Two-Component Sensor Required for Normal Symbiotic Colonization of *Euprymna scolopes* by *Vibrio fischeri*

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The light organ of the squid *Euprymna scolopes* is specifically colonized to a high density by the marine bacterium *Vibrio fischeri*. To date, only a few factors contributing to the specificity of this symbiosis have been identified. Using a genetic screen for random transposon mutants defective in initiating the symbiotic association or in colonizing the light organ to high density, we identified a mutant of *V. fischeri* that exhibited an apparent defect in symbiosis initiation. This mutant was not defective in motility, luminescence, or growth in minimal medium, suggesting that it lacks an essential, previously unidentified symbiotic function. By sequence analysis, we showed that the locus inactivated in this mutant encodes a predicted 927-amino-acid protein with a high degree of similarity to the sensor component of hybrid two-component regulatory systems. We have therefore designated this locus *rscS*, for regulator of symbiotic colonization—sensor. Sequence analysis revealed two hydrophobic regions which may result in the formation of a periplasmic loop involved in signal recognition; PhoA fusion data supported this proposed membrane topology. We have investigated the start site of *rscS* transcription by primer extension and identified a putative promoter region. We hypothesize that RscS recognizes a signal associated with the light organ environment and responds by stimulating a putative response regulator that controls protein function or gene expression to coordinate early colonization events. Further studies on RscS, its cognate response regulator, and the signaling conditions will provide important insight into the interaction between *V. fischeri* and *E. scolopes*.

The bioluminescent marine bacterium *Vibrio fischeri* forms an intimate symbiotic association with the Hawaiian squid *Euprymna scolopes*. Juvenile *E. scolopes* squid hatch from eggs uncolonized by *V. fischeri* cells but become rapidly and exclusively colonized by this bacterium despite potential competition from the many other bacterial species present in seawater (46, 48). Initiation of this mutualism typically requires only a short exposure (1 to 3 h) to *V. fischeri*, after which normal colonization will result even if the squid are removed to *Vibrio*-free seawater (40). Within 24 h, the *V. fischeri* cells reach a population of between 10^5 and 10^6 CFU in the symbiotic light organ, equivalent to approximately 10^8 CFU/cm^3 (40).

Because of the exclusive nature of the relationship between *V. fischeri* and *E. scolopes*, it is likely that both bacterial and host genes function in actively establishing this specific, long-term association. Through the study of bacterial mutants, three major stages of symbiotic colonization have been identified. These stages include (i) initiation of the association (initiation), (ii) colonization to the high cell density typically achieved by the wild type (accommodation), and (iii) persistence at a high cell density (persistence) (reviewed in reference 39).

Mutants defective for each of the stages have been identified. Initiation mutants include those defective for motility, because neither nonflagellated nor flagellated but nonmotile mutants can colonize the host (21). It is thought that a thick mucus layer present in the ducts leading to the light organ crypts, where colonization occurs, may prevent entry of non-

motile bacteria (47). Accommodation mutants include bacteria defective in one of several amino acid-biosynthetic genes. The ability of these strains to colonize, albeit at lower levels, suggests that the light organ environment is nutrient rich and that the required amino acids are being supplied by the squid host. The discovery of peptides present at high concentration in the fluid of the light organ supports this hypothesis (22).

Persistence mutants include two distinct subclasses. One subclass mutant lacks the ability to produce and take up siderophores. This mutant exhibited a decreasing level of colonization over the course of 3 days (23), suggesting that sequestration of iron from the light organ environment is an important factor in maintaining the symbiotic association. The second subclass includes bioluminescence mutants, defective in either structural or regulatory genes. The bioluminescence mutants colonize to a level comparable to the wild-type strain during the first day and thereafter show a decreased level of colonization (45, 47). At present, the exact relationship between defects in light production and reduced colonization remains unclear, but it has been speculated that utilization of oxygen by the bioluminescence machinery may reduce host-imposed oxidative stress (45).

Although these symbiosis-impaired mutants have provided valuable clues to the environmental conditions in the light organ, no comprehensive screen for bacterial genetic determinants of symbiotic competence has been described. We anticipate the existence of bacterial genes that are essential to initiate the symbiosis, including surface markers or receptors, as well as additional genes required for accommodation. It is also likely that regulatory factors controlling one or more regulons involved in the symbiosis will be found, particularly in view of the developmental events taking place in both partners as their association progresses (33, 39).

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In the present study, we have screened the ability of mutants generated by a random transposon insertion mutagenesis to colonize juvenile *E. scolopes*. We present here the characterization of one mutant with a severe symbiosis defect. This mutant colonizes poorly and thus may be defective in the ability to overcome a barrier(s) associated with early colonization events. We also present sequence data that predict that the defective gene interrupted by the transposon insertion in this mutant strain may encode a regulatory protein. We hypothesize that this putative regulator may play a key role in the expression of genes essential for, or even induced by, this bacterium-animal association.

MATERIALS AND METHODS

Strains and media. *V. fischeri* strain ES114 (9) and its rifampin-resistant derivative ESR1 (21) were the parent strains used in this study. *Escherichia coli* strains DH5α and CC118λ*pir* (27) were used as host strains for cloning, and S17-1λ*pir* (43) was used as the donor strain in conjugations.

