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The *lonS* Gene Regulates Swarmer Cell Differentiation of *Vibrio parahaemolyticus*

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Vibrio parahaemolyticus **differentiates from a polarly flagellated, short, rod-shaped cell known as the swimmer to the elongated, hyperflagellated, and multinucleated swarmer cell type when it is grown on a surface. The swarmer is adapted to movement over and colonization of surfaces. To understand the signal transduction mechanism by which the bacterium recognizes surfaces and reprograms gene expression, we isolated a new class of mutants defective in surface sensing. These mutants were constitutive for swarmer cell gene expression, inappropriately expressing high levels of a swarmer cell gene fusion product when grown in liquid. They showed no defect in the swimming motility system, unlike all previously isolated constitutive mutants which have defects in the alternate, polar motility system. The lesions in the majority of the newly isolated mutants were found to be in a gene, *lonS*, which encodes a polypeptide exhibiting 81% sequence identity to the *Escherichia coli* Lon protein, an ATP-dependent protease. Upstream sequences preceding the *lonS* coding region resemble a heat shock promoter, and the homology extends to sequences flanking *lonS*. The gene order appears to be *clpX lonS hupB*, like the organization of the *E. coli* locus. *V. parahaemolyticus lonS* complemented *E. coli lon* mutants to restore UV resistance and capsular polysaccharide regulation to that of the wild type. *Vibrio lonS* mutants were UV sensitive. In addition, when grown in liquid and examined in a light microscope, *lonS* mutant cells were extremely long and thus resembled swarmer cells harvested from a surface.**

The bacterium *Vibrio parahaemolyticus* possesses two cell types, each appropriate for life under different circumstances (1, 29). The swimmer cell is adapted to life in a liquid environment. It is rod shaped and approximately 2 μm long and has a single, sheathed polar flagellum. The flagellum is powered by the sodium motive force and can propel the cell at speeds as fast as 60 μm/s (4). The swarmer cell is adapted to life on a solid surface or in a slime layer, i.e., conditions under which the polar flagellum is not functional or is poorly functional. This cell type is extremely long (approximately 30 μm), multinucleate, and peritrichously flagellated. The peritrichous, or lateral, flagella are powered by the proton motive force and function to move the bacterium over surfaces or through viscous layers (4, 42). Swimming-negative mutants show no defect in swarming, and swarming-defective mutants swim as well as the wild type; therefore, the polar flagellar (Fla) and lateral flagellar (Laf) gene systems are distinct (27).

The gene systems that encode the two motility systems are large, each composed of 40 or more genes. As is the case for all studied flagellar systems (23, 25), the polar and lateral flagellar systems seem to be carefully regulated in hierarchies of control whereby regulation of gene expression is coupled to morphogenesis of the organelles (32, 33). In addition, the two gene systems interact. Performance of the polar organelle is in some way coupled to transcription of the swarmer cell gene system (27). We are interested in how the bacterium senses its presence on a surface and conveys this information to the genes controlling swarmer cell development. It is hypothesized that the polar flagellum functions as a tactile sensor or dynamometer measuring external forces influencing its motion (27). Swarmer cell genes are expressed only under conditions in which the polar flagellum is not functional. Physical conditions, such as a surface or a highly viscous environment, which impede rotation of the polar flagellum result in induction of the swarmer cell gene system. Similarly, perturbation of polar motor function with phenamil, a sodium ion channel inhibitor, results in a proportionate increase in lateral flagellar gene expression with a decrease in motor function (24). Genetic interference with the polar system also releases the lateral system from its normal control. All mutations in the *fla* system which eliminate swimming motility result in constitutive expression of *laf* genes (27).

How is the signal resulting from restriction of polar function transduced to reprogram gene expression? Recently, a search was initiated for a new class of mutants having a constitutive *laf* phenotype; in particular, mutants not defective in the *fla* system. Mutants in such a class would be candidates for possession of lesions in a swarmer cell-specific regulator. Previous work has established the outline of the lateral flagellar hierarchy of control, and the "highest" gene that has been identified in the pyramid of expression is *lafX*. The *lafX* gene has been sequenced and identified as the hook gene (30). Strain LM1017 contains a fusion between *lafX* and indicator luminescence (*lux*) genes. In this strain, luminescence is produced when the bacterium is grown on a surface and not when it is grown in liquid (27). After transposon mutagenesis of LM1017, mutants were identified as strains that were bright in liquid. In this report, we characterize the mutations yielding the Bright constitutive swarmer cell phenotype.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains and plasmids used in this work are described in Table 1. For propagation of *V. parahaemolyticus* strains, the following media were used: 2216 (28 g of marine broth 2216 [Difco Laboratories; the broth was filtered after autoclaving to remove precipitate] per liter); HI (25 g of heart infusion broth [Difco] and 15 g of NaCl per liter), and the minimal medium of Broach et al. (9) supplemented with 0.4% galactose, 20 mM NH_4Cl, and 2% NaCl (final concentrations). Solidified medium was prepared by

