JOURNAL OF BACTERIOLOGY, Mar. 2007, p. 2164–2169 Vol. 189, No. 5
0021-9193/07/$08.00+0 doi:10.1128/JB.01623-06


# Identification of Genes Involved in Swarming Motility Using a *Pseudomonas aeruginosa* PAO1 Mini-Tn*5*-*lux* Mutant Library[▽]

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Received 19 October 2006/Accepted 29 November 2006

**During a screening of a mini-Tn*5*-*luxCDABE* transposon mutant library of *Pseudomonas aeruginosa* PAO1 for alterations in swarming motility, 36 mutants were identified with Tn*5* insertions in genes for the synthesis or function of flagellin and type IV pilus, in genes for the Xcp-related type II secretion system, and in regulatory, metabolic, chemosensory, and hypothetical genes with unknown functions. These mutants were differentially affected in swimming and twitching motility but in most cases had only a minor additional motility defect. Our data provide evidence that swarming is a more complex type of motility, since it is influenced by a large number of different genes in *P. aeruginosa*. Conversely, many of the swarming-negative mutants also showed an impairment in biofilm formation, indicating a strong relationship between these types of growth states.**

*Pseudomonas aeruginosa* is an opportunistic human pathogen that can cause nosocomial pneumonia, catheter and urinary tract infections, and sepsis in burn wound and immunocompromised patients (14, 18, 23, 28, 34). Moreover, *P. aeruginosa* is the most prevalent and significant pulmonary pathogen and the most common cause of eventually fatal lung disease in patients with cystic fibrosis (16, 29). In addition to its intrinsic resistance to a broad spectrum of antimicrobial compounds, this organism is also noted for its metabolic diversity and extremely versatile lifestyle, which allows it to colonize a large number of different environments (33).

Motility has been strongly implicated in the virulence of *P. aeruginosa*. It plays an important role in mobilization to and colonization of different environments, attachment of the bacteria to surfaces, and biofilm formation (27). *P. aeruginosa* is unusual in that it is capable of three different types of motility: flagellum-mediated swimming in aqueous environments and at low agar concentrations (<0.3% [wt/vol]); type IV pilus-mediated twitching on solid surfaces or interfaces; and, most recently observed, swarming on semisolid (viscous) media (0.5 to 0.7% [wt/vol] agar) (7, 20, 30). Swarming is described as a social phenomenon involving the coordinated and rapid movement of bacteria across a semisolid surface, often typified by a dendritic-like colonial appearance. It is widespread among flagellated bacteria, including *Salmonella*, *Vibrio*, *Yersinia*, *Serratia*, and *Proteus* (4, 12, 31).

Recently, it was shown that swarming of *P. aeruginosa* is dependent on both flagella and type IV pili as well as the presence of rhamnolipids (2, 7, 20). It is induced under nitrogen limitation and in response to certain amino acids (e.g., glutamate, aspartate, histidine, or proline) when provided as the sole source of nitrogen (20). Like other swarming bacteria that differentiate from short, motile, vegetative swimmer cells into longer, hyperflagellated swarmer cells (10, 13), *P. aeruginosa* swarmer cells are elongated and can possess two polar flagella (20, 30). In addition to these physical changes, swarmer differentiation can also be coupled to increased expression of important virulence determinants in some species (10, 19, 31).

**Identification of *P. aeruginosa* mutants defective in swarming motility.** To understand more about swarming of *P. aeruginosa* PAO1, a mini-Tn*5*-*luxCDABE* transposon mutant library (22) was screened for alterations in swarming motility. Mutants were grown overnight in 96-well microtiter trays containing LB broth and transferred with a custom 96-well pin device onto the surfaces of brain heart infusion agar plates containing 0.5% (wt/vol) Difco agar (Becton-Dickinson Co.). To minimize the swarming of neighboring colonies into each other, plates were incubated overnight at room temperature. This screen lead to the identification of a total of 36 swarming-deficient mutants out of the approximately 5,000 Tn*5*-*luxCDABE* mutants tested (Table 1). Although two or more independently derived transposon mutants mapping to the same genes were found for 50% of the identified mutants (Table 1), only a single mutant (if available, with a transcriptional *luxCDABE* insertion) from each gene was chosen for further analyses. Phenotypes were verified by performing swarming experiments with all identified mutants on BM2 swarm agar plates (62 mM potassium phosphate buffer [pH 7], 2 mM $MgSO_4$, 10 μM FeSO4, 0.4% [wt/vol] glucose, 0.5% [wt/vol] Casamino Acids, and 0.5% [wt/vol] Difco agar) (Fig. 1A and B). Since swarming of *P. aeruginosa* PAO1 on BM2 swarm plates resulted in nondendritic, circle-like growth (Fig. 1A), we were able to accurately determine swarming of wild-type and mutant strains by measuring the colony diameter of the swarming zone after 20 h of incubation at 37°C. All 36 mutants exhibited significant ($P < 0.01$ by Student's *t* test) and strong (at least 50%) defects in swarming motility compared to wild-type *P. aeruginosa* PAO1 (Table

