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Swarming of *Pseudomonas aeruginosa* Is a Complex Adaptation Leading to Increased Production of Virulence Factors and Antibiotic Resistance▽†

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In addition to exhibiting swimming and twitching motility, *Pseudomonas aeruginosa* is able to swarm on semisolid (viscous) surfaces. Recent studies have indicated that swarming is a more complex type of motility influenced by a large number of different genes. To investigate the adaptation process involved in swarming motility, gene expression profiles were analyzed by performing microarrays on bacteria from the leading edge of a swarm zone compared to bacteria growing in identical medium under swimming conditions. Major shifts in gene expression patterns were observed under swarming conditions, including, among others, the overexpression of a large number of virulence-related genes such as those encoding the type III secretion system and its effectors, those encoding extracellular proteases, and those associated with iron transport. In addition, swarming cells exhibited adaptive antibiotic resistance against polymyxin B, gentamicin, and ciprofloxacin compared to what was seen for their planktonic (swimming) counterparts. By analyzing a large subset of up-regulated genes, we were able to show that two virulence genes, *lasB* and *pvdQ*, were required for swarming motility. These results clearly favored the conclusion that swarming of *P. aeruginosa* is a complex adaptation process in response to a viscous environment resulting in a substantial change in virulence gene expression and antibiotic resistance.

Swarming motility is a multicellular phenomenon involving the coordinated and rapid movement of a bacterial population across a semisolid surface (14). It is widespread among flagellated bacteria, including *Salmonella*, *Vibrio*, *Yersinia*, *Serratia*, and *Proteus* (9, 18). Swarming is highly dependent on bacterial cell density, nutrient growth medium, and surface condition moistness (53). In addition to physical changes such as an increase in the number of flagella or cell elongation, swarmer cell differentiation results in substantial alterations in metabolic bias and gene expression, indicating that swarming represents a complex lifestyle adaptation in response to particular medium conditions rather than merely a form of locomotion (18, 45).

Swarming of *Pseudomonas aeruginosa* is often typified by a dendritic colonial appearance. This gram-negative bacterium is a major cause of hospital-acquired bacterial infections and the most significant pulmonary pathogen in cystic fibrosis patients (17, 20, 44). It is one of the most difficult infections to treat due to its high natural (intrinsic) antibiotic resistance. It possesses three types of movement depending on medium viscosity, namely, swimming in aqueous environments, twitching on solid surfaces or interfaces, and swarming on semisolid, viscous media, such as those containing 0.4 to 0.7% (wt/vol) agar. It has been previously shown that swarming of *P. aeruginosa* is dependent on both flagella and type IV pili, which mediate actual movement, as well as on rhamnolipids, which are proposed to enable swarming cells to overcome the strong surface tension of the water surrounding swarming cells and thus modulate the swarming motility pattern (5, 10, 25, 35, 41). *P. aeruginosa* swarming is increased under nitrogen limitation and in response to certain amino acids (25).

Recently, we showed that swarming of *P. aeruginosa* represents an adaptation that is influenced by a large number of cooperating genes (41). Using a Tn*5-lux* mutant library (29), we were able to isolate more than three dozen swarming-negative mutants with transposon insertions in regulatory, metabolic, chemosensory, type II secretion system, hypothetical, motility, and quorum-sensing-related genes (41). Moreover, it was demonstrated that many mutants with altered swarming motility were also defective in biofilm formation, indicating that it may play a key role in early biofilm development (41, 48).

To understand the nature of the complex adaptation that accompanies swarming motility, we investigated here the influence of swarming motility on gene expression by performing microarrays of bacteria from the leading edge of a swarm zone compared to bacteria growing in identical medium under swimming conditions. Major shifts in gene expression patterns were observed under swarming conditions, including, among others, the up-regulation of a large number of virulence-related genes. Also, actively swarming cells exhibited adaptive antibiotic resistance against all tested antibiotics compared to what was seen for their planktonic counterparts. By analyzing a large subset of the up-regulated genes, we were able to show that two virulence genes, *lasB* and *pvdQ*, were required for swarming motility. These results clearly favor the conclusion that swarming of *P. aeruginosa* is a complex adaptation process

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in response to a viscous environment, resulting in a substantial change in virulence gene expression and antibiotic resistance.

MATERIALS AND METHODS

Bacterial strains, primers, and growth conditions. Bacteria included *P. aeruginosa* PAO1 (50) and *P. aeruginosa* PA14 (43), as well as a large number of derived Tn*5* mutants from the *P. aeruginosa* mutant libraries (29, 30) as described below. *Escherichia coli* XL1-Blue (4) and plasmids pUCP18 (47) and pUCP18-mini-Tn7-Gm (6) were used for cloning. The sequences of DNA primers (Alpha-DNA, Montreal, Canada) used in these studies are available from the authors upon request. Cultures were routinely grown in Luria-Bertani broth, BM2 minimal medium [62 mM potassium phosphate buffer, pH 7, 7 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 10 μM $FeSO_4$, 0.4% (wt/vol) glucose] or BM2-swarming medium, comprising BM2 with 0.1 or 0.5% (wt/vol) Casamino Acids substituted for 7 mM $(NH_4)_2SO_4$. Solid medium contained either 0.3% (wt/vol) agar for assessing swimming motility, 0.5% agar for swarming assessments, or 1% agar for twitching motility assessments (25, 41). When required for plasmid or transposon selection or maintenance, tetracycline, gentamicin, and carbenicillin were added to final concentrations of 50, 15, and 300 μg/ml, respectively. Routine genetic manipulations were carried out using standard procedures (32).