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source, parent strain, and/or reference
V. parahaemolyticus strains		
BB22	Wild type	R. Belas; 7
LM1017	*lafX313*::*lux*	BB22; 33
LM4170	*lafX313*::*lux motX118*::mini-Mu *lac* (Tet^r)	LM1017; 31
LM4400	*lafX313*::*lux lonS5*::mini-Mu *lac* (Tet^r)	LM1017; this work
LM4407	*lafX313*::*lux lonS12*::mini-Mu *lac* (Tet^r)	LM1017; this work
LM4417	*lafX313*::*lux lonS22*::mini-Mu *lac* (Tet^r)	LM1017; this work
LM4418	*lafX313*::*lux laf-23*::mini-Mu *lac* (Tet^r)	LM1017; this work
LM4420	*lafX313*::*lux lonS25*::mini-Mu *lac* (Tet^r)	LM1017; this work
LM4441	*lafX313*::*lux lonS5*::mini-Mu *lac* (Tet^r)/pLM1942	LM4400; this work
LM4442	*lafX313*::*lux lonS5*::mini-Mu *lac* (Tet^r)/pLM1944	LM4400; this work
LM4443	*lafX313*::*lux lonS12*::mini-Mu *lac* (Tet^r)/pLM1942	LM4407; this work
LM4444	*lafX313*::*lux lonS12*::mini-Mu *lac* (Tet^r)/pLM1944	LM4407; this work
E. coli strains		
DH5α	F$^-$ *endA1 hsdR17* (r_K^-) *supE44 thi-1* λ$^-$ *recA1 deoR gyrA96 relA1* Δ(*argF-lacZYA*)U169 ϕ80d*lacZ*ΔM15	Bethesda Research Laboratories
SG20780	Δ*lon-510 cpsB10*::*lac* Mu λ*imm*	S. Gottesman; 8
SG20781	*lon*$^+$ *cpsB10*::*lac* Mu λ*imm*	S. Gottesman; 8
Plasmids		
pACYC177	Ap^r	New England Biolabs
pLM1835	Cam^r Ap^r	pMMB66EH; this work
pLM1897	Ap^r; *Pst*I fragment encoding tetracycline resistance from LM4407 cloned into pACYC177	LM4407; this work
pLM1900	Ap^r; 0.75-kb *Hin*dIII fragment from pLM1897 cloned into pUC19	pLM1897; this work
pLM1942	Cam^r; 5.5-kb *Sal*I-*Bam*HI fragment cloned into pLM1835; *lonS*$^+$	This work
pLM1944	Cam^r; 5.5-kb *Sal*I-*Bam*HI fragment cloned into pLM1835; negative control with unrelated insert	This work

using 2% Bacto Agar (Difco). Tryptone motility medium was prepared with 10 g of tryptone, 20 g of NaCl, and 0.335% Bacto Agar. *Vibrio* strains were grown at 30°C. LB medium and MacConkey-lactose plates for propagation of *Escherichia coli* strains were prepared as described by Miller (35). Ampicillin, chloramphenicol, and tetracycline were used at final concentrations of 75, 10, and 10 μg/ml, respectively. Previous work has shown that iron starvation is required for full induction of *laf* genes (28). This condition has been achieved by addition of an iron chelator such as conalbumin (final concentration of 1 mg/ml; Sigma Chemical Co.) to growth medium prepared with double-distilled water; however, we have found that HI broth prepared with distilled water purified in a Milli-Q Water System (Millipore Corporation, Bedford, Mass.) without a chelator also supports *laf* induction.

Bioluminescence measurements. Bioluminescence was quantified in a Monolight 2010 luminometer by measuring 0.1- and 0.2-ml samples of cultures appropriately diluted to give a linear response. Luminescence is reported as specific light units which are relative light units per minute per milliliter per unit of optical density at 650 nm. Bioluminescence was also monitored by exposing cultures in microtiter plates to Kodak X-ray film (either X-Omat AR5 or Biomax MS).

Genetic and molecular techniques. General DNA manipulations were adapted from the methods of Sambrook et al. (38). Transposon mutagenesis with mini Mu *lac* (Tet^r) and the strategy for cloning the targeted gene have been described previously (6). Chromosomal DNA was prepared by the protocol of Woo et al. (46). Southern blot analysis of restricted genomic DNA (26) was performed on 0.45-μm-pore-size Magna Charge nylon membranes (Micron Separations Inc.; Westborough, Mass.). To be useful in vibrios, plasmid pMMB66EH (14), which is an ampicillin-resistant, broad-host-range expression vector, was made chloramphenicol resistant. Plasmid pLM1835 was constructed after cleavage of pMMB66EH with *Hin*dIII by the introduction of a chloramphenicol cassette from pUC18CMR (41), which was cut with *Bam*HI. Both the vector and the insert were blunt ended prior to ligation. A library was constructed by ligating *Bam*HI-*Sal*I-digested *V. parahaemolyticus* DNA in the size range of 5 to 6 kb to similarly digested pLM1835. After transformation into *E. coli* DH5α, recombinant colonies were gridded into arrays on LB chloramphenicol plates prior to transfer to filters for colony blotting and probing. The bank was probed with an oligonucleotide (5′ CCGCCTCAGCTAAGAAGAAATCAC 3′) derived from the sequence of clone pLM1900. Blots were prehybridized (6× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 5× Denhardt's reagent, 1% sodium dodecyl sulfate, 20 μg of denatured salmon sperm DNA per ml) and then hybridized (6× SSC, 5× Denhardt's reagent, 1% sodium dodecyl sulfate) at 42°C. All hybridization reagents were prepared as described by Sambrook et al. (38). Blots were washed in 6× SSC three times for 15 min each time at room temperature. Conjugative transfer of plasmids to vibrios has been described previously (43).

