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Enterobacterial Common Antigen Integrity Is a Checkpoint for Flagellar Biogenesis in *Serratia marcescens*▽

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Serratia marcescens **strains are ubiquitous bacteria isolated from environmental niches, such as soil, water, and air, and also constitute emergent nosocomial opportunistic pathogens. Among the numerous extracellular factors that *S. marcescens* is able to produce, the PhlA phospholipase is the only described exoprotein secreted by the flagellar apparatus while simultaneously being a member of the flagellar regulon. To gain insight into the regulatory mechanism that couples PhlA and flagellar expression, we conducted a generalized insertional mutagenesis and screened for PhlA-deficient strains. We found that three independent mutations in the *wec* cluster, which impaired the assembly of enterobacterial common antigen (ECA), provoked the inhibition of PhlA expression. Swimming and swarming assays showed that in these strains, motility was severely affected. Microscopic examination and flagellin immunodetection demonstrated that a strong defect in flagellum expression was responsible for the reduced motility in the *wec* mutant strains. Furthermore, we determined that in the ECA-defective strains, the transcriptional cascade that controls flagellar assembly was turned off due to the down-regulation of *flhDC* expression. These findings provide a new perspective on the physiological role of the ECA, providing evidence that in *S. marcescens*, its biosynthesis conditions the expression of the flagellar regulon.**

Serratia marcescens is an opportunistic human pathogen associated with urinary and respiratory tract as well as wound and eye infections, endocarditis, osteomyelitis, meningitis, and septicemia. Immunocompromised people and newborns are the most affected hosts. The incidence of *S. marcescens* infection has increased over the last years, mainly due to the acquisition of multiple antibiotic resistance (19, 20). *S. marcescens* produces numerous extracellular factors, including hemolysin, esterase, DNase, chloroperoxidase, S-layer, lipases, proteases, chitinases, and siderophohore (21, 24, 26, 27, 31, 38, 44, 45). These factors are predicted to play a role in bacterial environmental adaptive capacity, in either nonhost or host environments, contributing to its ambient persistence and to its pathogenic potential. In addition, an extracellular phospholipase named PhlA in *Serratia liquefaciens* MG1 (recently reclassified as *S. marcescens* MG1) and PlaA in *Serratia* sp. strain MK1 has been described previously (16, 48). The *phlAB* (or *plaAS*) locus codes for the PhlA (PlaA) phospholipase and for an accessory protein (PhlB/PlaS) that prevents the intracellular phospholipase enzymatic action. Interestingly, it has been shown not only that the *phlAB* promoter region displays homology to class III promoters of the flagellar transcriptional cascade controlled by the FliA sigma factor (15) but also that PhlA secretion depends on the integrity of the type III flagellar system export apparatus (15–17).

The flagellar structure has its dedicated type III protein export embedded apparatus that translocates proteins involved in the self-assembly process and is also able to function in the export of nonflagellar proteins. Indeed, *S. marcescens* PhlA and its orthologues, YplA from *Yersinia enterocolitica* and XlpA from *Xenorhabdus nematophila* (47, 54, 55), as well as the Cia proteins of *Campylobacter jejuni* (28) have been described as nonflagellar exoproteins exported by the flagellar secretome. Schmiel and coworkers (46) have shown that YplA displays a role in the pathogenicity of *Yersinia enterocolitica*. In contrast, the function for PhlA and the relevance of its coexpression with the flagellar appendage remain unclear.

To further explore the mechanism that underlies the PhlA-flagellum-concerted regulation, we carried out random transpositional mutagenesis, selecting for phospholipase-negative mutants in a *S. marcescens* clinical isolate. Strikingly, we found that phospholipase expression depended on the intactness of genes localized in the chromosomal *wec* cluster. The *wec* genes code for the proteins involved in the biosynthesis of the enterobacterial common antigen (ECA), a glycolipid detected in the outer leaflet of the outer membrane in gram-negative bacteria. The polysaccharide chains of the ECA consist of linear repetitive units of a trisaccharide composed of 4-acetamido-4,6-dideoxy-D-galactose, *N*-acetyl-D-mannosaminuronic acid, and *N*-acetyl-D-glucosamine. Three variants of ECA have been described for *Escherichia coli*. The major form of ECA is linked

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TABLE 1. Primers used in this study

Primer[a]	Sequence (5′→3′)
Tn5-I-end F	TTCGTCGACGGACTGCGGCC
PhlA P1	TCAGGTGCTCAATAAAAAGTCTATC GACAAGGAGTCGGCGTGTAGGCT GGAGCTGCTTCG
PhlB P2	TATGGGCGTTTACGGCTGCGCGTAG TGCGTGCCCAAGCGGCATATGAA TATCCTCCTTAG
FliC-F	ATGGCACAAGTAATC
FliC-R	CGTTCAGTGCGCCTTC
FliA-F	GTGAGCGATCTGTATAC
FliA-R	GCAGCTCGTCGAGCATC
FlhD-F	ATGGGGAATATGGGTAC
FlhD-R	CTTTGGTCAGGCGTTC
PhlA-R	CCTCCTGCGATTGCTGCC
PhlA-F	ATGAGTATGCCTTTAAG
16S-F	AAACTGGAGGAAGGTGGGGATGAC
16S-R	ATGGTGTGACGGGCGGTGTG

