

Vibrio parahaemolyticus *scrABC*, a Novel Operon Affecting Swarming and Capsular Polysaccharide Regulation

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Swarming is an adaptation of many bacteria to growth on surfaces. A search for genes controlling swarmer cell differentiation of *Vibrio parahaemolyticus* identified a novel three-gene operon that potentially encodes a pyridoxal-phosphate-dependent enzyme, an extracellular solute-binding protein, and a membrane-bound GG-DEF- and EAL-motif sensory protein. The functions of these motifs, which are named after conserved amino acid sequences, are unknown, although the domains are found singly and in combination in a variety of bacterial signaling proteins. Studies with translational fusions supported the predicted localization of the gene products. When the operon was overexpressed, swarmer cell gene transcription was induced in liquid culture. Mutants with defects in any of the three genes exhibited decreased swarming and lateral flagellar (*laf*) gene expression. Complementation studies confirmed an operon organization and suggested that all three genes participated in *laf* regulation. The lesions that decreased swarming increased capsular polysaccharide (CPS) production, and overexpression of the operon inhibited transcription of the CPS gene *cpsA*. Thus, the *scrABC* locus appears to inversely regulate two gene systems that are pertinent to colonization of surface swarming and CPS.

Vibrio parahaemolyticus is a ubiquitous marine bacterium and human pathogen that exists in multiple cell types appropriate for life under different circumstances (reviewed in reference 17). One type, the swimmer cell, is adapted to life in liquid environments. It is rod shaped and ca. 2 μm in length and possesses a single polar flagellum that propels it through liquid. Another cell type, the swarmer, is adapted to life on surfaces. It is an elongated cell ($\sim 30 \mu\text{m}$) and possesses numerous peritrichously arranged flagella, called lateral flagella. These lateral flagella enable the bacterium to move over and colonize surfaces in a process called swarming.

V. parahaemolyticus controls differentiation from a swimmer to a swarmer cell in two known ways: surface recognition resulting from interference with rotation of the polar flagellum and nutritional limitation for iron (reviewed in reference 14). How these physical and chemical signals are transduced to regulate gene expression remains to be discovered. Prior work suggested that positive regulators might be involved (22). A mutant with a defect in a gene, *lonS*, encoding a homolog to the *Escherichia coli* Lon protease constitutively expressed lateral flagellar genes and produced long cells in liquid. Because of this constitutive swarmer cell phenotype and by analogy to the role of the Lon protease in *E. coli*, it was hypothesized that LonS targets a transcriptional activator of lateral flagellar genes and a cell division inhibitor. This work describes a search for additional genes positively controlling swarmer cell differentiation. Putative DNA-binding transcriptional activators of transcription were not identified; however, a novel operon was discovered and characterized.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains and plasmids used in the present study are described in Table 1. All *V. parahaemolyticus* strains were derived from the wild-type strain BB22TR (3) and cultured at 30°C. HI broth contained 25 g of heart infusion broth (Difco) and 20 g of NaCl per liter. Swarm plates were prepared by adding 15 g of Bacto Agar (Difco) per liter to HI broth, and nonswarm plates contained 20 g of agar per liter. Antibiotics were used at final concentrations of 50 μg of kanamycin, 10 μg of tetracycline, 30 μg of gentamicin, and 10 μg of chloramphenicol/ml. The final IPTG (isopropyl- β -D-thiogalactopyranoside) concentration was 0.1 mM.

Genetic and molecular techniques. General DNA manipulations were adapted from the methods of Sambrook et al. (20). Insertion/deletion mutations were made by using a λ Red recombinase system to introduce the mutations onto cosmids in *E. coli* (6). The procedures for conjugation and gene replacement in *Vibrio parahaemolyticus* have been previously described (21). All strain constructions were confirmed by Southern blot analysis of restricted chromosomal DNA. Chromosomal DNA was prepared according to the protocol of Woo et al. (26). A locus encoding extracellular polysaccharide (*cps*) was identified in a screen, which will be described elsewhere, for biofilm-defective mutants. The *cps* locus was retrieved on a cosmid by complementation of the biofilm-defective mutant and a promoterless *lacZ* fusion (with kanamycin resistance) was introduced into the *cpsA* gene, which encodes a potential capsular polysaccharide (CPS) biosynthesis glycosyl transferase that is most homologous to VC0934 of *V. cholerae*.

The 15.7-kb *scrABC* subclone, pLM2796, was generated by digesting cosmid pLM2032 with *Eco47III* and ligating a 6-kb band into pLM1877 (Fig. 2). The *scrA* subclone, pLM2876, was made by digesting pLM2796 with *DrdI* and *PshAI*, removing the 2.9-kb band containing *scrBC* and religating the 12.7-kb band containing *scrA*. To create the *scrB* subclone, pLM2878, pLM2896 was digested with *XhoI* and *SmaI* to remove a 0.73-kb fragment containing *scrA* creating an in-frame deletion (pLM2879). A 2.5-kb DNA fragment containing *scrC* was then removed by an *AvrII* digest of pLM2879. The remaining 12.5-kb band containing only an intact *scrB* was ligated, creating pLM2878. The *scrC* gene was amplified by high-fidelity PCR (Boehringer Mannheim) and cloned into pLM1877. Plasmids were mutagenized with λ Tn*phoA*_m or λ Tn*lacZ*_m (11) or by restriction and insertion of a drug cassette. Active alkaline phosphatase or β -galactosidase fusion production was detected by screening for blue colonies on plates with the chromogenic substrates BCIP (5-bromo-4-chloro-3-indolylphosphate) *p*-toluidine salt or X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside). The precise location of each transposon insertion was determined by DNA sequencing with a primer derived from one end of the transposon. Sequence determination was performed by the DNA Core Facility of the University of Iowa. The GenBank nucleotide sequence accession number for the *scr* locus is AYO26362.