DNA sequencing analysis. Initial sequence information was obtained for clone pLM1900 by using the dideoxy-chain termination procedure of Sanger et al. (39), with the Sequenase 2.0 kit from United States Biochemical. The source of radioactivity was [α-^{35}S]dATP (Amersham). The 0.7-kb fragment from pLM1900 was isolated, self-ligated, and then sonicated to generate random subfragments approximately 300 to 600 bp long (5). After end repair with T4 DNA polymerase, the subfragments were ligated into the *Sma*I site of M13mp8 (34) and sequenced with universal primers. Additional sequence was obtained by the DNA Core Facility of the University of Iowa from cesium chloride-purified clone pLM1942 with a 373A stretch fluorescent automated sequencer (Applied Biosystems Division of Perkin Elmer, Foster City, Calif.). Synthetic oligonucleotides were prepared by Genosys Biotechnologies, Inc. (The Woodlands, Tex.). The DNA sequence for both strands was obtained. Sequence assembly was performed by using the Genetics Computer Group software package. Searches for homology were performed at the National Center for Biotechnology Information with the BLAST network service (2) and at the Block Server (blocks@howard.fhcrc.org; 21). Sequence information was obtained only for a single strand for approximately 500 bases beyond the end of the sequence presented in Fig. 3, i.e., beyond bp 2879.

UV sensitivity. Bacteria were grown to an optical density at 650 nm of approximately 0.4. Appropriate dilutions were made in growth medium, and 0.1 ml of cells was spread onto an agar plate (HI for *V. parahaemolyticus* and LB for *E. coli*). Plates were spread in triplicate, exposed in the energy mode for various settings in a Fisher Scientific FB-UVXL-1000 UV cross-linker, and incubated overnight, and survivors were counted.

Nucleotide sequence accession number. The nucleotide sequence reported here has been deposited in the GenBank database under accession number U66708.

RESULTS

Isolation of Bright (constitutive) mutants: the search for a master Laf regulator. *laf*::*lux* strain LM1017 exhibits surface-dependent production of light. A bank of approximately 12,000

TABLE 2. Light production of *lafX*::*lux* strains with Bright mutations

Strain	Specific light units[a] at approximate optical density at 650 nm[b] of:		
	0.5	1.0	1.5
LM1017 (parental control)	2.55×10^6	3.25×10^6	2.38×10^6
LM4170 (Mot$^-$ control)	8.87×10^8	2.17×10^9	4.01×10^9
LM4400	8.35×10^8	2.33×10^9	3.97×10^9
LM4407	1.15×10^8	3.90×10^8	1.40×10^9
LM4417	1.30×10^8	1.98×10^8	4.17×10^8
LM4418	2.00×10^8	5.63×10^8	1.67×10^9
LM4420	6.50×10^8	3.23×10^9	5.35×10^9

[a] Specific light units are relative light units (measured in a Monolight 2010 luminometer) per minute per milliliter per unit of optical density at 650 nm. Values are the average of at least four measurements from two or more growth experiments.

[b] Overnight cultures were diluted 1:500 in 2216 broth, grown with aeration at 30°C, and harvested for light measurements at points corresponding to the exponential and early and late stationary phases; i.e., approximate optical densities at 650 nm of 0.5, 1.0, and 1.5, respectively. All of the mutants grew at the same rate as the parent, LM1017.

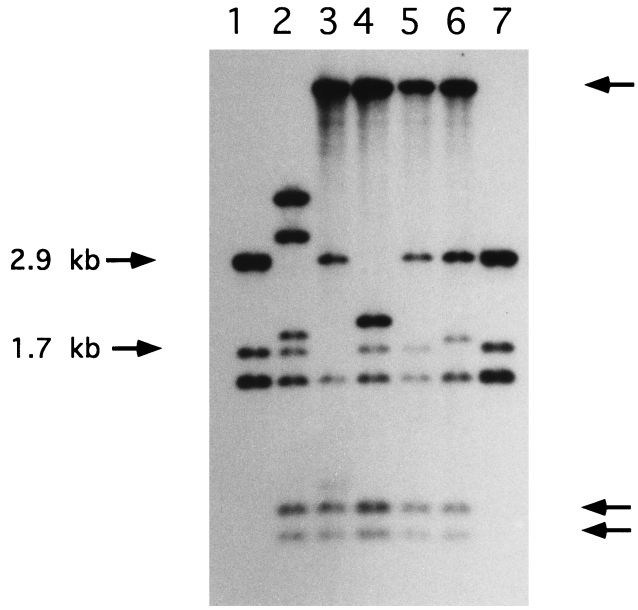

FIG. 1. Southern blot analysis of Bright mutants. Chromosomal DNAs from the following strains were prepared: lane 1, LM1017 (parental strain); lane 2, LM4400; lane 3, LM4407; lane 4, LM4417; lane 5, LM4418; lane 6, LM4420; lane 7, LM1017. DNA was digested with *Pst*I and *Hin*dIII, loaded in the indicated lanes of a 0.8% agarose gel, Southern blotted, and probed with pLM1897. All of the mutants except LM4418 show perturbation of either a 1.6- or 2.9-kb fragment, indicated by arrows at the left. The bands unique to mini Mu are indicated by the arrows at the right. LM4400 appears to have undergone an alteration in the large mini Mu fragment.

transposon mutants was assembled after mutagenizing LM1017 with transposon mini Mu *lac* (Tet^r). This bank was screened in microtiter dishes filled with liquid medium (HI broth with conalbumin) for Bright mutants producing light constitutively. The microtiter dishes were monitored for light emission by exposure to X-ray film for approximately 5 to 10 min at 7 and 14 h after inoculation into liquid medium. The bank was also examined for mutants with defects in swimming motility in semisolid tryptone marine motility medium, Dark (uninducible) mutants that failed to bioluminesce on solidified HI medium, and auxotrophs on minimal medium. Approximately 30 Bright Mot$^+$ mutants, 58 Bright Mot$^-$ mutants, 34 Dark Mot$^+$ mutants, and 154 auxotrophs were identified, corresponding to 0.25, 0.48, 0.28, and 1.28%, respectively.