[a] F, forward primer; R, reverse primer.

to diacylglycerol (ECA_{PG}), whereas minor amounts are found anchored to the lipopolysaccharide core structure (ECA_{LPS}) or as a periplasmic water-soluble cyclic form (ECA_{CYC}). Although highly conserved in all examined gram-negative enteric bacteria, the role of this exopolysaccharide remains largely uncharacterized (29, 43).

We found that flagellar assembly was precluded in *wec* mutants and determined that this effect corresponded with the down-regulation of the flagellar transcriptional cascade at the level of the master regulator $FlhD_2C_2$ expression. This study shows a novel physiological function of ECA, demonstrating that either the integrity of this polysaccharidic structure or the intactness of its biosynthetic pathway constitutes an essential checkpoint for the biogenesis of flagella in *S. marcescens*, profoundly affecting motility phenotypes and the expression of the PhlA phospholipase.

MATERIALS AND METHODS

Bacterial strains. *S. marcescens* RM66262 is a nonpigmented clinical isolate from a patient with urinary tract infection from the Bacteriology Service of the Facultad de Ciencias Bioquímicas y Farmacéuticas of the Rosario National University, Rosario, Argentina. This strain was biotyped as *Serratia* by classical biochemical tests. 16S rRNA gene sequencing, similarity analysis using the Ribosomal Database Project II (6), and the BLAST program search (www.ncbi.nlm.nih.gov/BLAST) allowed us to typify this isolate as *S. marcescens*. *wecD*::mini-Tn*5Km*, *wzxE*::mini-Tn*5Km*, and *wzyE*::mini-Tn*5Km* strains were obtained by random mutagenesis of *S. marcescens* RM66262 with pUT::mini-Tn*5Km* (8).

A complete deletion of *phlAB* was constructed in the pBBR1-MCS2::*phlAB* plasmid, containing a 3.0-kb chromosomal fragment, including the *phlAB* operon (sequence included in NCBI accession number EF152995), by linear DNA transformation (7) using primers PhlA P1 and PhlB P2 (Table 1). The *phlAB* operon was replaced by a chloramphenicol resistance cassette. The 3.2-kb fragment with the deleted *phlAB* operon was cloned in the suicidal plasmid pKNG101 (25) and conjugated into *S. marcescens* RM66262. Mutant strains were selected as chloramphenicol-resistant and streptomycin-sensitive colonies. The Δ*phlAB* deletion was confirmed by PCR and by Southern blot analysis.

Media and growth conditions. Cells were routinely grown in Miller's Luria-Bertani (LB) medium at 30°C or 37°C, as indicated. For the detection of phospholipase activity in growth plates, a lecithinase screening was performed with LB agar plate 5% egg yolk suspension (prepared with one egg yolk in 25 ml of sterile phosphate-buffered saline [PBS]). Lecithinase plates were incubated overnight (ON) at 30°C. Antibiotics used were kanamycin (Km; 50 μg/ml), ampicillin (Amp; 100 μg/ml), chloramphenicol (20 μg/ml), and streptomycin (100 μg/ml).

Transposon mutagenesis. *S. marcescens* RM66262 strain was mutagenized by conjugation with *E. coli* SM10(λpir)/pUT::mini-Tn*5Km*. Transconjugants were selected in LB agar plates supplemented with Km and Amp. The resulting colonies were replica plated in LB and LB-egg yolk plates, and colonies that did not have a precipitation halo in egg yolk plates were selected as lecithinase-negative clones. To identify the localization of the transposon insertion, chromosomal DNA from the selected clones was prepared, digested with SalI, ligated to SalI-digested pBluescript and transformed into *E. coli* XL1 Blue. Km- and Amp-resistant clones were selected and sequenced using primer Tn5-I-end F (Table 1).

Exoenzyme activity assays. Protease activity was assayed in LB agar plates containing nonfat dried milk to a final concentration of 2%. Lipase activity was determined in 1% tributyrin-LB agar plates. Protease and lipase activities were evident as a clearing zone around the growing colony within 12 h at 30°C. DNase test agar with toluidine blue was purchased from Difco Laboratories, and the test was performed according to the manufacturer's protocol.

For the hemolysin assay, *Serratia* strains were grown ON in LB at 37°C without aeration. Cultures were centrifuged, and the spent medium was filtered with a 0.2-μm cellulose acetate filter. Activity was measured by incubating 500 μl of serial dilutions of bacterial spent medium with 500 μl of 2% sheep red blood cells in PBS for 3 h at 37°C. The tubes were centrifuged, and the liberated hemoglobin in the supernatant was determined at 540 nm. One hundred percent activity was determined by incubating 500 μl of 2% sheep red blood cells with 500 μl of water.