V. fischeri cells were grown in SWT (9), LBS (15), or HEPES-minimal medium (41) containing ribose as a carbon source. *E. coli* strains were grown in Luria-Bertani (LB) medium (12). Conditioned medium (CM) was used to determine whether the colonization mutant KV712 was defective for bioluminescence in culture and was made as follows. *V. harveyi* B392 (38) was grown in LM (35) to an optical density of approximately 0.8 at 600 nm. The cells were removed by centrifugation followed by passage of the supernatant through a 0.2-μm-pore-sized filter. Where appropriate, antibiotics were added to the following final concentrations: ampicillin, 100 μg/ml; chloramphenicol (CHL), 1 to 5 μg/ml for *V. fischeri* and 30 μg/ml for *E. coli*; erythromycin, 1 to 5 μg/ml for *V. fischeri* and 150 μg/ml for *E. coli*; rifampin, 100 μg/ml; and tetracycline (TET), 5 μg/ml for *V. fischeri* and 15 μg/ml for *E. coli*. Agar was added to a final concentration of 1.5% for solid medium or 0.25% for motility plates.

Genetic techniques. Transposon mutagenesis was carried out using the Tn*10*-*lacZ* delivery plasmid pKV124 (see below). Conjugations were performed as follows. The donor strain (S17-1λ*pir* carrying the plasmid to be delivered) and the recipient *V. fischeri* strain (typically either ES114 or ESR1) were grown to mid-log phase at 37°C in LB and at 28°C in LBS, respectively. The strains were mixed, concentrated by centrifugation, and allowed to mate on solid medium. After an incubation of 6 to 14 h at 28°C, cells were diluted in LBS and transferred to selective medium. In some cases, triparental conjugations were performed using the appropriate *V. fischeri* recipient strain and two *E. coli* strains, DH5α carrying the plasmid to be transferred and DH5α carrying pRK2013 (13, 17), to supply conjugation functions.

Molecular techniques. All plasmid constructions were carried out by standard molecular biology techniques, using restriction and modifying enzymes obtained from New England Biolabs (Beverly, Mass.) or Promega (Madison, Wis.). The mini-Tn*10*-*lacZ* transposon in pKV124 was derived from pBSL181 (1) by the insertion of both a promoterless *lacZ* gene and *ori*R6K, the *pir*-dependent origin of replication from plasmid R6K (31). This plasmid is therefore a "suicide" plasmid in hosts lacking the *pir* gene, including *V. fischeri*. Cloning of DNA flanking the transposon insertion in *V. fischeri* strain KV712 was accomplished using the *ori*R6K and CHL resistance elements contained within the transposon. Chromosomal DNA was isolated and digested with a restriction enzyme that does not cleave within the insertion sequences. Fragments were then self-ligated using T4 DNA ligase and transformed into the permissive host CC118λ*pir* or S17-1λ*pir*. Clones carrying the transposon and flanking DNA were selected on CHL-containing LB plates. Two plasmids, pKV132 (containing about 3.8 kb of flanking DNA) and pKV133 (containing about 8.0 kb of flanking DNA), were obtained by digestion with *Nhe*I and *Bsr*GI, respectively. DNA fragments from clones pKV132 and pKV133 were subcloned into pBluescript (Stratagene, La Jolla, Calif.), pBC (Stratagene), or pEVS79 (E. V. Stabb and E. G. Ruby, submitted for publication). One of these subclones, pLMS22, contained a 2-kb *Hin*dIII-*Pst*I fragment; this plasmid was recombined into *V. fischeri* strain ESR1. Chromosomal DNA isolated from the resulting recombinant was used to clone the wild-type copy of the *rscS* gene and flanking DNA, resulting in pLMS25. Plasmid pLMS26 was derived from pLMS25 by the insertion of a 6-kb (*rscS*⁺) fragment into pKV69, a TET^r CHL^r mobilizable vector.

Sequencing was carried out using forward and reverse primers complementary to vector and insert sequences. Manual sequencing for primer extension was carried out using the Sequenase sequencing kit (Amersham-Pharmacia Biotech, Piscataway, N.J.). Similarity searches were performed using the NCBI Blast program (2). Oligonucleotides were obtained from either the Loyola Macromolecular Analysis Facility or Integrated DNA Technologies (Coralville, Iowa).

Southern blotting. Chromosomal DNA isolated from *V. fischeri* strain KV712 was digested with *Eco*RV, which cuts once within the mini-Tn*10*-*lacZ* transposon, or *Bsr*GI, which cuts outside the transposon. DNA fragments were separated using a 0.6% agarose gel, transferred onto a nylon membrane (Hybond XL; Amersham-Pharmacia Biotech), and UV cross-linked. Detection was carried out using the Boehringer Mannheim digoxigenin DNA labeling kit (Roche Molecular Biochemicals, Indianapolis, Ind.) as follows. Random priming was used to generate biotinylated DNA fragments complementary to the transposon sequences, and these fragments were hybridized to the chromosomal DNA in a buffer containing 50% formamide, 0.1% *N*-lauroylsarcosine, 0.02% sodium dodecyl sulfate, 750 mM NaCl, and 75 mM sodium citrate. Blocking reagent was added to 2% for the prehybridization step. Washing, blocking, treatment with primary and secondary antibodies, and colorimetric detection were carried out under conditions of high stringency according to the manufacturer's instructions.

