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Role for Phosphoglucomutase in *Vibrio fischeri-Euprymna scolopes* Symbiosis

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***Vibrio fischeri*, a luminescent marine bacterium, specifically colonizes the light organ of its symbiotic partner, the Hawaiian squid *Euprymna scolopes*. In a screen for *V. fischeri* colonization mutants, we identified a strain that exhibited on average a 10-fold decrease in colonization levels relative to that achieved by wild-type *V. fischeri*. Further characterization revealed that this defect did not result from reduced luminescence or motility, two processes required for normal colonization. We determined that the transposon in this mutant disrupted a gene with high sequence identity to the *pgm* (phosphoglucomutase) gene of *Escherichia coli*, which encodes an enzyme that functions in both galactose metabolism and the synthesis of UDP-glucose. The *V. fischeri* mutant grew poorly with galactose as a sole carbon source and was defective for phosphoglucomutase activity, suggesting functional identity between *E. coli* Pgm and the product of the *V. fischeri* gene, which was therefore designated *pgm*. In addition, lipopolysaccharide profiles of the mutant were distinct from that of the parent strain and the mutant exhibited increased sensitivity to various cationic agents and detergents. Chromosomal complementation with the wild-type *pgm* allele restored the colonization ability to the mutant and also complemented the other noted defects. Unlike the *pgm* mutant, a galactose-utilization mutant (*galK*) of *V. fischeri* colonized juvenile squid to wild-type levels, indicating that the symbiotic defect of the *pgm* mutant is not due to an inability to catabolize galactose. Thus, *pgm* represents a new gene required for promoting colonization of *E. scolopes* by *V. fischeri*.**

One of the most intimate interactions between organisms occurs during the establishment of a long-term symbiotic relationship between a microorganism and its host, as exemplified by the *Vibrio*-squid symbiosis. Upon hatching, juveniles of *Euprymna scolopes*, a nocturnal Hawaiian squid, quickly become colonized by the luminescent marine bacterium *Vibrio fischeri*, establishing a symbiosis that persists throughout the lifetime of the squid (see reference 40 for a review). The bacterial symbionts colonize a specialized structure in the squid host, the light organ, located within the mantle cavity of the animal. When a sufficient cell density is reached, the bacteria luminesce, providing the counterillumination ability believed to be an antipredation mechanism for the squid host (see reference 49 for a review). Various studies characterizing the *Vibrio*-squid symbiosis have identified several genetic determinants involved in establishing this association (2, 14, 48, 50, 52); however, much remains to be learned about how these symbiotic partners interact.

Despite the presence of other bacteria in the seawater, the light organ of the juvenile *E. scolopes* is colonized primarily by *V. fischeri* (6); even other *Vibrio* species are generally unable to colonize under lab conditions (29, 36). *V. fischeri* cells colonize the newly hatched aposymbiotic (symbiont-free) juveniles of *E. scolopes* within a few hours, reaching between 10^5 and 10^6 CFU per squid within 24 h (41). The bacteria are drawn to the vicinity of the light organ by currents created by specialized ciliated appendages of the organ as seawater is vented through the mantle cavity of the juvenile squid (see reference 49 for a review). The bacterial cells embed and aggregate in a mucus-like material sloughed off the appendages and secreted from pores located at the base of the light organ (36). As they enter the pores, they encounter mucus-filled ducts (27) and an outwardly directed current created by dense cilia lining the duct walls (see reference 49 for a review); not surprisingly, nonmotile *V. fischeri* are symbiosis defective (14, 36). The ducts ultimately lead to the site of colonization, the internal crypts, where the microbes likely encounter a relatively hostile environment due to the presence of macrophage-like cells (35) and halide peroxidase, an enzyme implicated in oxidative stress (53). A *V. fischeri* mutant that cannot produce catalase does not successfully compete with a catalase-positive strain for colonization (50).

Once inside the crypts of the light organ, the bacteria multiply to a high cell density, where the microbes are exposed to amino acids and peptides which likely serve as a nutrient source (15). Inside the crypts the microbes may attach by using fimbriae to sugar residues (such as mannose [28]) on the surfaces of host epithelial cells: certain *V. fischeri* mutants defective in the ability to hemagglutinate animal red blood cells (a characteristic correlated with the presence of fimbriae in some bacteria) showed a decreased ability to colonize juvenile squid (B. Feliciano and E. Ruby, Abstr. 99th Gen. Meet. Am. Soc. Microbiol. 1999, abstr. N-75, 1999). The presence of surface sugars may also serve as a nutrient source for the symbionts, a strategy employed in other host-microbe interactions. For example, *Bacteroides thetaiotaomicron*, a common mammalian gut commensal, induces fucosylation on the surface of ileal enterocytes in mice. The bacteria then use the fucose residues as an energy source (22).

Within hours of the onset of colonization, the squid under-

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goes bacterium-induced morphological changes, including increased microvillar density (25) and swelling of the epithelial lining of the crypts (30). The specialized ciliated appendages of the light organ also undergo bacterium-induced morphological changes, including lipopolysaccharide (LPS)-mediated apoptosis (12) and complete regression within 4 days of colonization (30). Thus, bacteria colonizing the light organ face continuously changing conditions, including stresses within the light organ and the dramatic morphological changes in the host, which may contribute to the specific colonization by *V. fischeri*. Interestingly, *V. fischeri* luminescence mutants, although able to establish the initial colonization, cannot induce swelling in the crypt epithelial cells and are also unable to maintain persistent symbiosis (48).

More recently identified colonization factors include RscS, a sensor kinase hypothesized to regulate symbiosis-specific genes (52), and OmpU, an outer membrane protein (2). Because colonization appears to require active participation on the part of both the bacterium and the squid host (36), it is likely that this exclusive association depends upon numerous additional host and bacterial genes. Other important bacterial factors may include those involved in chemotaxis, attachment, and protection against host stresses; however, the necessary genetic determinants for such processes have yet to be described.