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or description ^a	Source or parent strain and/or reference
Strains		
BB22TR	Wild type; translucent	R. Belas et al. (3)
LM1017	<i>lfgE313::lux</i> ; <i>lux</i> fusion in lateral flagellar hook gene	BB22TR (15)
LM4170	<i>motX118 lfgE313::lux</i>	LM1017 (16)
LM4851	LM1017/pLM1877	This work
LM4897	<i>scrB1::TnphoA_{in}</i>	BB22TR (this work)
LM5126	LM1017/pLM2032	This work
LM5139	LM1017/pLM2449	This work
LM5140	LM1017/pLM2502	This work
LM5153	LM1017/pLM2341	This work
LM5200	LM1017/pLM2102	This work
LM5435	LM1017/pLM2796	This work
LM5545	Δ <i>scrA1::Cam^r lfgE313::lux</i>	LM1017 (this work)
LM5547	<i>scrB1::TnphoA_{in} lfgE313::lux</i>	LM1017 (this work)
LM5549	Δ <i>scrC1::Kan^r lfgE313::lux</i>	LM1017 (this work)
LM5719	Δ <i>scrC1::Kan^r</i>	BB22TR (this work)
LM5733	Δ <i>scrA1::Cam^r</i>	BB22TR (this work)
LM5753	LM1017/pLM2936	This work
LM5789	<i>scrB1::TnphoA_{in} motX118 lfgE313::lux</i>	LM 4170 (this work)
LM5792	Δ <i>scrA2::Kan^r lfgE313::lux</i>	LM1017 (this work)
LM5793	Δ <i>scrA2::Kan^r</i>	BB22TR (this work)
LM5811	LM5818/pLM1877	This work
LM5812	LM5818/pLM2449	This work
LM5813	LM5818/pLM2796	This work
LM5818	<i>cpsA1::lacZ(Kan^r)</i>	BB22TR (this work)
LM5831	<i>cpsA1::lacZ(Kan^r)ΔscrA1::Cam^r</i>	LM5733 (this work)
Plasmids		
pKD3	Cam ^r template plasmid	6
pKD11	Kan ^r template plasmid	6
pBSL14	Kan ^r cloning vector	1
pLM1877	Gen ^r expression vector	5
pLM2032	Tet ^r cosmid containing <i>scr</i> locus	<i>V. parahaemolyticus</i> cosmid bank (13)
pLM2102	Tet ^r pLAFRII control cosmid from bank	7
pLM2341	Tet ^r Cam ^r cosmid pLM2032 with <i>scrB1::TnphoA_{in}</i> ; fusion at aa 195	pLM2032 (this work)
pLM2449	Gen ^r , IPTG-inducible <i>scrC⁺</i>	pLM1877 (this work)
pLM2502	Gen ^r Cam ^r pLM2449 carrying <i>scrC309::TnlacZ_{in}</i> ; fusion at aa 309	pLM2449 (this work)
pLM2796	Gen ^r , IPTG-inducible <i>scrA⁺B⁺C⁺</i>	pLM1877 and pLM2032 (this work)
pLM2797	Tet ^r Kan ^r cosmid pLM2032 with Δ <i>scrC1::Kan</i>	pLM2032 (this work)
pLM2811	Tet ^r Cam ^r cosmid pLM2032 with Δ <i>scrA1::Cam</i>	pLM2032 (this work)
pLM2876	Gen ^r , IPTG-inducible <i>scrA⁺</i>	pLM1877 and pLM2796 (this work)
pLM2878	Gen ^r , IPTG-inducible <i>scrB⁺</i>	pLM1877 and pLM2796 (this work)
pLM2879	Gen ^r , IPTG-inducible <i>scrB⁺C⁺</i>	pLM1877 and pLM2796 (this work)
pLM2916	Tet ^r Kan ^r cosmid pLM2032 with Δ <i>scrA2::Kan</i>	pLM2032 (this work)
pLM2936	Gen ^r Cam ^r pLM2976 with <i>scrA72::TnlacZ_{in}</i> ; fusion at aa 72	pLM2796 (this work)

^a Cam^r, chloramphenicol resistance; Kan^r, kanamycin resistance; Gen^r, gentamicin resistance; Tet^r, tetracycline resistance.

RNA was prepared as previously described (9), and reverse transcription-PCR (RT-PCR) performed according to Promega (Madison, Wis.).

Luminescence assays. Bioluminescence was monitored by exposing colonies or microtiter dishes in a Fujifilm luminescent image analyzer dark box (LAS-1000) for 1 to 5 s. Luminescence was quantified in a TD20/22 luminometer (Turner Designs) by measuring 0.1-ml samples of culture appropriately diluted to yield a linear response. Luminescence is reported as specific light units (SLU), which are relative light units per minute per milliliter per unit of optical density at 600 nm (OD₆₀₀). All light experiments were performed at least twice, with the activity of each sample measured in triplicate.

β -Galactosidase assays. Strains were grown on HI plates at 30°C for 12 h, harvested from the plates, and diluted to an OD₆₀₀ of 2. β -galactosidase enzyme activity was assayed by using the chloroform-sodium dodecyl sulfate (SDS) lysis procedure (18). Assays were performed at least twice, with the activity of each sample determined in triplicate.

Electron microscopy. Bacterial colonies were grown on HI plates at 30°C for 2 days and fixed by flooding the plate with 2.5% glutaraldehyde for 1 h at 4°C, followed by washing with 0.2 M cacodylate buffer (pH 7.2). Colonies were cut out of the agar plates and stained with 1% osmium tetroxide for 1 h, washed, and dehydrated in a series of acetone concentrations (25, 50, 75, 95, and 100%).

Samples were then dried chemically with hexamethyldisilazane, coated with tungsten for surface conductivity, and examined with a Hitachi S4000 scanning electron microscope.

Cell fractionation and immunoblot analysis. Cell fractions were prepared from 1-ml overnight cultures. To prepare whole membranes and cytoplasmic fractions, cells were pelleted and suspended in 0.5 ml of 50 mM Tris-HCl (pH 8)–50 mM EDTA–15% sucrose–0.3 mg of lysozyme per ml, incubated for 30 min on ice, and then centrifuged 5 min (13). The pellet was suspended in 0.25 ml of cold H₂O. The sample was passed through a 22-gauge needle to reduce DNA viscosity. After a 15-min centrifugation, the pellet was suspended in 500 μ l of Laemmli sample buffer (LSB) (10) and used as the whole membrane fraction. A 100- μ l sample of the supernatant was taken, diluted 1:2 in 2 \times LSB and used as the cytoplasmic fraction. To prepare periplasmic fractions (2), 1 ml of overnight culture was centrifuged and suspended in 100 μ l of 10 mM Tris-HCl (pH 8). To this, 20 μ l of chloroform was added, followed by incubation on ice for 5 min, and then 150 μ l of cold 10 mM Tris-HCl (pH 8) was added. The sample was centrifuged for 5 min, the supernatant was transferred to a new tube, and centrifuged of another 5 min. A 150- μ l volume of supernatant was mixed with 150 μ l of 2 \times LSB and used as the periplasmic fraction. Immunoblot analysis was performed as previously described (5) by using anti-alkaline phosphatase anti-

serum from 5-Prime 3-Prime (Boulder, Colo.) and anti- β -galactosidase anti-serum from Chemicon International (Temecula, Calif.).

RESULTS

Identification of a swarmer cell regulatory operon. To identify genes involved in the regulation of swarmer cell development, a luminescent reporter strain was used. This strain, LM1017, contains an insertion of the *luxCDABE* operon within the gene encoding the lateral flagellar hook protein, *lfgE*. The strain is unable to swarm or produce lateral flagella and makes light when grown on a surface but not when grown in liquid (15). Thus, strain LM1017 exhibits surface-regulated *lux* reporter gene expression. A cosmid library of *V. parahaemolyticus* DNA was introduced into LM1017 and cosmids were identified that increased *lfgE::lux* expression in liquid, i.e., constitutive lateral flagellar gene expression.

The cosmid library, which contained ~25-kb inserts of *V. parahaemolyticus* DNA (13), was conjugated with LM1017 and ~5,000 transconjugants were screened for the ability to produce light in liquid by monitoring light expression in microtiter wells. The cosmid backbone was pLAFRII, which contains a pLac promoter (7). Transconjugants appearing bright in microtiter wells were confirmed by measuring light of cultures grown in test tubes. Four classes of cosmids, based on DNA homology, were identified that caused constitutive *lfgE::lux* expression. One of these cosmids, pLM2032, which conferred the greatest *lfgE::lux* induction in strain LM5126, was selected for further studies. Induction of luminescence in this strain is shown in Fig. 1A.