Primer extension. A 25-ml culture of *V. fischeri* strain ES114 was grown in LBS to an optical density of 1 at 600 nm. The cells were lysed in GITCN lysis buffer (44), and mRNA was isolated by centrifugation through a cesium chloride gradient as previously described (44). An oligonucleotide primer, 10R3 (5′-GATT GTGATAAGGCTATAACG-3′), complementary to the 5′ end of the *rscS* locus was radiolabeled by treatment with T4 polynucleotide kinase (Amersham-Pharmacia Biotech) in the presence of [γ-³²P]ATP (Amersham-Pharmacia Biotech). The radiolabeled primer was hybridized to the mRNA and incubated with Moloney murine leukemia virus (MMLV) reverse transcriptase (Stratagene) and nucleotides per the manufacturer's instructions. Extension products were visualized by autoradiography after separation on an 8% polyacrylamide gel and compared to a sequencing ladder generated using the same oligonucleotide primer to sequence from plasmid pLMS35, containing the *rscS* locus and flanking DNA.

Colonization assays. To determine whether mutant *V. fischeri* strains were able to form a symbiotic association with *E. scolopes*, juvenile squid were placed in artificial seawater (Instant Ocean; Aquarium Systems, Mentor, Ohio) containing an inoculum of 1,000 to 5,000 cells of the desired *V. fischeri* strain per ml of fluid as previously described (39). Luminescence was monitored over the course of 16 to 24 h using a Packard Tri-carb 2100TR scintillation counter (Packard Instruments, Meriden, Conn.) modified to detect bioluminescence. At specific intervals, juvenile *E. scolopes* were sacrificed by homogenization to directly quantify the number of bacteria present in the light organ. Homogenates were serially diluted, and aliquots were spread on SWT agar for viable-cell counts. The limit of detection is 14 *V. fischeri* cells per squid.

Luminescence assays. KV712 and ESR1 were diluted 1:100 and grown in CM prepared as described above, in the presence or absence of a synthetic *V. fischeri* autoinducer (600 ng of 3-oxo-hexanoyl-L-homoserine lactone [Sigma, St. Louis, Mo.] per ml). At various times after inoculation, 1-ml samples were taken for luminescence and optical density measurements. A TD-20/20 luminometer (Turner Designs, Sunnyvale, Calif.) was used to determine the level of bioluminescence of KV712 and its parent.

Construction and analysis of *phoA* fusions. A mini-Tn*10* derivative carrying the *phoA* and *lacZ* genes fused in frame was obtained from M. Alexeyev. This transposon, carried by pMA651, was introduced by conjugation into DH5α carrying pBlueScript with a 3-kb *rscS*⁺ insert. After conjugation and selection for recipient strains carrying the transposon (carried out as described above for *V. fischeri*), the selected cells were pooled and subjected to a plasmid extraction procedure. The pooled plasmids were sequentially introduced into DH5α and CC118λ*pir* which were then grown on LB medium containing CHL and ampicillin to select for the presence of plasmids containing a transposon insertion. CC118λ*pir* recipient cells were plated on LB containing CHL and 5-bromo-4-chloro-3-indolylphosphate, an indicator of alkaline phosphatase activity. Colonies that turned blue on this medium (indicating an active fusion to PhoA) were identified; the plasmids from these colonies were analyzed by restriction digestion to ascertain the location of the transposon fusion. Fusions that mapped to the *rscS* gene were further localized by sequence analysis.

Nucleotide sequence accession number. The GenBank accession number assigned to the *rscS* sequence is AF319618.

RESULTS

Identification of a novel symbiotic locus. We have developed a genetic screen to identify bacterial genes important for the symbiotic interaction between *V. fischeri* and *E. scolopes*. Upon entering the light organ of a newly hatched juvenile squid,

wild-type *V. fischeri* rapidly reaches a high cell density (40). A cell density-dependent (quorum-sensing) mechanism regulates *V. fischeri* bioluminescence; therefore, the amount of light produced by the bacteria in the light organ serves as an indirect measure of colonization. We constructed transposon insertion mutants of *V. fischeri* strain ESR1 (see Materials and Methods) and inoculated newly hatched *E. scolopes* juveniles with individual mutant strains. Automated monitoring of luminescence over a 16- to 24-h period was used to determine the ability of these mutants to enter and proliferate within the light organ. This screen should identify mutants of the initiation class, which would be unable to colonize the light organ. The screen should also identify mutants of the accommodation class, which would be unable to reach normal cell densities after initial colonization.