In an effort to identify additional factors necessary for establishing the *Vibrio*-squid symbiosis, we screened a transposon mutant library and identified a colonization mutant that exhibited on average a 10-fold decrease in colonization ability relative to wild-type *V. fischeri*. We report here the characteristics of this symbiosis mutant of *V. fischeri*.

MATERIALS AND METHODS

Strains and media. The strains used in the present study are listed in Table 1. ESR1, the parent strain used here, was derived from *V. fischeri* ES114. *V. fischeri* cells were grown in SWT medium (6), LBS medium (9), or HEPES minimal medium (MM) (42) containing either glucose or galactose at a final concentration of 0.2%. *Escherichia coli* strains were grown in Luria-Bertani medium (8). Conditioned medium (CM), made from LM medium (31) as described previously (52), was utilized to determine whether the colonization mutant KV733 was defective for bioluminescence in culture. Where appropriate, antibiotics were added to the following final concentrations: chloramphenicol (CHL), 1 to 5 μg ml^{-1} for *V. fischeri* and 30 μg ml^{-1} for *E. coli*; erythromycin (ERY), 1 to 5 μg ml^{-1} for *V. fischeri* and 150 μg ml^{-1} for *E. coli*; rifampin, 100 μg ml^{-1}; kanamycin, 100 μg ml^{-1} for *V. fischeri* and 50 μg ml^{-1} for *E. coli*. Agar was added to a final concentration of 1.5% for solid media or 0.25% for motility plates.

Molecular techniques. All plasmid constructions were carried out by standard molecular biology techniques, with restriction and modifying enzymes obtained from New England Biolabs (Beverly, Mass.) or Promega (Madison, Wis.). Plasmids used or constructed in the present study are shown in Table 1. Cloning of DNA flanking the Tn*10* transposon insertion in *V. fischeri* strain KV733 was accomplished by using the *oriR6K* (the *pir*-dependent origin of replication from plasmid R6K [24]) and CHL resistance elements contained within the transposon.

Genetic techniques. Transposon mutagenesis by using the Tn*10* delivery plasmid pKV124 was described previously (52). Conjugations were performed as described previously (52) by using the appropriate *V. fischeri* recipient strain and two *E. coli* strains: DH5α carrying the plasmid to be transferred and DH5α carrying a conjugal plasmid (either pRK2013 or pEVS104).

The *pgm* and *galK* mutants were constructed with strains and plasmids as follows. The *pgm*::*erm* mutant was derived from pPS24 introduced into ESR1; a recombinant, KV1069, that was ERY resistant and CHL sensitive and grew poorly on MM containing galactose (MM-galactose) was subsequently identified. Similarly, the Δ*pgm* mutant was derived by introducing pPS33 into KV1069 by conjugation. An isolate, KV1177, was sensitive to both ERY and CHL and defective for growth on MM-galactose. The *galK* mutant was obtained by introducing pTMB9 into ESR1. KV1358, a CHL-sensitive strain unable to grow on MM-galactose, was identified.

The *pgm*::Tn*10* mutation was complemented with a wild-type copy of the *seqA-pgm* region inserted in single copy at the Tn*7* insertion site (*att*Tn*7*) in the *V. fischeri* chromosome (4; E. Stabb, unpublished data). Briefly, *E. coli* cells carrying the Tn*7*::*pgm*$^+$-containing plasmid pCD3 served as a donor in a tetraparental mating, which also included *E. coli* cells carrying pUX-BF13 and the *V. fischeri* recipient (ESR1 or KV733). Because an ERY resistance cassette was included within the Tn*7* ends along with *pgm*, ERY-resistant colonies were selected and screened for the loss of kanamycin resistance encoded by the Tn*7* vector. Strains with presumptive Tn*7*::*pgm* insertions were confirmed by Southern blot analysis.

Southern blotting. Chromosomal DNA isolated from *V. fischeri* strain KV733 was digested with *Eco*RV, which cuts once within the mini-Tn*10* transposon, or *Bsr*GI, which cuts outside the transposon. Chromosomal DNA isolated from *V. fischeri* strains KV733 (*pgm*::Tn*10*), KV1069 (*pgm*::*erm*), and KV1177 (Δ*pgm*) was digested with *Hin*dIII. DNA fragments were separated by using a 0.6% agarose gel, transferred onto a nylon membrane (Hybond XL; Amersham-Pharmacia Biotech, Piscataway, N.J.), and UV-cross-linked. Detection was carried out by using the Boehringer Mannheim DIG DNA labeling kit (Roche Molecular Biochemicals, Indianapolis, Ind.). Either random priming or a PCR-based technique was used to generate digoxigenin-labeled DNA complementary to the transposon or to the *pgm* gene. Hybridization and detection were carried out as described previously (52).

Colonization assays. To determine whether mutant *V. fischeri* strains were able to form a symbiotic association with *E. scolopes*, juvenile squids 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 and then analyzed for the presence of bacteria in the light organ as previously described (40). The limit of detection is 14 *V. fischeri* cells per squid.

Luminescence assays. KV733 and ESR1 were diluted 1:100 from an overnight culture and grown in CM in the presence or absence of 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 (OD) measurements. A TD-20/20 luminometer (Turner Designs, Sunnyvale, Calif.) was used to determine the level of bioluminescence of KV733 and its parent.

Phosphoglucomutase assay. Phosphoglucomutase activity was determined by the method of Adhya and Schwartz (1).