To identify genes responsible for the ability of pLM2032 to cause constitutive *lfgE::lux* expression, the cosmid was mutagenized with *TnphoA_{in}* and *TnlacZ_{in}*, transferred to LM1017, and screened for mutations that abolished constitutive *lfgE::lux* expression. Strain LM5153, carrying a *TnphoA_{in}*-mutated cosmid (pLM2341), was found to be unable to induce *lfgE::lux* in liquid (Fig. 1A). The point of transposon insertion on this cosmid, pLM2341, was sequenced and the insertion mapped in the second open reading frame (*scrB*) of a potential three-gene operon. To confirm whether this potential operon was responsible for *lfgE::lux* expression in liquid, an *Eco47III* fragment containing only the three open reading frames was subcloned from the cosmid into pLM1877 and named pLM2796. This clone contained sufficient genetic information to cause *lfgE::lux* expression in liquid (Fig. 1B). The potential operon it contained, *scrABC*, is depicted in Fig. 2. Although the maximal level of light produced by strain LM1017 containing pLM2796 (~35,000 SLU) was lower than that produced by the cosmid-containing strain (~350,000 SLU), it was significantly higher than strain LM1017 containing the vector (~500 SLU). The difference between light induced by the cosmid and the subclone may be due to copy number or promoter differences between the vectors. Furthermore, introduction of insertion mutations in *scrA* or *scrC* genes carried by pLM2796 abolished the ability of the subclone to cause light production in liquid (Fig. 1B).

The first gene, *scrA*, encodes a 468-aa potential protein that shares a domain present in several pyridoxal-phosphate-dependent enzymes (conserved domain Pfam00266, class V aminotransferases). The second gene, *scrB*, encodes a product

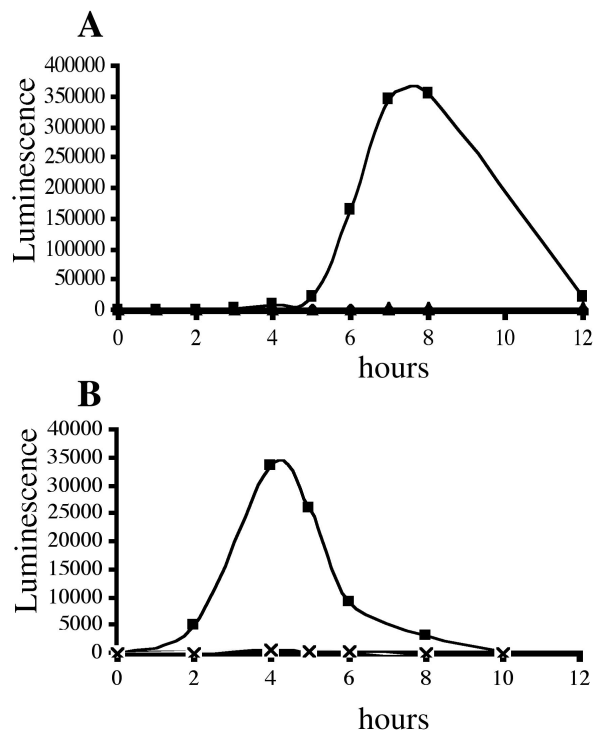


FIG. 1. Overexpression of *scrABC* induces *lfgE::lux* expression. Luminescence (expressed as SLU) was monitored during growth of cultures in HI liquid medium. (A) Cosmid-induced luminescence. Lux reporter strain LM1017 contained pLM2032 (■), pLM2032 carrying *scrB::TnphoA* (◇), and vector control (▲). (B) Subclone-induced luminescence. Lux reporter strain LM1017 contained pLM2796 (■), pLM2796 carrying *scrA::TnlacZ* (▲), pLM2796 carrying *scrC::TnlacZ* (×), and vector control (◆).

(321 aa) similar to many bacterial extracellular solute-binding proteins (family 3; ABC-type transporter components). For example, BLAST comparison with *Salmonella enterica* serovar Typhimurium FliY, which evidence suggests is an L-cystine binding protein (24), yielded a score of 70.1 bits, $E = 4e - 11$, with 25% identities and 42% similarities. The third gene, *scrC*, encodes a 774-aa potential sensory protein. The N terminus of ScrC has two predicted transmembrane domains flanking a potential periplasmic domain, which is ca. 300 aa in length. The C terminus contains two conserved domains of unknown function. The portion from aa 336 to 502 shows similarity to a number of proteins in the GGDEF family (Pfam00990; DUF9). The portion from aa 518 to 761 show similarity to an EAL domain (Pfam00563; DUF2). The GGDEF and EAL domains are often found in tandem in putative diguanylate cyclases and phosphodiesterases (8). A BLAST comparison of the C terminus of ScrC (300 to 774 aa) with diguanylate cyclase (from *Gluconacetobacter xylinus*; GenBank accession no. AF052518) yielded 28% identities and 47% positives (score of 177 bits, $E = 2e - 43$) and with phosphodiesterase A (from *G. xylinus*; GenBank accession no. AF052519) yielded 34% identities and 60% positives (score of 278 bits, $E = 1e - 73$).

The three potential open reading frames are transcribed in the same direction with very small intergenic regions, and *scrB* and *scrC* appear translationally coupled. RT-PCR analysis pro-

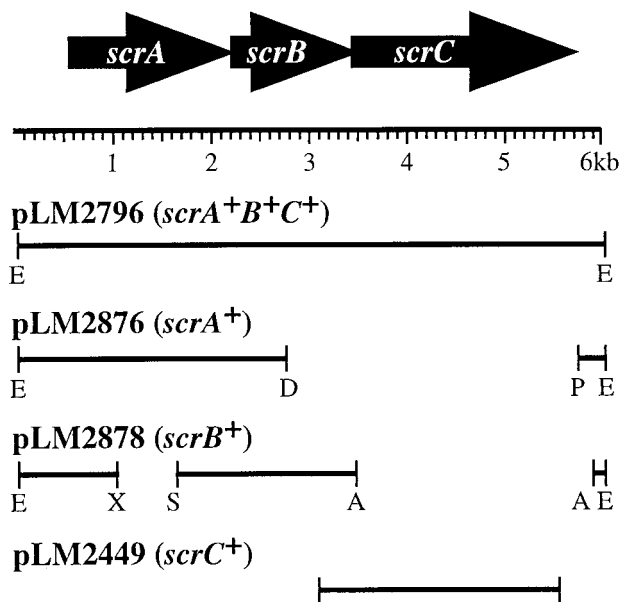


FIG. 2. Physical map of the *scr* locus and recombinant plasmids. Arrows point in the direction of transcription deduced from the open reading frames. The restriction sites relevant to constructs are indicated as follows: E, *Eco*47III; A, *Avr*II; X, *Xho*I; S, *Sma*I; D, *Drd*I; P, *Psh*AI. Plasmids pLM2876 and pLM2878 were derived from pLM2796. PCR amplification was used to generate the *scrC* clone pLM2449.

duced products with primers designed to reveal *scrAB* and *scrBC* transcription, whereas primers designed to show products upstream of *scrA* and downstream of *scrC* produced no products, although all primers could be shown to produce appropriately sized products with chromosomal DNA as a tem-

plate (data not shown). Thus, the evidence suggests that these three genes comprise an operon.