From a collection of approximately 2,000 transposon insertion mutants, we identified a strain, KV712, that failed to exhibit the wild-type pattern (Fig. 1A, top) of bioluminescence in the symbiotic association: during the first 16 h post inoculation, 7 of 10 juvenile squid exposed to this mutant failed to emit light, while 3 exhibited delayed onset of bioluminescence (Fig. 1A, bottom). However, KV712 showed no detectable defect relative to the parent strain ESR1 in producing bioluminescence in culture or in inducing luminescence in response to the addition of an autoinducer (3-oxo-hexanoyl L-homoserine lactone) (Fig. 1B). Thus, the symbiotic luminescence defect apparently did not result from a mutation in the *lux* operon, which encodes the structural genes for bioluminescence.

In addition to *lux* mutants, nonmotile mutants and some amino acid auxotrophs fail to establish normal levels of colonization (21, 23). When tested for motility in a soft agar plate, strain KV712 exhibited motility identical to that of the parent strain (data not shown). Growth in a minimal medium was also comparable to that of ESR1 (data not shown). These data suggested that KV712 carries a mutation in a previously unidentified locus that affects the symbiotic interaction.

To confirm a potential colonization defect of KV712, we determined the number of CFU present in the light organ of the juvenile squid at 19 h postinoculation (Fig. 2). Of the 10 juveniles inoculated with KV712, 4 exhibited no detectable colonization (the limit of detection in this experiment was approximately 14 CFU), while an additional 3 were colonized at a level approximately 100-fold reduced from that of juveniles exposed to the wild-type strain. The three remaining juveniles, which had exhibited a delay in onset of bioluminescence (Fig. 1A), contained *V. fischeri* cells at a level comparable to that of the wild-type-colonized squid.

These data suggest that although KV712 is capable of bioluminescence, in squid assays it fails to emit the typical wild-type pattern of bioluminescence due to a symbiotic defect. We hypothesize that this mutant lacks the ability to properly initiate the symbiotic association. Occasionally, however, some mutant cells succeed in bypassing the "block" to initiation, after which colonization (i.e., multiplication) within the squid proceeds normally. In support of this idea, we observed that squid in which mutant cells apparently overcame the block more quickly (as judged by luminescence [Fig. 1A]) exhibited higher colonization levels at the assay time than those that experienced a greater delay. Furthermore, in preliminary experi-
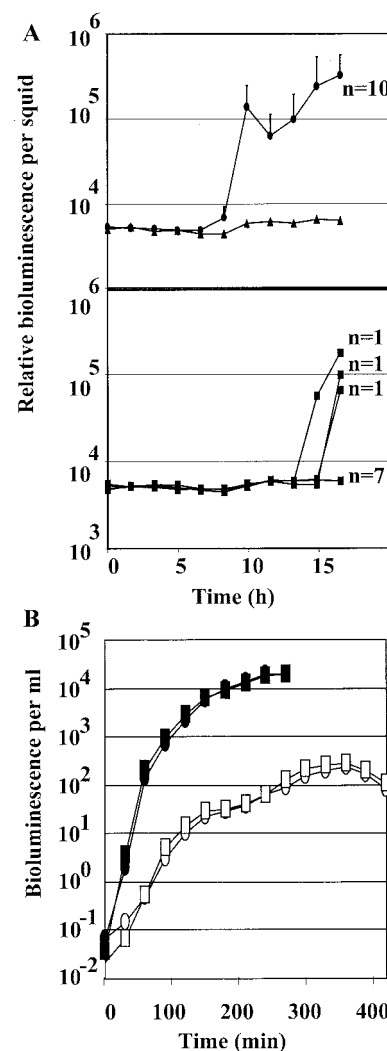

FIG. 1. Luminescence levels of colonization mutant KV712 and its parent. (A) Newly hatched juvenile *E. scolopes* squid were incubated in artificial seawater containing no *V. fischeri* (solid triangles, top panel) or either the parent strain ESR1 (solid circles, top panel) or the colonization mutant KV712 (solid squares, bottom panel). Bioluminescence was measured over time. The light levels of 10 animals inoculated with ESR1 (indicated by *n* = 10) and of 7 animals inoculated with KV712 (indicated by *n* = 7), were averaged, while three squid colonized by KV712 are represented singly (indicated by *n* = 1). Error bars are shown, representing the standard deviation of luminescence readings for ESR1-colonized animals; the level of light observed for seven of the KV712-inoculated squid was not above the limit of detection for the machine, and thus error bars are not displayed. (B) ESR1 (circles) and KV712 (squares) were grown in CM in the absence (open symbols) or presence (solid symbols) of an autoinducer of bioluminescence (3-oxo-hexanoyl-L-homoserine lactone). The level of luminescence was measured using a Turner 20/20 luminometer. The two strains grew at essentially the same rate.