Polymixin B, sodium dodecyl sulfate (SDS), and deoxycholate sensitivity determinations. Polymyxin B MIC determinations were made by using a protocol modified from that of Steinberg and Lehrer (46). Briefly, polymyxin B (Sigma) was diluted to a concentration of 1,280 μg ml^{-1} in 0.01% acetic acid and kept frozen at −20°C. Twofold serial dilutions of the stock antibiotic ranging from 0.04 to 80 μg ml^{-1} were prepared in the first row of a 96-well plate in 0.01% acetic acid–0.1% bovine serum albumin. Tenfold dilutions of these concentrations (0.004 to 8 μg ml^{-1}) were then made in SWT medium in the remaining wells. Log-phase cultures of each strain were inoculated into each dilution in triplicate to a starting concentration of 4×10^5 CFU ml^{-1}, incubated for 24 h, and visually examined for turbidity. The lowest concentration of polymyxin B inhibiting growth was designated the MIC.

We assayed for growth of the cells in the presence of SDS by first preparing SDS to a final concentration of 10% in SWT medium. This medium was inoculated to a starting concentration of 4×10^5 CFU ml^{-1} with log-phase cultures in triplicate which were grown at 28°C with shaking. Then, 1-ml samples were taken for OD measurements over time.

Sensitivity to deoxycholate was determined by diluting overnight cultures 1:1,000 in SWT containing 1% deoxycholate and incubating the cells with shaking at 28°C for 6 h. Growth of the cultures was monitored hourly by diluting and plating onto SWT and then counting the resulting CFU.

LPS gel analysis. LPS fractions were prepared by using a protocol modified from the method of B. L. Reuhs and J. S. Kim (as described in reference 39) with the following modifications: 1.5-ml samples were harvested from cultures grown to an OD at 600 nm of 0.5 to 0.7 in SWT medium, pelleted, decanted, and frozen at −80°C prior to a phenol-water extraction. The resulting LPS fractions were desalted by using MicroSpin G-25 columns (Amersham), and the volume was reduced by lyophilization in a Labconco freeze dryer 4.5 (Labconco, Kansas City, Mo.). The isolated LPS fractions were then resuspended in 20 μl of sample buffer, separated by deoxycholic acid-polyacrylamide gel electrophoresis (PAGE) on 14% acrylamide and silver stained with the Owl silver stain staining kit for electrophoresis (Owl Separation Systems, Portsmouth, N.H.) according to the manufacturer's instructions.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or characteristics[a]	Reference or source
Strains		
E. coli		
DH5α	*endA1 hsdR17* (r_K^- m_K^+) *glnV44 thi-1 recA1 gyrA* (Nal^r) *relA* Δ(*lacIZYA-argF*)*U169 deoR* [ϕ80d*lac*Δ(*lacZ*)M15]	54
CC118λ*pir*	Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB argE*(Am) *recA1* λ*pir*	20
S17-1λ*pir*	*thi pro hsdR hsdM*$^+$ *recA* λ*pir*	43
CGSC4982	*galK*	CGSC[b]
V. fischeri		
ES114	WT	6
ESR1	Rf^r	14
KV733	Rf^r *pgm*::Tn*10*	This study
KV828	Rf^r, carries pPS18 integrated into the chromosome	This study
KV1069	Rf^r *pgm*::*erm*	This study
KV1170	Rf^r *att*Tn*7*::*seqA-pgm*$^+$	This study
KV1171	Rf^r *pgm*::Tn*10 att*Tn*7*::*seqA-pgm*$^+$	This study
KV1177	Rf^r Δ*pgm*	This study
KV1306	Rf^r, carries pKV161 integrated into the chromosome	This study
KV1358	Rf^r, Δ*galK*	This study
Plasmids		
pBS	Blue-white screen cloning vector; Ap^r	Stratagene
pCD3	pEVS107 (*Spe*I) + 3-kb *Nhe*I fragment from pPS21, contains *seqA* and *pgm* inside the Tn*7* transposon	This study
pCNW1	pVO8 *Bam*HI + 5-kb *galK*$^+$ *Bgl*II fragment	This study
pEVS79	pBC + *mob*; Cm^r	44
pEVS104	Conjugal helper plasmid (*tra trb*); Kn^r	44
pEVS107	Tn*7* delivery plasmid; Kn^r Em^r	Eric Stabb
pKV25	pUC19::*erm*	50
pKV36	pUC19::*cat*	This study
pKV124	pBSL181 containing promoterless *lacZ* and *oriR6K* within Tn*10* ends	52
pKV161	pEVS79 *Hin*dIII + 2.8-kb *galK*$^+$ *Hin*dIII fragment from pCNW1	This study
pPS8	11-kb *Nhe*I fragment cloned from KV733, contains *pgm*::Tn*10* and flanking DNA	This study
pPS12	pBS (*Sac*I) + 6-kb *Sac*I fragment from pPS8, contains DNA flanking the Tn*10* insertion	This study
pPS15	pBS (*Xba*I) + 2.6-kb *Nhe*I fragment from pPS12, contains DNA upstream of *pgm*	This study
pPS18	pPS15 *Bgl*II + 1.1-kb *Bam*HI fragment encoding Cm^r from pKV36 (results in *fur*::*cat* mutation)	This study
pPS19	14-kb *Nsi*I fragment cloned from KV828, contains *seqA*$^+$ and a portion of *pgm*	This study
pPS20	pEVS79 (*Bam*HI) + 2.2-kb *Bam*HI/*Bgl*II fragment from pPS19, contains a portion of the *pgm* locus	This study
pPS21	20-kb *Xba*I fragment cloned from KV828, contains *seqA*$^+$ and *pgm*$^+$	This study
pPS24	pPS20 *Sph*I/filled + 1.2-kb *Sma*I/*Eco*RV fragment encoding Em^r from pKV25 (results in a *pgm*::*erm* mutation)	This study
pPS27	pEVS79 (*Xba*I) + 3-kb *Nhe* fragment from pPS21	This study
pPS33	pPS20 with two *Msc*I sites internal to *pgm* deleted (results in a 606-bp deletion)	This study
pRK2013	Conjugal helper plasmid (*tra trb*); Kn^r	10
pTMB7	9.6-kb *Cla*I fragment cloned from KV1306, contains *galK*$^+$ and flanking DNA	This study
pTMB9	pTMB7 deleted between two *Ase*I sites (results in 250-bp deletion at the 5′ end of *galK*)	This study
pUX-BF13	Encodes Tn*7* transposase (*tnsABCDE*); Ap^r	4
pVO8	Blue-white screen cloning vector; Cm^r Em^r	51

[a] Nal^r, nalidixic acid resistance; WT, wild type; Rf^r, rifampin resistance; Ap^r, ampicillin resistance; Kn^r, kanamycin resistance; Cm^r, CHL resistance; Em^r, ERY resistance.
[b] CGSC, *E. coli* Genetic Stock Center, Yale University, New Haven, Conn.

