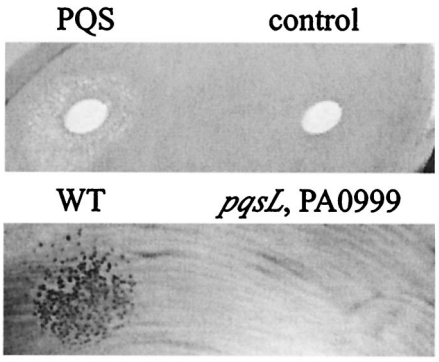

FIG. 10. Extracellular complementation of autolysis in a lawn of cells of a suppressed mutant. Fifteen microliters of solvent, either containing 15 μg of synthetic PQS (PQS) or alone (control), was added to a filter disk on the lawn (top row); 10 μl of sterile spent culture supernatant from 24-h cultures, either of *P. aeruginosa* strain PAO1 (WT) or of a double mutant in which autolysis is fully suppressed by an insertion in PA0999 (*pqsL*, PA0999), was added directly to the lawn (bottom row). The lysed area appears lighter in the top panel due to reflected light. The lawn was formed using a 12-h culture of a double mutant (in which autolysis caused by an insertion in *pqsL* is partially suppressed by an insertion in PA2587) that was diluted 10-fold to delay the density-dependent lysis during subsequent surface growth. One hundred microliters of the dilution was spread on LB agar, and after the various additions, the lawn was incubated for 24 h at 37°C. Three independent experiments yielded equivalent results.

aeruginosa, aggregation caused by the GGDEF-containing WspR is linked to regulation of *cup* genes (Fig. 2) that encode a putative fimbrial adhesin required in wild-type cells for biofilm formation (75). The role of WspR in biofilm formation could include controlling not only adhesion to foreign surfaces but also an alignment of bacterial cells that could act as a regulatory signal itself, as shown to occur during *Myxococcus xanthus* differentiation (38).

Proteins with a GGDEF domain control expression of a conserved set of products in cells forming wrinkled colonies, including a cellulose-like polymer (68, 83) and aggregative fimbriae (61, 62, 83). *P. aeruginosa* strain PAO1, however, appears not to have the known cellulose biosynthesis genes: the *P. fluorescens wss* operon (68) or the *P. putida* and *S. enterica* serovar Typhimurium *bcs* operon (83). Instead, WspR could participate in regulation of glycogen expression in *P. aeruginosa*. Glycogen is an intracellular polymer that is involved in *E. coli* and *Salmonella* biofilm formation (7, 34). As in these organisms, glycogen could function as a store of glucose (7, 34) for use as a precursor for the extracellular polysaccharides that comprise the protective matrix of *P. aeruginosa* biofilms (25, 29, 81). Glycogen metabolism and expression of aggregative fimbriae are coordinately regulated in *E. coli* (34). In *P. aeruginosa*, genes differentially regulated during biofilm growth (81) include PA2128 and PA2129 in the *cupA* fimbrial gene cluster (Fig. 2) and PA2160. DNA sequence analysis suggests that PA2160 encodes the glycogen catabolic enzyme GlgX (82), and several putative glycogen biosynthesis genes are closely linked to PA2160 on the chromosome (72).

The mechanism by which regulatory proteins with a GGDEF domain exert their effect is not yet clear, but there is growing evidence that the domain encodes a nucleotide cyclase (2, 55, 63, 73). The first such evidence was the requirement of this domain for biosynthesis of cyclic diguanylate, the intracellular signal regulating production of extracellular cellulose in *Gluconacetobacter xylinum* (63). The prediction that this novel signal might have more widespread functions in bacteria (63) was subsequently supported by bacterial genome sequences. GGDEF-containing proteins are not only widespread but also abundant in individual genomes, with 33 such proteins encoded in *P. aeruginosa* strain PAO1 alone (14, 23).

In this study, mutants with the wrinkled-colony phenotype carried insertions in three different loci (*wspF*, PA0171, and PA1121) that are each closely linked to a gene for a protein with a GGDEF domain (*wspR*, PA0169, and PA1120, respectively). Furthermore, PA2133 in the *cupA* operon (Fig. 2) is predicted to encode a protein in which the presence of an EAL domain (23, 50) suggests a phosphodiesterase activity for degrading the cyclic diguanylate signal (23, 73). An EAL domain is also present in the recently described regulator PvrR, which is involved in autoaggregation and the formation of a small, rough colony variant of *P. aeruginosa* strain PA14 (18). These observations support an intracellular signaling system in *P. aeruginosa* that is mediated by GGDEF-containing proteins and that plays a key role in controlling the adhesiveness of the bacterial cell surface.

