Loss of the WaaL O-Antigen Ligase Prevents Surface Activation of the Flagellar Gene Cascade in *Proteus mirabilis*[∇]

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Proteus mirabilis is a Gram-negative bacterium that undergoes a physical and biochemical change from a vegetative swimmer cell (a typical Gram-negative rod) to an elongated swarmer cell when grown on a solid surface. In this study, we report that a transposon insertion in the *waaL* gene, encoding O-antigen ligase, blocked swarming motility on solid surfaces but had little effect on swimming motility in soft agar. The *waaL* mutant was unable to differentiate into a swarmer cell. Differentiation was also prevented by a mutation in *wzz*, encoding a chain length determinant for O antigen, but not by a mutation in *wzyE*, encoding an enzyme that polymerizes enterobacterial common antigen, a surface polysaccharide different from the lipid A:core. In wild-type *P. mirabilis*, increased expression of the *flhDC* operon occurs after growth on solid surfaces and is required for the high-level expression of flagellin that is characteristic of swarmer cells. However, in both the *waaL* and the *wzz* mutants, the *flhDC* operon was not activated during growth on agar. A loss-of-function mutation in the *rcsB* response regulator or overexpression of *flhDC* restored swarming to the *waaL* mutant, despite the absence of O antigen. Therefore, although O antigen may serve a role in swarming by promoting wettability, the loss of O antigen blocks a regulatory pathway that links surface contact with the upregulation of *flhDC* expression.

Proteus mirabilis is a Gram-negative, rod-shaped bacterium that causes urinary tract infections in patients with catheters or abnormal urethras (50). It has been well studied for its ability to swarm, a flagellum-based, solid-surface-associated, social movement. In liquid broth *P. mirabilis* organisms are peritrichously flagellated swimmer cells with a few flagella. At 3 to 4 h after contact with a solid medium, the cells begin to differentiate into elongated rods that are 20- to 40-fold longer than their liquid counterparts and have a >50-fold increase in flagella. These cells are also multinucleated and aseptate (reviewed in references 21 and 41). The swarmer cells join together to form a swarming raft and, as a group, swarm out from a central inoculum until an unknown signal is sensed and the cells consolidate, or dedifferentiate, back into swimmer cells (24). This process repeats itself to form a characteristic bull's-eye pattern on an agar plate (42). At present, several known signals for differentiation have been identified, and these include the inhibition of flagellar rotation and the accumulation of putrescine. However, it is thought that there are additional unknown signals involved (4, 53).

Flagella play an important role in swarming as surface sensors and for propulsion; therefore, their expression is tightly regulated. The regulation of flagella in *Escherichia coli* and *Salmonella enterica* serovar Typhimurium has been well studied and is complex, involving a three-tiered regulatory cascade (reviewed in reference 11). The master regulator for flagellar synthesis is FlhD₂C₂, encoded by the *flhDC* operon. These genes are considered class I genes and control the expression

of the class II genes, which are involved in the hook and basal body construction. An alternative sigma factor, sigma 28, also a class II gene, controls the expression of class III genes such as *flaA*, the flagellin structural gene, and is activated only upon release of an anti-sigma factor, FlgM, which is exported through the completed hook/basal body structure (11). FlhD₂C₂ is necessary for swarming in *P. mirabilis* (12–14, 16, 52). During swarmer cell differentiation, the transcript levels of *flhDC* rise almost 50-fold; therefore, mutations in genes that regulate *flhDC* levels can have dramatic effects on swarming. For example, mutations in the leucine-responsive regulatory protein (a positive regulator of *flhDC*) block swarming, while mutations in components of the RcsBCD phosphorelay (a negative regulator of *flhDC*) result in hyperswarming and even elongation in liquid, a condition normally nonpermissive to cell differentiation (4, 22, 29).

The outer membrane of Gram-negative bacteria, especially the O antigen, is highly immunogenic, acts as a phage receptor and participates in development. *P. mirabilis* has a typical Gram-negative outer membrane in which there is a phospholipid monolayer on the periplasmic side and lipopolysaccharide (LPS) as the outermost leaflet of the membrane (48). The LPS contains a lipid A region, a core region, and the O-antigen region (43). Enteric bacteria such as *E. coli*, *S. enterica*, or *P. mirabilis* can have either the O antigen or another polysaccharide termed the enterobacterial common antigen (ECA) attached to the lipid A:core moiety (46, 47). These two antigens are synthesized via different pathways, but they are both attached to the lipid A:core by the same protein, WaaL, or O-antigen ligase (27).