Physiological characterization of Bright mutants. In a preliminary screen, 30 Bright strains were identified as candidates for mutants with lesions in a master lateral flagellar regulator. This set was then subjected to a more careful analysis, which included examination of motility phenotypes in liquid medium and in semisolid motility medium and *lux* induction measurements, i.e., measurement of light produced in a luminometer as a function of growth in liquid. Five mutants produced high levels of light in liquid and showed no motility or chemotaxis defect. Quantitation of the amount of light produced by these strains is shown in Table 2. Throughout the period of growth, Bright mutants produced approximately 2 orders of magnitude more light than strain LM1017. The amount of light emitted by these strains was comparable to the amount of light produced by a mutant strain which constitutively senses a surface, i.e., strain LM4170, which has polar flagellar function completely disrupted by a transposon in the gene encoding a motor component.

Bright mutations are physically linked in the chromosome. Chromosomal DNA was prepared from one of the mutants to clone DNA flanking the transposon insertion. The transposon mini Mu *lac* (Tet^r) contains a single *Pst*I site, which can be used to clone the tetracycline resistance gene from mini Mu and the flanking chromosomal DNA that stretches to the next *Pst*I site. By selecting for tetracycline resistance, approximately 4 kb of linked chromosomal DNA was cloned from LM4407. When the resultant clone, pLM1897, was used as a probe for a Southern blot of restricted chromosomal DNAs prepared from all of the Bright mutants, rearrangements were observed for four of the five mutants. Figure 1 shows a Southern blot analysis of chromosomal DNAs digested with *Pst*I and *Hin*dIII. The transposons in LM4407 and LM4420 appeared to map in a 1.7-kb fragment, while the insertions in LM4400 and LM4417 mapped in a 2.9-kb fragment. Thus, all but one of the transposons (in LM4418) were localized to the same region of the chromosome.

The cloning strategy described above retrieved the flanking sequence from only one side of the transposon, and so the intact gene needed to be cloned. A 700-bp *Hin*dIII fragment, containing approximately 200 bp of Mini Mu and the immediately adjacent 500 bp of chromosomal DNA, was subcloned into pUC19 to make pLM1900. By using pLM1900 to probe a Southern blot of variously restricted wild-type DNA, a 5.5-kb *Bam*HI-*Sal*I fragment was identified as potentially containing the entire gene, and a bank of sized, 5.5-kb fragments was constructed in the vector pLM1835. The bank was probed with an oligonucleotide derived from the nucleotide sequence of pLM1900, and a hybridizing clone, pLM1942, was identified. An oligonucleotide was used for probing because the 700-bp fragment from pLM1900 yielded a high background signal with *E. coli* colony blots. When the newly identified clone, pLM1942, was used as a probe against a Southern blot of restricted chromosomal DNAs, the same rearrangements were seen for the four mutants as in the Southern analysis shown in Fig. 1 (data not shown). Again, LM4418 failed to show any perturbation, suggesting that the mutation in this strain mapped in a distinct locus.

When clone pLM1942 was transferred to mutant strains LM4400 and LM4407, the Bright phenotype was lost. In contrast to parental strain LM1017 (Fig. 2, row 2), which was dark when grown in liquid medium, Bright mutants grown in liquid medium produced light (for example, LM4400; Fig. 2, row 3)

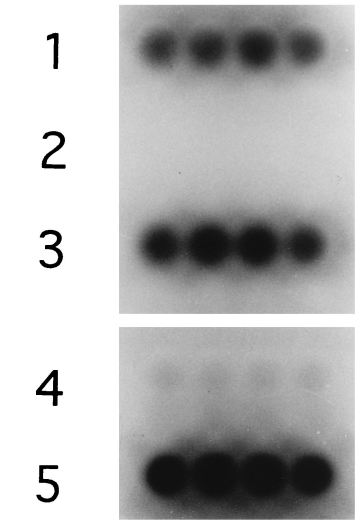

FIG. 2. Complementation of the Bright phenotype with clone pLM1942. *laf*::*lux* fusion strains were grown with aeration in HI broth or in HI broth with chloramphenicol, when appropriate, to an optical density at 650 nm of approximately 1.0, at which time four microtiter wells in a row were each filled with 0.1 ml of culture. The microtiter dishes were exposed to X-ray film for approximately 15 min. The strains were as follows: row 1, LM4170 (Mot$^-$); row 2, LM1017 (parent); row 3, LM4400; row 4, LM4400/pLM1942 (*lonS*$^+$); row 5, LM4400/pLM1944 (negative control plasmid).