ments we have observed that the relative frequency of high-level colonization by KV712 was reduced with smaller inoculum and a shorter duration of inoculation (Skoufos and Visick, unpublished data). Thus, we believe that the observed variation in the data represents random events during the interaction of a homogeneous population of mutants with an animal host.
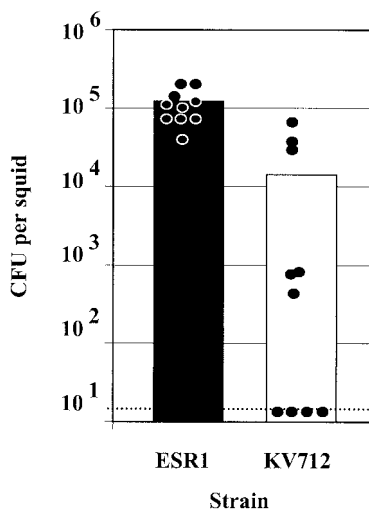
FIG. 2. Symbiotic colonization by *V. fischeri* strain KV712 and its parent, ESR1. Newly hatched juvenile *E. scolopes* were exposed for 15 h to either KV712 or ESR1. The level of colonization achieved by these strains was determined by homogenization and plating 19 h after the organisms were placed together. Each circle represents the number of *V. fischeri* cells in an individual squid, and the bar indicates the average colonization level of the 10 squid in each inoculation condition (2,300 cells per ml). The level of colonization of four squid inoculated with KV712 was below the limit of detection, as indicated by the dashed line.

Molecular characterization of KV712. Our data suggested that KV712 carries a mutation in a novel symbiosis locus. Because the mutation in this strain was generated by transposon mutagenesis, the number and location of transposon insertions could be easily determined by Southern blot analysis. DNA probes complementary to the transposon and its delivery plasmid were hybridized to chromosomal DNA isolated from KV712 and digested either with *Bsr*GI (which does not cut within the inserted sequences) or *Eco*RV (which recognizes a single site within the transposon). Consistent with a single transposon insertion event in KV712, we observed a single band for *Bsr*GI-digested DNA and two bands for *Eco*RV-digested DNA (approximately 5 and 7kb) (data not shown). When the same probe was hybridized to *Eco*RV-digested chromosomal DNA extracted from ESR1, no bands were observed (data not shown).

To further investigate the locus that was disrupted in KV712, we cloned the transposon and flanking chromosomal DNA. The presence of an origin of replication (*ori*R6K) and a CHL resistance marker within the transposon permitted rapid cloning by recircularization of chromosomal DNA fragments and transformation into a permissive *E. coli* host strain. Subcloning and sequencing revealed that the transposon had inserted into a large open reading frame (ORF), encoding a predicted 105-kDa protein of 927 amino acids.

Sequencing of flanking DNA demonstrated that the genes adjacent to this ORF possess a high degree of similarity to *E. coli* genes involved in glycerol metabolism and regulation. Located 581 nucleotides upstream of the putative translational start of the large ORF, there is an apparent homolog of the *glpR* gene (10), while 68 nucleotides downstream there is a *glpK* analog (37). Both genes are transcribed divergently rela-

tive to the large ORF (Fig. 3A), precluding the possibility that the ORF is part of an operon and suggesting that the transposon insertion in strain KV712 affects expression only of that single, large ORF. KV712 and its parent, ESR1, both fail to grow on glycerol as a carbon source, but a mutation of this ORF in ES114 (N. D. Montgomery and K. L. Visick, unpublished data) did not alter that strain's ability to grown on glycerol (data not shown).

Comparison of the ORF sequence to known sequences revealed significant amino acid sequence identity with the sensory component of hybrid two-component regulatory systems (Fig. 3B). Close matches included LuxQ (39% identity, 62% similarity) (7), ArcB (28% identity, 48% similarity) (30), and BvgS (26% identity, 45% similarity) (4), as well as a number of hypothetical *V. cholerae* sensor kinases (26). Each of these proteins (LuxQ, ArcB, and BvgS) plays a role in sensing an environmental signal and transducing the signal through a phosphorelay cascade (3, 29, 36) to a response regulator protein (LuxO [8], ArcA [14], and BvgA [4], respectively). Each response regulator protein then up- or downregulates transcription of a set of genes.

The domains involved in nucleotide binding and in the phosphorelay cascade are highly conserved among these proteins. The strongest similarity between the ORF disrupted in KV712 and these sensor kinases occurs in these domains (Fig. 3B). From these sequence alignments, we believe that this ORF likely encodes a hybrid two-component sensor kinase. Thus, we tentatively assign this locus the name *rscS*, for regulator of symbiotic colonization—sensor.

Complementation of the symbiotic defect. To determine whether the disruption of *rscS* caused the symbiotic defect of KV712, we cloned the wild-type copy of the locus. We performed complementation assays using a subclone, pLMS26, that contained *rscS*⁺ and approximately 3 kb of upstream DNA to ensure the presence of regulatory sequences. We infected juvenile *E. scolopes* with cells either wild-type (ESR1) or defective for *rscS* (KV712), carrying either the vector (pKV69) or the *rscS*⁺ complementing plasmid pLMS26. Plasmid pLMS26 but not pKV69 apparently restored symbiotic competence to KV712, as monitored indirectly by bioluminescence measurements over a 17-h period (data not shown). The presence of either plasmid did not affect symbiotic luminescence levels of the parent strain (data not shown).