LPS and ECA have been implicated to play roles in bacterial developmental signaling and motility. Bowden and Kaplan demonstrated a role for O antigen in *Myxococcus xanthus* so-

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cial motility and fruiting-body development where O-antigen mutants were defective in S motility and exhibited aggregation defects during development (6). Toguchi et al. demonstrated a role for LPS in *S. enterica* swarming motility (54). The O-antigen mutant failed to show a reduction in flagellar synthesis, leading these authors to hypothesize that the O antigen was part of an extracellular milieu that acted as a wettability agent to reduce surface friction (54). ECA has also been shown to be important in bacterial swarming. In *Serratia marcescens*, completed ECA was needed for the upregulation of the master regulator *flhDC*. Without functional ECA, swarming motility was abolished, while swimming motility was only decreased (9). Finally, recent studies in *E. coli* indicate that functional LPS is required for both swimming and swarming motility (18). In that study it was demonstrated that LPS-defective mutants failed to activate the *flhDC* operon, encoding the class I master activator. Interestingly, swarming could be restored by mutations that disrupt the Rcs pathway, suggesting that functional LPS was involved in relieving the RcsB-mediated repression during swarming on solid surfaces. In addition, that study revealed that truncations into the inner core of LPS did not prevent swarming when *flhDC* expression was maintained (18).

Additional surface components may also play a role in swarming in *P. mirabilis* (17, 51, 55). A slime layer behind a swarming raft was first observed by Fuscoe in 1973 (17). The production of slime was later found to be coordinated with swarmer cell development, but a direct link between this slime and swarming was never confirmed (51). More recently, a capsular polysaccharide (colony migration factor [20]) was shown to be needed for swarming and was proposed to reduce surface friction while possibly acting as a matrix for swarming raft formation (40). Along with Cmf, the O antigen has been implicated to have a role during swarming in *P. mirabilis*. A transposon library was screened for mutants that could not elongate, and insertions were found in the *cld* gene, encoding an O-antigen chain length determinant, and in the *waaD* and *waaC* (formerly *rfaD* and *rfaC*) genes, required for inner core LPS synthesis (3). Finally, using Fourier transform infrared spectroscopy, it was shown that different LPS forms are present on the cell surface during various stages of the swarm cycle and that the fatty acid composition of the membrane also changes during swarming (19). This indicates that *P. mirabilis* carefully controls its membranes during a swarming cycle. These data show that LPS is required for normal swarming, but the basis for this requirement is not understood.

We have found that a transposon insertion in the *P. mirabilis* *rfaL* (*waaL*) gene, encoding O-antigen ligase, blocks swarmer cell differentiation by preventing the increase in *flhDC* expression that occurs when vegetative cells are grown on solid surfaces. We show that the swarming defect in *P. mirabilis* was specific to the loss of O-antigen and not to ECA. Swarmer cell differentiation and swarming defects could be overcome in the *waaL* mutant by overexpressing *flhDC* in *trans*. In addition, swarming was restored by loss of function mutations in the *rscB* gene, encoding a response regulator in the RscBCD system that regulates *flhDC* and additional genes in response to growth on solid surfaces and cell envelope stress (28, 30). Finally, our data demonstrate that swarming in *P. mirabilis*, as in *E. coli*, can occur in the absence of O antigen.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used are listed in Table 1. Both *E. coli* and *P. mirabilis* were grown in modified Luria-Bertani (LB) broth (10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter) at 37°C or in LB medium on plates at 37°C. For swim and swarm assays, the agar concentrations were 0.3 and 1.5%, respectively. Antibiotics were used for selection at concentrations of 25 µg/ml for both chloramphenicol and streptomycin for *E. coli*. Antibiotic concentrations for the selection of *P. mirabilis* were 100 µg/ml for chloramphenicol, 35 µg/ml for streptomycin, 20 µg/ml for kanamycin, and 15 µg/ml for tetracycline.

Transposon mutagenesis. PM7002 was mated with SM10 *λpir* carrying pUT::mini-Tn5lacZ1, and the exconjugants with transposon insertions were selected on LB plates supplemented with kanamycin and tetracycline. After overnight incubation at 37°C, the cells were patched onto 2% LB medium to screen for the ability to swarm. To identify the insertion site of the transposon in nonswarming mutants, chromosomal DNA was digested with BamHI, ligated to pACYC184, and transformed into *E. coli* XL1. Kanamycin-resistant clones were sequenced by using a transposon-specific primer that read outward from one end into the flanking chromosomal DNA.

Southern blot analysis. To map the transposon insertion in PM942, chromosomal DNA was prepared, digested with BamHI, transferred to a nitrocellulose membrane, and probed with a digoxigenin-labeled probe to the kanamycin cassette of mini-Tn5lacZ1. To confirm the matings resulted in the appropriate gene disruptions, chromosomal DNA from the *wzz*, *wzyE*, and *rscB* mutants were extracted and separately digested with BglII, SalI, and EcoRI before being transferred to a nitrocellulose membrane; they were then probed with a gene-specific digoxigenin-labeled probe.