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▽ Published ahead of print on 8 December 2006.

TABLE 1. Swarming-deficient *P. aeruginosa* Tn*5-luxCDABE* transposon mutants identified in this study

| Locus of Tn*5* insertion | Function | Polar effect possible[a] | Hits of individual transposon mutants[b] | Colony diam (mm)[c] after 24 h | | |
|---|---|---|---|---|---|---|
| | | | | Swarming[d] | Swimming | Twitching |
| None (strain PAO1) | | | | 34 ± 4 | 23 ± 1 | 21 ± 1 |
| PA3817 | Unknown | Y | | 4 ± 1 | 11 ± 1 | 18 ± 1 |
| PA5208 | Unknown | Y | | 4 ± 1 | 23 ± 2 | 22 ± 1 |
| PA5261 (*algR*) | Transcriptional regulator | N | | 5 ± 1 | 21 ± 1 | 3 ± 1 |
| PA4554 (*pilY1*) | Type IV pilus biogenesis | Y | 5 | 5 ± 1 | 22 ± 2 | 3 ± 1 |
| PA2965 (*fabF1*) | Fatty acid metabolism | Y | 2 | 5 ± 1 | 7 ± 1 | 2 ± 1 |
| PA4551 (*pilV*) | Type IV pilus biogenesis | Y | 2 | 6 ± 1 | 24 ± 1 | 2 ± 1 |
| PA0186 (*atsR*) | Unknown | N | 2 | 6 ± 1 | 25 ± 2 | 18 ± 1 |
| PA1100 (*fliE*) | Flagellar biogenesis | Y | | 6 ± 1 | 4 ± 1 | 19 ± 1 |
| PA0944 (*purN*) | Nucleotide metabolism | Y | 2 | 7 ± 1 | 14 ± 1 | 20 ± 1 |
| PA4556 (*pilE*) | Type IV pilus biogenesis | Y | 3 | 7 ± 1 | 27 ± 2 | 2 ± 1 |
| PA5117 (*typA*) | Regulatory protein | N | | 7 ± 1 | 24 ± 1 | 21 ± 1 |
| PA4526 (*pilB*) | Type IV pilus biogenesis | Y | 4 | 7 ± 1 | 23 ± 1 | 3 ± 1 |
| PA1452 (*flhA*) | Flagellar biogenesis | Y | | 7 ± 1 | 6 ± 1 | 22 ± 1 |
| PA4547 (*pilR*) | Type IV pilus biogenesis | N | | 8 ± 2 | 22 ± 1 | 3 ± 1 |
| PA4546 (*pilS*) | Type IV pilus biogenesis | Y | 3 | 8 ± 1 | 25 ± 1 | 3 ± 1 |
| PA1087 (*flgL*) | Flagellar biogenesis | Y | | 8 ± 1 | 9 ± 1 | 21 ± 1 |
| PA3476 (*rhlI*) | Autoinducer synthesis protein | Y | | 9 ± 1 | 22 ± 2 | 19 ± 2 |
| PA2634 | Unknown | N | 2 | 10 ± 1 | 12 ± 1 | 18 ± 2 |
| PA0428 (*rhlE*) | ATP-dependent RNA helicase | N | 2 | 10 ± 2 | 21 ± 1 | 19 ± 1 |
| PA0413 (*pilL*) | Type IV pilus biogenesis | Y | 2 | 10 ± 1 | 22 ± 1 | 4 ± 1 |
| PA1084 (*flgI*) | Flagellar biogenesis | Y | 2 | 10 ± 1 | 4 ± 1 | 18 ± 1 |
| PA5197 (*rimK*) | RNA modification | N | | 10 ± 1 | 13 ± 1 | 19 ± 3 |
| PA1086 (*flgK*) | Flagellar biogenesis | Y | 4 | 12 ± 1 | 7 ± 1 | 20 ± 2 |
| PA1081 (*flgF*) | Flagellar biogenesis | Y | | 12 ± 1 | 8 ± 1 | 19 ± 1 |
| PA3100 (*xcpU*) | Protein secretion | Y | | 12 ± 1 | 24 ± 2 | 20 ± 1 |
| PA3912 | Unknown | Y | | 13 ± 4 | 13 ± 2 | 19 ± 1 |
| PA0583 (*folK*) | Cofactor biosynthesis | N | 3 | 13 ± 2 | 24 ± 1 | 21 ± 2 |
| PA1079 (*flgD*) | Flagellar biogenesis | Y | | 13 ± 3 | 5 ± 1 | 21 ± 1 |
| PA4701 | Unknown | N | | 14 ± 2 | 21 ± 1 | 22 ± 1 |
| PA4234 (*uvrA*) | DNA modification | N | 2 | 15 ± 3 | 21 ± 1 | 22 ± 1 |
| PA3352 (*flgN*) | Flagellar biogenesis | Y | 5 | 16 ± 3 | 8 ± 1 | 20 ± 2 |
| PA0005 (*lptA*) | Fatty acid metabolism | Y | | 16 ± 3 | 18 ± 2 | 16 ± 1 |
| PA4282 | Unknown | N | 5 | 17 ± 4 | 12 ± 1 | 21 ± 1 |
| PA0887 (*acsA*) | Central intermediary metabolism | N | | 17 ± 4 | 11 ± 1 | 19 ± 1 |
| PA2840 | RNA processing and degradation | N | | 18 ± 2 | 23 ± 1 | 21 ± 1 |
| PA0407 (*gshB*) | Amino acid metabolism | N | 2 | 20 ± 2 | 21 ± 1 | 20 ± 1 |