Zymogram assay. Twenty-five milliliters of saturated cultures grown in LB at 30°C was centrifuged for 5 min at 5,000 × *g*, and the supernatant was separated. Spent medium was filtered with 0.2-μm acetate-cellulose filters, precipitated with 12% trichloroacetic acid for 30 min at 4°C, and centrifuged for 30 min at 30,000 × *g*, and the precipitated proteins were resuspended in protein sample buffer. One milliliter of trichloroacetic acid-precipitated spent medium was loaded onto 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. Gels were washed twice for 5 min with 10 mM Tris-HCl (pH 8.0)-0.9% NaCl and subsequently overlaid with 5.0 ml of 5% egg yolk suspension in 100 mM Tris-HCl (pH 8.0)-0.9% NaCl-1% agarose. The gels were incubated ON at 37°C, and the lecithinase activity was visualized as a white precipitate band.

Lipopolysaccharide and ECA analysis. Exopolysaccharide preparation, electrophoresis, and detection were performed as previously described (36). Briefly, membrane fractions were prepared as described previously (3), boiled for 10 min, and then incubated ON at 60°C with 1.6 μg/μl proteinase K. Membranes were boiled again for 10 min, and sample buffer was added. Samples were separated on a 14% tricine SDS-PAGE gel and stained with the Silver stain kit (Bio-Rad) to detect lipopolysaccharide or transferred to nitrocellulose membranes. Blots were reacted with anti-O14 polyclonal antiserum to detect ECA.

Motility assays. Swimming medium consisted of LB-0.25% agar plates. A single colony was inoculated with a toothpick in the center of the plate. Swarming motility was observed on LB-0.6% agar plates. Swarm plates were dried for 30 min at 37°C and were inoculated by spotting 5.0 μl of a saturated liquid culture onto the plate. Swimming and swarming plates were incubated for 18 h at the indicated temperature.

Flagellin analysis. Flagellin levels were determined by Western blot analysis using *S. marcescens* anti-flagellin rabbit polyclonal antibodies. For the swimming assays, bacteria were grown in liquid LB medium, because in LB-0.25% agar plates, swimming takes place in the internal water-filled channels of agar, and it is experimentally cumbersome to extrude bacteria from this semisolid matrix. Two hundred microliters of bacterial cultures grown ON in LB at 30°C or 37°C was centrifuged and resuspended in a volume equal to an optical density at 630 nm/50 of protein sample buffer. For the swarming analysis, cells were sampled from the center or from the border of the colony of swarming plates (LB-0.6% agar) and bacteria were recovered with PBS and standardized as described above for liquid culture bacteria. Equal volumes (10 μl) of these whole-cell extracts were loaded onto 12% SDS-PAGE gels and transferred to nitrocellulose membranes.

Flagellin rabbit polyclonal antibodies. Flagella were purified essentially as described previously (14). Briefly, *S. marcescens* RM66262 was grown ON in 50 ml of LB at 30°C and harvested in 100 ml of PBS, pH 7.4. The flagella were detached from the bacterial cells by vigorous vortexing, and the supernatant containing the sheared flagella was separated by centrifugation at 8,000 × *g* for 30 min. The flagella were separated from outer membrane proteins and other contaminants by precipitation with 60% saturation of ammonium sulfate for 18 h at 4°C. After centrifugation at 12,000 × *g* for 30 min, the flagella were resuspended in PBS. The supernatant was subjected to a second cycle of 20% ammonium sulfate precipitation and resuspended in 10 mM Tris-HCl, pH 8.0. The

purity of the preparations was monitored by SDS-PAGE. The purified flagellin was electroeluted from the gels and used to immunize New Zealand rabbits (13).

Transmission electron microscopy. For examination of flagella by transmission electron microscopy (TEM), bacteria from mid-log-phase broth cultures (LB, 30°C or 37°C, 4 h) were adsorbed onto carbon-coated copper grids (1 min). Liquid excess was discarded, and the samples were negatively stained with 2% (wt/vol) phosphotungstic acid for 3 min. Bacteria from swarming plates (LB-0.6% agar, 30°C, 16 h) were recovered by adding a drop of PBS buffer on the agar surface. The cells were washed two times in PBS buffer before staining as above.