Swarming motility and biofilm assays. Except as otherwise noted, swarming of *P. aeruginosa* PAO1 was examined on BM2-swarming plates containing 0.5% (wt/vol) agar (Difco) and 0.5% (wt/vol) Casamino Acids as described previously (41), since these conditions result in a more circular swarm zone, permitting easier determination of swarm colony diameter. Since *P. aeruginosa* PA14 does not swarm consistently under these conditions, BM2-swarming plates supplemented with 0.1% (wt/vol) Casamino Acids were utilized, resulting in dendritic colonies that were analyzed by measuring the agar plate coverage as previously described (5). All swarm experiments were repeated five times independently. Bacteria were inoculated on swarm agar plates as 1- to 2-μl aliquots of an overnight culture grown in BM2 glucose, and plates were incubated for 18 h at 37°C.

Abiotic solid surface assay biofilm formation was analyzed in 96-well polystyrene microtiter plates after 20 h of incubation at 37°C as described previously (15, 40). After crystal violet staining, the absorbance was measured at 595 nm using a microtiter plate reader (Bio-Tek Instruments Inc.).

DNA microarray experiments. Microarray experiments were performed on four independent cultures. *P. aeruginosa* PAO1 was grown for 6 h to the late logarithmic growth phase (optical density at 600 nm, 0.9 to 1.0) in liquid BM2-swarming medium under shaking (swimming) conditions or for 18 h at 37°C on a BM2-swarming plate containing 0.5% (wt/vol) agar and 0.1% (wt/vol) Casamino Acids (to obtain dendritic swarm colony growth enabling actively swarming cells to be more easily harvested from the leading edge of the swarm colony [within 2 to 3 mm of the swarm zone edge]). Cells were harvested either by centrifugation (swimming) or by a sterile inoculating loop (swarming, within 2 to 3 mm of the swarm zone edge) and were resuspended in BM2-swarming media supplemented with RNAprotect reagent (Qiagen, Germany). RNA isolation, cDNA synthesis, and hybridization to microarray slides (The Institute for Genomic Research [TIGR] Pathogenic Functional Genomics Resource Center) along with analysis of DNA microarray slides using ArrayPipe version 1.7 and quantitative reverse transcription-PCRs (RT-PCRs) were performed as described previously (38). *P. aeruginosa* microarray slides from TIGR consist of 18,432 elements with 5,552 oligonucleotides and 3 oligonucleotide replicates (for more information, visit http://pfgrc.tigr.org/slide_html/array_descriptions/P_aeruginosa.shtml). Only genes that exhibited changes, compared to the swimming control, of twofold or more and *P* values of ≤0.05 were considered in this study.

Mutant complementation. For complementation of the *P. aeruginosa* PA14 *pvdQ* mutant ID27758 from the corresponding *P. aeruginosa* PA14 mutant library (30), the *pvdQ* gene was amplified by PCR and cloned as a HindIII fragment into the broad-host-range vector pUCP18. The resulting hybrid plasmid, pUCP18::*pvdQ*, was transferred into the *P. aeruginosa* PA14 *pvdQ* mutant ID27758 by electroporation (6), resulting in strain *P. aeruginosa* PA14 ID27758(pUCP18::*pvdQ*).

For complementation of the *P. aeruginosa* PAO1 *lasB* mutant 23_F8 from the corresponding *P. aeruginosa* PAO1 mutant library (29), the *lasB* gene including the promoter region was amplified by PCR and cloned as a BamHI fragment into plasmid pUC18-mini-Tn7-Gm (6). The resulting hybrid plasmid, pUCP18-mini-Tn7-Gm::*lasB*, was coelectroporated with pTNS2 into the *P. aeruginosa* PAO1 *lasB* mutant 23_F8 by electroporation (6), resulting in *P. aeruginosa* PAO1 strain 23_F8(*lasB*$^+$) with a chromosomally integrated *lasB*$^+$ gene.

Comparison of antibiotic resistance levels. *P. aeruginosa* PAO1 cultures were grown for 6 h in BM2 medium with 0.3% agar under swimming conditions or on BM2-swarming agar plates with 0.5% agar and 0.5% Casamino Acids for 18 h under swarming conditions. Swim plates containing BM2 medium were supplemented with different concentrations of antibiotics and inoculated with 1 μl of culture grown under swimming conditions (inoculum of 1×10^4 to 1×10^5 cells). Swarm agar plates containing BM2-swarming medium supplemented with different concentrations of antibiotics were inoculated with cells taken from the outside of a swarming colony (within 2 to 3 mm from the swarm zone edge) by use of a sterile toothpick (inoculum of 1×10^4 to 1×10^5 cells). After 18 h of incubation at 37°C, growth was determined by measuring the swimming or swarming colony diameter as previously described (41).

MIC. MICs were measured using standard broth microdilution procedures in BM2 and BM2-swarming medium (31). Growth was scored following 24 h and 48 h of incubation at 37°C. For measuring MICs against polymyxin B, a modified assay was used to prevent artificially high MICs due to aggregation of the antibiotic and binding to polystyrene (55).

Killing experiments. Cells of *P. aeruginosa* were grown to an optical density at 600 nm of 0.4 to 0.6 in BM2 or BM2-swarming liquid medium (i.e., supplemented with 0.1% Casamino Acids) or on BM2-swarming agar plates containing 0.1% (wt/vol) Casamino Acids for 18 h. These cultures were then diluted 1:100 into prewarmed 10 mM sodium phosphate buffer, pH 7.5, containing 2 μg/ml polymyxin B sulfate (Sigma). Samples were shaken at 37°C and aliquots were withdrawn at specified times, plated for survivors on LB agar, and grown overnight at 37°C. All experiments were repeated three times.

Microarray accession number. The ArrayExpress accession number is E-FPM1-10.