Localization of Scr proteins. To probe protein topology and determine the subcellular localization of the *scr* gene products, the *scrABC* locus was mutagenized with the Tn5-derived transposons Tn*phoA*_{in} and Tn*lacZ*_{in}. These transposons can be used to generate alkaline phosphatase and β-galactosidase gene fusions (11). Since active alkaline phosphatase fusions target exported protein sequences and active β-galactosidase fusions target cytoplasmic protein sequences they can be used to study protein localization and topology. Fusions producing active alkaline phosphatase or β-galactosidase were identified by screening on chromogenic substrates BCIP or X-Gal. Insertions in the *scr* region were mapped by restriction analysis and by sequencing. An alkaline phosphatase fusion with ScrB (pLM2341) and β-galactosidase fusions with ScrA (pLM2936) and ScrC (pLM2502) were identified. The fusion-containing plasmids were transferred to LM1017 and cytoplasmic, periplasmic, and whole membrane cell fractions were isolated. Immunoblots were performed and probed with anti-alkaline phosphatase or anti-β-galactosidase sera (Fig. 3). Although *V. parahaemolyticus* cannot utilize lactose as a carbon source and fails to hydrolyze X-Gal, there is a strong cross-reaction of the anti-β-galactosidase antibody with protein of low molecular mass (~30 kDa). Samples prepared from LM1017 carrying pLM2502 (ScrC-LacZ, lanes 2 to 5) showed a high-molecular-mass product in the whole-cell (lane 2) and membrane (lane 4) fractions corresponding to the ScrC-LacZ fusion protein (predicted molecular mass of ~204 kDa), which is not present in the parental strain (lane 1). Samples prepared from LM1017 carrying pLM2936 (ScrA-LacZ, lanes 6 to 9) showed a high-molecular-mass product in the whole-cell (lane 6) and cytoplasmic fractions corresponding to the ScrA-LacZ fusion pro-

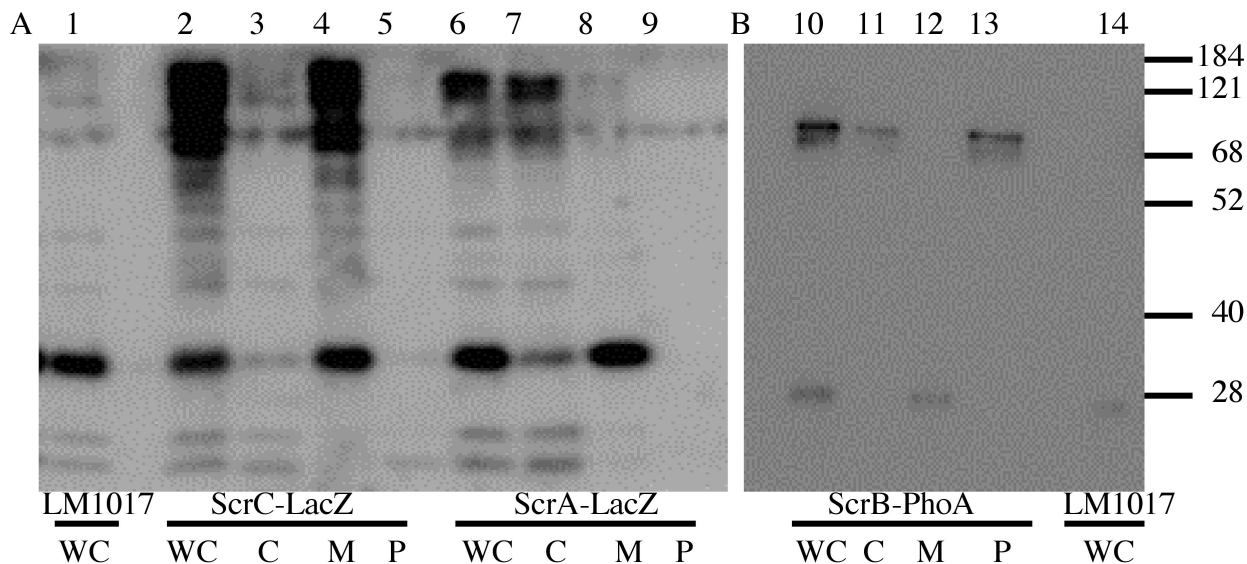


FIG. 3. Cellular localization of Scr proteins. Cellular fractions were isolated from strains producing the fusion proteins: ScrA-LacZ (LM5753), ScrB-PhoA (LM5153), and ScrC-LacZ (LM5140). Immunoblots were performed by probing with anti-β-galactosidase (A) or anti-alkaline phosphatase (B). Sample fractions are denoted as whole cells (WC), cytoplasm (C), membrane (M), or periplasm (P). Lanes: 1, LM1017 (WC); 2, LM5140 (WC); 3, LM5140 (C); 4, LM5140 (M); 5, LM5140 (P); 6, LM5753 (WC); 7, LM5753 (C); 8, LM5753 (M); 9, LM5753 (P); 10, LM5153 (WC); 11, LM5153 (C); 12, LM5153 (M); 13, LM5153 (P); 14, LM1017 (WC). The molecular size standards are given in kilodaltons on the right.

tein (predicted molecular mass of ~170 kDa) (lane 7). Samples prepared from LM1017 carrying pLM2341 (ScrB-PhoA, lanes 10 to 13) showed a high-molecular-mass product in the whole-cell (lane 10) and periplasmic (lane 13) fractions corresponding to the ScrB-PhoA fusion protein (predicted molecular mass of ~80 kDa). A small amount of ScrB fusion product was also detected in the cytoplasm (lane 11), which may be due to inefficient osmotic lysis. High-molecular-mass immunoreactive products were not seen with the parental strain LM1017 (lanes 1 and 14). To summarize, the ScrA-LacZ hybrid was found in the cytoplasm, the ScrB-PhoA hybrid was primarily detected in the periplasm, and the ScrC-LacZ hybrid was associated with the membrane fraction. The specific localization of each of the hybrid products was consistent with the enzyme activity of the reporter fusion. The ScrC-LacZ fusion joint occurred after two predicted transmembrane domains in ScrC and was predicted to place β -galactosidase activity in the cytoplasm.

scr mutants are defective in swarming. To initiate further studies on the *scr* operon, additional mutations in *scr* genes were constructed. Two antibiotic resistance cassettes were used to interrupt *scrA*, by deleting aa 108 to 385 and inserting a "polar" chloramphenicol cassette (from pKD3) whose transcriptional orientation was opposed to that of *scrA* to make pLM2811 (Δ *scrA1*) or inserting a "nonpolar" kanamycin cassette (from pKD11), whose transcription was in the same orientation as *scrA* and which possessed a ribosome-binding site and lacked a transcription terminator at its 3' terminus, to make pLM2916 (Δ *scrA2*). In *scrC*, aa 142 to 515 were deleted by *Bgl*II digestion, and a kanamycin cassette (from pLM1871) was inserted to make pLM2797 (Δ *scrC1*). These mutations, along with *scrB1::TnphoA* (pLM2341) were introduced onto the chromosome of reporter strain LM1017 and wild-type strain BB22TR by homologous recombination, and *lfgE::lux* expression and swarming motility were monitored. Mutations in the *scr* operon greatly reduced *lfgE::lux* expression in the LM1017 background when grown on plates (Fig. 4A). When the drug cassette in the *scrA2* allele was aligned with *scr* transcription, a direction that should not have been polar on downstream transcription, the phenotype was less severe than when transcription of the cassette was opposed to *scr* transcription (in *scrA1*). The *scrA1* mutation in strain LM5545 had the most profound effect (19,800 SLU) of all *scr* alleles on reducing light production ~70-fold compared to LM1017 (1,540,000 SLU), whereas the *scrA2* mutation in strain LM5792 produced 195,000 SLU. This suggests that *scrA* is required for operon function. Strains containing the *scrB1* mutation (LM5547) produced 47,300 SLU and the *scrC* mutation (LM5549) produced 47,500 SLU. Swarming motility was similarly perturbed in *scr* mutants in the BB22TR background (Fig. 4B). Mutants LM5793 (*scrA2*), LM4897 (*scrB1*), and LM5719 (*scrC1*) had much slower rates of swarming than the parent strain BB22TR. Mutant LM5733 (*scrA1*) had the most severe swarming defect with very little swarming movement. Western analysis confirmed that mutant strains produced significantly less lateral flagellin (data not shown). Swimming motility was unaffected in *scr* mutants.