Motility assays. To examine the ability of colonies to swim, cultures were grown overnight in LB media with appropriate antibiotics. All samples were normalized to the same optical density at 600 nm (OD₆₀₀). Droplets (5 µl) were placed on a 0.3% LB plate with chloramphenicol and incubated for 8 h at 37°C. Swarm assays were done identically, except the inoculum was spotted onto a 1.5% agar plate.

Mutant construction. Internal fragments of the *wzz*, *wzyE*, and *rscB* genes were generated by PCR using the primer sets intWzz.for/intWzz.rev, intWzyE.for/intWzyE.rev, and RcsB.for/RcsB.rev, respectively (Table 2). Products were digested with XbaI and SalI and ligated to pKNG101 cut with the same enzymes. Plasmids were initially electroporated into CC118 *λpir* and then electroporated into SM10 *λpir* for conjugal mating with PM7002 (*wzz* and *wzyE* mutations) and PM942 (*rscB* mutation) on LB plates. Exconjugants representing Campbell-type integration events that disrupted each gene were selected on LB plates with tetracycline and streptomycin, and mutations were confirmed by Southern blot analysis (see above).

Northern blot analysis. Cells were grown overnight in LB medium. All samples were normalized to the same OD₆₀₀. Droplets (150 µl) were spread onto 2% LB plates in parallel to produce cultures that were synchronously differentiating. The cells were collected from each plate at the indicated time points with LB media and normalized to an OD₆₀₀ of 0.7. One-milliliter portions of the cells were centrifuged at 12,000 rpm. Total RNA was isolated by using a Masterpure RNA purification kit (Epicentre, Madison, WI). Equal amounts of RNA were run on a 1.2% formaldehyde agarose gel and then transferred to a nitrocellulose membrane. A DNA probe specific to *flhDC* was labeled with digoxigenin and used to examine transcript levels by chemiluminescence using the CDP-Star substrate (Roche Applied Science).

Western blot analysis. Cells were grown overnight in LB medium. All samples were normalized to the same OD₆₀₀. Droplets (150 µl) were spread onto 2% LB plates in parallel to produce colonies that were synchronously differentiating. The cells were collected from each plate at the indicated time points with LB media and normalized to an OD₆₀₀ of 1.0. One-milliliter portions of cells were centrifuged at 12,000 rpm for 1 min. The pellet was resuspended in Laemmli sample buffer (Bio-Rad) with β-mercaptoethanol. Protein levels were normalized, run on an SDS-15% PAGE gel, and transferred to a nitrocellulose membrane. The primary antibody used was a rabbit anti-FlaA antibody. The secondary antibody was a donkey anti-rabbit antibody conjugated with peroxidase.

LPS purification and visualization. LPS was isolated from cells by using a modified version of the method of Marolda et al. (33). Briefly, cells were grown overnight in appropriate antibiotics. Equal amounts of cells were inoculated into fresh LB medium with antibiotics and allowed to grow to exponential phase. Droplets (100 µl) were spread onto a 1.5% LB plate and collected after 4 h of incubation at 37°C with phosphate-buffered saline (pH 7.2). After being lysed, the cells were treated with proteinase K overnight. In the morning, fresh proteinase K was added for 4 h, and the lysates were exposed to hot phenol. Ethyl

TABLE 1. Strains and plasmids

Strain or plasmid	Description or genotype ^a	Source or reference
Strains		
<i>E. coli</i>		
DH5 α	F ⁻ ϕ 80d <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF'</i>)U169 <i>endA1 recA1 hsdR17</i> (r _K ⁻ m _K ⁻) <i>deoR thi-1 supE44</i> λ ⁻ <i>gyrA96 relA1</i>	Laboratory stock
XL1	<i>endA1 gyrA96</i> (Nal ^r) <i>thi-1 recA1 relA1 lac glnV44 F' hsdR17</i> (r _K ⁻ m _K ⁺)	Laboratory stock
CC118 λ pir	<i>araD139</i> Δ (<i>ara-leu</i>)7697 Δ <i>lacZ74 phoA</i> Δ 20 <i>galE galK thi rpsE rpoB argE</i> (Am) <i>recA1</i>	32
SM10 λ pir	<i>thi thr leu tonA supE recA</i> RP4-2Tc::Mu Km ^r λ pir	35
<i>P. mirabilis</i>		
PM7002	Wild type; Tc ^r	ATCC ^b
PM942	<i>waaL</i> ::mini-Tn5 <i>lacZ1</i> -Km ^r	This study
RM5	PM942/pACYC184	This study
RM6	PM942/pFDCH1	This study
RM7	PM942 <i>rcsB</i> ::Sm ^r	This study
RM9	PM942/pACYC184 + <i>waaL</i>	This study
RM14	PM7002 <i>wzyE</i> ::Sm ^r	This study
RM16	PM7002 <i>wzz</i> ::Sm ^r	This study
Plasmids		
pACYC184	Low copy; Cm ^r	10
pBC	High copy; Cm ^r	Stratagene
pKNG101	R6K-derived suicide vector; Sm ^r	25
pRM5	pACYC184 + <i>waaL</i>	This study
pRM14	pKNG101::wzy (internal fragment)	This study
pRM17	pKNG101::wzz (internal fragment)	This study
pFDCH1	pACYC184 + <i>flhDC</i>	12
pRM18	pKNG101::rcsB (internal fragment)	13
pRM19	pACYC184 + wzz	This study