Sequencing. Automated sequencing was carried out with forward and reverse primers complementary to vector and insert sequences. Similarity searches were performed by using the National Center for Biotechnology Information BLAST program (3, 19). Oligonucleotides were obtained from the Loyola Macromolecular Analysis Facility, Integrated DNA Technologies (Coralville, Iowa), or MWG Biotech (High Point, N.C.).

Nucleotide sequence accession number. The following GenBank accession number was assigned to the *seqA-pgm-ybgI* sequence: AF474148.

RESULTS

Isolation of a symbiosis-defective *V. fischeri* mutant. Individual transposon insertion mutants of *V. fischeri* ESR1 were screened for their ability to form a symbiotic association with juveniles of the squid *E. scolopes* as described previously (52). Briefly, individual mutants were inoculated into seawater containing newly hatched juvenile *E. scolopes*. Colonization was monitored noninvasively by using bioluminescence as an indirect indicator of symbiont density in the light organ of *E. scolopes*. One mutant, KV733, could not achieve the high levels of luminescence sustained by ESR1 in the symbiosis with *E. scolopes* (Fig. 1A).

To confirm that the decreased level of symbiotic bioluminescence corresponded to decreased colonization, levels of colonization by KV733 and its parent were measured directly (Fig. 2). Juveniles inoculated with KV733 exhibited on average

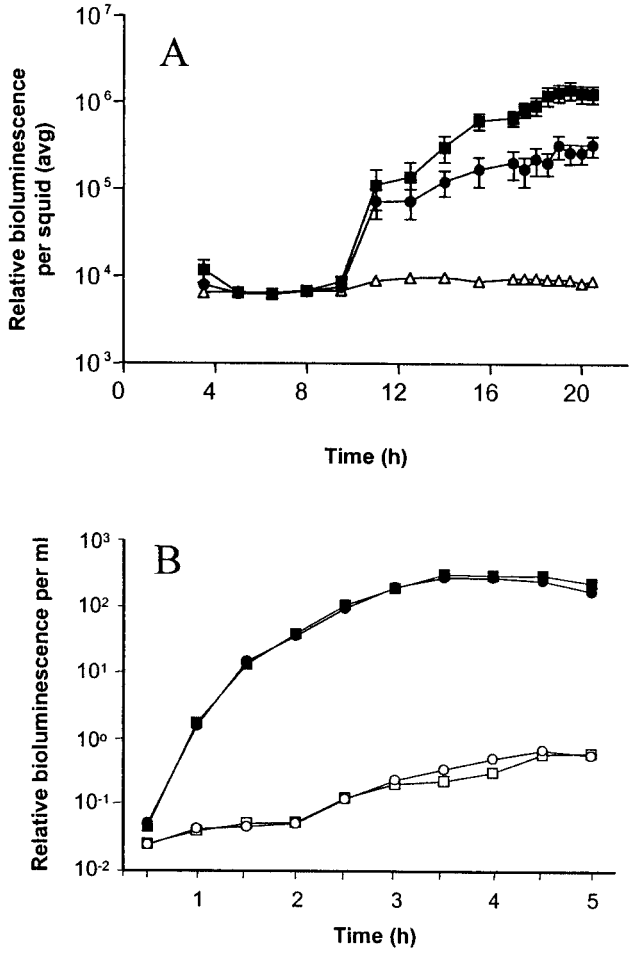

FIG. 1. Luminescence of colonization mutant KV733 and its parent. (A) Newly hatched juvenile *E. scolopes* squid were incubated in artificial seawater containing no *V. fischeri* (△), the parent strain ESR1 (■), or the colonization mutant KV733 (●). Bioluminescence emission was measured over time and represents an average of 11 animals for ESR1 and KV733. Error bars show the standard errors of the mean (SEM). (B) ESR1 (squares) and KV733 (circles) were grown in CM in the absence (open symbols) or presence (solid symbols) of autoinducer (3-oxo-hexanoy-L-homoserine lactone). The data shown are from a representative experiment of several replicates.

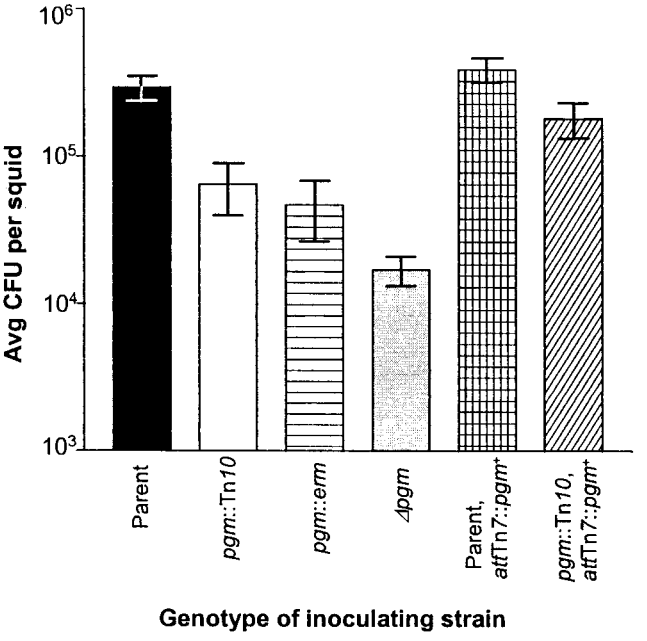

FIG. 2. Symbiotic colonization by *pgm* mutant and control strains. Newly hatched juvenile *E. scolopes* were inoculated with the following strains: ESR1 (■), KV733 (□), KV1069 (▤), KV1177 (▨), KV1170 (▩), or KV1171 (▨). The juveniles were exposed to ca. 1,000 cells of the parental and complemented strains or 2,000 cells of the mutant strains per ml of seawater for 3 h. The level of colonization achieved by these strains was determined by homogenization and plating 20 h after the organisms were placed together. The data shown are from a representative experiment, and the bars indicate an average of 11 squids. Error bars indicate the SEM.