RNA purification. Total RNA was extracted from mid-exponential-phase cultures grown in LB at 30°C. Briefly, 8 ml of ice-cold, 5% water-saturated phenol, pH 5.5, in ethanol was added to 50-ml cultures to stop the degradation of RNA. Cells were centrifuged at 7,000 × *g* for 5 min at 4°C, resuspended in 5 ml of 0.5 mg/ml lysozyme, Tris-EDTA, pH 8.0, 1% SDS, mixed, and placed in a water bath at 64°C for 2 min. After incubation, 5.5 ml of 1 M NaAc, pH 5.2, was added. The sample was extracted twice with an equal volume of water-saturated phenol, pH 5.5, and incubated at 64°C for 6 min. The aqueous layer was extracted with an equal volume of chloroform and precipitated with ethanol. RNA was resuspended in water, treated with RQ1 RNase-free DNase (Promega), and subjected to a final step of RNA cleanup using RNAeasy kit (Qiagen) according to the manufacturer's protocol. DNA contamination in RNA preparations was assessed by performing a control PCR prior to reverse transcription (RT)-PCR analysis.

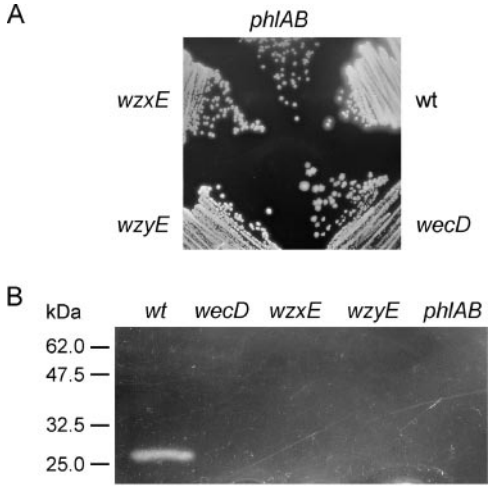
RT-PCR and qRT-PCR. cDNA synthesis was performed using random hexamers, 2 μg of total RNA, and 1 U of SuperScript II RnaseH2 reverse transcriptase (Invitrogen). Five microliters of a 1/10 dilution of each cDNA was used as the template for DNA amplification in RT-PCR or quantitative RT-PCR (qRT-PCR) (20 μl), using primers FlhD-F, FlhD-R, FliA-F, FliA-R, FliC-F, FliC-R, PhlA-F, and PhlA-R (Table 1). A primer set for the 16S rRNA was used as a control to confirm that equal amounts of total RNA were used in each reaction mixture. In every case, the amplified fragment was 250 bp. For RT-PCR, the number of cycles varied according to the level of expression of each mRNA to ensure that the comparison was performed in the linear range of the amplification.

For the quantitative real-time PCR of *flhD* and *fliC*, the reactions were carried out in the presence of the double-stranded DNA-specific dye SYBR green (Molecular Probes), and monitored in real time with the Mastercycler ep realplex real-time PCR system (Eppendorf). The relative expression was calculated using the threshold cycle values (Ct) obtained from each sample as follows: relative expression = $2^{-\Delta\Delta}$, being $\Delta^{Ct} = Ct_{sample} - Ct_{16S}$ and $\Delta\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{ref\ sample}$ (where sample is the mutant strain transcript, 16S is the 16S rRNA transcript, and ref sample is the *S. marcescens* RM66262 transcript). The reference sample was *S. marcescens* RM66262. The average values were calculated from triplicate samples.

Oligonucleotides used in this study. The sequences of the oligonucleotides used in this study are listed in Table 1.

RESULTS

Mutations in the *wec* cluster impair PhlA expression. In search of determinants involved in the coordinated expression of PhlA and the flagellar appendage, we undertook a random mini-Tn*5* mutagenesis approach in the *S. marcescens* RM66262 strain, followed by a phenotypic screen for phospholipase-negative mutants in egg yolk-LB agar plates (see Materials and Methods for details). The subsequent cloning, nucleotide sequencing of the regions adjacent to the transposon, and homology analysis using the GenBank database and the *S. marcescens* Db11 genome (www.sanger.ac.uk/Projects/S_marcescens/) indicated that none of the phospholipase-negative strains recovered from a total of 10,000 mutants harbored the mini-Tn*5* transposon insertion in the *phlAB* locus or the flagellar loci. Strikingly, independent clones containing insertions interrupting *wecD*, *wzxE*, and *wzyE* were isolated (Fig. 1A). These genes belong to the *wec* cluster, which is predicted to encode the proteins required for the biosynthesis of the ECA, a highly conserved cell surface glycolipid in gram-negative enteric bacteria (Fig. 2A presents a scheme of the *wec* cluster organization) (30). The three isolated mutants did not display significant phospholipase activities in zymogram assays when the spent culture medium was analyzed (Fig. 1B), suggesting that the secretion and/or expression of PhlA was inhibited in these strains.



C

Exoenzyme activity	*wt*	*wecD*	*wzxE*	*wzyE*
lecithinase	+	-	-	-
lipase	+	+	+	+
protease	+	+	+	+
DNase	+	+	+	+
haemolysin	+	+	+	+

FIG. 1. Phospholipase-negative mutant strains. (A) Egg yolk-LB agar plate showing the precipitation halo characteristic of lecithinase activity present in the wild-type (wt) strain and absent in the *wec* and Δ*phlAB* mutant strains. (B) Zymogram analysis of the spent culture medium from the wt and the *wec* mutant strains. Phospholipase activity is visualized as a white precipitate band of 26 kDa. The Δ*phlAB* strain was used as control. (C) Results of exoenzyme activity assays. Each activity assay was performed as described in Materials and Methods. −, negative result; +, positive result.

