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The Rcs Signal Transduction Pathway Is Triggered by Enterobacterial Common Antigen Structure Alterations in *Serratia marcescens*[▽]

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The enterobacterial common antigen (ECA) is a highly conserved exopolysaccharide in Gram-negative bacteria whose role remains largely uncharacterized. In a previous work, we have demonstrated that disrupting the integrity of the ECA biosynthetic pathway imposed severe deficiencies to the *Serratia marcescens* motile (swimming and swarming) capacity. In this work, we show that alterations in the ECA structure activate the Rcs phosphorelay, which results in the repression of the flagellar biogenesis regulatory cascade. In addition, a detailed analysis of *wec* cluster mutant strains, which provoke the disruption of the ECA biosynthesis at different levels of the pathway, suggests that the absence of the periplasmic ECA cyclic structure could constitute a potential signal detected by the RcsF-RcsCDB phosphorelay. We also identify SMA1167 as a member of the *S. marcescens* Rcs regulon and show that high osmolarity induces Rcs activity in this bacterium. These results provide a new perspective from which to understand the phylogenetic conservation of ECA among enterobacteria and the basis for the virulence attenuation detected in *wec* mutant strains in other pathogenic bacteria.

Serratia marcescens is a Gram-negative enteric bacterium that acts as a pathogen with a remarkably wide host range; it has been isolated from plants, insects, vertebrates, and humans (35). In humans, *S. marcescens* is an opportunistic pathogen causing infections in patients who are often immunocompromised or preventively treated with broad-spectrum antibiotics and subjected to diverse instrumentation. *Serratia* can produce urinary and respiratory tract infections, surgical wound infections, and septicemia or local infections, such as osteomyelitis and ocular or skin infections (35). The incidence of *Serratia* infections has increased in recent years mainly due to the acquisition of multiple-antibiotic resistance (19, 40, 59, 76).

Despite the clinical emergence of *Serratia*, the virulence mechanisms (adherence, invasion, dissemination, preferred niches) of this pathogen are unresolved. *S. marcescens* produces numerous exoproteins (phospholipase PhlA, DNases, chitinases, esterases, hemolysin, lipase, metalloproteases, and S-layer protein) which are predicted to play a role as virulence determinants (8, 33, 37, 44, 46). However, the regulatory strategies that govern the expression of these potential virulence factors remain poorly characterized.

Protein secretion plays a central role in modulating the interactions of pathogenic bacteria with host organisms. Gram-negative bacteria have evolved different secretion systems to transport proteins across their membranes. At least six general classes of protein secretion systems showing considerable diversity have been described (9, 28). Bioinformatic searches indicate that *Serratia* lacks a specialized type III secretion system (TTSS), which has been shown to be required for virulence in many Gram-negative pathogenic bacteria (18). The flagellar appendage has a tight phylogenetic and structural relatedness with the TTSS and can function as a secretion system to translocate not only proteins involved in the flagellar self-assembly process but also nonflagellar proteins. Indeed, there are several reports of nonflagellar proteins secreted through the flagellum that play a role in pathogenicity: the Cia proteins of *Campylobacter jejuni* (17, 49), HBL and PC-PLC virulence-associated proteins in *Bacillus thuringiensis* (11, 30), carocin S1 in *Pectobacterium carotovorum* (16), and YplA from *Yersinia enterocolitica* (72, 86, 87).

The extracellular *S. marcescens* phospholipase PhlA is the orthologue to YplA from *Yersinia* and, up to now, is the only known *Serratia* nonflagellar exoprotein that belongs to the flagellar regulon and that is also secreted through the flagellum. In a previous work, we demonstrated that mutations in the *wec* cluster, which impair the assembly of the enterobacterial common antigen (ECA), provoked the inhibition of PhlA expression due to the transcriptional downregulation of *flhDC*, the operon that codes for the master regulator which governs the flagellar biogenesis cascade (15).

ECA is a glycolipid present in the outer leaflet of the outer membrane in Gram-negative enteric bacteria. The polysaccharide chains of ECA consist of linear repetitive units of a trisaccharide composed of 4-acetamido-4,6-dideoxy-D-galactose (Fuc4NAc), *N*-acetyl-D-mannosaminuronic acid (ManNAcA), and *N*-acetyl-D-glucosamine (GlcNAc). Three variants of ECA have been described for *Escherichia coli*: ECA_{PG} linked to diacylglycerol (phosphatidylglycerol [PG]), ECA_{LPS} anchored to the lipopolysaccharide (LPS) core structure, and ECA_{CYC} as a periplasmic water-soluble cyclic (CYC) form (24, 43).

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Although it is present in the cell surface of all Gram-negative enteric bacteria, the function of ECA remains to be established.

The assembly and function of the flagellar and chemotaxis system require the expression of more than 50 genes codified in at least 17 operons that constitute the large and coordinately expressed flagellar regulon (56, 61). FlhDC is the flagellar master regulator which activates the transcription of class 2 genes, including genes coding for proteins required in the structure and assembly of the hook-basal body and the sigma and antisigma factors FliA and FlgM. FliA in turn regulates the transcription of class 3 genes, which include *fliC* (coding for flagellin) and, in *Serratia*, *phlA* (32).

The transcription of *flhDC* is a crucial regulatory point in flagellum biogenesis, being regulated in response to global regulatory factors and several environmental conditions. The regulators include cyclic AMP-CRP (cyclic AMP receptor protein) (80), OmpR (47, 77), RcsB (26), the histone-like nucleoid-structuring (H-NS) protein (51), the two-component system QseCB that responds to quorum-sensing signals (79), the LysR-type regulator LrhA (55), and heat shock proteins DnaK, DnaJ, and GrpE (75), while signals comprise high concentrations of either inorganic salts, carbohydrates, or alcohols (74), surface-liquid transition (22), phosphatidylethanolamine (73) and phosphatidylglycerol (63) synthesis, temperature (38, 52), and growth phase (62, 67).

In order to identify the regulatory mechanism responsible for the downregulation of *flhDC* in the *wec* cluster mutant strains, we performed a random mini-Tn*5* mutagenesis and screened for colonies with restored PhlA secretion. We show that the insertional mutations located in genes that code for components of the Rcs signal transduction system suppress the *flhDC* downregulation observed in the *wec* mutant strains.

The Rcs system constitutes a signal transduction phosphorelay that was first identified in *E. coli* as the regulator of capsule synthesis (34). The system is basically composed of two inner membrane proteins of the two-component family RcsC and RcsD (also called YojN) and an orthodox-associated response regulator (RR), RcsB. Apart from the sensor histidine kinase (HK) H1 module, RcsC harbors an additional D1 receiver domain. RcsD is a membrane protein with a histidine phosphotransfer domain. Phosphorylated RcsB binds to the promoter regions of the genes that regulate, activating or repressing their transcription. In addition, two proteins encoded in distant chromosomal regions, RcsF and RcsA, form part of the system. RcsF is an outer membrane lipoprotein that senses perturbations in the cellular surfaces and activates RcsC. RcsA is an accessory DNA binding protein that binds together with RcsB to the promoter region of a subset of genes in the regulon. Most genes that belong to the regulon are activated, while it has been reported that the flagellar regulator *flhDC* is repressed by the Rcs phosphorelay (57).

To rule out that the *rcs* mutations were merely alleviating or counteracting Rcs-independent *flhDC* transcriptional downregulation, we verified that the RcsCDB system activity was triggered by the signal originated by the *wec* deletion mutants. For this purpose and because no *rcs* regulon members were established in *S. marcescens*, a bioinformatics approach was performed using a motif search along the *S. marcescens* Db11 genome to identify Rcs targets other than *flhDC*. SMA1167, predicted to code for a UDP-glucose 4-epimerase, was found to be an Rcs-activated gene. Quantitative real-time reverse transcription-PCR (RT-PCR) assays showed that expression of SMA1167 was indeed highly activated in the *wec* mutant background, while it was repressed in the single *rcs* or double *wec*/*rcs* mutants assayed.