a 10-fold reduction in colonization levels relative to those inoculated with ESR1. This putative symbiosis-defective mutant was further characterized in culture by examining motility on soft agar plates (data not shown), growth on minimal medium (data not shown), natural bioluminescence, and response to autoinducer (Fig. 1B). In these assays, KV733 behaved like its parent, suggesting that the symbiotic defect in KV733 was not due to deficiency in motility, amino acid biosynthesis, or luminescence, all factors previously identified as important in the symbiosis. It therefore seemed likely that KV733 was defective for an as-yet-unknown symbiotic locus.

Identification of the locus disrupted in KV733. To identify the genetic defect in KV733, we determined the number and location of the transposon insertion(s) in this strain by Southern analysis. A single, large (>12-kb) *Bsr*GI fragment that carried the transposon was identified by probing with DNA complementary to the transposon delivery plasmid, providing evidence that the strain carried a single transposon insertion (data not shown). Further analysis of KV733 chromosomal DNA revealed that the entire transposon delivery plasmid had incorporated into the chromosome at this site (data not shown).

We cloned the transposon and flanking chromosomal DNA. Sequence analysis revealed that the transposon had inserted after codon 189 of a 548-codon open reading frame (ORF). This putative protein exhibited high sequence similarity (69% identity, 83% similarity) to Pgm (phosphoglucomutase) of *E. coli* (26) and *V. cholerae* (80% identity, 89% similarity [17]). Directly upstream of the putative *pgm* gene in *V. fischeri* was an ORF that encoded a putative protein with high sequence similarity to SeqA, encoded by a gene upstream of *pgm* in both *E. coli* (51% identity, 71% similarity [5]) and *V. cholerae* (65% identity, 79% similarity [17]). Directly downstream of *pgm* in *V. fischeri* was an ORF encoding a predicted protein similar to proteins of unknown function in *V. cholerae* (74% identity, 81% similarity [17]) and *Haemophilus influenzae* (YbgI, 62% identity, 78% similarity [11]). Directly downstream of this gene and in the opposite orientation is an ORF encoding a product with high sequence similarity, based on partial sequence data, to GltA encoded by the *gltA* gene of *E. coli* (5).

In *E. coli*, phosphoglucomutase interconverts glucose-1-phosphate and glucose-6-phosphate and thus plays roles in both galactose metabolism and polysaccharide biosynthesis

(13). The protein is part of a family of phosphoserine enzymes which contain a highly conserved serine residue within a conserved 5-amino-acid sequence critical for enzyme activity (38, 55). The deduced amino acid sequence of this region in *V. fischeri*, *V. cholerae*, and *E. coli* is Thr-Pro-Ser-His-Asn at amino acids 144 to 148, differing in a single Ala-Pro change from the rabbit muscle phosphoglucomutase consensus sequence Thr-Ala-Ser-His-Asn (38). On the basis of the presence of this conserved active site region in the putative *V. fischeri pgm* gene, as well as on the overall high sequence similarity to the *pgm* genes of both *V. cholerae* and *E. coli*, we conclude that the locus disrupted in KV733 is the *V. fischeri pgm* gene. The phenotypic and biochemical data described below further support this conclusion.

Role for *pgm* in the symbiosis. Because the transposon insertion in KV733 also contains the delivery plasmid (encoding transposase), it is conceivable that, during symbiotic colonization, additional insertions that might inhibit the ability of KV733 to colonize could be generated. To determine whether the transposon insertion in KV733 was retained in the *pgm* gene during colonization, chromosomal DNA extracted from KV733 harvested after symbiotic colonization was subjected to Southern analysis. In each of 10 independent clones from individual animals, the transposon was present only in the *pgm* gene (data not shown).

Additionally, it was formally possible that KV733 carried a secondary mutation; therefore, in order to confirm that the *pgm* gene itself plays a role in the symbiosis, we constructed additional *pgm* mutants (Table 1). A *pgm*::*erm* insertion mutant (KV1069) and a Δ*pgm* (in-frame deletion) mutant (KV1177 with a region that includes the conserved 5-amino-acid active-site sequence deleted) also exhibited defects in the ability to colonize juvenile squid similar to that of the original transposon insertion mutant, as shown in Fig. 2 (the mean colonization levels of the three *pgm* mutants were not statistically different [$P = 0.2$] as determined by one-way analysis of variance analysis). Additionally, when a pgm^+ gene was inserted in single copy into the chromosome of KV733 (KV1171), the complemented strain achieved a colonization level similar to that of the wild-type parent (Fig. 2). These data indicate that the *pgm* gene plays a critical role in symbiotic colonization.

Phosphoglucomutase assay. To determine whether our sequence analysis correctly predicted the function of the putative Pgm protein, we measured phosphoglucomutase activity of our wild-type and mutant strains. Whereas wild-type extracts displayed Pgm activity, the *pgm* mutants tested were deficient (Fig. 3). Complementation restored Pgm activity to the *pgm* mutant and, not surprisingly, the strain with two wild-type copies of *pgm* exhibited approximately twice as much Pgm activity.