RESULTS

Microarray analyses of *P. aeruginosa* swarmer cells. Based on mutant studies, it would appear likely that *P. aeruginosa* swarming is a complex adaptation to movement in a viscous environment in that it is influenced by a substantial number of cooperating genes and exhibits a strong interrelation with biofilm formation (41, 48). To further understand swarming motility, gene regulation events associated with swarming were investigated, comparing bacteria at the leading edge of a swarm zone (within 2 to 3 mm of the swarm zone edge) to those grown under swimming conditions. Analysis of four independent experiments demonstrated the statistically significant ($P < 0.05$ by Student's *t* test) greater-than-twofold change in expression levels of 417 genes (7.5% of all *P. aeruginosa* genes) (see Table S1 in the supplemental material; also see Table 1 for selected examples). Of these, 309 were up-regulated and 108 down-regulated, including 40% hypothetical or conserved hypothetical genes with no currently defined function. RT-PCR experiments confirmed the microarray data for genes PA0044 (*exoT*), PA1706 (*pcrV*), PA3478 (*rhlB*), PA3841 (*exoS*), and PA4225 (*pchF*) (data not shown).

The 243 annotated genes were analyzed by functional category according to the *Pseudomonas* genome database V2 (www.pseudomonas.com). Under swarming conditions, differential expression of genes from 21 different functional classes was observed, demonstrating the complex differences accompanying growth under swarming compared to swimming conditions (Fig. 1). Most interestingly, we observed under swarming conditions the up-regulation, by 2- to 13-fold, of a large number of genes associated with virulence factor synthesis and export. Among these were genes coding for the type III secretion system (TTSS) and related secreted factors *exoS*, *exoT*, and *exoY*; genes *aprA*, *lasB*, and *pIV*, encoding the extracellular proteases alkaline protease, elastase, and protease IV, respectively; the rhamnosyltransferase gene *rhlB*; and genes encoding the biosynthesis of the siderophores pyochelin and pyoverdine as well as redox-active phenazine compounds (Table 1).

TABLE 1. Selected *P. aeruginosa* genes that were up-regulated under swarming motility conditions cf. swimming conditions

Identification	Gene designation	Fold change	*P* value	Protein
Secreted factors				
PA0044	*exoT*	12.7	0.0075	Exoenzyme T
PA2191	*exoY*	5.5	0.0138	Adenylate cyclase Y
PA3841	*exoS*	8.7	0.0177	Exoenzyme S
PA1249	*aprA*	2.5	0.0046	Alkaline metalloproteinase precursor
PA3724	*lasB*	4.9	0.0023	Elastase LasB
PA4175	*pIV*	5.2	0.0011	Protease IV
PA3478	*rhlB*	6.9	0.0049	Rhamnosyltransferase chain B
Alkaline protease secretion				
PA1245	*aprX*	3.8	0.0008	Hypothetical protein
PA1246	*aprD*	2.4	0.0036	Alkaline protease secretion protein AprD
PA1247	*aprE*	2.5	0.0017	Alkaline protease secretion protein AprE
TTSS				
PA1692	*pscS*	2.3	0.0256	Translocation protein in TTSS
PA1694	*pscQ*	2.2	0.0270	Translocation protein in TTSS
PA1695	*pscP*	3.6	0.0090	Translocation protein in TTSS
PA1697		2.7	0.0270	ATP synthase in TTSS
PA1698	*popN*	5.2	0.0030	TTSS outer membrane protein precursor
PA1699		2.2	0.0030	Hypothetical protein in TTSS
PA1700		4.2	0.0115	Hypothetical protein in TTSS
PA1701		4.8	0.0037	Hypothetical protein in TTSS
PA1702		2.0	0.0133	Hypothetical protein in TTSS
PA1703	*pcrD*	4.8	0.0156	TTSS protein PcrD
PA1704	*pcrR*	3.4	0.0266	Transcriptional regulator PcrR
PA1705	*pcrG*	10.6	0.0022	Regulator in TTSS
PA1706	*pcrV*	9.6	0.0057	TTSS protein PcrV
PA1707	*pcrH*	8.4	0.0040	Regulatory protein PcrH
PA1708	*popB*	10.0	0.0054	Translocator protein PopB
PA1711	*exsE*	5.1	0.0008	Regulator in TTSS
PA1712	*exsB*	8.5	0.0004	Exoenzyme S synthesis protein B
PA1713	*exsA*	4.6	0.0059	Transcriptional regulator ExsA
PA1714	*exsD*	2.5	0.0472	Hypothetical protein
PA1715	*pscB*	2.0	0.0300	TTSS apparatus protein
PA1717	*pscD*	4.4	0.0139	TTSS export protein PscD
PA1719	*pscF*	7.8	0.0244	TTSS export protein PscF
PA1720	*pscG*	3.7	0.0441	TTSS export protein PscG
PA1722	*pscI*	3.1	0.0480	TTSS export protein PscI
PA1723	*pscJ*	2.2	0.0036	TTSS export protein PscDJ
PA1724	*pscK*	2.8	0.0440	TTSS export protein PscK
PA1725	*pscL*	2.2	0.0281	TTSS export protein PscL
Phenazine biosynthesis				
PA1899	*phzA2*	4.7	0.0023	Phenazine biosynthesis protein
PA1900	*phzB2*	4.3	0.0083	Phenazine biosynthesis protein
PA1901	*phzC2*	6.7	0.0002	Phenazine biosynthesis protein PhzC
PA1902	*phzD2*	3.0	0.0081	Phenazine biosynthesis protein PhzD
PA1903	*phzE2*	2.4	0.0087	Phenazine biosynthesis protein PhzE
PA1904	*phzF2*	4.7	0.0008	Phenazine biosynthesis protein
PA1905	*phzG2*	4.1	0.0001	Pyrodoxamine 5′-phosphate oxidase
Pyoverdine biosynthesis				
PA2385	*pvdQ*	3.1	0.0145	PvdQ
PA2392	*pvdP*	3.3	0.0244	PvdP (pyoverdine biosynthesis)
PA2394	*pvdN*	5.4	0.0250	PvdN (pyoverdine biosynthesis)
PA2397	*pvdE*	3.3	0.0204	Pyoverdine biosynthesis protein PvdE
PA2399	*pvdD*	4.1	0.0454	Pyoverdine synthase D
Pyochelin biosynthesis				
PA4220	*fptB*	3.3	0.0383	Hypothetical protein
PA4221	*fptA*	6.0	0.0087	Fe(III)-pyochelin outer membrane receptor
PA4223	*pchH*	5.2	0.0139	ATP-binding component of ABC transporter
PA4224	*pchG*	3.8	0.0112	Pyochelin biosynthetic protein PchG
PA4225	*pchF*	14.6	0.0096	Pyochelin synthetase
PA4226	*pchE*	7.9	0.0036	Dihydroaeruginoic acid synthetase
PA4228	*pchD*	5.5	0.0111	Pyochelin biosynthesis protein PchD