Our results demonstrate that the absence of the ECA polysaccharidic structure constitutes an inducing signal for the Rcs transduction system, provoking alterations in the motile capacity of the bacteria. Because most signals identified for the Rcs system were found to be linked to cell surface perturbations, these findings also suggest that ECA plays a relevant role in the integrity and/or stability of the enterobacterial envelope.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Serratia marcescens* RM66262 is a nonpigmented clinical isolate from a patient with a urinary tract infection from the Bacteriology Service of the Facultad de Ciencias Bioquímicas y Farmacéuticas of the Universidad Nacional de Rosario, Rosario, Argentina (15).

wecD::mini-Tn*5* Km, *wzxE*::mini-Tn*5* Km, and *wzyE*::mini-Tn*5* Km strains were previously obtained by random mutagenesis of *S. marcescens* RM66262 with pUT::mini-Tn*5* Km (20).

Insertion mutations in *wecG*, *rcsB*, *rcsC*, *rcsA*, *rcsF*, *wecA*, and *wzzE* were constructed with the pKnock suicide plasmid (3). For each gene, an internal 400-bp region was amplified using primers wecG12396 Fw and wecG12796 Rv, wecA402 BamHI and wecA802 Rv PstI, WzzE Fw 300 Xba and WzzE Rv 728 XhoI, RcsB Fw 5014 BamHI and RcsB Rv 5314 XhoI, RcsC Fw 7781 BamHI and RcsC Rv 7382 XhoI, RcsA Fw 663 BamHI and RcsA Rv 961 XbaI, and RcsF Fw 557 BamHI and RcsF Rv 758 XbaI. The amplified products were digested with the restriction enzymes indicated in the primer names and cloned into the pKnock-Cm plasmid (*wecA* and *wecG*) or pKnock-Gm plasmid (*rcsB*, *rcsC*, *rcsA*, and *rcsF*). The resulting pKnock derivatives were conjugated into *S. marcescens* RM66262, and mutant strains were selected as chloramphenicol (Cm)- or gentamicin (Gm)-resistant colonies. As a result of homologous recombination between the cloned 400-bp internal region of each gene and the chromosomal copy of the gene, the 5′ region of the gene is separated from the 3′ region by insertion of the pKnock plasmid (3). The mutations were confirmed by PCR and by Southern blot analysis.

To construct the pBBR1MCS-5::*wzyE wecG* plasmid (pYG), the *wzyE* and *wecG* genes were amplified from the *S. marcescens* RM66262 strain chromosome by PCR, using primers wzyE-NTF and wecG-CTR, respectively. The 2,123-bp fragment obtained was cloned into the BamHI-HindIII-digested pBBR1MCS-5 plasmid (50). Plasmid DNA was introduced into bacterial strains by conjugation.

Media and growth conditions. Cells were routinely grown in Miller's Luria-Bertani (LB) medium at 30°C or 37°C, as indicated. For detection of the phospholipase activity in growth plates, bacteria were grown in LB agar plates with 5% egg yolk suspension (prepared with one egg yolk in 25 ml of sterile phosphate-buffered saline [PBS]). Lecithinase plates were incubated overnight at 37°C. The antibiotics used were kanamycin (Km; 50 μg/ml), ampicillin (Amp; 100 μg/ml), chloramphenicol (20 μg/ml), and gentamicin (10 μg/ml).

Zymogram assay. The zymogram assay was performed as previously described (15).

Transposon mutagenesis. The *S. marcescens wecG* strain was mutagenized by conjugation with *E. coli* SM10(λpir)/pUT::mini-Tn*5* Km (20). 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 had a precipitation halo in the egg yolk plates were selected as lecithinase-positive 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.

Motility assays and flagellin analysis. Swimming and swarming assays and immunodetection of flagellin in total cell extracts from swimming or swarmer cells were performed as previously described (15). Densitometric analysis of Western blots was performed with ImajeJ software (1).

Swarmer cell isolation. Plates used for swarming analysis (swarming plates) were inoculated and incubated overnight at 30°C. Swarmer cells were isolated with a sterile spatula from the edge of the swarming analysis plate and

TABLE 1. Sequences of primers used in this study

Primer	Sequence
wecG12396 Fw BamHI	5′-CGGGATCCGGCGCTGCATGCGTTGC-3′
wecG12796 Rv PstI	5′-GACTGCAGTACAGCGCCTGCGGATG-3′
wecA402 BamHI	5′-CGGGATCCGCGCAGCTTCGGCCATG-3′
wecA802 Rv PstI	5′-GACTGCAGAGCGATGATCCACAGC-3′
WzzE Fw 300 Xba	5′-TCCTCTAGAGCGTACAATGAGTTCATC-3′
WzzE Rv 728 XhoI	5′-GACCTCGAGGAGTGTCGGTTTGCGTGC-3′
RcsB Fw 5014 BamHI	5′-CGGGATCCGTGACAAGTATGGCG−3′
RcsB Rv 5314 XhoI	5′-CCGCTCGAGCAGACGCAGCACTTC-3′
RcsC Fw 7781 BamHI	5′-CGGGATCCTGTATGTACTGACGC-3′
RcsC Rv 7382 XhoI	5′-CCGCTCGAGTTGCGCTCAAACAGC-3′
RcsA Fw 663 BamHI	5′-CGGGATCCGAGGACTGCTTCATC-3′
RcsA Rv 961 XbaI	5′-GCTCTAGACCGGACATCCACATG−3′
RcsF Fw 557 BamHI	5′-CGGGATCCACGTATCGCGCCATC-3′
RcsF Rv 758 XbaI	5′-GCTCTAGAAGCCAGATTCGGTGG-3′
Tn5-I-end F	5′-GGAATTCGGCCTAGGTGGCCAG-3′
FlhD-F	5′-TCGCCCGGGATGGGGAATATGGGTAC-3′
FlhD-R	5′-ACGCCCGGGCTTTGGTCAGGCGTTC-3′
1167-F	5′-ATGGATAAGCGTAAAG-3′
1167-R	5′-AATGGGATCGACGTTG-3′
16S-F	5′-AAACTGGAGGAAGGTGGGGATGAC−3′
16S-R	5′-ATGGTGTGACGGGCGGTGTG-3′
wzyE-NTF	5′-GAGGATCCATGACGTTGGCGC-3′
wecG-CTR	5′-TCCAAGCTTACATCTTGCCGCTG-3′

resuspended in PBS. Cells from four swarming plates were pooled for each strain. The bacterial suspensions were standardized by measuring the absorbance at 600 nm.

RNA purification. Total RNA was extracted from mid-exponential-phase cultures grown in LB medium at 37°C or from swarmer cells isolated as described above. 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-HCl (10 mM)–EDTA (1 mM), 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 sodium acetate, 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 an RNeasy kit (Qiagen) according to the manufacturer's protocol. DNA contamination in RNA preparations was assessed by performing a control PCR prior to RT-PCR analysis.

Quantitative real-time RT-PCR. cDNA synthesis was performed using random hexamers, 2 μg of total RNA, and 1 U of SuperScript II RNase H2 reverse transcriptase (Invitrogen). Five microliters of a 1/10 dilution of each cDNA was used as the template for DNA amplification in RT-PCRs (reaction mixture, 20 μl), using primers FlhD-F, FlhD-R, 1167-F, and 1167-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 of 250 bp.

For the quantitative real-time PCR, 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 a Mastercyclerep Realplex real-time PCR system (Eppendorf). The relative expression (RE) was calculated using the threshold cycle (C_T) values obtained for each sample, as follows: $2^{-\Delta\Delta CT}$, with $\Delta C_T = C_{T,\text{ sample}} - C_{T,\text{ 16S}}$ and $\Delta\Delta C_T = \Delta C_{T,\text{ sample}} - \Delta C_{T,\text{ ref sample}}$, where ref sample is the reference sample, which was *S. marcescens* RM66262 grown in LB liquid medium. 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.