Extracellular signaling and autolysis. The *pqsL64* mutant, identified because of its pronounced autolysis, overproduces PQS. The link between PQS and the monooxygenase that *pqsL* is predicted to encode is not yet clear. The fact that autolysis is partially suppressed by mutation of the homologous monooxygenase predicted to be encoded by PA2587 is suggestive. PA2587 could encode the enzyme for the final step in PQS biosynthesis, addition of the hydroxyl group (Fig. 9). Therefore, the homologous *pqsL* could encode an enzyme that also acts on PQS, and the *pqsL64* mutation could increase PQS levels by interrupting a pathway for PQS degradation (49) or modification.

Although there is a correlation between suppression of autolysis and decreased PQS production by the *pqsL64* mutant, the mechanism of autolysis also remains unknown. *P. aeruginosa* quinolones have antibacterial activity, a property discovered because of the ability of this bacterium to prevent anthrax in a mixed infection, a line of investigation begun by Pasteur (8, 27). The efficacy of quinolone antibiotics may be due in part to their induction of endogenous prophage via the bacterial SOS response (20, 57), perhaps the same phenomenon triggered by overproduction of PQS in the *pqsL64* mutant. The genome of *P. aeruginosa* strain PAO1 contains a filamentous bacteriophage (72) whose genes are highly upregulated during biofilm growth (81). In addition, inserted between the two anthranilate synthase subunit genes, *trpE* and *trpG*, is a gene cluster encoding bacteriocins believed to be evolved phage tails (52) and whose induction shares elements with the SOS response (47). Closely linked to this gene cluster (PA0616 to PA0648) is *vfr* (PA0652). The *vfr* gene is the other locus in this study whose mutation is associated with visible autolysis (Fig. 5). This is perhaps analogous to the situation in *E. coli* where mutation of *crp* shifts the balance from lysogeny to lysis (33), given that *vfr* and *crp* are homologs (4, 78), and suggests an alternate pathway for autolysis in *P. aeruginosa*.

The autolysis associated with PQS overproduction notwithstanding, PQS is likely to play a fundamental role in tuning cellular physiology. PQS strongly induces the *rhl* quorum-sensing system (49), and a balance between expression of the *las* and *rhl* systems weighted towards *rhl* is associated with growth of *P. aeruginosa* cells as a resistant biofilm outside of and within a human host (66). Furthermore, PQS was originally discovered because it induced expression of the quorum-sensing-regulated *lasB* gene that encodes the secreted virulence factor elastase (56). The LasB protease, however, is also required within the bacterial cell to activate the nucleotide diphosphate kinase Ndk (36). In the activated form, Ndk generates GTP, used as the substrate for RelA to generate the signal for the stringent response to starvation (11, 36, 37). Further supporting such a metabolic link, the quorum-sensing system can be activated by starvation either physiologically (77) or by mutation (76). This situation may underlie the selection pressure for compensatory mutations in certain quorum-sensing mutants (3, 4).

GTP is in particularly high demand during biosynthesis of alginate (11, 36, 37), which is secreted as part of a stress response, and which contributes to persistence of mucoid *P. aeruginosa* cells in a biofilm in chronic infections (25, 29). Consistent with a delicate equilibrium (26) between this protective response and autolysis, each controlled by PQS levels, *P. aeruginosa* strain PAO1 cells forming colonies with visible lysis (74) were repeatedly recovered during chemostat growth with media that stimulated alginate production. Such an equi-

librium could be maintained in part at the level of transport. PQS appears to be the substrate of at least one multidrug efflux pump (39, 54), including a transport system whose expression varies between isolates of strain PAO1 depending on the allele of *mexT* (39, 46).

Even autolysis, which might seem unambiguously detrimental to a unicellular organism, may be an adaptive behavior mediated by PQS. There is increasing evidence for programmed death pathways in bacteria, some based on an extracellular signal and influenced by the stringent response (19, 43, 70). Whether or not cell death is their primary function (24) remains uncertain, but an outcome in common between these pathways is persistence of a fraction of the bacterial population (19, 43, 70). Such persistence may be reflected in the cycles of lysis seen for the *pqsL* mutant in this study (Fig. 7). Persisting cells could benefit from the DNA released by lysed cells, since extracellular DNA was recently shown to be required by *P. aeruginosa* for formation of a protective biofilm (79).

***P. aeruginosa* diversity.** The myriad capabilities of *P. aeruginosa* have been revealed in part by examining single strains under a variety of conditions, including models of infection in diverse hosts. In a complementary approach applied in this and other *Pseudomonas* studies (17, 59, 68), mutants with a gain of function were characterized on the basis of their unique colony morphology. The readily visible phenotype associated with *P. aeruginosa* mutants with enhanced autolysis and enhanced autoaggregation will facilitate analysis of extracellular signaling by PQS and intracellular signaling mediated by WspR and perhaps other GGDEF domain-containing regulators. It remains a continual challenge to reveal the breadth and sources of *Pseudomonas* diversity (67), but the knowledge gained is likely to fundamentally contribute to our understanding of the evolutionary arms race between bacteria and their hosts.

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