Continued on following page

TABLE 1—*Continued*

Identification	Gene designation	Fold change	*P* value	Protein
PA4229	*pchC*	10.1	0.0059	Pyochelin biosynthesis protein PchC
PA4230	*pchB*	7.6	0.0097	Salicylate biosynthesis protein PchB
Efflux transport system				
PA4205	*mexG*	3.3	0.0032	Hypothetical protein
PA4206	*mexH*	2.2	0.0252	RND efflux membrane fusion protein
PA4208	*opmD*	3.1	0.0110	Outer membrane protein
Transcriptional regulator				
PA1430	*lasR*	2.1	0.0090	Transcriptional regulator LasR
PA2259	*ptxS*	3.0	0.0178	Transcriptional regulator PtxS
PA3477	*rhlR*	6.0	0.0050	Transcriptional regulator RhlR
Others				
PA2570	*lecA*	8.2	0.0057	LecA
PA3361	*lecB*	5.0	0.0011	Fucose-binding lectin PA-IIL

Although we and others have shown recently that swarming of *P. aeruginosa* PAO1 is dependent on both flagella and type IV pili (25, 41) and swarm cells of *P. aeruginosa* are described to be hyperflagellated and to possess two polar flagella (25), genes related to flagella and type IV pili biosynthesis and function were not up-regulated under swarming conditions. In contrast, genes *fimU*, *pilW*, *pilX*, *pilY1*, and *pilY2*, comprising one of the gene clusters involved in type IV pilus assembly and biosynthesis, were down-regulated by three- to eightfold.

Interestingly, with more than 70 differentially regulated genes relating to various types of bacterial metabolism, including cofactor biosynthesis, energy, carbon compounding, and central intermediary, fatty acid, and amino acid metabolism (see Table S1 in the supplemental material), actively swarming cells exhibited a striking difference in the gene expression profile of metabolic genes compared to what was seen for swimming cells. For example, various genes associated with glucose metabolism and uptake, including those for the carbohydrate-selective sugar porin OprB and other carbohydrate transporters, were down-regulated, whereas genes involved in fatty acid metabolism and nitrite reduction were up-regulated in swarmer cells. In this context it is worth mentioning that nitrite, which is present in the cystic fibrosis lung, promotes swarming and rhamnolipid production (49) and that mutants of *P. aeruginosa* with defects in nitrite reduction exhibit a swarming-negative phenotype (51).

Furthermore, no fewer than 18 predicted or known transcriptional regulatory genes were observed to be differentially regulated under swarming conditions, indicating that a complex regulatory network was involved in the swarmer differentiation process. These included *pcrR*, *pcrG*, *pcrH*, *exsB*, *exsA*, *ptxS*, *gltR*, *rhlR*, *ntrB*, *ybbL*, *rnk*, *glnK*, *lasR*, PA0610, PA2897,

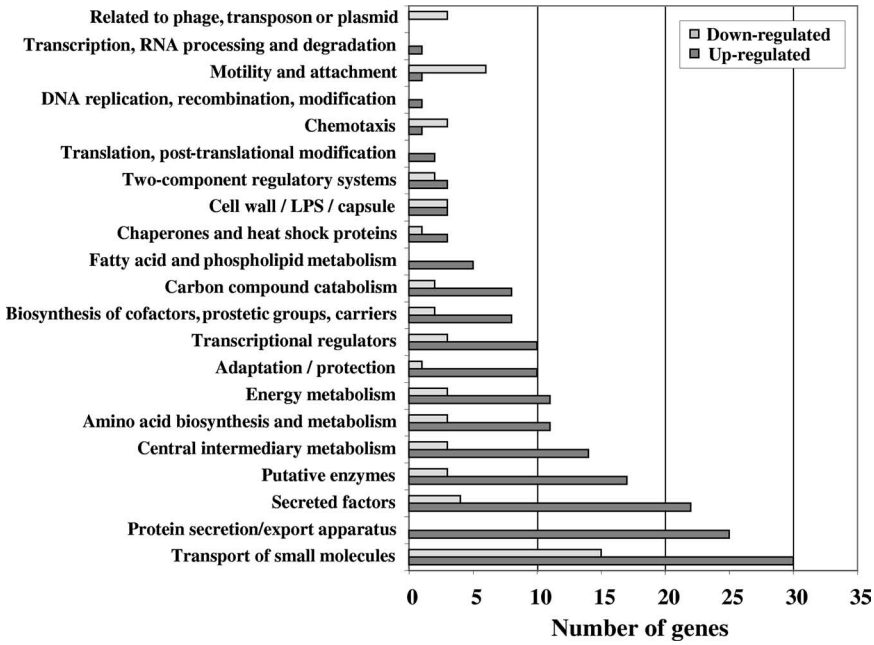

FIG. 1. Functional classification of nonhypothetical genes with a change in expression level of greater than twofold.