Growth of the *pgm* mutant in culture. An *E. coli pgm* mutant exhibits a decreased ability to grow on minimal medium containing galactose as the sole carbon source (26). We hypothesized that if Pgm has the same function in *V. fischeri*, then the *pgm* mutants would be similarly defective. This was the case for all three *pgm* mutants (KV733, KV1069, and KV1177), whereas the complemented strain (KV1171) exhibited a restored ability to grow on MM-galactose as the sole carbon source (data not shown).

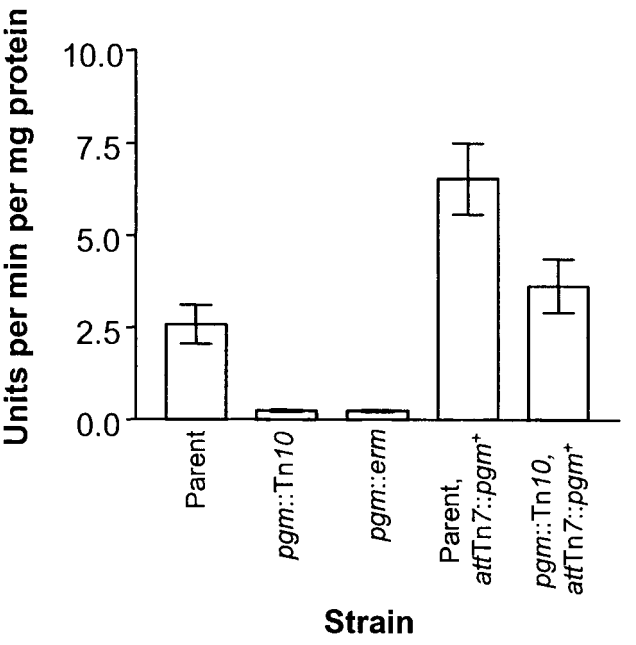

FIG. 3. Phosphoglucomutase assay. Phosphoglucomutase activity of strains ESR1 (parent), KV733 (*pgm*::Tn*10*), KV1069 (*pgm*::*erm*), KV1170 (ESR1 with chromosomal pgm^+ complementation), and KV1171 (*pgm*::Tn*10* with chromosomal pgm^+ complementation). The data shown are averages from four independent experiments. Error bars show the SEM.

We also examined the ability of the *pgm* mutant KV733 to grow in different complex nutrient media. When grown in SWT (a seawater-based medium), the *pgm* mutant KV733 grew identically to its parent, ESR1, suggesting that KV733 does not possess a general growth defect (Fig. 4A). In contrast, when grown in LBS medium, a medium containing NaCl as the only added salt, KV733 displayed a growth defect when inoculated to a starting cell density of $<10^6$ CFU ml^{-1}. As shown in Fig. 4B, when the *pgm* mutant KV733 was inoculated to a starting density of 10^5 CFU ml^{-1}, its growth leveled off at an OD of ca. 0.1.

Given the differences in the ability of the mutant to grow in the two complex media under conditions of a low inoculum, we examined the possibility that the salts present in SWT medium may be rescuing the growth defect of the *pgm* mutant in LBS medium. SWT medium contains NaCl, KCl, $MgSO_4$, and $CaCl_2$, whereas LBS medium contains NaCl as the only added salt. The growth experiments were repeated with LBS medium supplemented with the individual SWT salts (Fig. 4C). The addition of either $MgSO_4$ or $CaCl_2$, both salts containing divalent cations, to LBS medium allowed the *pgm* mutant to grow to wild-type levels when it was inoculated to a starting density of 10^5 CFU ml^{-1}, whereas the addition of the monovalent salt KCl did not affect the mutant's growth. The growth of ESR1 was not affected by the addition of any of the three salts (data not shown). The consequences of this ion requirement in nature are unclear because seawater and most likely the internal crypts of the light organ contain plentiful amounts of Mg^{2+} and Ca^{2+}.