LPS and ECA analysis. Exopolysaccharide preparation, electrophoresis, and detection were performed as previously described (15). Anti-O14 polyclonal antiserum (Statens Serum Institut) was used to detect ECA.

Bioinformatics analysis. To search for the putative Rcs-binding sites in the *S. marcescens* genome, the MEME/MAST tools were used. In brief, MEME is an algorithm which, given multiple input sequences, identifies one or more candidate motifs. MEME outputs the motif with the lowest (estimated) *E* value. Then, MAST searches for the motif in sequence databases using motifs with the position-specific probability matrix created by MEME (6, 7).

RESULTS

Identification of mechanism that mediates *flhDC* downregulation in *wec* mutant strains. In order to screen for the signal transduction mechanism responsible for the detection of ECA deficiency and the consequent inhibition of *flhDC* expression in the *wec* mutant strains, we performed a generalized mutagenesis using the mini-Tn*5* Km transposon (20). For this purpose, we first constructed a *wecG* mutant strain using the pKnock-Cm suicide plasmid (see Materials and Methods for details) (3). The *wecG* mutant displayed all the phenotypes detected in the previously reported strains carrying mini-Tn*5* Km insertions in *wecD*, *wzxE*, or *wzyE*: it showed very low levels of flagellin expression (Fig. 1A), lacked phospholipase activity (Fig. 1B), and presented reduced swimming and swarming compared to the wild-type strain (Fig. 1C). All these phenotypes could be complemented by transforming this mutant strain with a plasmid (pYG) carrying a copy of the *wecG* gene under the control of the *E. coli lac* promoter (Fig. 1).

We carried out the mini-Tn*5* Km mutagenesis on this *wecG* mutant and screened for the restored expression of the se-

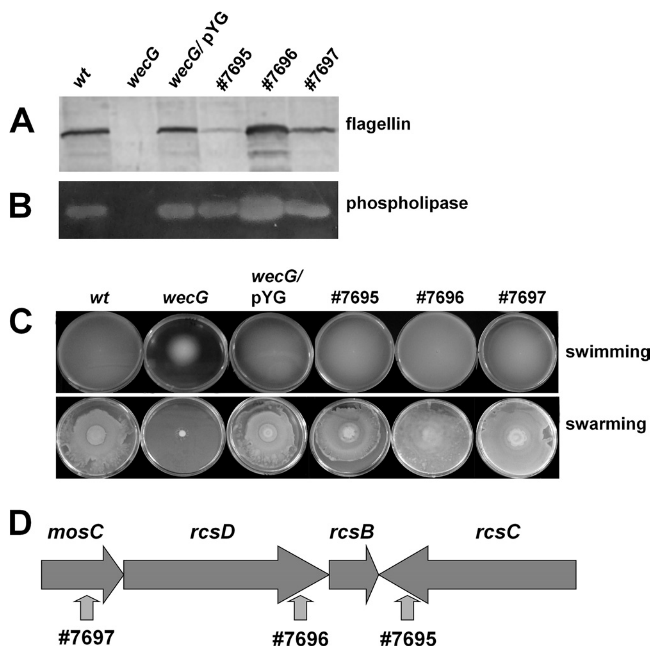

FIG. 1. (A) Flagellin immunodetection. Whole-cell extracts from cells grown overnight in LB medium at 37°C were analyzed by Western blotting, in which the blots were developed with antiflagellin polyclonal antibodies. (B) Zymogram analysis. The spent culture medium from strains grown overnight in LB medium at 37°C was analyzed. Samples were prepared and standardized as described in Materials and Methods. Phospholipase activity is visualized as a white precipitate band that comigrates with the 26-kDa marker. (C) Motility assays. Swimming (LB medium, 0.25% agar) plates were incubated overnight at 37°C (upper panel), and swarming (LB medium, 0.6% agar) plates were incubated overnight at 30°C (lower panel). Results are representative of those from three individual assays with identical results. The following strains were analyzed: *S. marcescens* RM66262 (wild type [wt]); derived pKnock-Cm *wecG* mutant; complemented *wecG* mutant (*wecG*/pYG); and double mutants 7695, 7696, and 7697. (D) Schematic representation of the *rcs* genes in *Serratia marcescens*. The sites of the mini-Tn*5* Km transposon insertions in strains 7695, 7696, and 7697 are indicated with arrows.

creted phospholipase activity in egg yolk-Km-LB medium-agar plates at 37°C, a condition in which phospholipase activity is detected as a white halo surrounding the colony for the wild-type strain. We screened approximately 10,000 colonies and selected three independent mutants that were positive for phospholipase activity on plates (mutants 7695, 7696, and 7697). We analyzed phospholipase secretion by performing a zymogram analysis with spent culture medium and found that the PhlA secretion levels in mutants 7695 and 7697 were similar to the level in the wild-type strain, while strain 7696 presented higher levels of secreted phospholipase than the wild-type strain (Fig. 1B). We then performed swimming and swarming assays and observed that the three double-mutant strains suppressed the motility deficiency of the *wecG* parental strain in both assays (Fig. 1C). In addition, the analysis of flagellin expression levels by immunodetection showed that strain 7696 had higher flagellin expression levels than the wild-type strain, while the flagellin expression levels of strains 7695 and 7697 were reduced compared to the level for the wild-type strain but increased compared to the level for the parental *wecG* strain (Fig. 1A).

These results demonstrate that mutations 7695, 7696, and 7697 suppress the flagellar inhibition observed in the *wecG* mutant strain, restoring motility, flagellin, and PhlA expression.

To identify the transposon insertion sites, the DNA regions flanking the transposon were cloned and sequenced. Mutants 7695, 7696, and 7697 had insertions disrupting the *rcsC*, *rcsD*, and *mosC* genes, respectively (Fig. 1D). According to their intergenic distances, *mosC*, *rcsD*, and *rcsB* might form an operon, while *rcsC* resides downstream of *rcsB* but in the opposite direction. Mini-Tn*5* Km insertions are known to be polar, and therefore, we should take into account the possibility that *rcsB* expression could be affected in both strain 7696 and strain 7697.

RcsF but not RcsA is involved in the flagellar repression observed in *wec* mutant strains. In order to analyze the effect of *rcs* insertions in a wild-type strain genetic background, we constructed independent insertional mutant *rcsC* and *rcsB* strains using the suicide plasmid pKnock-Gm. In addition, we obtained *rcsF* and *rcsA* pKnock-Gm mutants to examine the role of each Rcs system component in the flagellar regulation under study.

The *rcsC*, *rcsB*, *rcsF*, and *rcsA* pKnock-Gm mutants were constructed either in the wild-type or in the *wecD* or *wzyE* mini-Tn*5* Km genetic background previously described (15) in order to analyze the double-mutant phenotypes. We did not further use the *wecG* pKnock-Gm mutant strain because of the impossibility of constructing double pKnock insertions.

As shown in Fig. 2A, the swimming motility behavior of the *rcsA*, *rcsB*, *rcsC*, and *rcsF* mutant strains was similar to that of the wild-type strain. Inactivation of *rcsB*, *rcsC*, or *rcsF* suppressed the reduced swimming phenotype of the *wecD* and *wzyE* mutants. In contrast, *rcsA* inactivation had no effect on the motility of *wecD* or *wzyE* mutant strains.

When the strains were tested for the swarming phenotype, *rcsB*, *rcsC*, or *rcsF* inactivation suppressed the swarming deficiency of *wecD* and *wzyE* mutant strains, although to different extents, depending on each *wec* mutation background (Fig. 2B). In contrast, the *rcsA* mutation did not suppress the swarming defect of *wecD* or *wzyE* strains. When the strains harboring single *rcs* mutations were analyzed, only the *rcsA* mutant strain showed reduced swarming compared with that of the wild-type strain (Fig. 2B; see Discussion).