Complementation analysis was performed to confirm operon structure and gain insight into *scr* gene function (Fig. 5). Plasmid pLM2796, containing *scrABC*, restored *lfgE::lux*

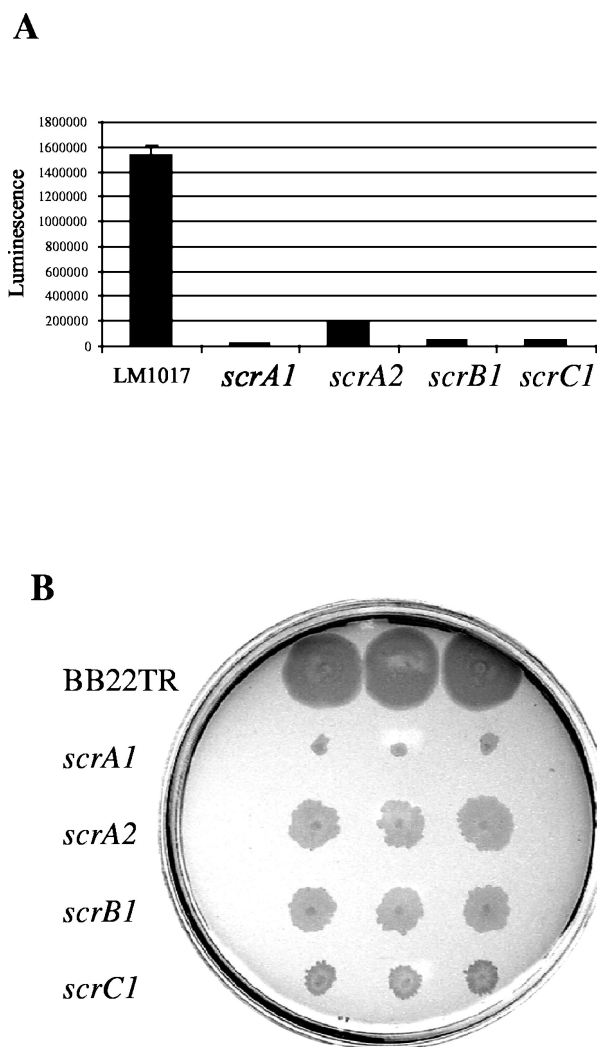


FIG. 4. *scr* mutants are defective in lateral flagellar gene expression and swarming. (A) Luminescence (reported as SLU) was measured from HI plate-grown samples after 9 h at 30°C. The values used for light production were as follows: LM1017 (*lfgE::lux*), 1,540,000 ± 75,000 SLU; LM5545 (*scrA1*), 19,800 ± 800 SLU; LM5792 (*scrA2*), 195,000 ± 9,000 SLU; LM5547 (*scrB1*), 47,300 ± 3,100 SLU; and LM5549 (*scrC1*), 47,500 ± 2,200 SLU. (B) Swarming motility of *scr* mutants after 9 h at 30°C. The strains included BB22TR (wild type), LM5733 (*scrA1*), LM5793 (*scrA2*), LM4897 (*scrB1*), and LM5719 (*scrC1*).

expression to all of the *scr* mutants and increased expression in LM1017 when grown on HI plates containing gentamicin to select maintenance of the plasmid (Fig. 5A). Plasmid pLM2876, containing *scrA*, was unable to restore *lfgE::lux* expression in LM5545 (*scrA1*), LM5547 (*scrB1*), and LM5549 (*scrC1*) but did increase *lfgE::lux* expression threefold in LM5792 (*scrA2*). This indicates that the *scrA1* allele in LM5545 was more polar than the *scrA2* mutation in LM5792. Plasmid pLM2878, containing *scrB*, did not restore *lfgE::lux* expression in any of the mutants. pLM2878 was derived from pLM2879 (*scrB⁺C⁺*), which was able to significantly increase *lfgE::lux* expression in liquid-grown LM1017 compared to liquid-grown LM1017 carrying a vector control or pLM2449 (*scrC⁺*) (data

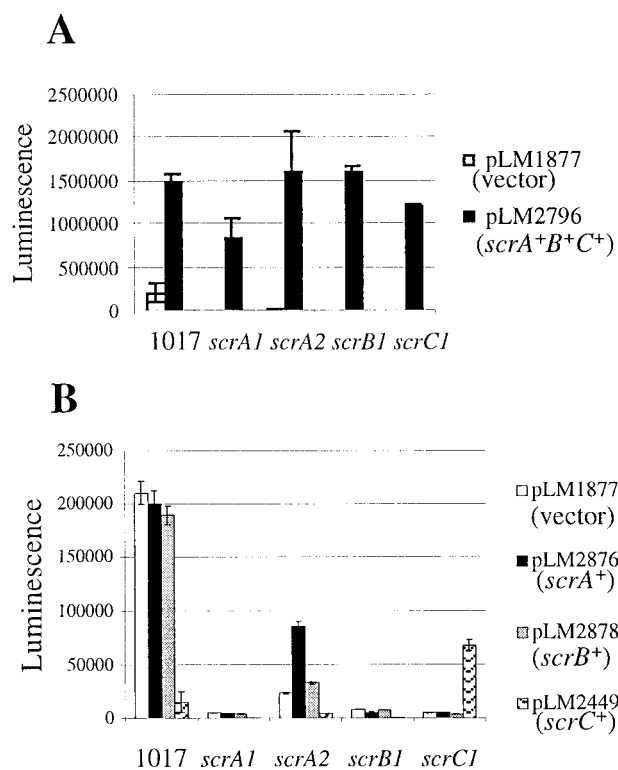


FIG. 5. Complementation of lateral flagellar gene expression defects in *scr* mutants. (A) Plasmids pLM2796 (*scrA⁺B⁺C⁺*) and pLM1877 (vector) in LM1017 and *scr*-defective backgrounds. (B) Plasmids pLM1877 (vector), pLM2876 (*scrA⁺*), pLM2878 (*scrB⁺*), and pLM2449 (*scrC⁺*) in LM1017 and *scr*-defective backgrounds. Luminescence (reported in SLU) was measured from HI plate-grown samples after 10 h of growth at 30°C.

not shown), suggesting that the *scrB* gene can be expressed from pLM2879 (and consequently pLM2878). Therefore, *scrB* on a plasmid does not complement the *scrB* mutant. Plasmid pLM2449, containing *scrC*, increased *lfgE::lux* in LM5549 (*scrC1*) by 12-fold. To summarize the mutant phenotype and complementation results, *scrA* was found to be required for operon function. Expression of *scrA* from a plasmid resulted in partial complementation of *lfgE::lux* expression in the *scrA* mutant containing the aligned cassette but not with the opposed cassette. Expression of *scrB* resulted in no complementation of *scr* mutants. Expression of *scrC* restored *lfgE::lux* expression in an *scrC* but not an *scrB* mutant strain. The inability of *scrC* to complement the *scrB* mutant suggests *scrB* is required for normal functioning of the operon. These data are consistent with an operon organization for the *scr* genes. It also indicates that the product of each gene has an essential role in the functioning of this operon.