To exclude the possibility that the mutations in the *wec* cluster provoked a major envelope disturbance, globally affecting protein secretion processes, we screened the *wec* mutant strains for the secretion of other *Serratia* exoenzymes (see Materials and Methods for details). We found that the secretion of lipase, DNase, proteases, and hemolysin was not affected in any of the three *wec* mutants relative to the wild-type strain (Fig. 1C). Furthermore, the growth rate of the three *wec* mutant strains was comparable to that of the wild-type strain (not shown).

The synthesis of ECA in *Serratia* has not been investigated yet; however, the *Serratia wec* cluster organization and sequence are highly conserved compared to those of other enterobacteria. (42, 43). The mini-Tn*5-Km* transposon has strong terminators at both ends (8). Thus, the resultant transposon insertions are expected to have polar effects, inactivating downstream genes. Therefore, although the transcriptional units in the *wec* cluster have not yet been well defined, the ECA pathway could be blocked at early biosynthetic stages in

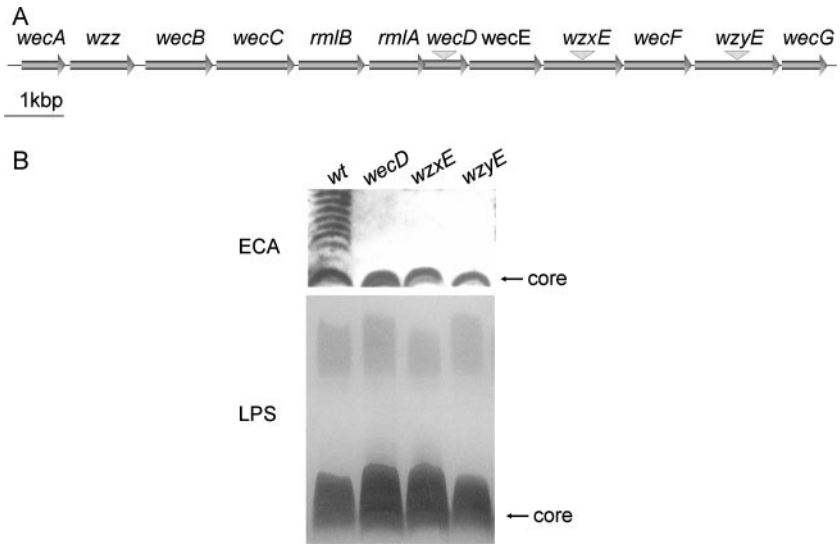


FIG. 2. (A) Schematic representation of the *wec* cluster in *S. marcescens*. The sites of the mini-Tn*5-Km* transposon insertions are indicated with arrows. (B) Exopolysaccharide preparations of the wild-type and mutant strains were analyzed by immunodetection with anti-O14 antiserum to detect ECA (upper panel) or by silver staining to examine LPS (lower panel).

these mutants. The ECA exopolysaccharide was isolated, analyzed by SDS-PAGE, and detected with anti-O14 antiserum that specifically recognizes ECA epitopes (Fig. 2B, upper panel) (36). The characteristic modal pattern of ECA was absent in the *wecD*, *wzxE*, and *wzyE* mutant strains. In contrast, LPS analysis indicated that the three *wec* mutants displayed patterns identical to that of the wild type (Fig. 2B, lower panel).

These results indicated that either the altered ECA structure or the cellular imbalance of ECA biosynthetic intermediates was somehow affecting the ability of *S. marcescens* to express and/or secrete PhlA.

***wec* mutations affect flagellum-dependent phenotypes.** Previous work indicated that PhlA contains a putative FliA recognition motif, being part of the flagellar regulon (15, 47). FliA, the alternative sigma 28 transcriptional factor, is specifically required for the control of the final stages of flagellar assembly once the hook-basal body structure is completed. In the flagellar regulation cascade, *fliA* is a class 2 (middle) gene whose transcription depends on class 1 (early) *flhDC* expression (4).