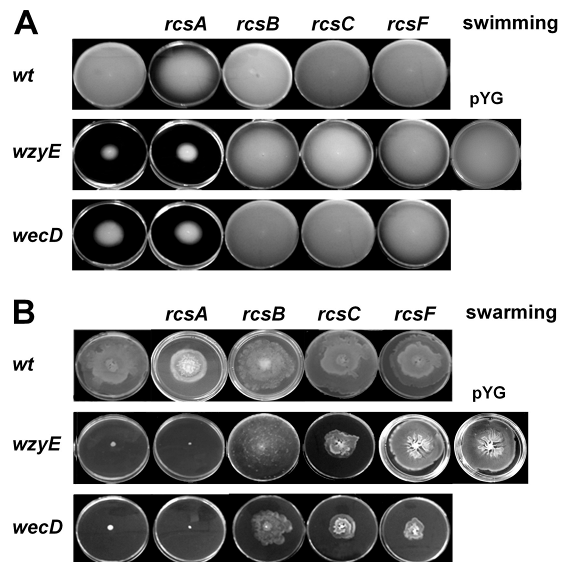

FIG. 2. Motility assays. Swimming (LB medium, 0.25% agar) plates (A) and swarming (LB medium, 0.6% agar) plates (B) were incubated overnight at 37°C and 30°C, respectively. The following strains were analyzed: *S. marcescens* RM66262 (wild type [wt]); derived pKnock-Gm *rcsB*, *rcsA*, *rcsC*, and *rcsF* insertion mutants; mini-Tn*5* Km mutants *wecD* and *wzyE*; complemented *wzyE* mutant (*wzyE*/pYG); and double mutants *wecD rcsA*, *wecD rcsB*, *wecD rcsC*, *wecD rcsF*, *wzyE rcsA*, *wzyE rcsB*, *wzyE rcsC*, and *wzyE rcsF*. Results are representative of those from three individual assays with identical results.

As mentioned above, the mini-Tn*5* Km insertions in *wecD* and *wzyE* are polar on downstream genes. For this reason, the *wecD* insertion could not be complemented with a plasmid carrying a copy of only *wecD* (data not shown). The mini-Tn*5* Km insertion in the *wzyE* mutant is polar on *wecG*, coded downstream of *wzyE* (the last gene in the operon; see Fig. 6 for a scheme of the *wec* cluster), so we constructed a plasmid carrying a copy of both genes, *wecG* and *wzyE*, under the control of the *E. coli lac* promoter (pYG) to complement the *wzyE* mutant (see the assay results shown in Fig. 2, 3, and 5).

Flagellin contents from all the strains under study were analyzed with an immunodetection assay using whole-cell extracts of either swimming cells grown in LB liquid medium or swarmer cells obtained from swarming plates (Fig. 3). The wild-type strain showed a 3-fold induction of flagellin expression levels when it was grown on swarming plates relative to that when it was grown in liquid medium. This result was in accordance with the results of previously described experiments that show that *Serratia* swarmer cells induce the expression of flagella (15, 22). The *wecD* and *wzyE* mutant strains showed very low flagellin expression levels when they were grown under either condition. In accordance with the results of the swimming and swarming assays, the flagellin expression levels of *wecD* and *wzyE* mutant strains were not altered by *rcsA* inactivation. Double-mutant strains *wecD rcsB* and *wzyE rcsB* showed flagellin expression levels 1.6- to 1.7-fold higher than the expression level for the wild-type strain in liquid

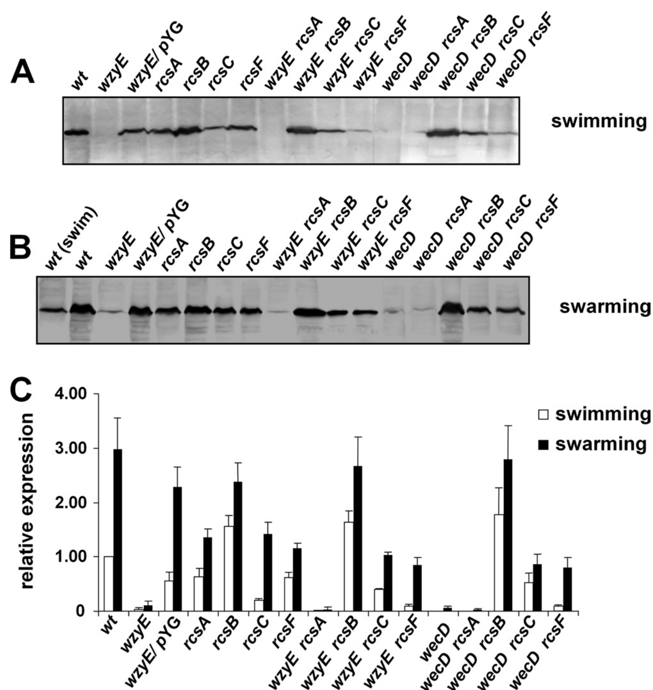

FIG. 3. Flagellin immunodetection. Whole-cell extracts from cultures of the indicated *S. marcescens* strains grown overnight in liquid LB medium (swimming) (A) or obtained from the edge of LB medium–0.6% agar plates (swarming) (B) were analyzed by Western blotting in which the blots were developed with antiflagellin polyclonal antibodies. Samples were standardized as described in Materials and Methods. (C) Densitometric analysis of the flagellin immunodetections shown in panels A and B. Results were calculated from three independent immunodetection assays and are expressed in units relative to the value determined for the wild-type (wt) strain (swimming). Error bars indicate standard deviations.

medium and flagellin expression levels similar to the level for the wild-type strain in swarm plates. Inactivation of *rcsC* in the *wecD* or *wzyE* background led to an increase in flagellin expression levels compared to the level for either the *wecD* or *wzyE* single mutant under both conditions. This increment was lower than the one resulting from *rcsB* inactivation. It has been previously described that RcsB can accept phosphate from donors other than RcsC, such as acetyl phosphate (27), and that RcsC is able to act as a phosphatase on phosphorylated RcsB, rendering RcsB partially activated in an *rcsC* mutant background. This could explain the difference observed between *rcsB* and *rcsC* mutants. In accordance with this hypothesis, inactivation of *rcsC* in a wild-type background also led to a decrease in flagellin expression levels. Double mutants *wecD rcsF* and *wzyE rcsF* restored the flagellin expression under both growth conditions, although flagellin expression levels were lower than the level for the wild-type strain (12.5-fold lower for the *wzyE rcsF* strain and 10-fold lower for the *wecD rcsF* strain relative to that for the wild-type strain in liquid medium and 3.7- and 3.6-fold lower, respectively, relative to that for the wild-type strain in swarm plates). This may suggest that in the *wec* mutants the signal might not exclusively require RcsF to be channeled to the downstream components of the phosphorelay. In either *rcsC* or *rcsF* double mutants, although flagellin expression levels were lower than the level in the wild-type strain, the motility assays showed a complete restoration of swimming and a partial restoration of swarming, suggesting that this flagellin expression level would suffice to provide motility to the cell.

On the basis of the observed suppression of flagellar inhibition in the *wec* mutants by *rcs* inactivation, we can postulate a model in which either the absence of ECA or the accumulation of an intermediate metabolite of its biosynthesis activates the Rcs phosphorelay system. Phosphorylated RcsB would in turn repress *flhDC* and in this way diminish the flagellar regulon expression levels. This would explain the deficiency in swimming and swarming phenotypes and the reduction both in flagellin expression and in phospholipase activity observed in the *wec* mutant strains.