After 17 h, we directly determined the level of colonization achieved by each strain. As predicted from the luminescence patterns, pLMS26 complemented the disrupted *rscS* gene in KV712: the complemented strain reached colonization levels comparable to those of the parent strain carrying either vector or pLMS26 (Fig. 4). We also observed wild-type levels of colonization when we inoculated juvenile squid with KV712 complemented with a construct containing only the 3-kb *rscS*⁺ locus (data not shown). In contrast, KV712 carrying the vector alone remained defective in properly initiating the symbiotic interaction. These results are consistent with the conclusion that the transposon insertion in *rscS* caused the symbiotic defect displayed by KV712.

Analysis of the *rscS* promoter region. To confirm transcription of the *rscS* gene and to identify a putative promoter region, we mapped the transcriptional start of *rscS* using primer extension. A radiolabeled primer complementary to the 5′
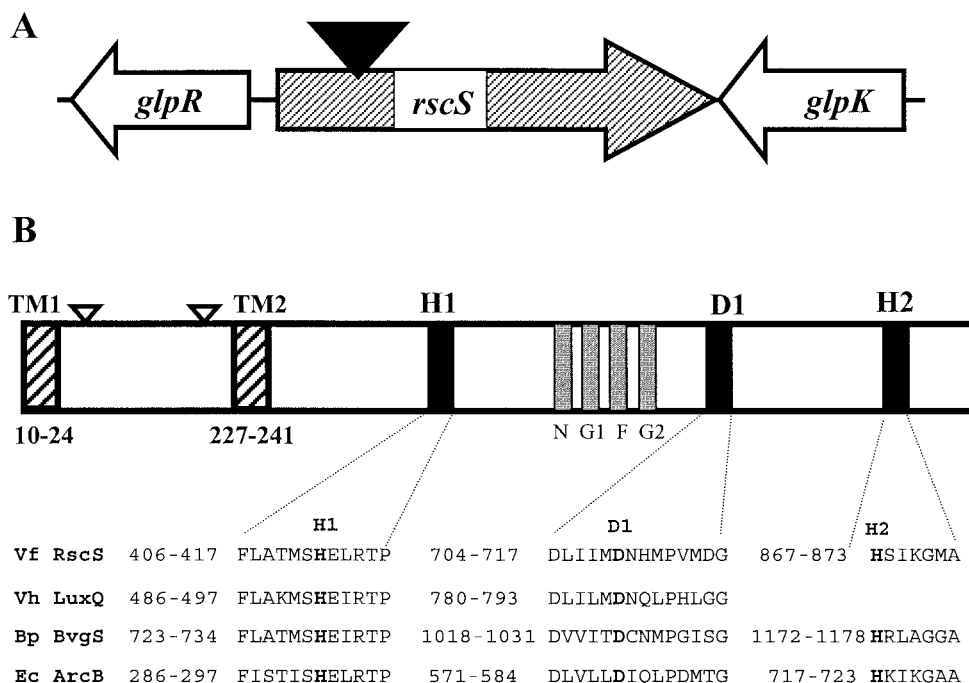
FIG. 3. Schematic diagram of the *rscS* locus and flanking genes. (A) The region of the chromosome flanking the transposon insertion in KV712 is indicated. The transposon (solid triangle) inserted in a large ORF, *rscS*, which is flanked by genes with high sequence similiarity to the *E. coli glpR* and *glpK* genes. The direction of transcription is indicated. (B) Noteworthy regions of the 927-amino-acid RscS protein are diagrammed. RscS exhibits sequence similarity to the *V. harveyi* (Vh) LuxQ, *Bordetella pertussis* (Bp) BvgS, and *E. coli* (Ec) ArcB proteins, particularly, as indicated below the black bars, in the regions flanking the histidine and aspartic acid residues (H1, D1, and H2) thought to be involved in the phosphorelay cascade. RscS also exhibits sequence similarity to the nucleotide-binding sites depicted by the gray boxes and labeled N, G1, F, and G2. Two putative transmembrane (TM) regions are indicated by striped bars and are designated TM1 and TM2. The locations of active *phoA* transposon insertions are indicated by open triangles.

region of the putative *rscS* ORF (spanning nucleotides 19 to 39 downstream from the putative translational start) was hybridized to mRNA isolated from the wild-type *V. fischeri* strain ES114. This served as the template for reverse transcription by MMLV reverse transcriptase. The resulting product was compared to a sequencing ladder generated from the same primer. We observed a major band (Fig. 5A), which places the transcriptional start site (+1) at the A residue located 34 nucleotides upstream of the predicted ATG translational start codon (Fig. 5B), and a minor band, which maps to an A residue 46 bases further upstream. The significance of the minor transcriptional start is unclear.

We have identified possible regulatory elements upstream of the putative translational start, based on the location of the major transcriptional start site and consensus sequences found in *E. coli*. These assignments are tentative because there has been little research identifying such elements in *V. fischeri*. A possible Shine-Dalgarno sequence, AGGAGC (shown in boldface and underlined), is located 8 bases upstream of the proposed translational start and matches five of six bases of the consensus *E. coli* sequence AGGAGG (42). A putative −10 promoter sequence (dotted underline), TAAAAT, is centered at base −11 with respect to the transcriptional start. This sequence is similar to that identified by primer extension mapping of the *V. fischeri lux* operon (16) and is identical at five of six bases to the canonical *E. coli* −10 sequence, TATAAT (25). Fifteen nucleotides upstream of this sequence (dotted under-

line) is a potential −35 sequence, TTGTAA, that matches the *E. coli* −35 consensus (TTGACA) (25) at four of six bases. In addition to predicting a possible promoter, these primer extension results provide evidence that the *rscS* locus is transcriptionally active in cells grown in laboratory culture.