[a] Possibility of Tn5 insertion affecting expression on downstream genes. Y, yes; N, no.
[b] Hits of individual Tn5 insertion mutations mapped to the same gene identified during the screening.
[c] The means ± standard deviations were determined from five different experiments.
[d] All swarming-deficient mutants exhibited a *t* test *P* value of <0.01 compared to the wild type, which was statistically significant.

1 [in which mutants are arranged by increasing swarming motility efficiency]).

While previous work clearly demonstrated the involvement of flagella and rhamnolipids in *P. aeruginosa* swarming (8, 20, 30, 36), the requirement for type IV pili of this kind of motility seems to vary among different *P. aeruginosa* strains. For example, *P. aeruginosa* PA14 does not apparently require type IV pili for swarming, since pilus synthesis mutations had no effect on swarming motility (30, 36), whereas Köhler et al. (20) demonstrated that a *P. aeruginosa* PAO1 *pilA* mutant was defective in swarming motility. We have confirmed and extended these conclusions to demonstrate that pili are essential for swarming in *P. aeruginosa* PAO1, in that mutants with Tn*5* insertions in a variety of type IV pilus synthesis genes (*pilB*, *pilE*, *pilL*, *pilR*, *pilS*, *pilV*, and *pilY1*) all displayed similarly strong swarming-deficient phenotypes (Table 1). The reason for the strain specificity of pilus dependence is unclear, although these two strains vary in several virulence-related features (15) and differ substantially in the type IV pilus-related genes *pilA* and *pilC* (3, 32). It is still unknown whether these pili are involved in partly directing surface propagation or, alternatively, whether they sense the viscosity of the surface and send a signal for initiation of swarming (20).

In addition to flagellum- and type IV pilus-related mutants, 21 mutants were found to have Tn*5* transposon insertions in regulatory, metabolic, chemosensory, or secretion genes or in hypothetical genes with unknown function (Table 1) not related to any phenotype found to date, suggesting a more complex motility mechanism.