SMA1167 belongs to the Rcs regulon in *Serratia*. According to our proposed model, *wec* mutants should display upregulation of the Rcs-activated genes. However, the *S. marcescens* Rcs regulon has not yet been characterized. Therefore, in order to identify Rcs-regulated genes, we performed a bioinformatic search of genes with a putative RcsB-binding site in their promoter regions in the *S. marcescens* Db11 strain genome using the MEME/MAST motif detection programs (see Materials and Methods and references 6 and 7). With this approach we generated an RcsB recognition motif using a collection of previously defined RcsB-dependent promoters in *E. coli* (81), *Salmonella enterica* serovar Typhi (36), *Klebsiella pneumoniae* (4), *Erwinia amylovora* (13), *Pantoea stewartii* (85), and *Bordetella pertussis* and *B. parapertussis* (71) as the database.

As predicted, we found that *S. marcescens flhDC* displayed a putative RcsB motif in its regulatory region, which validated the search procedure (Fig. 4B). We also identified an Rcs-binding motif sequence in the promoter region of SMA1167 (Fig. 4B), annotated as UDP-glucose 4-epimerase in the *S. marcescens* Db11 genome (65). SMA1167 is the first gene of a cluster of 11 genes. Among these genes, three show homology to conserved genes (*wza*, *wzc*, and *wzx*) present in all group 1 capsule loci and four are annotated as glycosyltransferases (65). Taking into consideration the fact that the Rcs system activates the expression of genes necessary for the synthesis of polysaccharidic capsules in *E. coli*, *Klebsiella*, *Salmonella*, *Erwinia*, and *Pantoea* (57), we chose this gene to further examine if it could be used as a suitable reporter of the Rcs-regulated activity, independent from the flagellar cascade.

To determine whether SMA1167 belonged to the Rcs regulon, we quantitatively analyzed its transcriptional activity by real-time RT-PCR in a wild-type versus an *rcsB* background. We also evaluated high osmolarity, as it is a condition known to activate RcsCDB in other bacteria (78), and performed the same analysis to examine *flhD* expression levels.

As shown in Fig. 4A, the *rcsB* mutant strain showed higher levels of *flhD* expression than the wild-type strain (3.68-fold induction), indicating that, in *S. marcescens*, RcsB represses *flhD* as was previously described in *E. coli* and also as predicted by our *in silico* analysis with *Serratia*. In addition, *flhD* expression was repressed by high osmolarity in an RcsB-dependent fashion, showing that high osmolarity can act as an Rcs system-inducing signal in *Serratia*. The expression level of SMA1167 was reduced (11-fold) in the *rcsB* mutant relative to that for the wild-type strain. Under high-osmolarity conditions, SMA1167

B

RcsB consensus motif TRVGAAW:AWTSYGR

SMA1167 *S. marcescens* TCGGATT:AATCTCA

RcsB motif

flhD *E. coli* CGTTGTATGTGCGTGTAGTGACGAGTACAGTTGCGTCGATTTAGGAAAAATCTTAGATA..176b..ATG

flhD *S. enterica* CGTTGTATGTCACGAAGCTGACGAGTAGAGTTGCGTCGAATTAGGAAAAATCTTAGGCA..182b..ATG

flhD *S. marcescens* ATTGTGTGTGCTAATGGTTCGGGGGTAGAGTTGCGCTGCTTTAGGAATAATCTTATTAA..231b..ATG

-35 -10

FIG. 4. (A) Quantitative RT-PCR analysis of *flhD* and SMA1167 expression. Total RNA was extracted from the indicated strains grown to mid-exponential phase in LB medium or LB medium–0.45 M NaCl (indicated NaCl) at 37°C. *flhD* and SMA1167 expression was analyzed by real-time RT-PCR using specific primers (Table 1), and 16S rRNA was used as the internal control. Average relative expression and fold change in gene expression in each mutant strain compared with the expression of the wild-type (wt) strain grown in LB medium were calculated from triplicate samples as described in Materials and Methods. Error bars indicate standard deviations. Note the different scales for the *y* axes. (B) Predicted RcsB-binding motif in the promoter regions of *flhD* and SMA1167. The consensus RcsB motif is compared to the region detected in *S. marcescens* SMA1167 promoter. (Lower panel) Alignment of *E. coli*, *S. enterica*, and *S. marcescens flhD* promoters. In *E. coli* the identified RcsB-binding site is shaded in gray, the transcription initiation site is indicated with an arrow, and the corresponding −35 and −10 regions are underlined. In *S. enterica* and *S. marcescens*, these putative elements are also indicated to highlight the high degree of conservation with the *E. coli* sequence.

was induced 14-fold, and this induction was RcsB dependent. These results show that SMA1167 is an RcsB-activated gene in *S. marcescens*.

RcsB is highly activated in *wec* mutant strains. To analyze if, as predicted in our model, an RcsB-activated gene was induced in the *wec* mutants, we quantitatively analyzed SMA1167 transcriptional activity by real-time RT-PCR in different *Serratia* strains (wild type, *wecD*, *wzyE*, *wzyE*/pYG, *rcsB*, *wecD rcsB*, and *wzyE rcsB*) grown to exponential phase in LB liquid medium and from swarmer cells.

As we have previously described, *flhD* transcription was repressed (for *wecD* and *wzyE*, 3.8- and 4.8-fold repression, respectively, under swimming conditions and 10- and 12.5-fold repression, respectively, under swarming conditions) in the *wec* mutant strains relative to that in the wild-type strain (15) (Fig. 5A). *rcsB* inactivation in the *wecD* and *wzyE* mutant strains suppressed the *flhD* repression, indicating that, in the *wec* mutant strains, *flhD* repression is RcsB dependent.

Conversely, SMA1167 transcription was highly induced (41-fold induction under swimming conditions and 68-fold under swarming conditions) in the *wecD* mutant strain and (38-fold induction under swimming conditions and 56-fold under swarming conditions) in the *wzyE* mutant strain, and this induction was lost when the mutation in *rcsB* was introduced (i.e., in the *wecD rcsB* or in the *wzyE rcsB* double-mutant strains) (Fig. 5B). These results clearly corroborate the idea that RcsCDB system activity is triggered by the signal originated by the blockage in the expression of the ECA biosynthetic pathway genes and rule out the possibility that the *rcs* mutations were merely alleviating or counteracting *flhDC* transcriptional downregulation by an independent pathway.

In addition, the analysis of the transcriptional activity of *flhD* and SMA1167 also indicates that while *flhD* is induced in swarming plates independently of RcsB, SMA1167 transcription was not altered by the swimming or swarming growth condition.

The Rcs system is induced in *wecA* mutants. The ECA trisaccharide repeat unit is synthesized in stepwise fashion at the inner membrane by the successive transfer of the component amino sugars to the lipid carrier, undecaprenyl monophosphate (Und-P) (Fig. 5B) (24, 69). WecA catalyzes the transfer of GlcNAc-1-phosphate from UDP-GlcNAc onto Und-P, yielding Und-P-P-GlcNAc, also known as lipid I. It is important to highlight that this lipid intermediate is involved not only in the synthesis of ECA but also in the synthesis of O-antigen polysaccharides containing GlcNAc as the first carbohydrate (2). ManNAc is then transferred from UDP-ManNAcA to lipid I to yield Und-PP-GlcNAc-ManNAcA (lipid II). Finally, Fuc4NAc is transferred from TDP-Fuc4NAc to lipid II to yield a complete lipid-linked trimer, Und-PP-GlcNAc-ManNAcA-Fuc4NAc (lipid III). Subsequent steps involve membrane translocation, polymerization, transfer of the polymer to a phospholipid aglycone or to the LPS core, and translocation to the outer membrane. However, the mechanisms of these events have not yet been completely established.