The *scr* operon is not required for polar flagellum-mediated surface sensing. Surface sensing and induction of swarmer cell differentiation is hypothesized to occur upon restriction of polar flagellar function. Mutants with defects in the polar flagellar gene system constitutively express lateral flagellar genes (5). To determine whether the *scr* operon participated in surface sensing via the polar flagellar pathway, the mutant strain LM5789, containing *motX* and *scrB* alleles, was con-

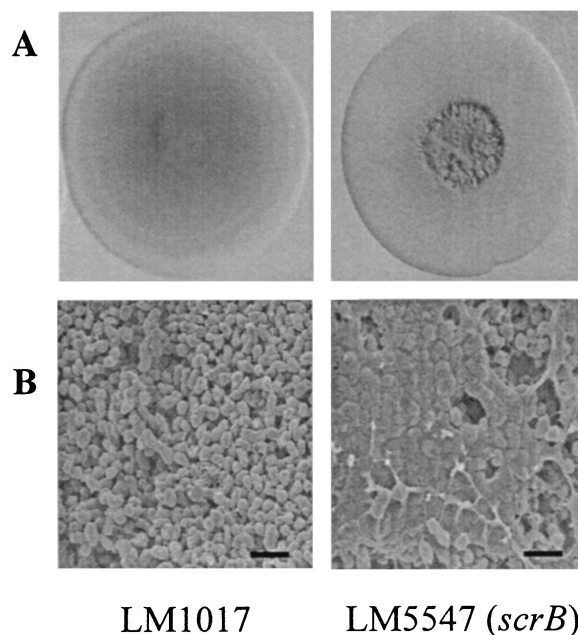


FIG. 6. *scr* mutants display a rugose colony morphology and produce an extracellular matrix. (A) Colony morphology of a smooth parent strain, LM1017, and a rough *scrB* mutant, LM5547. (B) Scanning electron microscopy of a smooth LM1017 colony and rugose LM5547 colony. Colonies were grown on HI plates at 30°C for 2 days. Bar, 2 μ m.

structed in the LM1017 background. Strains were grown in HI broth with light measurements taken from late-logarithmic-phase cultures when maximal light was observed. LM1017 produced little light when grown in liquid ($1,200 \pm 55$ SLU), as did LM5547 (*scrB1*) ($1,500 \pm 59$ SLU). In contrast, LM4170 (*motX118*) produced $1,200,000 \pm 40,000$ SLU. Introduction of a *scrB* mutation into LM4170 (to make LM5789) caused little effect on light production ($600,000 \pm 22,000$ SLU), indicating that the *scr* operon is not required for induction of the swarmer cell program via the polar flagellum-mediated surface sensing pathway.

The *scr* operon controls CPS production. The *scr* mutants displayed abnormal colony morphologies that were more rugose than the smooth, translucent colony type of the parental strains (BB22TR or LM1017) (Fig. 6A). Colony roughness was not simply a result of lack of lateral flagella because mutants bearing lesions in *laf* genes, i.e., LM1017, do not form rugose colonies. To more closely examine the rough colony morphology, scanning electron microscopy was performed on rugose colonies of *scrB* mutant LM5547 and on smooth colonies of the parent LM1017 (Fig. 6B). In micrographs of the rough LM5547 colonies, cells appeared encased in an electron-dense extracellular matrix, whereas smooth LM1017 colonies contained cells with no extracellular matrixes.

CPS seemed a likely candidate for the extracellular matrix observed in the rugose colony by scanning electron microscopy. To test this idea, the expression of a CPS synthase gene, *cpsA*, fused to *lacZ* (*cpsA::lacZ*) was monitored in wild-type and *scr* mutant backgrounds. Strain LM5818, which contains the *cpsA::lacZ* mutation, produced 54 ± 1.1 Miller units when β -galactosidase assays were performed on cells harvested from

HI plates grown for 12 h. In comparison, the double-mutant strain LM5831, which carries the *cpsA::lacZ* and Δ *scrA::Cam* mutations, produced 158 ± 2.1 Miller units. Thus, the *scr* mutations appear to have pleiotropic defects, inversely affecting lateral flagellar expression and CPS expression. Consistent with these results, strain LM5831 lacked the rugose colony phenotype normally seen in *scr* mutants. LM5831 (*scrA1 cpsA1*) formed smooth colonies that resembled the colonies of LM5818 (*cpsA1*) and the wild-type strain BB22TR. The reporter gene data and the colony morphology are consistent with the hypothesis that *scr* mutants have an increased level of CPS production that contributes to the rugose colony morphology. Furthermore, the *cps* lesion had no effect on swarming. Strain LM5818 swarmed as well as wild-type parental strain LM5674. In contrast, LM5831 showed a profound swarming defect that was equivalent to the defect seen for strain LM5733 (*scrA1*). Thus, the *scr* allele was dominant to the *cps* allele with respect to *laf* gene expression.

Overexpression of *scrABC* and *scrC*. Surface-grown mutants with *scr* defects displayed reduced *lfgE::lux* levels but increased *cpsA::lacZ* levels. Overexpressing *scrABC* from a plasmid slightly increased *lfgE::lux* levels (1,400,000 SLU) compared to LM1017 carrying a vector control (600,000 SLU) (Fig. 7A). In contrast, expressing only *scrC* from a plasmid greatly decreased *lfgE::lux* levels (25 SLU) (Fig. 7A). The effect of ScrC overproduction on CPS was also examined (Fig. 7B). Strain LM5813 (*cpsA::lacZ*) expressing *scrABC* from a plasmid produced 25 Miller units, LM5812 expressing *scrC* from a plasmid produced 278 Miller units, and LM5811 containing the plasmid control produced 35 Miller units. Thus, overexpression of *scrC* significantly increased expression of a CPS gene (~8-fold).

DISCUSSION

When grown on a surface *V. parahaemolyticus* differentiates from a swimmer cell to a swarmer cell. Differentiation allows efficient movement over and colonization of surfaces. One large gene set that is induced in response to growth on surfaces encodes the lateral flagellar motility system. In an attempt to discover regulators of lateral flagella gene expression, a cosmid library of *V. parahaemolyticus* DNA was screened for the ability to induce constitutive expression of the lateral flagellar system by monitoring *lfgE::lux* expression in liquid. One cosmid, which was chosen for these studies, contained a 6-kb, three-gene operon that was determined to be responsible for inducing constitutive *lfgE::lux* expression and repressing CPS production. This operon, which appears unique to *V. parahaemolyticus*, was named the "swarming and CPS regulatory" operon (*scr*), and the gene products are depicted in Fig. 8. None of the genes encode potential transcriptional activators, but instead the gene products appear to have the capacity to form a sensory signaling cascade. The first gene in the operon, *scrA*, encodes a polypeptide that contains a signature domain characteristic of pyridoxal-phosphate-dependent enzymes. ScrB appears to be an extracellular solute-binding protein whose closest homologs are bacterial extracellular amino acid solute-binding proteins. ScrC contains a potential periplasmic receptor domain and a cytoplasmic domain containing GGDEF and EAL domains. Localization studies revealed ScrA is found in the cytoplasm, ScrB is detected in the periplasm and