**Characterization of swarming-deficient Tn*5* mutants.** To further characterize the identified swarming-deficient mutants with respect to motility, all isolates were tested for their ability to swim and twitch as described elsewhere (23a), using BM2 glucose minimal medium containing 0.3% (wt/vol) agar or LB containing 1.0% (wt/vol) agar, respectively (Table 1). All flagellum- and type IV pilus-related Tn*5* mutants showed the expected swimming or twitching phenotypes, in that flagellum-related mutants exhibited normal twitching and decreased

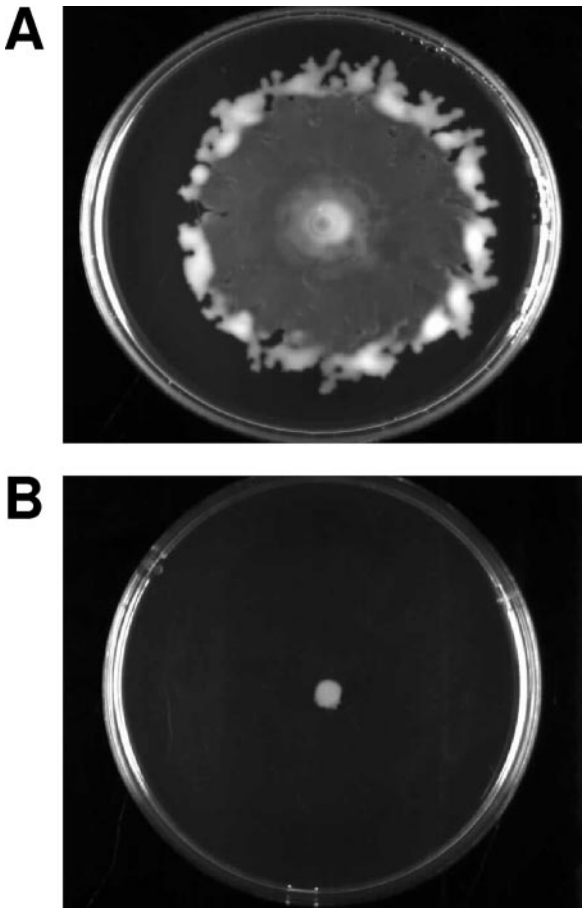


FIG. 1. Swarming of *P. aeruginosa* PAO1. Cells of *P. aeruginosa* were spot inoculated on swarm plates containing BM2 swarm medium. (A) Swarming of wild-type cells on BM2 swarm medium solidified with 0.5% agar at pH 7.4; (B) swarming of *algR*::Tn*5-luxCDABE* mutant cells on BM2 swarm medium solidified with 0.5% agar at pH 7.4.

swimming motility, while type IV pilus-related mutants were defected in twitching but showed a normal swimming phenotype compared to the wild type (Table 1). The remaining 21 mutants were differentially affected in swimming and twitching motility (Table 1). A *fabF1* mutant, which is affected in fatty acid/phospholipid metabolism, was the only strain defective in all three kinds of motility. In addition to their defects in swarming, seven mutants (with mutations in PA3817, *purN*, PA2634, *rimK*, PA3912, PA4282, and *acsA*) also exhibited reductions in swimming, while one mutant (with a mutation in *algR*) had reduced twitching motility. The remaining 12 mutants were limited only in swarming and revealed no further restrictions in swimming or twitching motility.

To investigate whether the deficiencies in twitching observed in the *algR* and *fabF1* mutants were related to generation of nonfunctional type IV pili, sensitivity to the pilus-specific phage PO4 was examined as described previously (23a) for all 21 swarming mutants with mutations in genes that were not flagellum or pilus related (Table 1). Only the *algR* mutant was resistant to phage PO4 treatment, indicating an abnormal pilus generation, whereas the other 20 mutants, including the *fabF1* twitching-deficient mutant, were highly sensitive to phage PO4

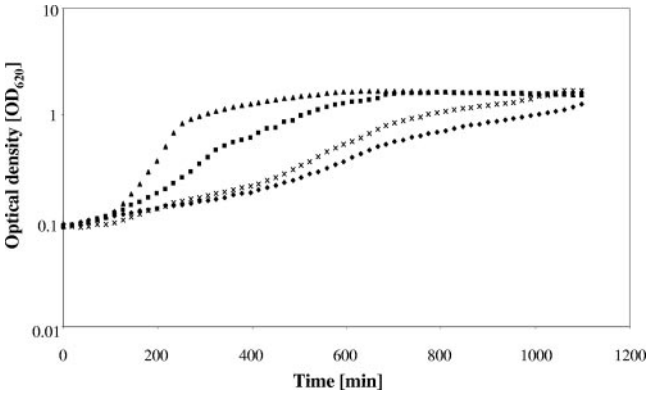


FIG. 2. Growth of wild-type *P. aeruginosa* PAO1 and swarming-deficient mutants in liquid BM2 swarm medium. Growth was measured at 37°C using a TECAN Spectrafluor Plus. Symbols: ▲, wild-type PAO1; ■, PA5208 mutant; ×, *folK* mutant; ◆, PA3817 mutant.

treatment, indicating their ability to generate a functional type IV pilus.