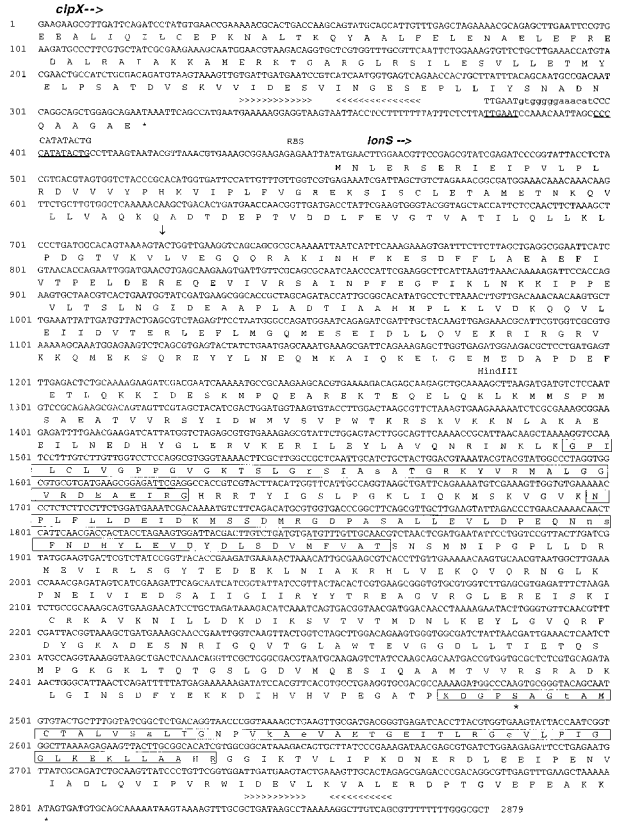

FIG. 3. DNA nucleotide and deduced amino acid sequences of the *lonS* locus. The features of a potential heat shock promoter are underlined, above which are shown the homologous regions of the *E. coli lon* promoter. A site resembling the Shine-Dalgarno sequence of the *E. coli* ribosome-binding site (RBS) is indicated, and potential regions that could serve as rho-independent transcriptional terminators are marked with chevrons. The approximate point of insertion of the transposon in strain LM4407 is indicated by an arrow at bp 722. Clone pLM1900 originated at this point and extended to the indicated *Hin*dIII site at bp 1277. Conserved domains in the deduced protein sequence with the family of ATP-dependent proteases are boxed. The first two boxes encompass the presumed nucleotide-binding pocket. The third box contains the predicted active-site serine at position 678 (indicated by the asterisk). Deviations within these domains from the *E. coli* Lon sequence are shown in lowercase letters. Preceding the *lonS* gene is a coding region whose deduced polypeptide product resembles ClpX-like proteins. Although the sequence is not presented, downstream approximately 200 bases beyond *lonS* begins the coding region for a gene whose product resembles histone-binding protein HU-1.

and the level of luminescence was comparable to the level emitted by Mot$^-$ strain LM4170 (Fig. 2, row 1). Plasmid pLM1942 appeared to contain a functional gene that complemented the defects causing the Bright phenotype in both mutants. Strains with pLM1942 (for example, LM4400/pLM1942; Fig. 2, row 4) produced significantly less light than strains carrying a control plasmid with an unrelated insert, pLM1944 (for example, LM4400/pLM1944; Fig. 2, row 5).

Nucleotide sequence analysis: identification of a *lon* gene. The DNA sequence is presented in Fig. 3 along with deduced amino acid sequences. The initial sequence was obtained from pLM1900, containing approximately 500 bp of chromosomal DNA linked directly to the transposon insertion. The remainder of the sequence was obtained from clone pLM1942 by priming with oligonucleotides directed 5′ and 3′ to the sequence obtained for pLM1900. The mutation mapped within a very large open reading frame. The deduced product encodes a polypeptide 783 amino acids long with a calculated molecular mass of 87,695 Da. The predicted amino acid sequence shows exceptionally high homology to the *E. coli lon* gene. BESTFIT analysis with the Genetics Computer Group sequence analysis program calculated the *Vibrio* product to be 89.8% similar and 80.7% identical to *E. coli* Lon (10; GenBank accession no. L20572). It is equally related to the *lon* gene product of *Erwinia amylovora* (13; GenBank accession no. X77706). The Lon protease has a serine in its active site and belongs to a family of proteases which are dependent on ATP hydrolysis for their activity (3, 19). The *Vibrio* gene product contains the signature domains of this family, and these are indicated by the boxes in Fig. 3 (10, 44). Sequences in the boxes are identical to *E. coli* Lon, with exceptions denoted by the lowercase amino acid designation. The putative active-site serine is marked by an asterisk. Also, the gene may be under similar transcriptional controls: preceding the coding region for *lonS* are sequences, which are underlined, resembling heat shock promoter elements and exactly matching relevant upstream sequences for *E. coli lon* (11, 15).

The entire region is similar to *lon* loci of other bacteria. There are potential rho-independent transcriptional terminators flanking the gene. Preceding the *lonS* gene, BLASTX analysis of the nucleotide sequence identified a coding region for a ClpX-like protein. The deduced polypeptide showed 83% positives and 73% identities with respect to *E. coli* ClpX (GenBank accession no. L18867) over 106 amino acids at the C terminus. Downstream of *lonS* is a coding region for an HU-1-like protein, which exhibited 85% positives and 67% identities over the length of *E. coli* HU-1 (90 amino acid residues; GenBank accession no. X16540). Thus, the organization of genes in this region resembles that found in *E. coli*, *Azospirillum brasilense*, and *Bacillus subtilis* (18, 37, and GenBank accession no. U18229, respectively).

***V. parahaemolyticus lonS* complements an *E. coli lon* mutant.** In *E. coli*, loss of the *lon* gene has pleiotropic effects, including overproduction of colanic acid (encoded by *cps* genes) and sensitivity to UV damage (8, 17). The *lon* mutant *E. coli*