TABLE 2. Gene expression of selected *P. aeruginosa* genes under swarming conditions cf. nonswarming conditions

Gene	Gene expression under swarming conditions in comparison to:	
	Swimming conditions[a]	Surface growth[b]
exoT	12.7	3.3 ± 1.1
aprD	2.4	3.8 ± 1.4
pcrV	9.6	4.9 ± 1.9
exoY	5.5	3.9 ± 1.4
rhlB	6.9	14.9 ± 6.3
lasB	4.9	3.7 ± 1.5
exoS	8.6	2.0 ± 0.9
pchF	14.6	2.8 ± 1.1

[a] Results obtained by microarrays.
[b] Results obtained by RT-PCR.

PA4288, PA4843, and PA5116, several of which are known to have a role in the regulation of virulence. As anticipated for a social-behavior-like swarming, these included the two major quorum-sensing regulators *lasR* and *rhlR*, with two- and sixfold up-regulated gene expression levels, respectively. This up-regulation of *lasR* and *rhlR* under swarming conditions is consistent with the concept that swarming motility is a social bacterial phenomenon and explains the overlaps of the swarming transcriptome with the LasR and RhlR transcription profiles that were described previously (46).

To demonstrate that changes in gene expression are specific to swarming and do not reflect a response to surface growth under nonswarming conditions, RT-PCR was performed for a subset of genes by use of RNA from actively swarming cells in comparison to RNA isolated from cells grown on BM2-swarming media for 5 h under nonswarming conditions (BM2 plus 1.5% agar). As shown in Table 2, all analyzed genes showed differential gene expression, with an up-regulation in the range of 2- to 15-fold under swarming conditions in comparison to what was seen for nonswarming surface growth. These findings underline the swarming-specific gene profile obtained by the microarray experiment described above.

Elastase LasB and PvdQ are essential for normal swarming behavior. Since such a large number of virulence-related genes were up-regulated under swarming conditions by more than twofold, we were interested in whether these virulence-associated genes were required for swarming motility. Using the opportunity to analyze gene functions afforded by two *P. aeruginosa* mutant libraries, namely, the PAO1 *lux* fusion library (29) and the PA14 mutant library (30), which comprise nearly every nonessential gene, we tested strains with mutations in up-regulated, virulence-associated genes for their swarming abilities (Table 3). No swarming defects relative to the parental wild-type strains were observed for tested mutants with transposon insertions in the TTSS or effectors *exoT* or *exoY*, alkaline protease secretion apparatus, or pyochelin, phenazine, and pyoverdine biosynthesis pathways. However, a known virulence-related gene, *lasB*, was shown to play an important role in swarming motility. Four independently derived *lasB* mutants from both transposon mutant libraries (Table 3 and Fig. 2A) showed strong swarming defects but normal swimming and twitching motility phenotypes compared to their corresponding wild-type strains. Moreover, all four tested *lasB* mutants also showed a strong impairment in biofilm formation with about 50% less biomass in the respective microtiter plate assays (Fig. 2B). The swarming as well as the biofilm phenotype could be completely restored by the expression of the chromosomally integrated *lasB*$^+$ gene (Fig. 2).

TABLE 3. Swarming motility of selected *P. aeruginosa* mutants

Locus of Tn*5* insertion	*P. aeruginosa* mutant library	Mutant identification no.	Swarming phenotype[a]
Secreted factors			
exoT	PA14	36955	+
exoY	PA14	36309	+
aprA	PA14	23768	+
lasB	PA14	31938	−
lasB	PA14	45691	−
lasB	PAO1	23_F8	−
lasB	PAO1	75_H11	−
rhlB	PA14	27130	−
Alkaline protease secretion			
aprD	PA14	53795	+
aprE	PA14	29480	+
TTSS			
pcrD	PA14	23060	+
pcrH	PA14	48586	+
popB	PA14	34677	+
exsA	PA14	23567	+
exsB	PA14	31713	+
pscD	PA14	38075	+
Phenazine biosynthesis			
phzB2	PA14	48282	+
phzC2	PAO1	83_A9	+
Pyoverdine biosynthesis			
pvdQ	PA14	27758	−
pvdP	PA14	31341	+
pvdN	PA14	6127	+
pvdD	PA14	40342	+
Pyochelin biosynthesis			
pchF	PA14	24493	+
pchE	PAO1	73_F1	+

[a] +, swarm zone coverage was >90% of that of wild-type *P. aeruginosa*; −, swarm zone coverage was <50% of that of wild-type *P. aeruginosa*.

Since high cell density and quorum sensing are described to play an important role in swarming motility, we analyzed the *pvdQ* gene (PA2385) in more detail. In addition to its involvement in pyoverdine biosynthesis (26), PvdQ exhibits an acyl-homoserine lactone (HSL) acyclase activity and is known to degrade the quorum-sensing signal 3-oxododecanoyl-HSL ($3OC_{12}$HSL), among other long-chain acyl-HSLs (19). The *pvdQ* gene was up-regulated threefold under swarming conditions (Table 1). We tested the respective *pvdQ* mutant, *P. aeruginosa* PA14 ID27758 from the PA14 mutant library, for its swarming ability and demonstrated a strong decrease in swarming motility of about 75% compared to what was seen for wild-type *P. aeruginosa* PA14 (Table 3 and Fig. 3). In contrast, this *pvdQ* mutant revealed normal swimming and twitching motility as well as normal biofilm formation (data not shown). The swarming phenotype could be completely restored by expression of the cloned *pvdQ*$^+$ gene (Fig. 3).