Since swarming motility involves the rapid movement of growing cells, all isolates were tested to see if the swarming-deficient phenotype was related to growth inhibition. Growth was measured in liquid BM2 swarm minimal medium at 37°C, using a TECAN Spectrafluor Plus, by measuring the $A_{620}$ every 20 min for 20 h under shaking conditions. These studies demonstrated that except for the PA3817 and *folK* mutants, which had severe growth defects, and a PA5208 mutant that had a slight growth defect (Fig. 2), all other mutants (data not shown) were able to grow like the wild-type *P. aeruginosa* PAO1.

It was previously reported that rhamnolipid excretion into the medium is necessary for swarming motility of *P. aeruginosa*, and mutants with defects in rhamnolipid synthesis genes showed an abnormal swarming pattern or even a major defect in swarming motility (2, 7). To test whether the swarming-deficient phenotype of the isolated transposon mutants is related to a lack of rhamnolipid biosynthesis, agar plate assays were performed as described previously (5, 6, 7) using BM2 swarm plates to analyze rhamnolipid production. All except one mutant showed the same formation of a dark blue halo around the colony as the wild-type strain, indicating efficient production of this biosurfactant; the only exception was a *rhlI* mutant with a disrupted regulatory gene that is involved in the biosynthesis of the quorum-sensing molecule *N*-butanoyl-L-homoserine lactone, which is known to influence rhamnolipid biosynthesis (17, 21).

Twelve of 20 isolated nonflagellum, nonpilus *Tn*5 mutants exhibited a colinear integration of the *luxCDABE* operon into the disrupted gene (Table 2), resulting in transcriptional *lux* fusions of the corresponding genes. These mutants were used to test gene expression under swarming conditions by measuring luminescence as described previously (22). All analyzed mutants showed luminescence during growth on swarming medium, indicating gene expression of the corresponding disrupted gene under these growth conditions (Table 2).

**Analysis of biofilm formation by swarming mutants.** Swarming motility and biofilm formation have common features, including the generation of large bacterial masses with close

TABLE 2. Determination of luminescence of Tn*5* mutants with transcriptional *luxCDABE* fusions

| Locus of transcriptional *luxCDABE* fusion | Luminescence under: | |
|---|---|---|
| | Swimming conditions | Swarming conditions |
| PA5208 | + | + |
| PA5261 (*algR*) | + | + |
| PA0944 (*purN*) | + | ++ |
| PA5117 (*typA*) | ++ | +++ |
| PA2634 | + | + |
| PA0428 (*rhlE*) | ++ | +++ |
| PA3912 | + | + |
| PA0583 (*folK*) | + | + |
| PA4701 | + | ++ |
| PA0005 | + | + |
| PA2840 | ++ | ++ |
| PA0407 | + | + |

interaction of cells and the generation of slime that promotes associations (25). To investigate whether the identified swarming-deficient mutants also exhibited defects in biofilm formation, all mutants were tested for their ability to form biofilms by using an abiotic solid surface assay (11, 26). All 15 flagellum- and pilus-related mutants showed the expected strong impairment in biofilm formation, with an optical density at 595 nm ($OD_{595}$) of the extracted crystal violet in the range of 0.19 to 0.44, corresponding to 20 to 50% of the wild-type level ($OD_{595}$ = 0.89), which is consistent with the known involvement of flagella and pili in adhesion and colonization of solid surfaces as early steps of biofilm formation.