^a Cm^r, chloramphenicol resistance; Tc^r, tetracycline resistance; Sm^r, streptomycin resistance; Km^r, kanamycin resistance; Nal^r, nalidixic acid resistance.

^b ATCC, American Type Culture Collection.

ether was used to remove any phenol. Once the ether was removed, the LPS was suspended in loading buffer and run on an SDS–12% PAGE gel. The LPS was visualized by using the method of Kittelberger and Hilbink (26).

Complementation of *waaL* and *wzz* mutations. To ensure that the phenotypes seen in the *waaL* and *wzz* mutant backgrounds were due to the specific mutations and not polar effects or second site mutations, the full-length version of each gene, including the native ribosome binding site, was generated by PCR. The *waaL* gene was cloned into pACYC184 at the BamHI and SalI sites by using the primers 942.for and 942.rev, while *wzz* was cloned into pACYC184 at the EcoRV and BamHI sites by using Wzz.for and Wzz.rev (see Table 2 for primer sequences).

RESULTS

A mini-Tn5Cm insertion in the *waaL* gene blocks swarming, but not swimming motility. Swarming and swimming in *P. mirabilis* both require proper flagellar function and a functional chemotaxis system (7, 21, 41). Swimming occurs in liquid

and in soft motility agar (0.2 to 0.4%), whereas swarming only occurs on a solid surface, such as 1.5% agar plates. In addition, swarming is characterized by the differentiation of vegetative cells to elongated swarmer cells, which then interact to form swarming rafts (24, 41). In order to further elucidate the pathway(s) required for surface recognition and swarmer cell differentiation in *P. mirabilis*, mini-Tn5*lacZ1* transposon mutagenesis was performed on PM7002, a wild-type strain of *P. mirabilis*, and a mutant, PM942, that was unable to swarm but maintained the ability to swim was isolated.

The site of the mini-Tn5*lacZ* transposon insertion in PM942 was determined as described in Materials and Methods. The insertion was at a position corresponding to amino acid 183 within an open reading frame encoding a 422-amino-acid protein. A BLAST search of the deduced protein from PM7002

TABLE 2. Primers

Primer	Sequence (5'–3')	Purpose
942.for	ATCGAGGATCCTTATTGTATGATGAGCCATTC	Complementation
942.rev	ATGACATGTCGACTTAGCTAACGGATGTATCTTC	Complementation
intWzyE.for	ATGACATGTCGACAGCCTCTAGCGAGCCTTCTAGG	Deletion mutant
intWzyE.rev	ACAGTCTAGAACGACATCTGGTCTTTGTGGC	Deletion mutant
intWzz.for	ATGACTAGTCGACATGTTGATACTGGCGTAAATG	Deletion mutant
intWzz.rev	ACAGTCTAGATTCAGAACTGACCGTTGTAGG	Deletion mutant
Wzz.for	AATACTGATATCACGATTATCGGATTAGG	Complementation
Wzz.rev	ATCGTGGATCCACCTACTTTTATTGTGG	Complementation
RcsB.for	GTACAGTCGACTCACCGACCTATCTATGCCT	Deletion mutant
RcsB.rev	GTACAGTCGACTCACCGACCTATCTATGCCT	Deletion mutant

FIG. 1. Kyte-Doolittle hydropathy profiles of WaaL proteins. The Kyte-Doolittle hydropathy profile of the PMI3163 open reading frame from *P. mirabilis* was compared to WaaL proteins from other Gram-negative bacteria. The hydropathy profiles were generated by using the Biology Workbench program (<http://seqtool.sdsc.edu/CGI/BW.cgi>).

exhibited 100% identity over the sequenced region to PMI3163 from the genome of HI4320 (36). Additional BLAST searches revealed homology to putative WaaL (RfaL) orthologs from *Photorhabdus luminescens* (50% amino acid identity) and *S. enterica* serovars Heidelberg, Agona, and Schwarzengrund (39% identity). The WaaL protein functions as an O-antigen ligase that links undecaprenol-bound O-antigen subunits to the outer core of LPS (34). The Kyte-Doolittle hydropathy profile of the putative *P. mirabilis* WaaL ortholog was highly similar to WaaL proteins from *S. enterica* serovar Typhi, *E. coli* O157:H7 and *Pseudomonas aeruginosa* PAO1 (Fig. 1). The similarity in hydropathy profiles between various WaaL proteins from Gram-negative bacteria has been noted previously and used to identify potential WaaL-encoding genes, despite the large differences in amino acid similarity between the WaaL proteins (1). Based on the data presented above and the analysis of O antigen presented below, the PMI3163 gene is hereinafter designated *waaL* (44).