The diverse mutations in the *wec* cluster identified in this study interfere at different levels of the ECA biosynthetic pathway (Fig. 6B). The insertion in *wecD* blocks the conversion of lipid II to lipid III, while inactivation of *wecG* blocks the synthesis of lipid II. Mutations in *wzxE* and *wzyE* preclude the translocation or subsequent *en bloc* polymerization of the undecaprenylpyrophosphate-linked trisaccharide, which takes place at the periplasmic side of the bacterial inner membrane. As described, the *wecG*, *wecD*, *wzxE*, and *wzyE* mutant strains

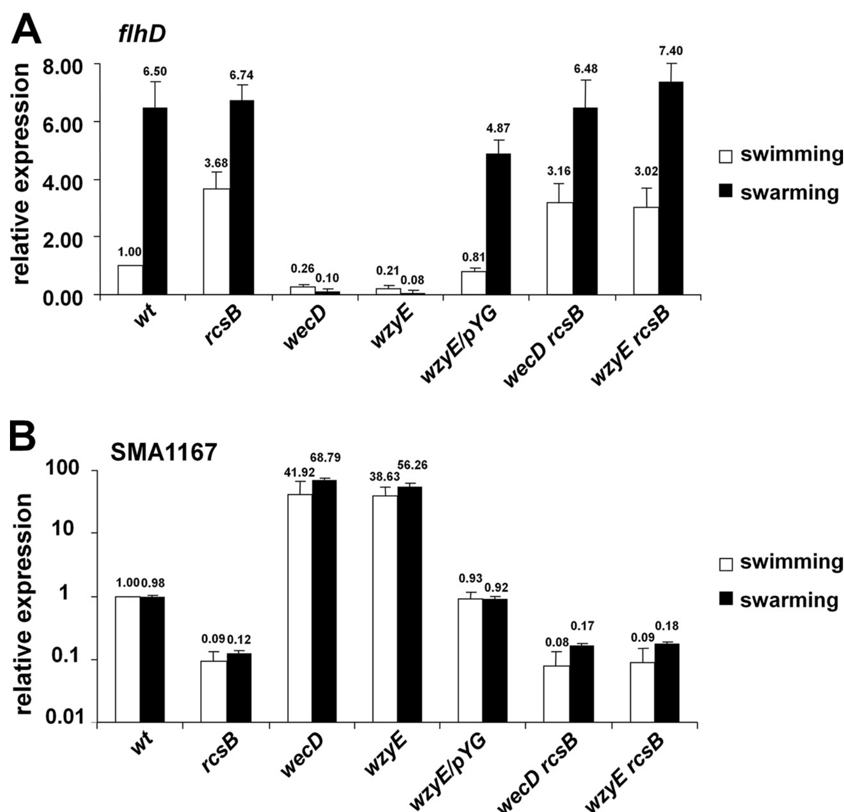

FIG. 5. Quantitative RT-PCR analysis of *flhD* (A) and SMA1167 (B) expression under swimming and swarming conditions. Total RNA was extracted from the indicated strains grown to mid-exponential phase in liquid LB medium (swimming) or obtained from the edge of LB medium–0.6% agar plates (swarming). *flhD* and SMA1167 expression was analyzed by real-time RT-PCR using specific primers (Table 1), and 16S rRNA was used as the internal control. Average relative expression and fold change in gene expression in each mutant strain compared with the expression of the wild-type (wt) strain grown in LB medium were calculated from triplicate samples as described in Materials and Methods. Error bars indicate standard deviations. Note the different scales for the *y* axes.

show the same phenotype of flagellar downregulation; and in the case of the *wecG*, *wecD*, and *wzyE* mutants, we have shown that this repression is due to the induction of the Rcs phosphorelay.

To analyze the blockage of ECA synthesis at its earliest step precluding ECA assembly and at the same time to examine whether accumulation of intermediate metabolites could be responsible for Rcs induction, we constructed a *wecA* mutant using the pKnock-Cm suicide plasmid (see Fig. 6B for the *wecA* role in the ECA pathway). The immunodetection of ECA and the results of LPS analysis shown in Fig. 7A and B, respectively, demonstrate that, as predicted, this mutant lacked both ECA and O-antigen moieties. The *wecA* mutant strain showed almost undetectable levels of flagellin expression, as shown by immunodetection assay (Fig. 7C). Using real-time RT-PCR, we determined that transcription of *flhD* was repressed (3.44-fold repression) and that transcription of SMA1167 was highly induced (40.32-fold induction) in the *wecA* mutant relative to their expression in the wild-type strain (Fig. 7D). This result shows that abolishing ECA biosynthesis at the earliest step in the pathway also induces the Rcs system.

In addition, the fact that strains with different insertion mutations causing blockage of ECA synthesis at different steps exhibit the same phenotype suggests that the structural lack of ECA in the bacterial envelope rather than the accumulation of biosynthetic intermediates, such as lipid I (accumulated in *wecG*) or lipid II (accumulated in *wecD*), could constitute the Rcs system input signal.

The potential role of ECA_{CYC} in induction of Rcs system. The polysaccharidic moieties of ECA_{PG} and ECA_{CYC} are assembled by a common biosynthetic pathway. However, no details concerning the mechanism of the cyclization reaction are known (43). WzzE modulates the ECA polysaccharide chain length to yield a modal population of polymers (Fig. 6). In *E. coli wzzE* mutants, ECA_{PG} molecules show a random nonmodal distribution of polysaccharide chain lengths. Intriguingly, it was previously found that nonpolar mutations in *wzzE* block the synthesis of ECA_{CYC}, indicating that chain length regulation is a prerequisite for the assembly of ECA_{CYC} molecules (43).

To explore if the observed phenotypes of the *Serratia wec* mutants could be the consequence of the ECA_{CYC} absence, we analyzed a *wzzE* insertion mutant. The ECA exopolysaccharide was isolated, analyzed by SDS-PAGE, and detected with anti-O14 antiserum that specifically recognizes ECA epitopes (Fig. 7A). We observed that, as in *E. coli*, the *wzzE* mutant strain was able to synthesize ECA_{PG}, although with a different modal distribution, showing multiple high-molecular-weight bands not present in the pattern for the wild-type strain (Fig. 7A; compare the bands in lanes *wt* and *wzzE* indicated with an