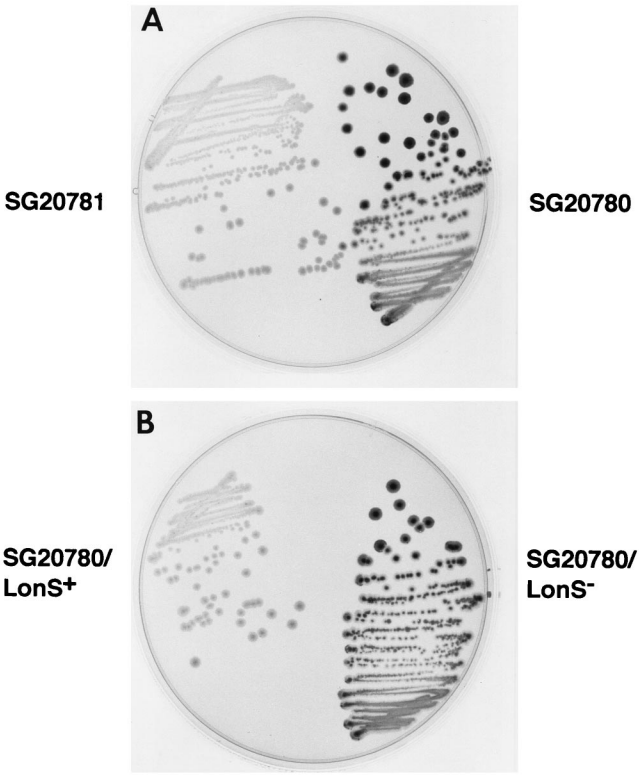

FIG. 4. Complementation with *V. parahaemolyticus lonS* of an *E. coli lon* mutant strain containing an *rcsB*::*lac* fusion on MacConkey-lactose indicator plates. Red Lac^+ colonies appear dark, and White Lac^- colonies appear light. In panel A, lon^+ *E. coli* SG20781 is on the left and *lon* mutant SG20780 is on the right. In panel B, *E. coli lon* mutant SG20780 with pLM1942 ($lonS^+$) is on the left and SG20780 with pLM1944 (control plasmid) is on the right.

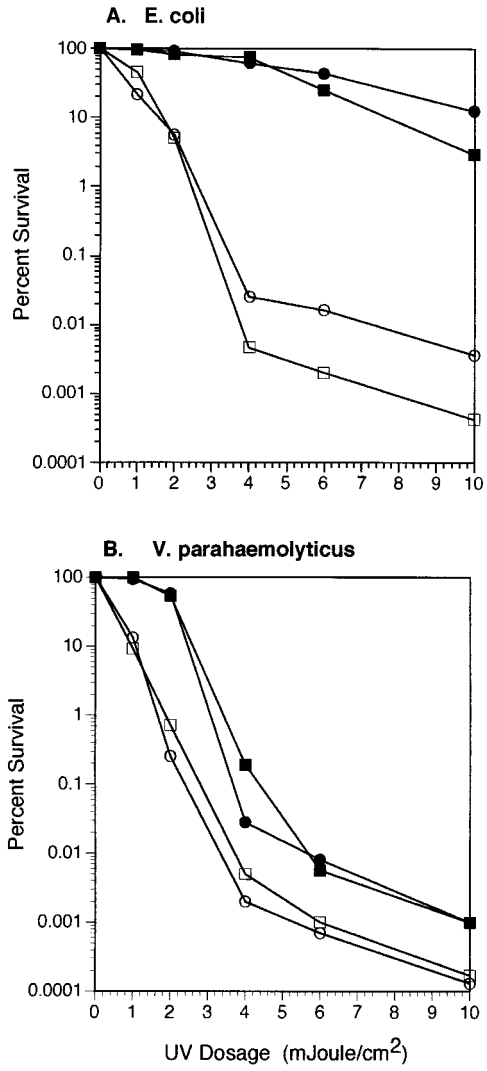

FIG. 5. UV survival of an *E. coli lon* mutant strain complemented with *V. parahaemolyticus lonS* and of *V. parahaemolyticus* strains. Cultures were grown to an optical density at 650 nm of approximately 0.4, placed on ice, and diluted for plating. Cells at various dilutions were spread on plates in triplicate, and the plates were exposed to UV for the indicated energy levels. After overnight incubation, the survivors were counted. Numbers of survivors are expressed as fractions of the nonirradiated samples and are averages of at least two experiments. Panel A shows the UV survival of *E. coli*. pLM1942 carries $lonS^+$, and pLM1944 is a negative control plasmid. Symbols: ●, SG207081 (lon^+); ■, SG207080/pLM1942; ○, SG207080 (*lon*); □, SG207080/pLM1944. Panel B shows the UV survival of the indicated *V. parahaemolyticus* strains. Symbols: ●, LM1017 ($lonS^+$); ■, LM4418 ($lonS^+$); ○, LM4407 (*lonS*); □, LM4400 (*lonS*).

SG20780 carries a *lac* fusion in the *cpsB* gene. On MacConkey-lactose indicator plates, this strain is Lac^+ (Red), while lon^+ *cpsB*::*lac* strain SG20781 is Lac^- (White) on indicator plates (Fig. 4, plate A). Introduction of clone pLM1942, which carries *V. parahaemolyticus lonS*, into SG20780 converts the strain to a Lac^- phenotype on MacConkey-lactose plates (Fig. 4, plate B). For comparison, introduction of control plasmid pLM1944 into SG20780 had no effect on the Lac^+ phenotype. Thus, the *V. parahaemolyticus lonS* clone complements the *lon* defect in *E. coli* with respect to regulation of capsular polysaccharide gene expression.

E. coli lon mutants were originally isolated as a class of UV-sensitive mutants (22). To further examine the functionality of the *Vibrio lonS* gene product and the interchangeability of the gene with *E. coli lon*, UV survival rates were examined. The dramatic contrast in UV sensitivity between SG20780 (*lon*) and SG20781 (lon^+) is shown in Fig. 5A. When $lonS^+$ *Vibrio* clone pLM1942 was introduced into SG20780, the response to UV irradiation was restored to a level of resistance equivalent to that which was observed for SG20781, while introduction of control plasmid pLM1944 had no effect.

Pleiotropy of the *lonS* mutation in *V. parahaemolyticus*. Since transposons causing the Bright phenotype were inserted into a gene encoding an ATP-dependent protease that was extraordinarily homologous to and functionally interchangeable with the *E. coli* Lon protease, we examined the responses of *V. parahaemolyticus* mutant strains to UV exposure. All of the mutant strains with transposons mapping in the *lonS* locus, i.e., LM4400, LM4407, LM4417, and LM4420, were more sensitive to UV damage than the wild-type strain or LM4418, the one Bright strain that appeared to have a lesion in an unlinked gene. The UV responses of LM4407, LM4400, LM4418, and LM1017 are shown in Fig. 5B. *V. parahaemolyticus* appeared to be more sensitive to UV irradiation than *E. coli*, and so the difference between *lonS* and $lonS^+$ strains is not as great as that observed for *lon* and lon^+ *E. coli* strains; however, the *lonS* mutants were clearly more UV sensitive than the parental strain. Introduction of pLM1942 into LM4400 and LM4407 restored UV survival to the wild-type level (data not shown).