Interestingly, all but five of the non-flagellum- or non-pilus-related mutants showed a statistically significant ($P < 0.01$ by Student's *t* test) decrease in biofilm formation in comparison to the wild type (Fig. 3). Only four mutants (the PA4701, *rimK*, PA2840, and *atsR* mutants) formed a normal level of biofilm compared to the wild-type PAO1, whereas one mutant (the PA3912 mutant) showed a strong increase in biofilm formation. Six mutants (the *lptA*, *xcpU*, *acsA*, *algR*, *rhlE* and *gshB* mutants) exhibited strong defects in biofilms formation, with approximately 50% of wild-type levels of biofilm formation; it is worth noting that the strong biofilm defects in the *acsA* and *algR* mutants were likely related to their deficiencies in swimming motility and pili, respectively. Ten mutants had more modest, but statistically significant ($P < 0.01$ by Student's *t* test), defects in biofilm formation, leading to approximately 60 to 80% of wild-type levels of biofilm formation. Overall, although there was evidently considerable overlap between swarming and biofilm formation, there was poor correspondence, as, e.g., the *gshB* and *acsA* mutants had considerable defects in biofilm formation but weaker swarming defects than many other strains; conversely, the *atsR* strain was one of the most defective swarming mutants but had no biofilm defect.

**Swarming motility and biofilm formation are dependent on the Xcp-related type II secretion system.** The *xcpU* gene is part of the Xcp-related type II secretion system (also called pseudopilus), which is required for secretion of exotoxin A, lipases,

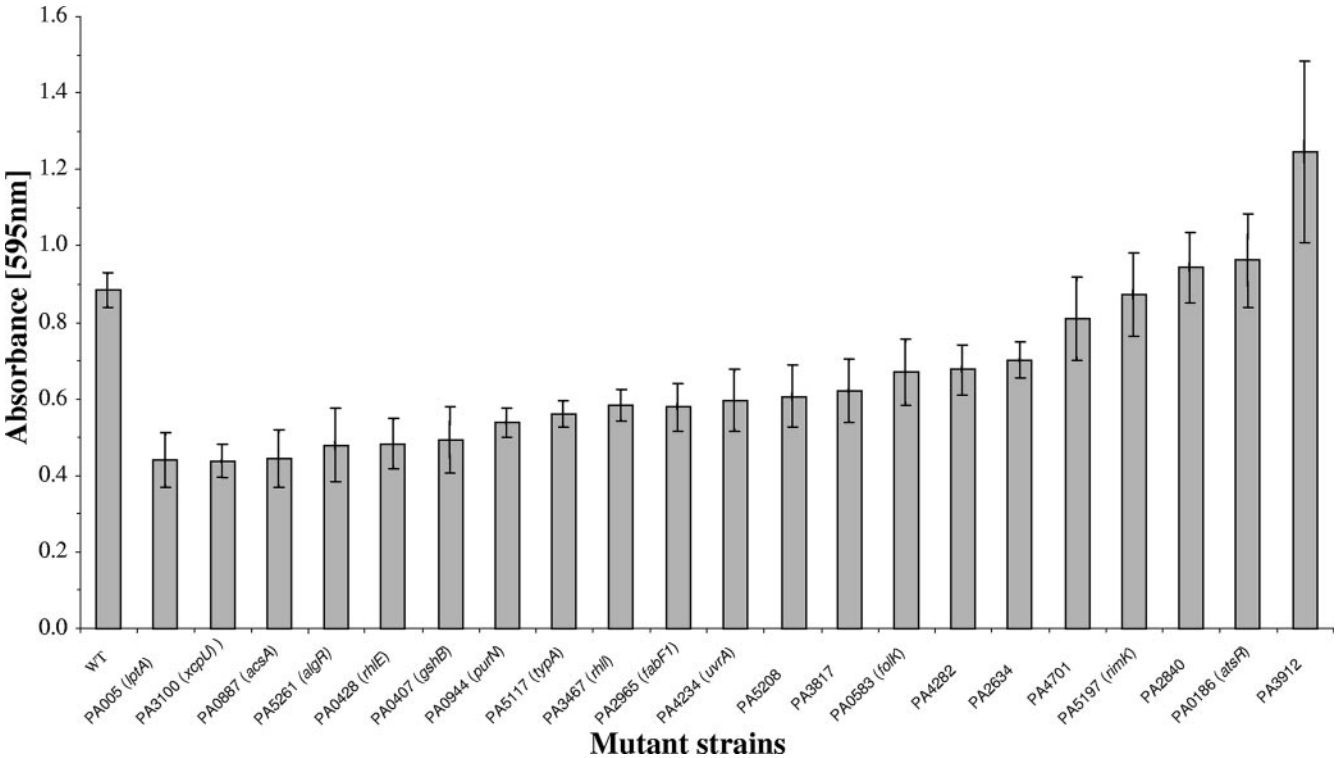


FIG. 3. Biofilm formation by *P. aeruginosa* PAO1 swarming mutants. Cells were grown for 20 h at 37°C in 96-well microtiter plates containing LB, and surface-associated biofilm formation was analyzed by crystal violet staining of the adherent biofilm, extraction of the crystal violet with ethanol, and measurement of the absorbance ($OD_{595}$). All experiments were done in triplicate with six technical repeats.