Since the genome of *P. aeruginosa* reveals three open read-

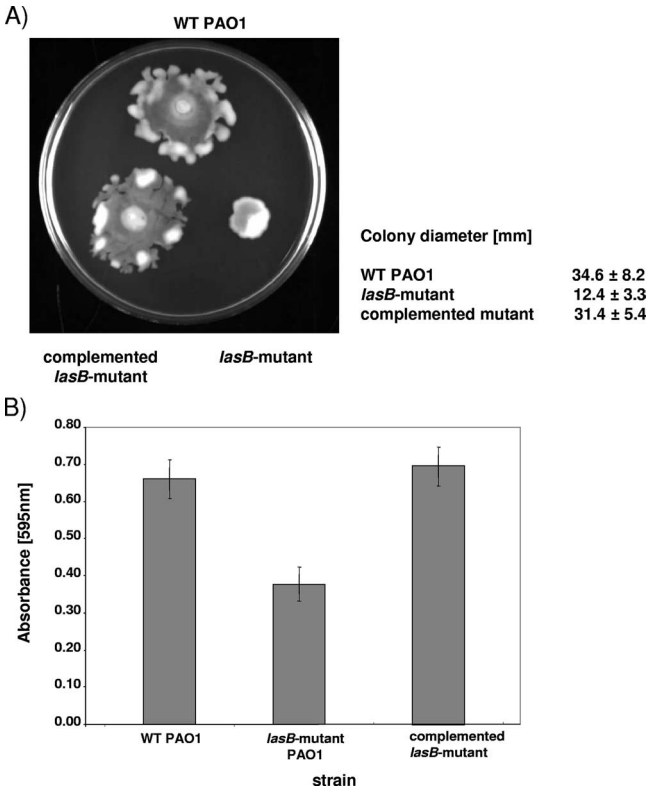

FIG. 2. Swarming (A) and biofilm formation (B) of wild-type *P. aeruginosa* PAO1, *P. aeruginosa* PAO1 *lasB* mutant 23_F8, and the complemented *lasB* mutant. (A) Shown is a representative swarm plate and quantification (five plates) after 18 h of incubation. (B) Biofilm formation was determined after 16 h of incubation using the microtiter plate assay. WT, wild type.

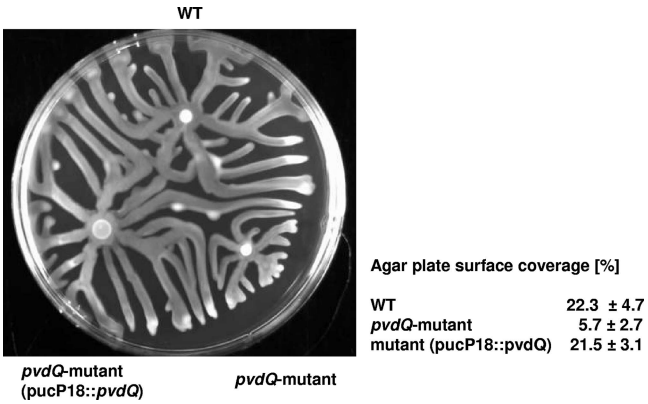

FIG. 3. The *pvdQ* mutant exhibits a strong swarming motility defect. Swarming levels of wild-type *P. aeruginosa* PA14, the *pvdQ* mutant, and the complemented *pvdQ* mutant PA14 ID27758(pUCP18::*pvdQ*) were assayed on swarm plates after 18 h of incubation, and agar plate surface coverage was determined from five replicates.

ing frames that exhibit homologies to *pvdQ*, namely, PA0305, PA1032, and PA1893, all corresponding mutants from the PA14 and PAO1 mutant libraries were analyzed for their swarming phenotype. However, none of the tested mutants showed any swarming defect compared to the wild-type strains (data not shown). Thus, these homologs do not seem to be necessary for normal swarming behavior under the tested conditions.

Swarmer cells exhibit enhanced antibiotic resistance. Recently, it was demonstrated that swarm-cell differentiation in *Salmonella enterica* serovar Typhimurium results in elevated resistance to various antibiotics (22). To test whether *P. aeruginosa* also reveals altered antibiotic susceptibility under swarming conditions, we compared the antibiotic resistance of actively swarming cells with that of swimming cells by use of media solidified with appropriate agar concentrations.

As shown in Fig. 4, swarmer cells of *P. aeruginosa* PAO1 grown on BM2-swarming agar plates supplemented with 0.5% Casamino Acids and 0.5% agar exhibited a 4- to 16-fold increase in antibiotic resistance toward polymyxin B, gentamicin, and ciprofloxacin compared to what was seen for swimming cells on BM2 medium with 0.3% agar. To analyze if the determined antibiotic resistance is due to the slightly different medium compositions for swarm and swim agar plates as opposed to the swarming growth state, we performed MIC experiments in liquid cultures as described previously (37). Identical MICs of 0.5, 2, and 0.1 μg/ml for polymyxin B, gentamicin, and ciprofloxacin, respectively, were recorded for BM2 medium with 0.5% Casamino Acids and BM2-swarming medium supplemented with 0.5% (wt/vol) Casamino Acids, using either swarming or swimming cells as precultures. This experiment demonstrated clearly that the observed antibiotic resistance is a result of swarmer differentiation of *P. aeruginosa* cells and is not due to a medium effect.

To verify these results, kill curves were performed. Cells of *P. aeruginosa* PAO1 were examined for sensitivity to killing by 2 μg/ml polymyxin B after growth under swimming conditions in BM2 or BM2-swarming liquid medium in comparison to what was seen for swarmer cells grown on BM2-swarming agar plates. Very high susceptibility to polymyxin B, with 5-log orders of killing occurring in 5 min, was observed with cells grown under swimming conditions in BM2 medium, whereas cells grown under swarming conditions showed high polymyxin resistance, with less than a 1-log order of killing after 10 min.

Subinhibitory concentrations of aminoglycosides do not influence swarming motility. It has been shown previously by our group that subinhibitory concentrations of aminoglycoside antibiotics induce the gene expression of various motility genes, including genes for the biosynthesis and function of flagella and pili (33). With this in mind, we examined the swarming motility of *P. aeruginosa* PAO1 in the presence of subinhibitory concentrations of aminoglycosides. Using BM2-swarming medium supplemented with 0.5% Casamino Acids and subinhibitory concentrations of gentamicin in the range of 0.1 to 0.5 μg/ml, we determined swarm zones after 18 h of inoculation. No statistically significant change in swarming motility was detectable using subinhibitory concentrations of gentamicin or tobramycin, demonstrating that subinhibitory concentrations of aminoglycosides do not increase swarming motility under the tested conditions.