We hypothesized that PhlA deficiency in the *wec* mutants could be linked to a flagellar system malfunction. In *S. marcescens*, either swarming or swimming is a motility trait strictly dependent on flagellar expression (18). In contrast to swimming, swarming implies a multicellular process in which bacteria differentiate from typical rod-shaped cells (at the center of the colony) into long, multinucleated, hyperflagellated cells (at the rim of the colony), which spread on top of the growth medium surface (11, 18). In *Serratia*, flagellar expression is induced at 30°C and no swarming motility is detected at 37°C (10). Therefore, we analyzed the effect of the mutations in the *wec* cluster on the two motility phenotypes at both temperatures. As shown in Fig. 3, swarming was completely abolished in the three *wec* mutants at both 30°C and 37°C. Furthermore, the *wec* mutants were unable to swim at 37°C and they displayed strongly diminished swimming capacities at 30°C, with an estimated 50% to 75% reduction of the swimming halo, relative to that of the wild-type strain (Fig. 3). To distinguish whether the observed defect in motility was due to a restriction in flagellar motion or to a deficiency in flagellar expression, we examined cellular morphology by using TEM (Fig. 4). As shown in Fig. 4, wild-type *Serratia* displayed the expected swarming differentiation phenotype and typical morphology and flagellation in the swimming assay. In contrast, the *wec* mutants, taken from either the swimming or the swarming assay, did not exhibit flagellar appendages at either temperature analyzed (Fig. 4). Furthermore, flagellin contents in whole cells were assayed by SDS-PAGE, followed by immunodetection (Fig. 5). In the *wec* mutants, flagellin was barely detectable

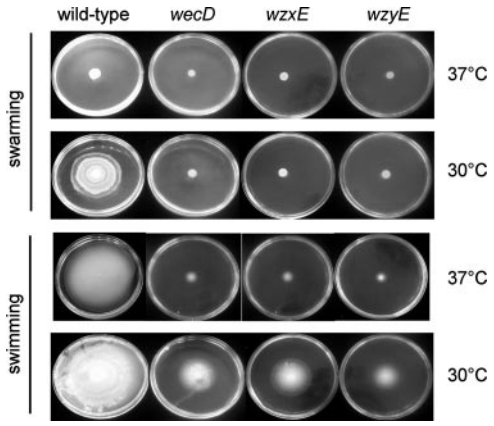


FIG. 3. Motility assays. Swarming (LB-0.6% agar) and swimming (LB-0.25% agar) plates were incubated ON at 37°C or 30°C, as indicated in Materials and Methods, and photographed. The strains analyzed were *S. marcescens* RM66262 (wild-type) and *wecD*-, *wzxE*-, and *wzyE*-derived mini-Tn*5-Km* mutants. Results are representative of four identical individual assays.

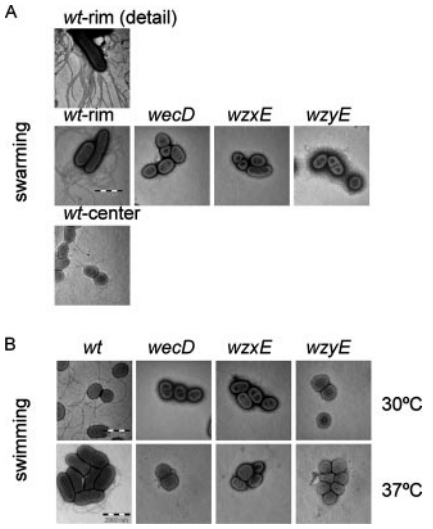


FIG. 4. TEM. Representative TEM observations of wild-type (wt) *S. marcescens* and mutants illustrating phenotype differences when cells were taken from (A) swarming plates (rim or center of the colony, as indicated) or (B) LB broth cultures, at the indicated growth temperatures. The strains analyzed were *S. marcescens* RM66262 (wild type) and *wecD*-, *wzxE*-, and *wzyE*-derived mini-Tn*5*-*Km* mutants. A detail of a hyperflagellated wild-type cell taken from the swarm center is shown. Cells were grown and stained for TEM as described in Materials and Methods. Bars = 2.0 μm (magnification is identical for all images, and the scale is included in the first image of each series).

compared to levels observed in the wild-type cells, taken from either the center or the colony rim of the swarming plates. Flagellin levels were also drastically reduced in the three *wec* strains taken from the swimming assay. The reduction was more pronounced in those cells grown at 37°C compared to those obtained from cells grown at 30°C (average reductions of 97% and 90%, respectively, relative to the flagellin levels in the wild type, as estimated by densitometric analysis) (Fig. 5).

Taken together, these results clearly show that the altered motility phenotypes observed in the *wec* mutant strains are due to a diminished flagellin expression.

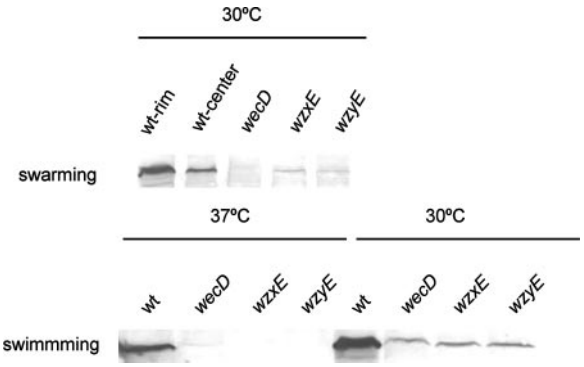


FIG. 5. Flagellin immunodetection. Whole-cell extracts from the wild-type (wt) and mutant strains isolated from the rim or the center of the swarming colony grown at 30°C (upper panel) or liquid LB cultures grown at 37°C or 30°C (lower panel) were analyzed by Western blotting developed with anti-flagellin polyclonal antibodies. Samples were standardized as described in Materials and Methods.