phospholipases C, alkaline phosphatase, or elastase (LasB) in *P. aeruginosa* (9). The *xcpU* mutant exhibited a defect in both swarming motility and biofilm formation. To verify these investigations, we tested other mutants of this machinery with Tn*5* insertions in *xcpS*, *xcpT*, *xcpY*, and *xcpQ*, all available in our mutant library. All mutants except the *xcpQ* mutant showed the same swarming and biofilm-deficient phenotype, like the *xcpU* mutant (data not shown), whereas the three independently tested *xcpQ* mutants revealed no defect at all. However, the unaltered swarming phenotype of the *xcpQ* mutants was not surprising, since it has been shown recently that a mutation in the *xcpQ* gene is functionally suppressed by the homolog *xqhA* (24). For further confirmation, semiquantitative reverse transcription-PCR was performed as described previously (35) using RNA isolated from actively swarming cells from the outer part of a swarming colony of *P. aeruginosa* PA01 and PCR primers for amplification of *xcpR* and *xcpQ*. Gene expression could be determined for both Xcp gene-containing clusters. It still remains whether the excretion of an exoproduct of the Xcp machinery is necessary for swarming and biofilm formation or whether the pseudopilus itself is involved in these phenotypes e.g., as a sensor; however, investigations of the attachment behavior of *xcp* mutants revealed no differences from the wild type (data not shown).

To test whether the recently identified Hxc type II secretion system, which is required for the secretion of the low-molecular-weight alkaline phosphatase LapA (1), is also involved in swarming motility and/or biofilm formation, we analyzed two mutants of this machinery with Tn*5* insertions in the *hxcP* (PA0679) and *hxcV* (PA0680) genes, respectively. No defect in either motility or biofilm formation could be detected (data not shown). Furthermore, no semiquantitative reverse transcription-PCR product could be obtained using swarmer cell RNA and PCR primers for the amplification of *hrxP* and *hrxW*, indicating that this system is most likely not involved in swarming motility or biofilm formation.

**Conclusions.** During our screen for mutants with alterations in swarming motility, we were able to isolate a large number of transposon mutants with Tn*5* insertions in many different genes. Besides mutations in flagellin, type IV pilus, rhamnolipid biosynthesis, and regulators, we found Tn*5* insertions in genes encoding metabolic proteins involved in amino acid, nucleotide, fatty acid/phospholipid, and central intermediary metabolism, as well as in genes encoding proteins involved in DNA replication/modification and RNA processing/degradation which are linked to adaptation and differentiation. In addition to the mutants with strong inhibition of swarming motility described here, we found many mutants with mutations in metabolic processes with more modest but still statistically significant defects in swarming (data not shown). Several of the identified genes are of unknown function and have not been related to any phenotype to date. Nevertheless most of the strongly affected mutants were able to grow as well as the wild type, produced normal amounts of rhamnolipid, and were sensitive to phage PO4, indicating a functional pilus. These mutants were differentially affected in swimming and twitching motility but in most cases had only a minor additional motility defect. It is unlikely that we have found every mutant altered in swarming motility, although the observation of independent selection of multiple strains with the same mutation indicated that the screen was relatively effective in determining swarming motility deficiencies. Overall, these results provide evidence that it is possible to obtain mutants with defects in swarming motility but without alterations in the known requirements for swarming, namely, flagella, type IV pili, and rhamnolipids. Conversely, many but not all of the swarming-negative mutants also showed an impairment in the ability to form simple biofilms, a surface-related differentiation process which presumably shares many important common features with swarming motility. Similar conclusions were also made for *Salmonella* (25). Thus, our data suggest that swarming is a more complex motility mechanism influenced by a large number of cooperating genes.

We acknowledge funding from the Canadian Institutes of Health Research. J.O. was supported by postdoctoral fellowships from the Alexander von Humboldt Foundation and the Canadian Cystic Fibrosis Foundation. S.L. received a fellowship from the Canadian Cystic Fibrosis Foundation. R.E.W.H. holds a Canada Research Chair.

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