PhoA fusions predict a periplasmic loop. LuxQ and BvgS hypothetically recognize their respective environmental signals by means of an amino-terminal periplasmic loop (4, 7). These periplasmic loops exhibit no sequence similarity to each other despite the strong conservation of other domains in the proteins, suggesting that this region gives each protein its unique function. Similarly, the amino terminus of the putative RscS shows no significant similarity to any other known protein.

Initial support for the hypothesis that RscS may possess a periplasmic loop resulted from a hydrophobicity analysis (dense alignment surface method) (11). Two highly hydrophobic regions were found in the N-terminal third of the protein (amino acids 10 to 24 and 227 to 241). A second program (SOUSI) (28) predicted two transmembrane (TM) regions, from 6 to 28 and 222 to 244, as well as a third TM from 312 to 334.

To investigate whether these hydrophobic regions result in a periplasmic loop, we mutagenized an *E. coli* strain (Materials and Methods) carrying a plasmid-borne copy of *rscS* with a mini-Tn*phoA* transposon. Insertion of this transposon in frame into the *rscS* gene would result in synthesis of an RscS-PhoA fusion protein, but the PhoA portion would be active only if
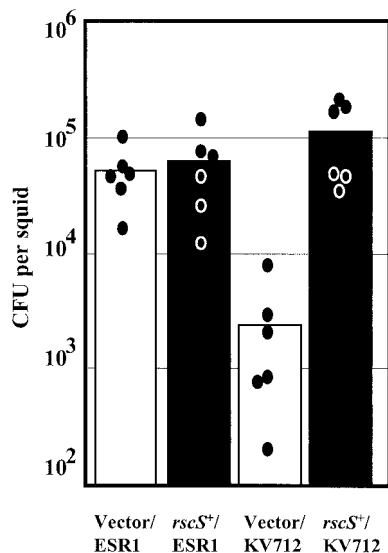
FIG. 4. Complementation of the colonization defect in KV712. Newly hatched juvenile *E. scolopes* were inoculated with one of four strains: KV712 or its parent ESR1, each carrying either vector pKV69 or *rscS*+ plasmid pLMS26. The squid were exposed to approximately 1,000 cells of *V. fischeri* per ml of seawater for 3 h. The level of colonization achieved by these strains 17 h after the organisms were placed together was determined by homogenization and plating as described in Materials and Methods. Each circle represents the number of *V. fischeri* cells in an individual squid, and the bar indicates the average colonization level of the six squid in each inoculation condition.
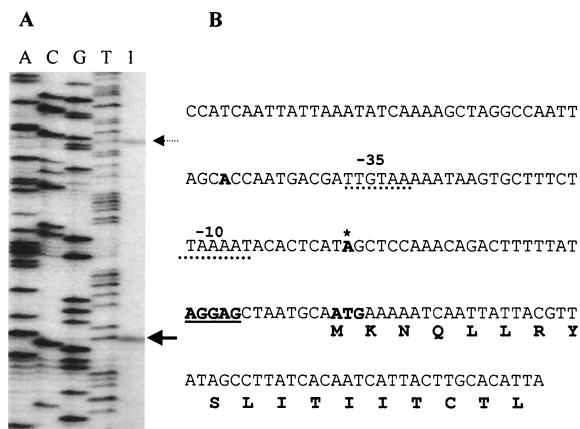

FIG. 5. Promoter mapping by primer extension. (A) The primer extension products obtained using a primer complementary to the *rscS* coding sequence (see Materials and Methods) appear in lane 1 and are indicated by arrows. Contained within the adjacent lanes, labeled A, C, G, and T, are the bands obtained by DNA sequencing using the same primer. (B) The DNA sequence at the beginning of the *rscS* ORF and upstream is shown. The putative ATG translational start is indicated by the bold ATG. Centered 11 bp upstream of the possible translational start is a potential Shine-Dalgarno site, boldface and underlined. The possible transcriptional starts identified by primer extension are Mboldface; the more significant product is also indicated by an asterisk. A possible promoter region is indicated by a dashed underline and labeled above with −10 and −35.

localized to the periplasm (32). An alkaline phosphatase indicator medium was used to identify fusions with alkaline phosphatase activity; four independent insertions which had this activity were identified. Two of the four active insertions were located after amino acid 40 of RscS, and the other two were both inserted after amino acid 200. Both of these sites are within the region flanked by the two putative membrane-spanning segments, providing strong evidence that this region lies in the periplasm. These data also demonstrate that *rscS* is not only transcribed but also translated in vivo.