PM942 *waaL*::mini-Tn5*lacZ*1 exhibited essentially wild-type levels of swimming when assayed on 0.3% agar, indicating it possessed functional flagellar and chemotactic systems, but was unable to swarm (Fig. 2). To determine whether the observed swarming defect in PM942 was due to loss of *waaL* function

FIG. 2. WaaL is necessary for swarming but not for swimming. The swimming (left panel) and swarming (right panel) phenotypes of the following strains are shown: PM7002 wild type/pACYC184, PM942 *waaL*::mini-Tn5*lacZ*1/pACYC184, and PM942 *waaL*::mini-Tn5*lacZ*1/pRM5 (pACYC184 + *waaL*). For both swarming and swimming assays, strains were grown overnight in the appropriate antibiotics, followed by adjusting each culture to the same optical density, and 5- μ l drops were placed on each plate. Swimming assays were performed in LB medium with 0.3% agar, and swarming was done on LB plates with 1.5% agar that were predried for 30 min at 37°C. Plates were photographed after 12 h of incubation at 37°C.

FIG. 3. SDS-PAGE analysis of O-antigen production. O-antigen length was determined by SDS-PAGE analysis of LPS preparations followed by silver staining. The designated strains are indicated in the figure as follows: WT, PM7002/pACYC184; *waaL*⁻, PM942 *waaL*::mini-Tn5 *lacZ*/pACYC184; *waaL*⁻/*waaL*⁺, PM942 *waaL*::mini-Tn5 *lacZ*/pACYC184 + *waaL*; *wzz*⁻, RM16 *wzz*::Sm^r/pACYC184 vector only; and *wzz*⁻/*wzz*⁺, RM16 *wzz*::Sm^r/pACYC184 + *wzz*.

and not to a polar effect or a secondary unlinked mutation, the *waaL* gene was amplified from the chromosome of PM7002 and cloned into pACYC184, resulting in plasmid pRM5. In PM942 containing pRM5, swarming motility was restored to wild-type levels (Fig. 2).

O-antigen profile of the PM942 mutant. To test whether the *waaL* gene product functioned in a manner consistent with an O-antigen ligase and was a WaaL (RfaL) ortholog, LPS was isolated from the wild-type PM7002, PM942 *waaL*::mini-Tn5 *lacZ*/pACYC184, and PM942/pRM5 (pACYC184 + *waaL*) and analyzed by SDS-PAGE. Figure 3 shows that the wild-type *P. mirabilis* PM7002 strain exhibited core and core+1 bands. In addition, there was also a ladder of bands that represented the O antigen of various lengths. In PM942 *waaL*::mini-Tn5 *lacZ*, the core+1 band was absent, and there was no ladder of O antigen in the short-to-mid-size length. However, there was a faint banding pattern of material that extended much higher in the gel than the material from wild-type cells. In PM942/pRM5, the LPS profile was restored back to that of wild-type PM7002 (Fig. 3).

The presence of the high-molecular-weight material in PM942 varied in individual LPS preparations, and we hypothesized that this material might represent O-antigen subunits that remained linked to undecaprenol-PP due to the absence of WaaL activity. Alternatively, this material could represent an undefined polymer whose production is induced in the absence of O antigen. To test whether this material was O antigen, a mutation was made in the gene (*wzz*) encoding the O-antigen chain length determinant in PM942. This mutation is predicted to reduce the length of O antigen whether it is linked to undecaprenol-PP or the LPS core. The *waaL wzz* double mutant exhibited an LPS profile that only consisted of core, indicating the material was likely composed of O antigen linked to undecaprenol-PP (data not shown). The above data, taken together with the concomitant loss of core+1 and the

FIG. 4. A *wzz* mutation prevents swarming. The swarming phenotypes of the indicated strains were determined by growing cells overnight in LB broth with the appropriate antibiotics and adjusting cultures to the same optical density. For the swarming assays, three individual 5- μ l drops for each strain were placed on the same LB plate, and migration distances were measured at 30-min intervals. The starting diameter of each spot was 5 mm. The average of three measurements is shown for PM7002 (wild type), RM16 *wzz*::Sm^r/pACYC184 vector, and RM16 *wzz*::Sm^r/pRM19 (pACYC184 + *wzz*).

short to intermediate O-antigen chains is consistent with loss of O-antigen ligase activity in PM942.