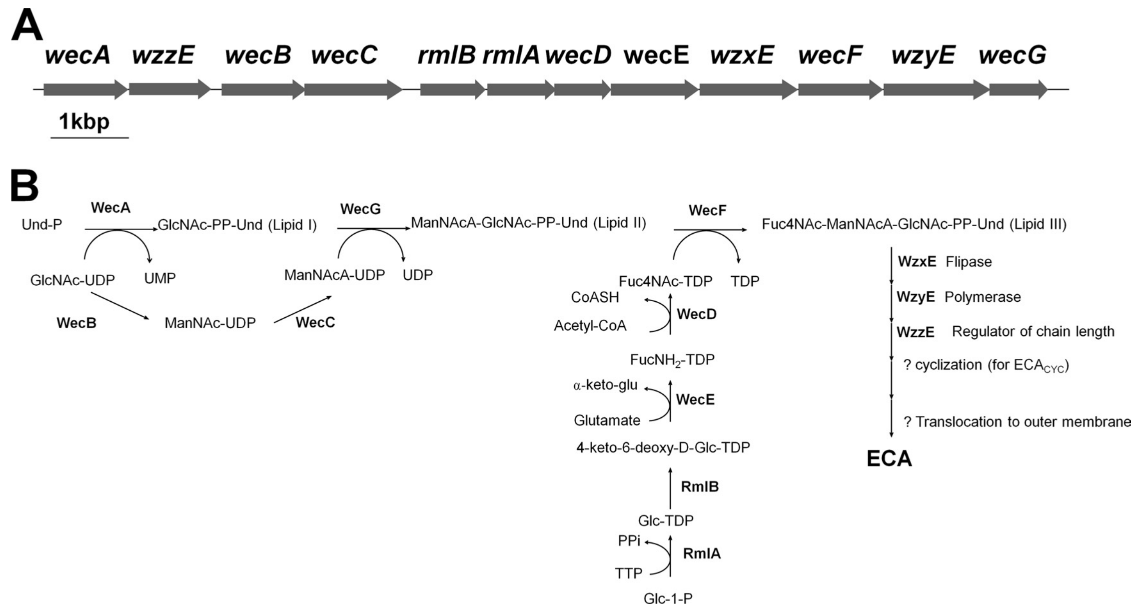

FIG. 6. Biosynthetic pathway for the assembly of ECA. (A) Schematic representation of the *wec* cluster in *S. marcescens*; (B) enzymatic reactions and enzymes involved in the biosynthesis of ECA. Abbreviations: Und-P, undecaprenyl monophosphate; Und-PP, undecaprenyl pyrophosphate; TTP, dTTP; TDP, dTDP; PP_i, inorganic pyrophosphate; Glc-1-P, glucose-1-phosphate; α-keto-glu, α-ketoglutarate; 4-keto-6-deoxy-D-Glc, 4-keto-6-deoxy-D-glucose, acetyl-CoA, acetyl coenzyme A; CoASH, coenzyme A; GlcNAc, *N*-acetylglucosamine; ManNAc, *N*-acetylmannose; ManNAcA, *N*-acetyl-D-mannosaminuronic acid; FucN, fucosamine; Fuc4NAc, 4-acetamido-4,6-dideoxy-D-galactose.

asterisk). LPS analysis showed that the *wzzE* mutant displayed a pattern identical to that observed in the wild-type strain (Fig. 7B). We next examined the flagellin expression levels and observed that the *wzzE* mutant strain showed lower levels of flagellin expression than the wild-type strain (Fig. 7C). In addition, we performed real-time RT-PCR on both *flhD* and SMA1167 to determine Rcs induction levels in the *wzzE* mutant background. As shown in Fig. 7D, *flhD* expression was downregulated (4.16-fold repression) and SMA1167 was highly induced (18-fold induction) in the *wzzE* mutant relative to their expression in the wild-type strain. Although direct determination of ECA_{CYC} was not performed, these results allowed us to speculate that the absence of ECA_{CYC} may be responsible for RcsCDB induction in the *wec* mutant strains analyzed here. However, we cannot exclude the possibility that in the *wzzE* mutant, instead of the absence of ECA_{CYC}, the presence of an altered ECA_{PG} structure with an atypical chain length pattern could disturb the envelope integrity and signal on Rcs.

DISCUSSION

In this work we determine that disruption of the ECA biosynthetic pathway in *S. marcescens* leads to the activation of the phosphorelay RcsCDB signal transduction system. Previously, we have demonstrated that the expression and secretion of the PhlA phospholipase was inhibited in *Serratia* strains with mutations in the *wec* cluster. PhlA is part of the flagellar regulon, and the observed inhibition was found to be due to the transcriptional downregulation of *fhlDC*, which codes for the master flagellar regulator (15). We now identify the regulatory mechanism involved in the detection and transduction of the signal. We show that mutations in *rcsC*, *rcsB*, and *rcsF* suppress the *flhDC* downregulation determined in the *wec* mutant strains, restoring motility and phospholipase secretion.

The RcsCDB phosphorelay is known to act as a global regulatory network, controlling multiple cellular pathways, including capsule synthesis, cell division, motility, biofilm formation, and virulence mechanisms (57). It has been reported that the Rcs phosphorelay system regulates the *flhDC* flagellar master operon in *E. coli* and *Salmonella.* This regulation is negative and requires an RcsB box located downstream of the *flhD* transcription initiation site (26, 84). In *S. marcescens*, an RcsB conserved motif is present in the promoter region of *flhD* at a position equivalent to that of the RcsB-binding box identified in *E. coli* and *Salmonella* (Fig. 4B).

The observed Rcs activation in the ECA-deficient strains involves RcsF. RcsF has motifs characteristic of a lipoprotein anchored in the outer membrane. In the Rcs signal transduction pathway, it has been shown that RcsF acts upstream of RcsC; however, not all Rcs-inducing signals require RcsF to be detected by the system (29, 58). Besides, the mechanism by which this lipoprotein is activated by upstream signals and how the signal is transduced to RcsC remain to be explored. The engagement of RcsF suggests that the blockage of ECA biosynthesis induces a stress that originates either in the outer membrane or in the periplasmic compartment rather than in the cytoplasm or cytoplasmic membrane.

There are contradicting reports about the role of the auxiliary protein RcsA in the repression of *flhDC*. Using *in vitro* footprinting, RcsA was found to bind an RcsAB box in the promoter region of *flhDC* in *E. coli* (26). However, RcsA is not involved in motility regulation in *E. coli* (27). In *S. enterica*, microarray data did not show a requirement for RcsA in the repression of the flagellar genes by RcsB (84), and earlier

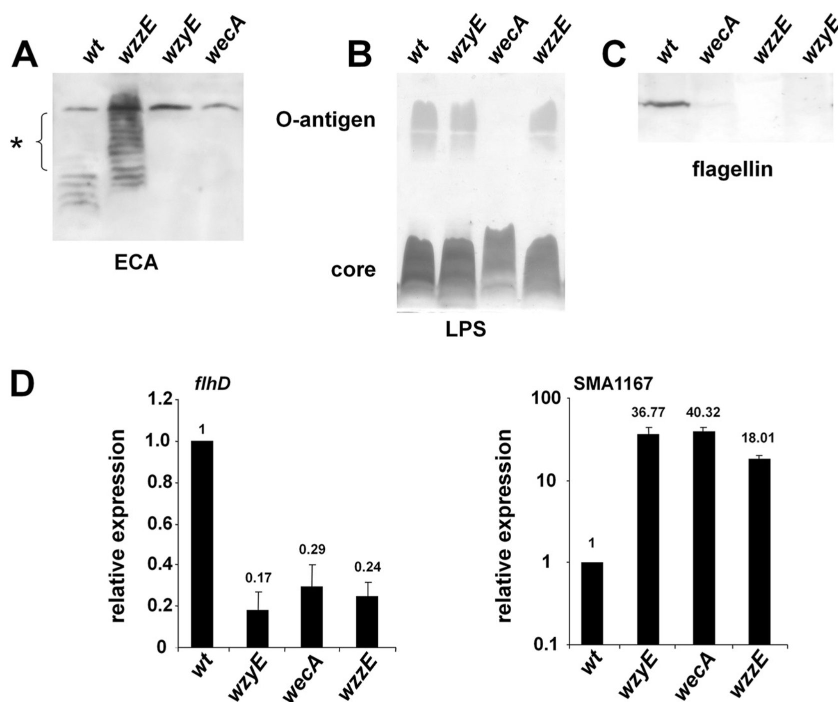

FIG. 7. Phenotypic analysis of *S. marcescens wecA* and *wzzE* mutant strains. Exopolysaccharide preparations of the wild-type (wt) and mutant strains were analyzed by immunodetection with anti-O14 antiserum to detect ECA (A) or by silver staining to examine LPS (B). The localization of the high-molecular-weight bands pattern in ECA (*) and the core and O antigen in LPS are indicated. (C) Flagellin immunodetection. Whole-cell extracts from cultures of the indicated strains grown in LB medium overnight at 37°C were analyzed by Western blotting, in which the blots were developed with antiflagellin polyclonal antibodies. Loaded samples were standardized as described in Materials and Methods. (D) Quantitative RT-PCR analysis of *flhD* and SMA1167 expression. Total RNA was extracted from the indicated strains grown to mid-exponential phase in LB medium at 37°C. *flhD* and SMA1167 expression was analyzed by real-time RT-PCR using specific primers (Table 1), and 16S rRNA was used as the internal control. Average relative expression and fold change in gene expression in each mutant strain compared with the expression of the wild-type strain grown in LB were calculated from triplicate samples as described in Materials and Methods. Error bars indicate standard deviations. Note the different scales for the *y* axes.

studies showed no effect of an *rcsA* mutation on the transcription of *flhD* (14). Moreover, in *S. enterica*, swarming is apparently more sensitive than swimming to changes in the transcription profile of flagellar genes. An *rcsA* mutation affected swarming at the initiation stage of this motile behavior, and it has been speculated that this could be due to enhanced inhibition of *flhDC* by RcsB in the absence of RcsA (84). Our results indicate that in *S. marcescens* RcsA would not act together with RcsB in *flhDC* repression. The effect of *rcsA* observed in the swarming phenotype could be due to RcsA action on exopolysaccharide synthesis or on the expression of other factors that might influence motility on the surface. This is consistent with the notion that, in contrast to swimming, swarming motility involves a complex cellular differentiation process (48, 83).