DISCUSSION

Microbiological and functional genomic studies have led to the concept that *P. aeruginosa* can adapt its lifestyle to various

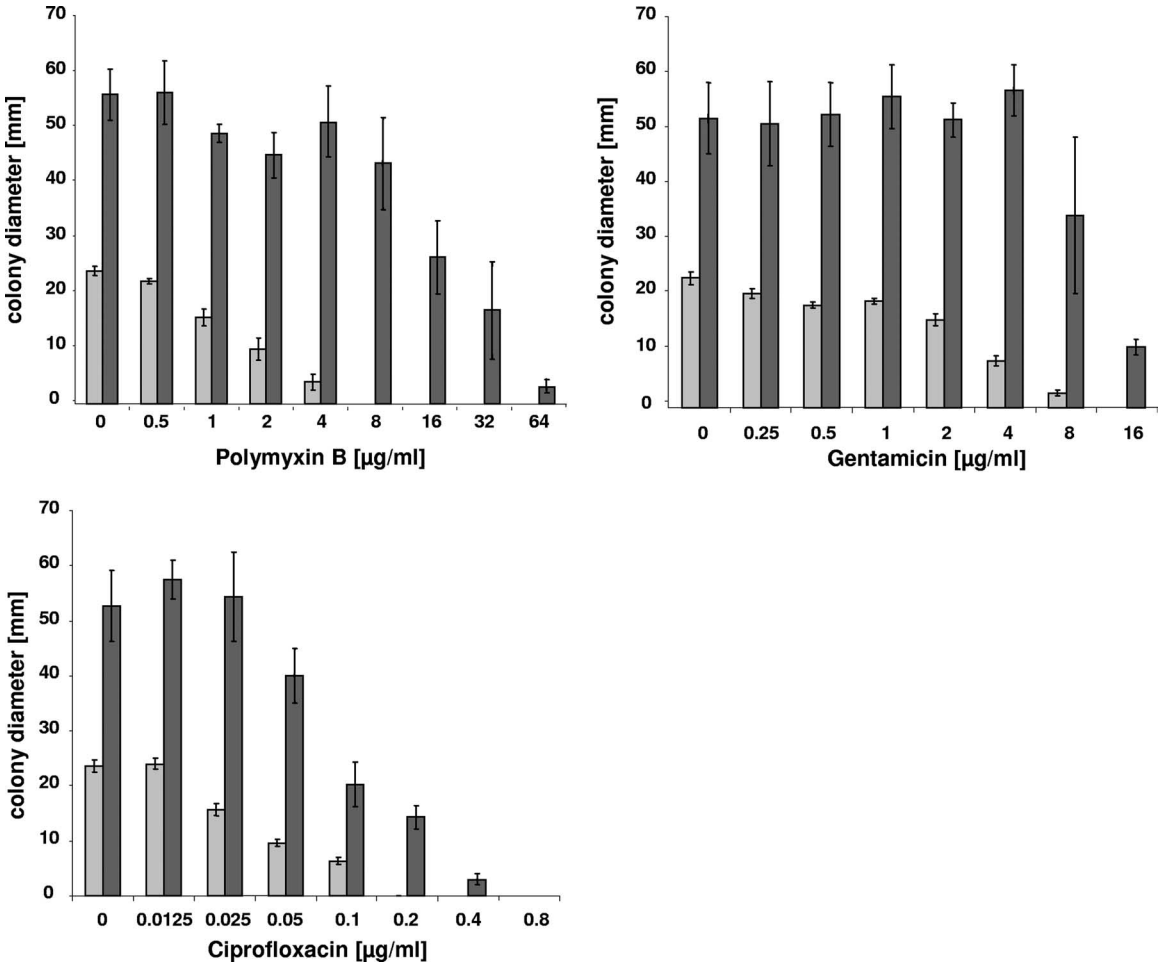

FIG. 4. Swarmer cells of *P. aeruginosa* exhibit enhanced antibiotic resistance. Cells of *P. aeruginosa* were spot inoculated on swim and swarm agar plates containing different concentrations of polymyxin B, gentamicin, and ciprofloxacin. Growth was determined by measuring the colony diameter after 18 h of incubation at 37°C. Dark gray, swarming cells of *P. aeruginosa* PAO1; light gray, swimming cells of *P. aeruginosa* PAO1.

different circumstances through the up-regulation of large subsets of genes located around the genome. Indeed, its prominence as an opportunistic pathogen and widespread environmental isolate is likely to reflect this capability in part. Known complex adaptations in *P. aeruginosa* include the formation of organized structured communities termed biofilms (16, 24), the adaptation to nutrient starvation by iron, phosphate, and sulfur (39), the switch to anaerobic growth (13), and a phenomenon known as quorum sensing (52). In this study, we present the first global approach to investigating the *P. aeruginosa* swarming phenotype, which reflects life in viscous environments such as mucosal surfaces, and present evidence that confirms that it represents another type of complex adaptation of *P. aeruginosa*.

Our microarray data clearly revealed that *P. aeruginosa* gene expression during swarming is substantially different from that observed during growth under lower viscosities (swimming/planktonic conditions). More than 400 genes, approximately 7.5% of the genome, were altered in expression. In addition to a large number of hypothetical genes with unknown functions, there was a broad distribution of known genes with altered expression into 21 functional classes, including various metabolic pathways, transport and secretion, regulation, adaptation, and motility among others. The differential expression of such a large and diverse set of genes indicates that there was a striking physiological diversity between planktonic growth and growth on a viscous, semisolid surface. Conceptually similar results were recently made with *Salmonella enterica* serovar Typhimurium by use of genomic and proteomic approaches (23, 53), although the details differed from those observed here.