Examination of the Bright mutants in a light microscope revealed a striking contrast between *lonS* mutants and the

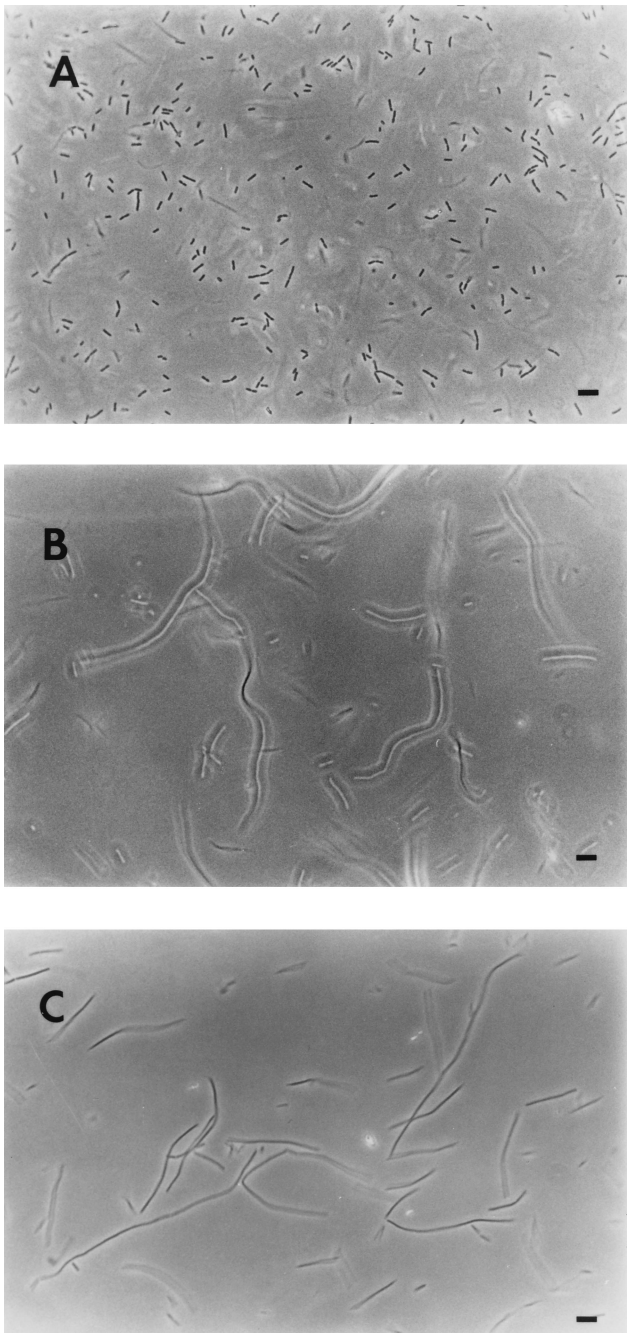

FIG. 6. Size of a *lonS* mutant grown in liquid. Overnight liquid cultures were diluted 1:250 into fresh HI broth and grown with aeration until the optical density at 650 nm was approximately 0.4. Overnight HI plate-grown cells were resuspended and replated on fresh HI plates and grown for 4 h. Liquid- and plate-grown cultures were examined in an Olympus BH2-RFCA phase-contrast microscope with a 40× objective lens. Photographic exposures were recorded on Kodak Tmax 400 film with an automatic exposure meter. The size of swimmer cells is 2 to 3 μm, and the bar in each panel is approximately 5 μm long. Strains examined: A, LM1017 swimmer cells in HI broth; B, LM4407 *lonS* mutant cells in HI broth; C, BB22 swarmer cells from an HI plate.

parental strain. Exponentially growing liquid cultures of *lonS* strains were composed primarily of very long bacteria (Fig. 6B) equivalent in size to plate-grown swarmer cells (Fig. 6C) and in sharp contrast to the length of liquid-grown swimmer cells (Fig. 6A). LM4418 was approximately the same length as LM1017. Introduction of $lonS^+$ plasmid pLM1942, but not that of the control pLM1944, into LM4407 restored the length to that of liquid-grown LM1017.

DISCUSSION

Development to the swarmer cell is signaled by growth on a surface, and the swarmer cell is highly adapted for colonization of surfaces or slime layers. Movement over surfaces can be very fast, e.g., cells inoculated into the center of an 85-mm-diameter HI petri plate solidified with 1.5% agar move to the perimeter within 10 h when incubated at 30°C. Swarmer cell differentiation represents a sizable cellular commitment in terms of energy expenditure and gene expression. Genetic analysis suggests that there are more than 40 swarmer cell genes and that they are carefully regulated in a hierarchy of expression (33). Moreover, we know that there are multiple inputs essential for initiation of differentiation (28). The developmental switch is tightly controlled and is subject to multiple regulatory signals.