Loss of O antigen and not ECA is responsible for the swarming defect of a *waaL* mutant. In enteric bacteria, another outer membrane component exists called the ECA (27, 47). This can exist on the outer surface in two forms: linked to diacylglycerol or linked to the lipid A:core. In *E. coli*, WaaL can link either O-antigen or ECA subunits to the lipid A:core (27, 45). In *S. marcescens* ECA, completion acts as a checkpoint for *flhDC* activation (9). To test whether the lack of ECA or O antigen due to the *waaL* mutation was causing the swarming defect, a mutation in *wzyE* (PMI3326) encoding the ECA polymerase was made in PM7002. The *wzyE* mutant exhibited a significant growth defect but was still able to swarm after overnight growth, indicating that ECA is not needed for swarming (data not shown).

Loss of full-length O-antigen in a *wzz* mutant confers a swarming defect. To independently confirm the requirement of full-length O antigen in swarming, a mutation was made in the O-antigen chain length determinant (*clb*), *wzz* (PMI2182), by insertion of the suicide plasmid pKNG101, hereinafter designated *wzz*::Sm^r (25, 39). This mutation should result in O-antigen subunits with reduced chain length being connected to the lipid A:core (39). The mutation in *wzz* abolished swarming in RM16 (*wzz*::Sm^r) and could be complemented by the cloned *wzz* gene in pACYC184 (pRM19) (Fig. 4). Analysis of LPS profiles by SDS-PAGE gel demonstrated that in the *wzz* mutant (*wzz*::Sm^r/pACYC184), O antigen of very short chain length was present, and in the *wzz*-complemented strain (*wzz*::Sm^r/pACYC184 + *wzz*), the wild-type pattern of O-antigen distribution was restored (Fig. 3). These data provide additional evidence that full-length O antigen is needed for swarming.

***waaL* and *wzz* mutants fail to activate the flagellar gene cascade.** The basis for the decreased swarming in *waaL* and *wzz* mutants was investigated. The most obvious explanation was that loss of surface O antigen prevented swarming because it normally acted as a lubricant or increased "wettability" by

FIG. 5. Analysis of FlaA and *flhDC* expression in wild-type and *waaL* mutant strains. Cells were grown overnight to an OD_{600} of 1.8, and 150 μ l of the culture was spread onto separate 2% agar plates for hourly collection up to 6 h. The overnight culture was diluted to an OD_{600} of 1.0 for the T_0 sample. Cells were collected off plates as described previously (19), and duplicate samples were lysed in Laemmli buffer or used for RNA isolation. The amounts of protein and RNA were standardized and equal amounts were loaded for each sample. (A) Western blot analysis of FlaA protein levels. The T_4 to T_6 samples were run on a separate gel and processed at the same time as the T_0 to T_3 samples (B) Northern blot analysis of *flhDC* mRNA accumulation of RNA collected from the same cell sample as in panel A. Ethidium bromide staining of the RNA used in panel B is shown. (C) Northern blot analysis of *flhDC* mRNA accumulation and Western blot analysis of FlaA protein expression in cells harvested at T_4 during swarmer cell differentiation. The same cell pellets from each strain were split and used for each analysis.

extracting water from the agar. A similar role for O antigen has been proposed in *S. enterica* serovar Typhimurium (54). However, the addition of a surfactant, such as surfactin from *B. subtilis* or purified LPS from wild-type *P. mirabilis*, to the agar did not restore swarming to PM942 (data not shown). When *waaL* or *wzz* mutant cells were examined microscopically at the outside edge of growth, there were no swarmer cells observed (data not shown). To examine the basis for this lack of differentiation, we examined the expression of flagellin, encoded by the *flaA* gene, which is a hallmark of the differentiation process. Analysis of FlaA (flagellin) levels at various times during swarmer cell differentiation in the wild type and *waaL* mutant (PM942) indicated that the characteristic rise in flagellin expression beginning at 3 h postinoculation on LB plates (T_3) in the wild-type strain was not observed in PM942 even after 6 h of growth on agar plates (T_6) (Fig. 5A). However, liquid-grown cells exhibited similar amounts of flagellin (Fig. 5A, T_0 sample).

To determine whether the failure of PM942 to activate FlaA (flagellin) during swarmer cell differentiation was due to insuf-

FIG. 6. Suppression of the swarming defect in a *waaL* mutant. In panel A, the swarming phenotype of wild-type PM7002/pACYC184, PM942 *waaL*::mini-Tn5lacZ1/pACYC184, PM942 *waaL*::mini-Tn5lacZ1/pFDCH1, and RM7 (*waaL*::mini-Tn5lacZ1, *rcsB*::Sm^r) strains is shown on a 1.5% agar plate after growth at 37°C for 8 h. In panel B, the swarming distance of the strains used in panel A is shown as a function of time.

ficient levels of the class 1 activator FlhD₂C₂, Northern blots were used to examine *flhDC* mRNA accumulation in wild-type and PM942 cells at hourly time points representing various stages of swarmer cell differentiation. In PM942, there was no detectable activation of *flhDC* expression at any point during swarmer cell differentiation (T_2 to T_6) as seen in Fig. 5B.