The biochemical identity of the inducer leading to activation of the RcsCDB phosphorelay has remained elusive. However, a variety of environmental stimuli (high osmolarity, chlorpromazine treatment) and genetic mutations (*igaA*, *dsbA*, *rfa*, *mdoH*, *opg*) and the overexpression of some proteins (DjlA, LolA, OmpG) have been demonstrated to promote the activity of the system. A common feature of these inducing conditions is that they all produce changes in the properties of the cell envelope (57).

Different reports have previously linked LPS synthesis with Rcs induction. Mutations in *rfa* genes in *E. coli*, required for the synthesis of lipopolysaccharide, induce colanic acid synthesis, and this induction is dependent on *rcsF* and *rcsC* (66). Girgis et al. (31) performed a genome-wide screen of loci involved in motility in *E. coli* and identified that mutants with large LPS truncations were defective in both swimming and swarming. In addition, they found that deletion of *rcsB*, *rcsD*, or *rcsF* was sufficient to rescue motility. Other observations indicate that accumulation of dTDP-glucose due to mutations in *pgi*, rendering an *E. coli* strain unable to properly metabolize glucose-6-phosphate, induces capsule synthesis in an Rcs-dependent fashion (23). dTDP-glucose is a common precursor for O antigen and ECA oligosaccharides, and it has been suggested that its accumulation could perturb LPS synthesis, inducing capsule expression as in the above-mentioned *rfa* mutants (57). In *Proteus mirabilis*, the loss of O antigen due to a mutation in either *wzz* (encoding a chain length determinant for O antigen) or *waaL* (coding for the O-antigen ligase) blocked swarming motility on solid surfaces but had little effect on swimming motility in soft agar (64). A mutation in *rcsB* or the overexpression of *flhDC* restored swarming to the *waaL* mutant, despite the absence of O antigen. Interestingly, a mutation in *wzyE*, which encodes the enzyme that polymerizes ECA, had no effect on motility in this bacterium. The fact that we demonstrate here that a *wecA* mutant strain which simul-

taneously displays deficient ECA and O-antigen assembly shows identical Rcs-associated phenotypes as the *wec* mutants, which disrupt only ECA biosynthesis, clearly indicates that in *S. marcescens*, alteration of ECA is sufficient to induce the Rcs regulatory system.

In addition, mutations in another component of the cell surface, the osmoregulated periplasmic glucans (OPGs), similarly induce the Rcs system (21). The OPGs maintain the periplasmic osmolarity when Gram-negative bacteria are subjected to low-osmolarity stress, preventing the entrance of water to the cytoplasm and subsequent cell lysis (54). In either *Salmonella* or *E. coli*, disruption of the OPG biosynthetic genes (i.e., the *opgGH* operon) results in pleiotropic phenotypes such as a slower growth rate under hyposmotic growth conditions and reduced motility (25, 31, 45). In *E. coli*, inactivation of *rcsB*, *rcsD*, or *rcsF* leads to restoration of motility in *opg* mutants (21, 31). Similarly, *Dickeya dadantii opg*-negative mutants display a loss of virulence, decreased motility, and increased exopolysaccharide synthesis; and this pleiotropic phenotype can be restored by mutations in the RcsCDB phosphorelay system (10).

Three variants of ECA have been described: ECA_{PG} and ECA_{LPS}, both in the outer leaflet of the outer membrane, and soluble ECA_{CYC}, exclusively in the periplasm (43). Although the three forms of ECA are composed of the same polysaccharide unit, their physicochemical properties are quite different. According to the described biosynthetic pathway, mutations in *wecA*, *wecD*, *wzyE*, *wzxE*, or *wecG* should impede the synthesis of the three forms of ECA. Our analysis of these mutations in *Serratia* suggests that the absence of any of the three different ECA molecules could be a signaling on Rcs. On the contrary, a nonpolar *E. coli wzzE* mutant synthesizes ECA_{PG} molecules with a random nonmodal distribution of polysaccharide chain lengths and blocks the synthesis of ECA_{CYC} (43). We constructed an *S. marcescens wzzE* mutant and determined that *flhD* was repressed, while SMA1167 was highly induced compared to the expression level for the wild-type strain, indicating induction of the Rcs system. As in *E. coli*, the *Serratia wzzE* mutant synthesizes ECA_{PG} with a modal distribution different from the wild-type strain distribution pattern. In addition, ECA_{CYC} localizes in the periplasm, exhibiting a cyclic structure similar to that of OPGs synthesized by Gram-negative bacteria. As mentioned above, absence of OPGs has been demonstrated to activate the Rcs system. Therefore, it is tempting to speculate that both types of molecules could have similar functions in the bacterial periplasm, acting as a signal to the Rcs system, although further analysis will be required to corroborate the absence of the ECA_{CYC} in the *S. marcescens wzzE* mutant strain.

Interestingly, one of the mini-Tn*5* Km insertions identified in this study was located in *mosC*, a gene located upstream of *rcsD*, presumably forming part of an operon together with *rcsD* and *rcsB*. MosC has homology to transporters of the major facilitator superfamily (MFS) and is also coded by a sequence adjacent to the *rcs* genes, possibly forming an operon with these genes in other bacteria, such as *Serratia proteamaculans*, *Yersinia enterocolitica*, *Yersinia pestis*, *Yersinia pseudotuberculosis*, and *Erwinia carotovora*. There is increasing evidence of auxiliary factors different from HK RR influencing two-component system (TCS) activity (12, 82). In many cases sensors employ transport proteins as cosensors (82). In fact, there are about 70 examples of genes encoding TCSs located adjacent to genes encoding ABC transporters in *Bacillus* and *Clostridium*. It has been proposed that TCSs and their cognate ABC transporters evolved as a unit and that both systems participate in a physiological process (42, 60). Other interesting examples include DcuB fumarate/succinate antiporter and the TCS DcuS/DcuR (41), the PstSCAB2 ABC transporter and the TCS PhoR/PhoB (39), and the bacitracin ABC transporter BceAB and the TCS BceS/BceR (70). In all these examples, direct interactions between transporter and sensor are postulated, and it is proposed that these interactions influence the HK enzymatic activity. However, the mechanistic and structural details of cosensing are still missing. In this context, we are currently analyzing whether MosC performs an analogous role as an auxiliary component of the Rcs transduction system.

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 (5, 53, 68). In light of our results, the virulence defects detected can be attributed to the inability of these pathogens to adequately modulate the activity status of the Rcs regulon, which determines essential virulence-related phenotypes such as motility and exopolysaccharide production.

In sum, our findings underscore a new role for ECA in the control of Rcs phosphorelay system activity, suggesting that this exopolysaccharidic structure is relevant for the integrity of the bacterial cell envelope and also providing a basis to further understand the significance for the phylogenetic conservation of the *wec* gene cluster among a broad range of enterobacteria.

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