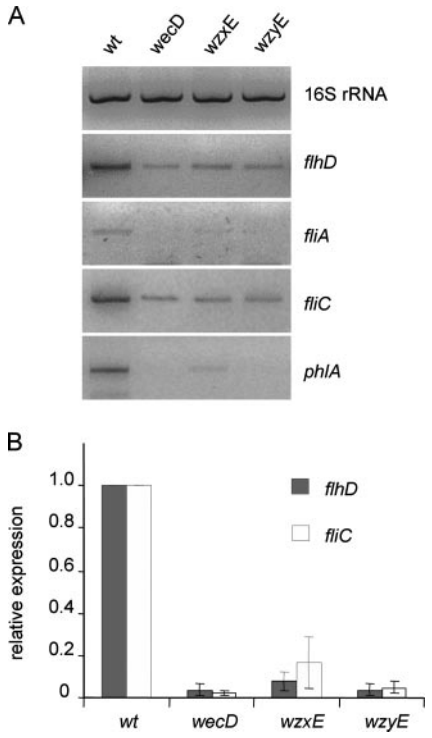


FIG. 6. RT-PCR and qRT-PCR analysis of flagellar cascade genes and *phlA* expression. (A) Total RNA was extracted from the wild-type (wt) and the *wecD*, *wzxE*, and *wzyE* mutant strains. *flhD*, *fliA*, *fliC*, and *phlA* expression was analyzed by RT-PCR using specific primers (Table 1). 16S rRNA was used as an internal control. The PCR was carried out for 25 cycles for *flhD*, *fliC*, and 16S rRNA and 35 cycles for *fliA* and *phlA*. (B) Quantitative RT-PCR analysis of *flhD* and *fliC* expression. Average Ct values and *n*-fold changes in gene expression in each mutant strain compared with those of the wild-type strain were calculated from triplicate samples as described in Materials and Methods. Error bars indicate standard deviations.

***wec* mutations provoke a class I level repression of the flagellar regulatory cascade.** The transcriptional levels of class I, II, and III representative genes were analyzed to determine whether the mutations in the *wec* cluster inhibited flagellar expression at the transcriptional level and, if this was the case, at which stage in the flagellar regulatory cascade the inhibited flagellar expression occurred.

With this aim, RT-PCR was performed using total RNA derived from cells grown to exponential phase in LB liquid medium at 30°C, the condition which conveyed the highest levels of flagellin expression (Fig. 5). As shown in Fig. 6A, the transcript levels of *flhD*, *fliA*, and *fliC* were significantly reduced in the *wec* mutant strains relative to the wild-type strain. Accordingly, and as predicted, the transcription of *phlA* was also severely diminished.

Although *fliA* and *phlA* could not be analyzed by real-time RT-PCR due to very low amplification levels achieved using the *wec* mutant samples, the quantification of *flhD* and *fliC* expression levels confirmed that the mutations in the *wec* cluster caused a strong inhibition of *flhD* transcription, with the concomitant down-regulation of *fliC* expression (Fig. 6B).

These findings reveal that the blockage in the ECA biosynthetic pathway provokes a transcriptional repression of the

Serratia flagellar regulon, including *phlAB*, by inhibiting the expression of the master operon *flhDC*.

DISCUSSION

The strategies that an opportunistic pathogen such as *S. marcescens* displays to adapt to different environments, ranging from a free-living to a host-associated lifestyle, are complex and not yet fully understood. A remarkable attribute of *S. marcescens* resides in its capacity to express a wide array of extracellular proteins with distinct enzymatic activities that are predicted to strongly contribute to the bacterial adaptive capacity. Besides, the flagellum provides the bacteria with the capacity to translocate to new niches, functioning as an adhesin and triggering immunomodulatory responses in the host (40, 50). Consequently, these secreted or extracellularly exposed determinants are predicted to play a major role in the interaction of the bacterium with its environment, requiring tight and coordinated regulation over their expression.

It has previously been shown that the expression of the extracellular phospholipase PhlA is coupled to the flagellar expression. With the aim of finding regulatory factors that would participate in this mechanism, we undertook a random mutagenesis strategy selecting for phospholipase-deficient clones. Mutant strains harboring independent transposon insertions localized to the *wec* cluster. The inactivation of *wecD* is predicted to block the metabolic route, with accumulation of the undecaprenylpyrophosphate-linked disaccharide precursor in the inner membrane and TDP-4-amino-4,6-dideoxy-D-galactose. Two downstream mutations were located in *wzxE* and *wzyE*. These mutations preclude the translocation and subsequent en bloc polymerization of the undecaprenylpyrophosphate-linked trisaccharide, which takes place at the periplasmic side of the bacterial inner membrane. Accordingly, we show that in the three analyzed mutants, the assembly of ECA is prevented (Fig. 2).