These data indicated that the *waaL* mutant was defective in activating the *flhDC* operon when grown on solid surfaces. To further investigate whether this was a consequence of altered surface O antigen, we examined the ability of the *wzz*::Sm^r mutant, exhibiting O antigen of reduced chain length, to activate flagellin expression on solid surfaces. Like the *waaL* mutation, the *wzz* mutation also resulted in the failure to activate both *flhDC* and flagellin expression after growth on solid surfaces (Fig. 5C).

Restoring *flhDC* expression in the *waaL* mutant rescues swarming. To test whether the failure of the *waaL* mutant to activate *flhDC* on solid surfaces was primarily, if not exclusively, responsible for the inability of PM942 to swarm, *flhDC* was expressed from a constitutive promoter (*E. coli lac_p*) on the plasmid pFDCH1 (12). In PM942/pFDCH1, swarming motility was restored and was actually increased compared to the wild type (Fig. 6). This was likely due to the increased expression of FlhDC. The LPS profile of PM942/pFDCH1 was identical to PM942, indicating that overexpression of *flhDC* did not restore O-antigen synthesis (data not shown). Taken together, these data indicated there was not an intrinsic inability of the *waaL* mutant to swarm, but that a signaling defect resulting from the *waaL* mutation prevented *flhDC* activation, which is

critical for activation of the *flaA* flagellin gene and additional genes involved in differentiation.

Inactivation of the RcsB response regulator restores swarmer cell differentiation to the *waaL* mutant. The data described above suggested that surface O antigen is required to relay a signal that leads to *flhDC* activation. It was hypothesized that this signal may be mediated by surface contact and involve the RcsBCD phosphorelay, since this system has previously been implicated in sensing solid surfaces and membrane stress. In addition, mutations in this pathway result in hyperswarming in *P. mirabilis* due, in part, to overexpression of *flhDC* (4, 13, 29). To test a possible role of RcsB in relaying a signal from the O antigen, a *waaL rcsB* double mutant, designated RM7, was constructed and demonstrated that swarming was restored when *rcsB* was inactivated in PM942 *waaL::mini-Tn5lacZ1* (Fig. 6). The *waaL rcsB* double mutant, like the *flhDC* overexpressing strain, did not restore O-antigen addition to LPS (data not shown).

DISCUSSION

Previous studies have implicated a role for the outer surface of *P. mirabilis* in swarming, where both a slime layer and a capsular polysaccharide, designated colony migration factor (Cmf), are required (17, 20, 40, 51). In the present study, an additional role for the cell surface in swarming was identified. A transposon insertion in a putative *waaL* ortholog (PMI3163), involved in both O-antigen and ECA synthesis, blocked the ability of *P. mirabilis* to swarm. Our study is not the first to report a role for *waaL* in swarming. Studies by Toguchi et al. in *S. enterica* serovar Typhimurium demonstrated that *waaL* was required for swarming motility (54). However, in contrast to *P. mirabilis*, the loss of *waaL* in *S. enterica* was not associated with a failure to upregulate flagellin expression during swarming (54).

The LPS profile of the *P. mirabilis waaL* mutant in Fig. 3 is consistent with loss of O-antigen ligase activity, with a concomitant loss of the O-antigen + 1 band and the ladder of O-antigen repeats. Although the *P. mirabilis* WaaL protein shared limited sequence homology with other WaaL proteins, this is a common feature among WaaL proteins from different bacteria and even among WaaL proteins from different serotypes of the same species (1, 23, 37, 38, 49). For example, WaaL proteins between *E. coli* K-12 and R3 share limited sequence homology and those between different serotypes of *Vibrio cholerae* O1 and V194 share only 24% identity and are not capable of cross-complementing the respective mutations (23, 49). The *P. mirabilis* WaaL protein displayed a high degree of similarity in Kyte-Doolittle hydropathy profiles to WaaL proteins from other Gram-negative bacteria (Fig. 1).

Since the *waaL* mutation alters both O-antigen and ECA addition to the lipid A core, individual mutations were made in the *wzyE* and *wzz* genes, encoding ECA polymerase and an O-antigen chain length determinant, respectively (27, 39, 44). Swarming was abolished in the *wzz* mutant, where the O-antigen is synthesized in a truncated form and ECA is unaffected, but the *wzyE* mutant, defective in ECA synthesis, was still able to swarm. Therefore, O-antigen, but not ECA, is required for swarming. This contrasts to the proposed role of ECA in *Serratia marcescens*, where it was necessary for swarm-

ing and *flhDC* activation and was suggested to act as a checkpoint for *flhDC* activation (9). An analogous role for O antigen in *P. mirabilis* seems unlikely, since liquid-grown cultures at early stationary phase do not upregulate *flhDC* but have complete O antigen (data not shown).