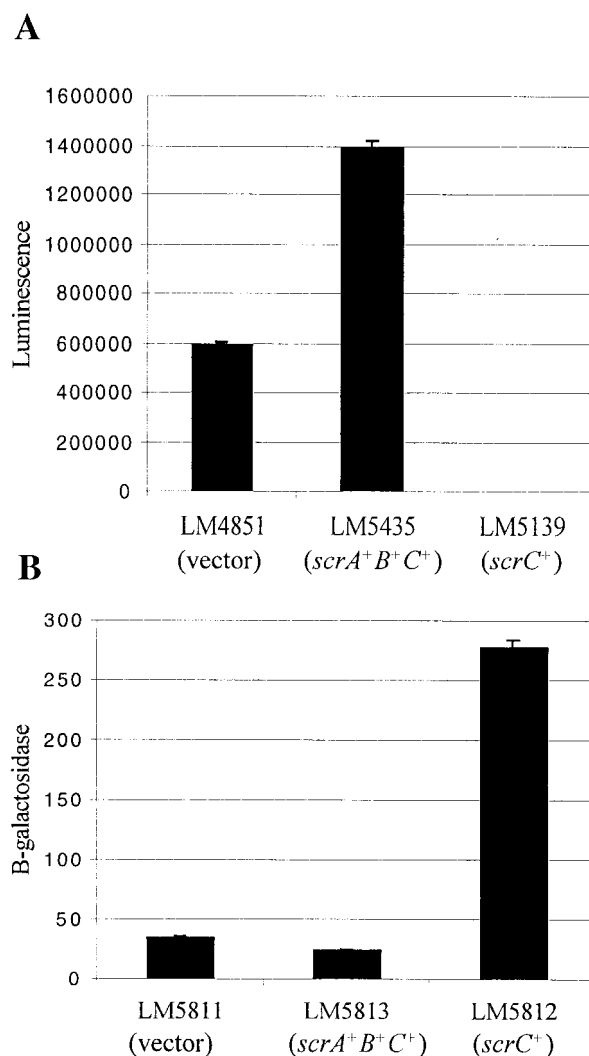


FIG. 7. *scrABC* and *scrC* overexpression inversely affects lateral flagellar and CPS gene expression. (A) The amounts of luminescence produced were as follows: $600,000 \pm 11,200$ SLU in LM4851 (carrying the vector), $1,400,000 \pm 25,500$ SLU in LM5435 (carrying *scrA⁺B⁺C⁺*), and 25 ± 4 SLU in LM5139 (*scrC⁺*). (B) The amounts of β -galactosidase produced (expressed as Miller units) were as follows: 35 ± 1.2 in LM5811 (vector), 25 ± 1 in LM5813 (*scrA⁺B⁺C⁺*), and 278 ± 6 in LM5812 (*scrC⁺*). Samples were harvested from HI-gentamicin plates with IPTG after 12 h of growth.

ScrC is associated with the membrane fraction. The two potential transmembrane domains of ScrC in combination with membrane localization of an active β -galactosidase fusion positioned at aa 309 of ScrC suggest that the GGDEF and EAL domains are in the cytoplasm.

To probe the function of these proteins, mutations in each gene were constructed and transferred to the chromosome. When grown on surfaces, the *scr* mutants displayed greatly reduced levels of *lfgE::lux* expression and were impaired in their ability to swarm. RT-PCR and complementation studies demonstrated that the *scrABC* genes are organized in an operon, and each gene appears to contribute to functioning of the operon. The *scr* mutants displayed a rugose colony phenotype. Examination by scanning electron microscopy revealed

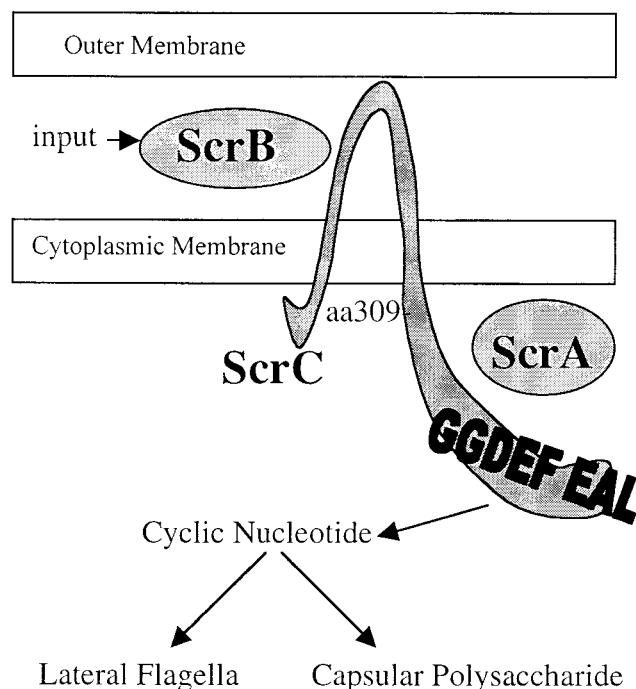


FIG. 8. Model for the ScrABC sensory transduction system. ScrB has similarity to solute-binding proteins and is hypothesized to receive an input signal and interact with the periplasmic domain of ScrC. This interaction could modulate the activity of the cytoplasmic portion of ScrC, possibly controlling levels of a cyclic nucleotide signaling molecule by acting as a cyclic nucleotide phosphodiesterase or cyclase. ScrC aa 309 is the site of an active β -galactosidase fusion, suggesting that the GGDEF and EAL domains reside in the cytoplasm. ScrA contains a domain shared by pyridoxal-phosphate-dependent enzymes and is required for signal transduction, but its role is unclear. Levels of the small signaling molecule would ultimately determine output by affecting the expression of lateral flagella and CPS in an inverse manner.

an extracellular matrix encasing the cells of the rough colonies. To determine whether this was a result of CPS production, a *cpsA::lacZ* and *scrA* double mutant was constructed. This double mutant had a fivefold increase in *cpsA::lacZ* expression compared to the strain containing only the *cpsA::lacZ* allele. Moreover, introduction of the *cpsA::lacZ* mutation into the rugose *scrA* strain caused loss of the rough colony phenotype.

Overexpression of *scrABC* increased *lfgE::lux* expression and decreased *cpsA::lacZ* expression. The overexpression of *scrC* had the opposite effect; decreasing *lfgE::lux* expression and increasing *cpsA::lacZ* expression. These results, along with mutant data, localization results, and sequence homology, lead us to propose the following model. ScrB may be a periplasmic binding protein involved in signal reception and interacts with the periplasmic domain of ScrC. The interaction could modulate the activity of the cytoplasmic GGDEF and EAL domains, which possibly control the intracellular concentration of a small signaling molecule. Although GGDEF and EAL domains are found widely in bacteria (8), little is known about their function. The two domains are found in tandem in the diguanylate cyclases and phosphodiesterases that regulate cellulose synthesis in *G. xylinus* (23). In this organism, it is thought that a cyclic nucleotide (cyclic di-GMP), whose level is controlled by numerous GGDEF and EAL domain-containing

proteins, directly stimulates cellulose biosynthesis. By analogy to *G. xylinus*, Scr signaling may influence the level of a small regulatory molecule, e.g., interaction of ScrB with ScrC may activate a cyclic nucleotide phosphodiesterase (or cyclase) activity. The level of the small signaling molecule could then modulate the activity of a transcription factor (or factors) such as cyclic AMP activates CAP. The role of ScrA is less clear, although it does contain a domain found in many pyridoxal-phosphate-dependent enzymes. Final output of this signal transduction pathway influences both lateral gene expression and CPS expression, two gene systems important to life on a surface. Because a *scr* allele is dominant to the *cps* allele with respect to swarming and the *cps* allele is dominant to an *scr* allele with respect to CPS production, it does not seem likely that overproduction of one extracellular factor (e.g., CPS) negatively controls production of the second factor (e.g., lateral flagella or vice versa).