This diversity in the transcriptomes of these two physiological states of a *P. aeruginosa* cell is remarkable given that another complex adaptation, biofilm formation, was associated with only 73 differentially regulated genes compared to planktonic cells (54). These genes were largely related to phages, motility, and translation, while genes associated with metabolic functions were hardly affected at all. Thus, despite the recently reported relationship between swarming motility and biofilm formation (41, 48), a comparison of our microarray data with those published for biofilm formation strongly indicate that the physiological states of cells adapted to swarming motility and to biofilm formation differ substantially.

Keeping in mind that a viscous or semisolid environment is

likely to reflect bacterial life in the mucous blanket covering epithelial surfaces or the viscous hyperabundant mucous in the cystic fibrosis lung, one of the most interesting observations of our studies is the up-regulated expression of various virulence factors during swarming. Besides genes coding for extracellular proteases or biosynthesis of the siderophores pyoverdine and pyochelin, which are involved in iron acquisition in the host (42), genes for the complete TTSS apparatus, including those for the related effector proteins required for colonization and dissemination (28), were up-regulated in swarming cells. Furthermore, genes coding for the biosynthesis of the redox-active compound phenazine, which plays an important role in pulmonary tissue damage and other effects in chronic lung infections by *P. aeruginosa* (36), as well as the multidrug efflux pump MexGHI-OpmD, which confers antimicrobial resistance (1, 2), were up-regulated in swarming cells. This phenomenon of increased virulence factor expression in swarming cells is consistent with observations with other bacteria such as *Proteus* and *Salmonella* (53) but has not to date been described for *P. aeruginosa*. The up-regulated expression of a variety of virulence factors would benefit the population of bacterial cells in the process of colonizing new environments, including host mucosal surfaces.

Since our microarray data revealed virulence expression as a dominant phenotype of *P. aeruginosa* swarm cells, we analyzed to what extent these virulence factors are necessary for normal swarming behavior. Although the swarm cell invests a lot of energy in virulence factor synthesis, most of these processes do not appear to play obligatory roles in swarming motility. No mutants affected either in iron acquisition or the biosynthesis of phenazine or in the TTSS apparatus and effectors were found to be deficient in swarming motility. However, two genes associated with virulence, *pvdQ* and *lasB*, were newly identified here to play important roles in swarming behavior.

The protein produced by the *pvdQ* gene, which is involved in pyoverdine biosynthesis (26), also exhibits acyl-HSL acylase activity (19). Since other mutants with transposon insertions in genes necessary for pyoverdine biosynthesis did not reveal a swarming defect, the acylase activity rather than its involvement in pyoverdine synthesis seems likely to be the important function influencing the swarming defect. One of the preferred substrates of the PvdQ protein is one of two major quorum-sensing signals, $3OC_{12}HSL$, which affects both the LasR and QscR systems (7). This observation is consistent with previous conclusions that the $3OC_{12}HSL$ signal is necessary for normal swarming and mutants and that defects in the synthesis of this signal lead to deficiencies in swarming motility (25). Since it was demonstrated that the *pvdQ* mutant with defects in signal degradation also exhibited swarming defects, it seems likely that a specific concentration of signal is important for this type of multicellular behavior. Given our previous demonstration that an *rhlR* mutant influencing the expression of the second signal, butanoyl-HSL, was also defective in swarming, a specific balance of these signals might be important or conversely a degradation product induced by *pvdQ* might act as a signal during swarmer differentiation. Regardless, we hypothesize that quorum regulation of swarming is uniquely regulated through degradation rather than synthesis of quorum-sensing signals.

The extracellular elastase LasB, encoded by the *lasB* gene, is one of the most studied virulence factors of *P. aeruginosa*. This protease exhibits tissue-damaging activity, is capable of degrading various plasma proteins among other proteins, and is highly expressed during tissue colonization and infection, particularly in the lungs (27, 34). Interestingly, *lasB* mutants of *P. aeruginosa* also showed a strong defect in biofilm formation. Our results are consistent with the secretion of LasB by the type II secretion system, since mutants with defects in this secretion apparatus also exhibit strong swarming and biofilm defects (41). The involvement of extracellular proteases in swarming motility has been also demonstrated for strains of *Bacillus subtilis* and *Vibrio vulnificus* (8, 11, 12, 21). However, the functions of these processes are still unknown.

The diversity between swarmer and planktonic cells was also reflected in differential susceptibility to antimicrobial compounds. Our studies revealed that swarmer cells of *P. aeruginosa* are up to 16 times more resistant to the tested antibiotics than are planktonic cells. The mechanisms for this elevated antibiotic resistance were not investigated in detail here but are likely to be complex. For example, altered expression of the ATP-dependent protease Lon might be responsible in part for ciprofloxacin resistance (3), while the decreased expression of outer membrane components, including certain porins, the increased expression of efflux pump systems, such as the multidrug efflux pump MexGHI-OpmD (2), and the differential expression of genes associated with lipopolysaccharide (see Table S1 in the supplemental material) are all likely to have influenced susceptibility. Similarly, elevated resistance to multiple antibiotics was previously ascribed to complex changes in cell physiology and a more positively charged lipopolysaccharide (23, 53).

Overall, our studies clearly demonstrate that swarmer cells of *P. aeruginosa* respond and adapt to a viscous, semisolid environment through altered gene expression, resulting in a unique physiological state (complex adaptation) that differs from both planktonic and biofilm growth. The substantial overexpression of numerous virulence factors benefits the swarmer population in the process of environmental and/or host colonization and infection. Finally, our data indicate two new possibilities for cell-to-cell signaling involved in swarming motility of *P. aeruginosa*, which will be analyzed in more detail in the future.

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