In the *waaL* mutant, mRNA for *flhDC* and the flagellin protein (FlaA) failed to increase during growth on solid surfaces (Fig. 5). However, swarming in the *waaL* mutant was restored by artificially raising the levels of *flhDC* or by a loss-of-function mutation in the RcsB response regulator (Fig. 6) and neither condition restored O-antigen synthesis (data not shown). This indicated that the failure of *waaL* mutants to differentiate and swarm on solid surfaces was not due to an intrinsic structural or physical defect resulting from the absence of O-antigen but may have resulted from the interruption of a surface signaling pathway that increased expression of the master regulator *flhDC*. This does not preclude a second role for O antigen in facilitating movement by promoting wetability in a manner similar to that proposed for O antigen in *Salmonella enterica* serovar Typhimurium (54).

At this time, the mechanism by which a *waaL* mutation results in a failure to activate *flhDC* on solid surfaces is unclear, but several possibilities exist. In *E. coli*, mutations that truncate the inner core of LPS activate the Rcs system, resulting in greater repression of *flhDC* and inhibition of swimming and swarming (18). Based on this, the loss of O-antigen ligase activity in *P. mirabilis* may lead to cell envelope stress via the accumulation of unligated O-antigen intermediates in the periplasm. In turn, this may activate the RcsBCD system, resulting in greater repression of *flhDC*. However, this seems unlikely based on the following: (i) the levels of *flhDC*-dependent FlaA expression are similar in liquid-grown wild-type and PM942 cells (T_0 sample in Fig. 5A), (ii) both strains swim at equal efficiencies (Fig. 2), and (iii) a *wzz* mutant, which contains short O-antigen length without the accumulation of unligated O-antigen precursors in the periplasm, is also unable to activate *flhDC* and *flaA* on surfaces (Fig. 5C).

A second possibility is that O-antigen is acting as a sensor to monitor a solid surface. If O-antigen functions in surface sensing, it may require the RcsBCD phosphorelay, composed of the RcsC sensor kinase, RcsD (RsbA, YojN) and the RcsB response regulator, which has a role in surface sensing in other bacteria, such as *E. coli* (15). This model is also based on previous studies, where mutations in the *P. mirabilis rcsB*, *rcsC*, or *rcsD* (*rsbA*) gene result in overexpression of *flhDC*, differentiation under nonpermissive conditions (liquid), and hyperswarming on agar surfaces (4, 13, 29). Upon contact of O antigen with solid surfaces, it is hypothesized that a change in the outer membrane occurs that decreases the RcsC kinase and/or increases phosphatase activity, resulting in lower levels of phosphorylated RcsB and derepression of *flhDC*. A mediator of this signal between the outer membrane and RcsC may be an outer membrane protein such as RcsF and/or the putative inner membrane protein UmoB (IgaA), both of which regulate RcsC activity (8, 14, 18, 31).

The present study adds to the complexity of how *P. mirabilis* senses a solid surface and regulates differentiation. One mechanism has been proposed by Belas and coworkers and involves a role for flagella in sensing solid surfaces (2, 5). In these studies, when *P. mirabilis* was grown in liquid culture, condi-

tions that inhibited flagellar rotation, such as the addition of anti-FlaA antibodies or a thickening agent, resulted in the formation of swarmer cells (5). Although it is possible that the loss of O antigen alters flagellar synthesis or function, this seems unlikely because the *waaL* mutant exhibited swimming motility that was similar to the wild type (Fig. 2). Therefore, flagellar inhibition should be relayed in similar manners for both wild-type and *waaL* mutant strains upon placement on solid media. It also seems unlikely that the *waaL* mutation specifically decreases flagellar function on solid surfaces because then the *waaL* mutant would be predicted to differentiate more efficiently than the wild type, since inhibition of flagellar rotation is a signal for differentiation. Based on this information, it seems likely that two distinct sensing mechanisms operate in *P. mirabilis* to regulate the ability to swarm, one involving the inhibition of flagellar rotation and a second mechanism that requires O antigen. We propose that the inhibition of flagellar rotation activates an undefined pathway that regulates genes required for swarmer cell elongation. In a separate pathway, O-antigen contact with solid surfaces activates the flagellar gene cascade needed for the copious amounts of flagella required for movement of a swarmer cell. In support of the two pathways for swarmer cell differentiation, unpublished studies from our lab have shown that a *P. mirabilis* *motA* mutant, unable to rotate its flagella, fails to differentiate but correctly activates *flhDC* expression upon contact with a solid surface. Future work will investigate which genes are controlled by flagellar inhibition and which are controlled by the O-antigen-mediated surface signaling pathway.

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