We have found a gene, *lonS*, encoding an ATP-dependent protease that plays a key regulatory role in swarmer cell differentiation. By using a luminescence reporter gene system fused to the lateral flagellar gene *lafX*, transposon-induced constitutive mutants producing luminescence in liquid were isolated. Bright mutants produced at least 2 orders of magnitude more light in liquid than the parental strain from which they were derived. Four of five transposons inducing the Bright phenotype were found to be inserted in a gene, *lonS*, that encodes a protein similar to the *E. coli* Lon protease. The gene containing the fifth transposon insertion remains to be identified. In this work, we have initiated characterization of *lonS* and the role its product plays in *V. parahaemolyticus*. Nucleotide sequence analysis revealed a deduced gene product remarkably similar to *E. coli* Lon, showing 81% identity throughout the length of the polypeptide. Moreover, preceding the coding region are sequences reminiscent of *E. coli* heat shock promoters and highly similar to the sequences preceding *lon* (11, 13). The entire locus resembles other *lon* loci: flanking sequences encode ClpX-like and HU-1-like polypeptides. Thus, the molecular organization of the genetic locus is well conserved.

The function of the gene product also seems to be conserved. The LonS protease of *V. parahaemolyticus* substitutes well for the Lon protein of *E. coli*. Mutants of *E. coli* with *lon* defects overexpress capsular polysaccharide due to accumulation of RcsA, a positive transcriptional regulator of *cps* genes (20). Lon degrades RscA and thus limits the accumulation of the transcriptional activator. LonS appears to be able to target RcsA, for overexpression of the *cpsB* gene was kept in check in an *E. coli lon* mutant by the introduction of a plasmid carrying the *Vibrio* gene encoding the Lon homolog. The *lonS* gene could also cure the sensitivity of *lon* mutants to UV light. DNA damage caused by UV exposure induces the SOS response in *E. coli*. One of the induced genes is *sulA*, which encodes a cell division inhibitor (36). As a result, cells that overproduce SulA fail to septate. Wild-type cells form filaments after UV exposure, but with time the filaments resolve. Mutants with defects in *lon* cannot recover from elongation. They produce long, nonviable filaments on exposure to UV due to an accumulation of SulA. The *lonS* gene rescued a *lon* mutant from the lethal effects of SulA. Thus, the *Vibrio* protease targets multiple *E. coli* proteins.

What are the *Vibrio* targets of the LonS protease? Gottesman has remarked that regulation by proteolysis seems to be a mechanism ideally suited for rapid control of critical regulatory proteins, i.e., timing proteins (17). The pleiotropic phenotype

(*laf*::*lux* constitutivity and UV sensitivity) of the *Vibrio* mutants suggests that, as is the case for *E. coli* Lon, there are multiple targets of LonS, including a transcriptional activator of lateral flagellar genes and a cell division inhibitor. Perhaps the master swarmer cell regulator, which is stabilized in *lonS*-deficient strains, is a positive transcription factor analogous to RcsA or a swarmer cell-specific sigma factor. Examples of protease-controlled steps in cellular development include fruiting body formation of *Myxococcus xanthus* (16, 44) and sporulation of *Bacillus subtilis* (40). Mutants to be investigated as candidates for possession of lesions in a master swarmer cell regulator are the uninducible Dark mutants, which were also isolated in this study.

The most intriguing characteristic of the Bright mutants is their long phenotype. It should be emphasized that *E. coli lon* mutants are not constitutively long: they become long after exposure to UV. When grown in liquid, *lonS* mutants not only express the *laf*::*lux* fusion inappropriately but are filamentous, resembling swarmer cells harvested from a plate. In *E. coli*, cell growth, DNA replication, and cell division are precisely balanced (12). In *V. parahaemolyticus*, cell division does not occur with the same regularity in swimmer and swarmer cells. It seems that the timing of cell division, i.e., the coordination or coupling of DNA replication and division, might be under a different or additional layer of control compared with that of *E. coli*. This work provides a clue to how the timing of cell division might be linked to swarmer cell differentiation. Thus, it is attractive to hypothesize the existence of a swarmer cell-inducible cell division inhibitor. Moreover, the inhibition of cell division during the swarmer cell cycle must be very carefully regulated, for prolonged repression of cell division would be a terminal event, and so the cell must be able to escape from filamentation. Swarmer cell differentiation does seem to be a transient state. Differentiation is reversible: swarmer cells removed from an agar plate and suspended in liquid growth medium form septa, separate into short cells, and cease synthesis of lateral flagella (42). More importantly, dedifferentiation also occurs on a plate: cells at the leading edge of a swarming colony are long, while those in the center are short (45). Cloning and examination of the genes encoding the *Vibrio* counterparts to SulA and its target FtsZ may be very informative.

In future work, it will be critical to construct *lon* knockout mutants by allelic replacement to further examine the role of *lonS* in swarmer cell differentiation. The transposon-induced mutants examined in this work may have accumulated additional mutations suppressing detrimental effects of lesions in *lonS*. Mutants will be constructed in the wild-type background, as well as the *lafX*::*lux* background. The *lafX*::*lux* strain cannot swarm and fails to express lateral flagellar genes that occur later in the flagellar hierarchy, for example, the flagellin, motor, and sigma genes (33). So, to examine the level at which LonS exerts its effect upon swarmer cell differentiation, the mutations must be transferred into the wild-type background. Interestingly, preliminary attempts to construct a Swarm$^+$ *lonS* mutant have been unsuccessful.

How does *lonS* fit into the scheme of swarmer cell gene control? Perhaps the gene directly participates in the tactile sensing pathway; however, it could also function to control swarming by an independent mechanism. In many ways, the gene so closely resembles *E. coli lon* that it seems possible that the role LonS plays in the swarmer cell gene system is in its general capacity as a policeman, keeping in check the regulatory proteins that mediate surface sensing. Alternatively, LonS may play a more specialized role in swarmer cell gene expression. To test this hypothesis, it will be interesting to determine whether the expression of *lonS* or the activity of the gene product is regulated by growth on a surface.

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