What is the role of the *scr* operon with respect to control of swarmer cell development? Loss of function in *scr* genes greatly reduces but does not entirely eliminate swarming; therefore, the operon is not absolutely required for swarmer cell differentiation. Supporting this is the observation that when an *scr* mutation is introduced into a polar flagellum mutant, which constitutively expresses lateral flagella in liquid, *lfgE::lux* expression is not significantly reduced in liquid. Thus, the dominance of the polar motility allele also suggests that the *scrABC* operon is not required for the flagellum-sensing pathway. Epistasis of the polar motility allele to an *scr* allele could reflect participation in a common pathway initiating surface-induced gene expression and swarmer cell differentiation; however, because *scr* lesions do not completely abolish induction of swarmer cell differentiation, we favor the hypothesis that the *scr* operon plays a role at a later point of surface colonization. Swarmer cell differentiation is a transient phenomenon. The initial contact with a surface results in the restriction of polar flagellum rotation. Through the polar flagellum-mediated surface sensing pathway, this leads to cell elongation and production of hundreds of lateral flagella per cell. At some point the swarmer cells must dedifferentiate in order to divide and grow (22, 25). In addition, lateral flagellar production is a costly process that is not beneficial unless the cells are on the leading edge of a swarming colony. Studies on another swarming bacterium, *Proteus mirabilis*, indicate that swarming involves a highly regulated, complex sequence of multicellular events organized into distinct periodic cycles of differentiation, migration, and dedifferentiation (4, 12, 19). There are probably times during surface colonization when it is advantageous to be an elongated, hyperflagellated motile swarmer cell (e.g., when initially establishing the colonization of a surface). There are also probably instances on a surface when it is advantageous to be a less motile, more adhesive cell type producing CPS (e.g., during biofilm development). The *scr* operon, which has control over lateral gene expression and CPS expression, may play a role in mediating the switch between these cell types.

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REFERENCES

- Alexyev, M. F. 1995. Three kanamycin resistant gene cassettes with different polylinkers. *BioTechniques* **18**:52–55.
- Ames, G. F., C. Prody, and S. Kustu. 1984. Simple, rapid, and quantitative release of periplasmic proteins by chloroform. *J. Bacteriol.* **160**:1181–1183.
- Belas, R., M. Simon, and M. Silverman. 1986. Regulation of lateral flagella gene transcription in *Vibrio parahaemolyticus*. *J. Bacteriol.* **167**:210–218.
- Belas, R. 1996. *Proteus mirabilis* and other swarming bacteria, p. 183–219. In J. A. Shapiro and M. Dworkin (ed.), *Bacteria as multicellular organisms*. Oxford University Press, Oxford, England.
- Boles, B. R., and L. L. McCarter. 2000. Insertional inactivation of genes encoding components of the sodium-type flagellar motor and switch of *Vibrio parahaemolyticus*. *J. Bacteriol.* **182**:1035–1045.
- Datsenko, K. A., and B. L. Wanner. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. USA* **97**:6640–6645.
- Friedman, A., S. R. Long, S. E. Brown, W. J. Buikema, and F. Ausubel. 1982. Construction of a broad-host-range cosmid cloning vector and its use in the genetic analysis of *Rhizobium* mutants. *Gene* **18**:289–296.
- Galperin, M. Y., A. N. Nikolskaya, and E. V. Koonin. 2001. Novel domains of the prokaryotic two-component signal transduction systems. *FEMS Microbiol. Lett.* **203**:11–21.
- Kim, Y. K., and L. L. McCarter. 2000. Analysis of the polar flagellar gene system of *Vibrio parahaemolyticus*. *J. Bacteriol.* **182**:3693–3704.
- Laemmli, U. K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* **227**:680–685.
- Manoil, C., and J. Bailey. 1997. A simple screen for permissive sites in proteins: analysis of *Escherichia coli lac* permease. *J. Mol. Biol.* **267**:250–263.
- Matsuyama, T., Y. Takagi, Y. Nakagawa, H. Itoh, J. Wakita, and M. Matsushita. 2000. Dynamic aspects of the structured cell population in a swarming colony of *Proteus mirabilis*. *J. Bacteriol.* **182**:385–393.
- McCarter, L. L., and M. Silverman. 1987. Phosphate regulation of gene expression in *Vibrio parahaemolyticus*. *J. Bacteriol.* **169**:3441–3449.
- McCarter, L. L., and M. Silverman. 1990. Surface-induced swarmer cell differentiation of *Vibrio parahaemolyticus*. *Mol. Microbiol.* **4**:1057–1062.
- McCarter, L. L., and M. E. Wright. 1993. Identification of genes encoding components of the swarmer cell flagellar motor and propeller and a sigma factor controlling differentiation of *Vibrio parahaemolyticus*. *J. Bacteriol.* **175**:3361–3371.
- McCarter, L. L. 1994. MotX, the channel component of the sodium-type flagellar motor. *J. Bacteriol.* **176**:5988–5998.
- McCarter, L. L. 1999. The multiple identities of *Vibrio parahaemolyticus*. *J. Mol. Microbiol. Biotechnol.* **1**:1–7.
- Miller, J. H. 1972. *Experiments in molecular genetics*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Rauprich, O., M. Matsushita, C. J. Weijer, F. Siefert, S. E. Esipov, and J. A. Shapiro. 1996. Periodic phenomena in *Proteus mirabilis* swarm colony development. *J. Bacteriol.* **178**:6525–6538.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Silverman, M., R. Showalter, and L. L. McCarter. 1991. Genetic analysis in *Vibrio*. *Methods Enzymol.* **204**:515–536.
- Stewart, B. J., J. L. Enos-Berlage, and L. L. McCarter. 1997. The *lonS* gene regulates swarmer cell differentiation of *Vibrio parahaemolyticus*. *J. Bacteriol.* **179**:107–114.
- Tal, R., H. C. Wong, R. Calhoun, D. Gelfand, A. L. Fear, G. Volman, R. Mayer, P. Ross, D. Amikam, H. Weinhouse, A. Cohen, S. Sapir, P. Ohana, and M. Benziman. 1998. Three *cdg* operons control cellular turnover of cyclic di-GMP in *Acetobacter xylinum*: genetic organization and occurrence of conserved domains in isoenzymes. *J. Bacteriol.* **180**:4416–4425.
- Turner, M. S., T. Woodberry, L. M. Hafner, and P. M. Giffard. 1999. The *bspA* locus of *Lactobacillus fermentum* BR11 encodes an L-cysteine uptake system. *J. Bacteriol.* **181**:2192–2198.
- Ulitzur, S. 1974. Induction of swarming in *Vibrio parahaemolyticus*. *Arch. Microbiol.* **101**:357–363.
- Woo, T. H. S., A. F. Cheng, and J. M. Ling. 1992. An application of a simple method for the preparation of bacterial DNA. *BioTechniques* **13**:696–697.