Susceptibility of the *pgm* mutant to polymyxin B, deoxy-

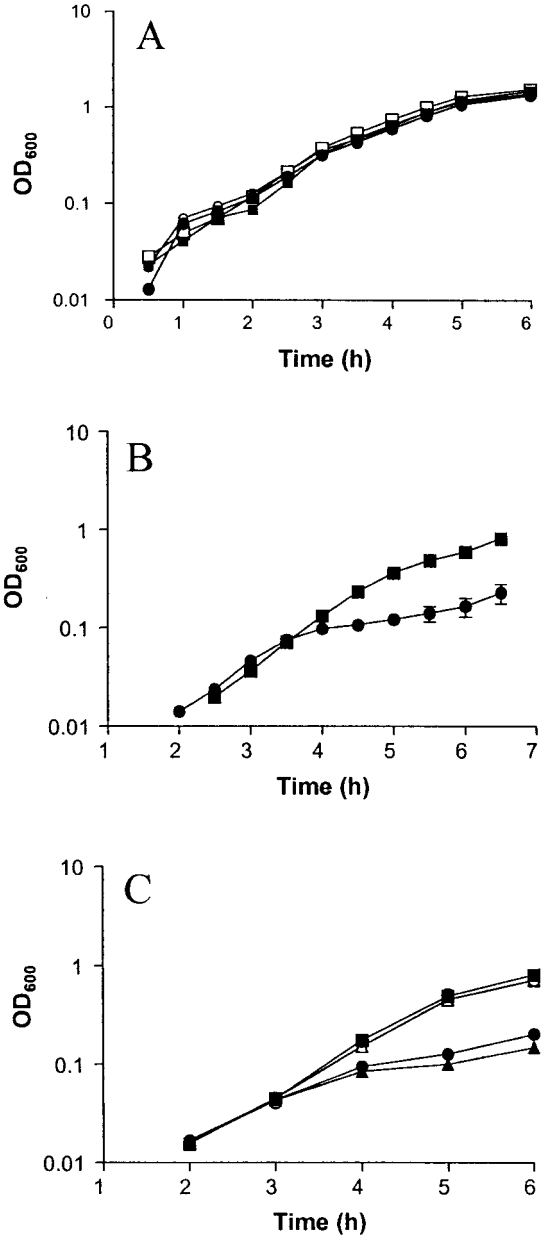

FIG. 4. Growth of ESR1 and KV733 in liquid media. (A) ESR1 (squares) and KV733 (circles) were inoculated to a starting concentration of 10^6 CFU ml^{-1} in either SWT (solid symbols) or LBS (open symbols) medium and grown for 6.0 h. (B) ESR1 (■) and KV733 (●) were inoculated to a starting concentration of 10^5 CFU ml^{-1} and grown in LBS medium for 6.5 h. Error bars indicate the SEM. (C) ESR1 (■) was inoculated to a starting concentration of 10^5 CFU ml^{-1} and grown in LBS medium for 6 h. In parallel, KV733 was inoculated to a starting concentration of 10^5 CFU ml^{-1} and grown in LBS medium (●), LBS medium supplemented with $MgSO_4$ (○), LBS medium supplemented with $CaCl_2$ (△), or LBS medium supplemented with KCl (▲) for 6 h. The data from ESR1 grown in LBS medium supplemented with individual salts were omitted for clarity.

cholate, and SDS. An *E. coli pgm* mutant exhibits increased susceptibility to environmental stresses (26). To investigate whether the *V. fischeri pgm* mutants also exhibited this phenotype, we determined the MIC of the cationic antibiotic polymyxin B for each *pgm* mutant and for ESR1 grown in SWT medium. All three *pgm* mutants had an MIC of 2 μg ml^{-1}, a threefold increase in susceptibility relative to the MIC for ESR1 of 6 μg ml^{-1}. Complementation with *pgm*$^+$ restored the MIC to 5 to 6 μg ml^{-1}.

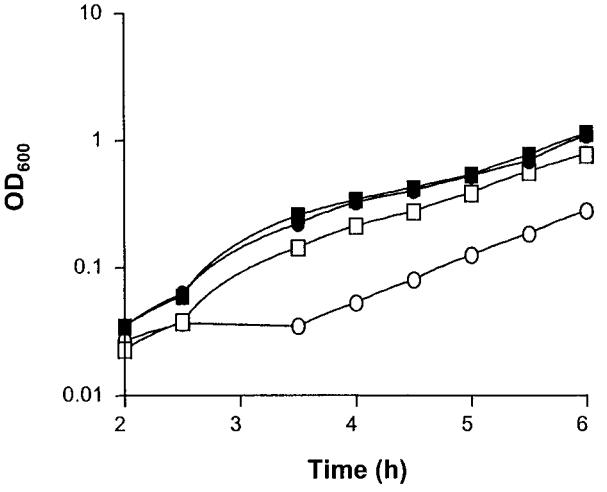

FIG. 5. Growth of ESR1 and KV733 in the presence of SDS. ESR1 (squares) and KV733 (circles) were inoculated in triplicate to a starting density of 4×10^5 CFU ml^{-1} in either SWT medium (solid symbols) or SWT medium with 10% SDS (open symbols) and grown for 6.0 h. Error bars show the SEM. The data shown are from a representative experiment.

We also examined the ability of the *pgm* mutant to grow in SWT in the presence of either 10% SDS or 1% deoxycholate. The presence of SDS interfered with the growth of KV733 to a greater extent than it did the parent strain (Fig. 5). The growth of KV733 (but not the parent strain) was similarly affected in the presence of 1% deoxycholate (data not shown). Taken together, these data indicate that the *pgm* mutant is more susceptible to environmental stresses.

LPS structure of the *pgm* mutant. *E. coli* (1) and *Agrobacterium tumefaciens* (47) *pgm* mutants exhibit defects in LPS production due to an inability to produce sufficient amounts of UDP-glucose. To determine whether the *V. fischeri pgm* mutant had a similar defect in LPS biosynthesis, we analyzed LPS from the *pgm* mutant and wild-type strains by separating the LPS molecules with deoxycholic acid-PAGE and then visualizing them by silver staining (Fig. 6). The LPS extracted from the *pgm* mutant KV733 had a banding pattern distinct from that of ESR1, containing additional species that migrated faster than that of the smallest LPS band of ESR1 (Fig. 6). An altered banding pattern was also observed with LPS extracted from the other two *pgm* mutant strains (data not shown). Complementation restored the LPS banding pattern to that of the parent strain (Fig. 6 and data not shown), suggesting that the altered LPS profile of the mutant is due to the loss of *pgm*.

Colonization assay of the *V. fischeri galK* mutant. To test whether the inability of the *pgm* mutants to utilize galactose accounts for the colonizaton defect of these strains, we constructed a mutant defective for galactokinase (encoded by the *galK* gene) and examined the strain's ability to form a symbiotic association with *E. scolopes*. The *galK* mutant, KV1358, colonized to wild-type levels (Fig. 7). These data indicate that an

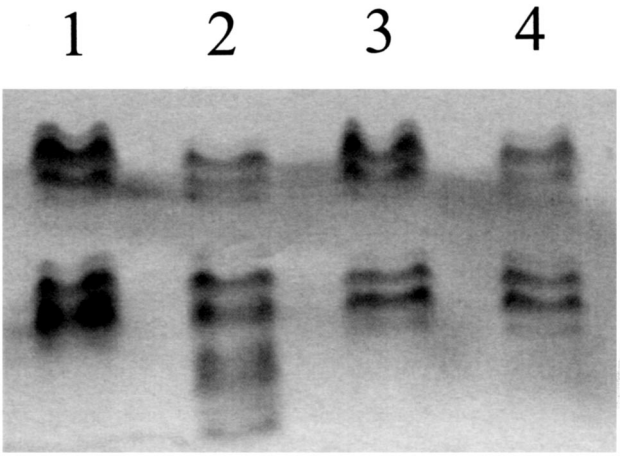

FIG. 6. LPS profiles of the *pgm* mutant and wild-type strains. LPS fractions were prepared as described in Materials and Methods, separated by deoxycholic acid-PAGE, and visualized by silver staining. Lane 1, ESR1 (parent); lane 2, KV733 (*pgm*::Tn*10*); lane 3, KV1170 (ESR1 with chromosomal pgm^+ complementation); and lane 4, KV1171 (*pgm*:: Tn*10* with chromosomal pgm^+ complementation). The gel is from a representative experiment.

inability to catabolize galactose does not account for the symbiotic defect in KV733.