DISCUSSION

This paper reports the first major result of a genetic screen for *V. fischeri* mutants defective in symbiotic initiation and accommodation: a novel symbiotic locus, *rscS*, that appears to play an important role in symbiotic initiation. A single transposon insertion in *rscS* severely impaired the ability of *V. fischeri* to initiate a symbiotic association with juvenile *E. scolopes* (Fig. 2), a defect that could be complemented by a wild-type copy of the *rscS* gene. We identified putative promoter and ribosome-binding sequences on the basis of the transcriptional start site and *E. coli* consensus sequences. Given a paucity of data for regulatory elements in *V. fischeri* genes, however, positive confirmation of these sequences will require mutational and other analyses.

Molecular characterization of *rscS* revealed a predicted 927-amino-acid protein, RscS, that exhibited strong sequence similarity (Fig. 3B) to hybrid two-component sensor kinases. Each of these kinases participates in a series of four phosphorelay reactions that occur in response to particular environmental conditions (such as a specific level of osmolarity or the presence of a particular ion [3, 36]). Initially, autophosphorylation occurs on a histidine residue (H1). This phosphate sequentially transfers to an aspartic acid (D1), another histidine (H2), and finally to another aspartic acid residue (D2).

Although as many as four distinct proteins may be utilized in this phosphorelay, there are typically two key proteins: a sensor that recognizes the environmental cue and begins the phosphorelay, and a response regulator that contains the conserved final aspartic acid residue and effects changes in gene expression or protein function (3, 29, 36). The module arrangement of the RscS protein most resembles that of BvgS and ArcB, both of which contain three of the four conserved phosphorylation domains (H1, D1, and H2). RscS therefore appears to be a sensor component belonging to the subclass of hybrid two-component regulators. The amino acid sequence in the periplasmic loop domain, however, diverges from other known sequences, suggesting that it may respond to a distinct environmental condition.

The predicted RscS protein most resembles LuxQ of *V. harveyi* (39% identity). LuxQ functions as one of two "redundant" quorum-sensing proteins that sense cell density and regulate luminescence through the recognition of an autoinducer signaling molecule (6, 7). Individually, LuxQ and a second sensor, LuxN, funnel their signals through a common histidine phosphotransferase protein, LuxU, which then modulates phosphorylation of LuxO, a DNA-binding protein that represses transcription of the *V. harveyi lux* operon (8, 18, 19). In recent years, proteins with sequence similarity to these and other *V. harveyi* quorum-sensing components have been identified in *V. fischeri*. The *V. fischeri* AinR protein (20) shows

some sequence similarity with a portion of the *V. harveyi* LuxN sensor kinase. Furthermore, proteins with high sequence identity to the *V. harveyi* LuxO (65%) and LuxU proteins have recently been identified in *V. fischeri* (34). The *V. fischeri* LuxO plays a role similar to that of the *V. harveyi* protein in repressing luminescence (34).

The identification of a *V. fischeri* protein, RscS, with sequence similarity to LuxQ makes it tempting to speculate that RscS plays a role in recognizing an autoinducer and participating in *lux* gene regulation. Unlike LuxQ, which carries only two of the modules predicted to be involved in a phosphorelay cascade (H1 and D1), RscS contains three of the four modules (H1, D1, and H2). Thus, a LuxU intermediate would likely be unnecessary. The two proteins, RscS and LuxQ, show no apparent sequence similarity in the proposed periplasmic loops, suggesting that they recognize different signals. This prediction is supported by a lack of cross-stimulation of *V. harveyi* bioluminescence by *V. fischeri* culture supernatants (5, 24). Furthermore, our data to date indicate no apparent defect in luminescence gene regulation (Fig. 1B). It is formally possible that, similar to the *V. harveyi* system, RscS and a second sensor kinase, such as AinR, may function redundantly to control *lux* expression. Thus, we would be unable to detect a defect by removing only one of these two proteins. Further work needs to be performed to test this hypothesis by constructing *ainR* and possibly *luxO* mutations singly and in combination with an *rscS* mutation in our squid-symbiont strain of *V. fischeri*. These mutants can then be tested for both luminescence in culture and colonization in the symbiosis.

Regardless of a hypothetical additional role for RscS in controlling luminescence, normal symbiotic colonization by *V. fischeri* requires this protein. Given the sequence similarity of RscS to two-component sensors and the colonization defect observed in the *rscS* mutant strain, we believe that RscS may be a key regulatory factor in the *Vibrio-Euprymna* symbiosis. We propose that RscS responds to some factor unique to the light organ environment and subsequently communicates to a hypothetical "RscR" the information that the cell now occupies a special niche. The phosphorelay hypothetically initiated by RscS could then alter gene expression and/or protein activity, switching the bacteria into symbiotic mode and perhaps activating the developmental programs that permit *V. fischeri* to permanently colonize its host.

We expect that further characterization of the role of RscS will contribute to a better understanding of the mechanism(s) by which *V. fischeri* senses the symbiotic environment and of the nature of the response that permits these bacteria to colonize their host. We anticipate the discovery of a cognate response regulator that acts in conjunction with RscS to control a symbiotic gene function(s) and the identification of these downstream targets. In so doing, we may learn the environmental cues that permit *V. fischeri* to sense that it has entered the light organ and modulate specific functions required for the interaction between the bacteria and their animal host.

ACKNOWLEDGMENTS

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