Because PhlA belongs to the flagellar regulon, we predicted that flagellar assembly could also be affected in the *wec* mutants. Motility assays showed that the *wec* mutants either are nonmotile or exhibit a marked reduction in their translocation capacities. Microscopic analysis of the cells revealed the absence of detectable flagella at the surfaces of the bacteria. Consistently, the *wec* mutants exhibited severely diminished flagellin expression.

A recent genome-wide screening using an *E. coli* K-12 knockout mutant collection (22) showed that swarming, but not swimming motility, was affected in all of the *wec* mutants analyzed, with exception of the *wzzE* strain. These results support and extend our findings, suggesting that ECA strongly influences motility not only in *S. marcescens* but also in other enterobacteria. Although our results could provide the genetic basis for the observed phenotypes, further analysis needs to be performed with *E. coli* K-12 in order to understand the differential behavior with *S. marcescens.*

It is reasonable to assume that *S. marcescens* exhibits the same regulatory mechanism for flagellar biogenesis, as it has been extensively described for other *Enterobacteriaceae*, such as *E. coli* and *Salmonella enterica*. In this scenario, FliA expression depends on the expression and transcriptional activity of the master regulator $FlhD_2C_2$. Indeed, we determined that *fhlDC* transcription was strongly down-regulated, indicating that flagellar expression was repressed at the earliest level in the three *wec* mutants. This inhibition extended to the downstream hierarchical levels in the flagellar regulatory cascade, as *fhlDC* down-regulation prevented *fliA* expression and resulted in the repression of *fliC* and *phlA* expression (Fig. 6).

What is the mechanism that senses the blockage in the ECA biosynthetic pathway and transduces it into *flhDC* repression? Several regulatory factors have been identified as influencing *fhlDC* transcriptional activity in diverse bacterial species. They include the EnvZ/OmpR two-component system, the CAP-AMPc complex, the histone-like H-NS protein, the LysR-type LhrA regulator, the *umo* proteins, the RcsCBD phosphorelay, and the QseCB quorum-sensing system (5, 9, 12, 34, 39, 49) and could conceivably be directly or indirectly affected by the metabolic or structural cellular anomaly generated by the disruption in the ECA biosynthetic pathway. A more detailed analysis of the influence of the ECA pathway on the flagellar biogenesis is under way in our laboratory in order to define the targets of the ECA-triggered signals.

Alternatively, Kutsukake (32) and Clarke and Sperandio (5) found evidence that both in *Salmonella* and in enterohemorrhagic *E. coli*, FliA was able to up-regulate *flhDC* transcription, giving rise to a feedback regulatory loop. These results open the possibility that FliA might alternatively be the primary target of the ECA-dependent effect. Wang et al. (52) demonstrated that the flagellar appendage monitors environmental wetness. In this model, when suboptimal hydration is detected, the secretion of the anti-sigma factor FlgM is halted, raising intracellular FlgM levels that inhibit FliA transcriptional activity. FliA inhibition would in turn prevent the up-regulation of *flhDC* transcription. Additionally, previous work suggested that extracellular polysaccharides, acting as water-retaining agents, favor the motion of flagella by lowering frictional forces (1, 23, 37, 51). Our results showed that the O-antigen pattern was not altered by the analyzed *wec* mutations (Fig. 2). In this context, our findings suggest that in *S. marcescens*, ECA deficiency would alter the hydration status of the cell, even in the presence of the O-antigen, a major polysaccharidic structure.

In *Serratia*, both swimming and swarming motilities are induced at 30°C and swarming, but not swimming, is completely inhibited at 37°C (10) (Fig. 3). Apart from the fact that production of the surfactant serrawettin, which favors swarming motility, depends on a quorum-sensing process that is induced at 30°C (35, 53), the regulatory mechanism that underlies temperature-controlled motility in *Serratia* remains unclear. The fact that the mutations in the *wec* genes did not completely preclude swimming and flagellin expression at 30°C indicates that this down-regulation does not consist of an on-off mechanism and that at 30°C, the expression of other factors might counteract or modulate the inhibition.

Lastly, our findings underscore a novel role for the *wec* cluster and for its encoded biosynthetic pathway, which is conserved in a broad range of enterobacteria. Previous reports demonstrated that in *Salmonella enterica*, *Klebsiella pneumoniae*, and uropathogenic *E. coli*, mutations in genes localized in the *wec* cluster rendered the bacteria attenuated for virulence in mouse infection models (2, 33, 41). Therefore, it is tempting to speculate that these virulence defects are due to the inability of the pathogens to adequately control the expres-

sion of the flagellar appendage and of nonflagellar exoproteins, which are members of the flagellar regulon.

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