DISCUSSION

In the present study we identified a mutant of *V. fischeri* that failed to colonize *E. scolopes* to wild-type levels. This mutant, KV733, carried a transposon insertion in an ORF with high identity to the *pgm* (phosphoglucomutase) gene of *E. coli*. Our data suggest that the locus interrupted in KV733 encodes a functional homolog of the *E. coli* Pgm protein and that this locus, which we have designated *pgm*, plays a role in symbiotic colonization.

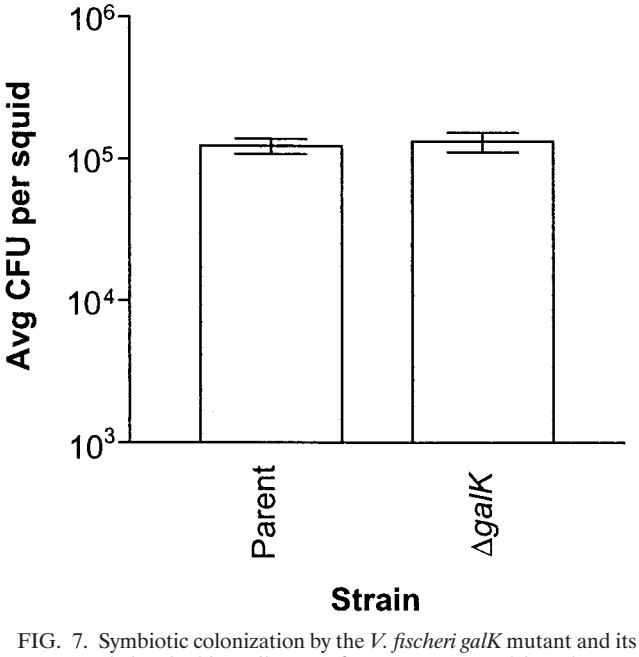

FIG. 7. Symbiotic colonization by the *V. fischeri galK* mutant and its parent. Newly hatched juvenile *E. scolopes* were exposed for 3 h at ca. 3,000 cells per ml of either ESR1 or KV1358. The level of colonization was determined by homogenization and plating 20 h after the organisms were placed together. The data shown are from a representative experiment. The bars indicate the average colonization level of 12 squids. Error bars show the SEM.

In *E. coli*, Pgm plays roles both in catabolizing galactose and in promoting the production of UDP-glucose, an activated form of glucose that serves as a building block for a number of polysaccharides, including LPS. Strains with a deficiency in UDP-glucose production exhibit a number of phenotypes, including an altered LPS profile (21, 32) and increased susceptibility to hydrophobic agents, bile salts, and cationic antimicrobial peptides (32). Similarly, the *V. fischeri pgm* mutant displayed a decrease in phosphoglucomutase activity, grew poorly on MM-galactose, exhibited an altered LPS profile, and displayed an increased susceptibility to polymyxin B, deoxycholate, and SDS. Taken together, our data indicate that KV733 exhibits phenotypes consistent with a defect in a gene with the same function as that of the *E. coli pgm* gene.

Interestingly, in the seawater-based SWT medium, wild-type *V. fischeri* grew to high cell densities despite the presence of 10% SDS. This is in contrast to a recent report indicating that wild-type *V. fischeri* cells grown in LBS medium were susceptible to 0.009% SDS (2). It is possible that the three additional salts found in SWT medium ($MgSO_4$, $CaCl_2$, and KCl) protect the cells against lysis by the detergent. In support of this hypothesis, the addition of Mg^{2+} to growth medium has been shown to stabilize the outer membrane of a *Salmonella enterica* serovar Typhimurium LPS mutant (45). Similarly, we saw that divalent cations were required for optimal growth of the *pgm* mutant in LBS medium.

In addition, our data suggested that the *pgm* gene itself is required for symbiotic colonization: the original transposon insertion mutant and two other *pgm* mutants that we constructed exhibited similar defects in colonization, and complementation restored the ability of KV733 to colonize. The colonization defect conferred by the mutation in *pgm* is similar to that caused by some amino acid auxotrophies (15). It is more severe than either a mutation in the *luxA* gene, which causes a fourfold decrease only after the first day of colonization (48), or a mutation in catalase, which results in a competitive defect (50). We have observed that the *pgm* mutant is at least 10-fold reduced in colonization as early as 12 h postinoculation, a time at which the wild-type strain has not yet achieved its population maximum (C. R. DeLoney and K. L. Visick, unpublished observations). These data suggest that the *pgm* mutant is not simply growing normally until it reaches a population maximum that is 10-fold reduced from that which can be achieved by the wild-type strain. Rather, it appears that this mutant is deficient at even earlier stages of colonization. This result is consistent with our hypothesis that the *pgm* mutant fails to resist host-imposed stresses (see below).

What role does *pgm* play in symbiotic colonization? Given the pleiotrophic effects of *pgm* mutants, several explanations could be proposed to explain the symbiotic defect. One possibility was that the symbiotic defect resulted from the inability of the *pgm* mutant to use galactose as a carbon source. We speculated that galactose moieties on the surfaces of cells in the light organ might serve as a carbon source for the bacteria.

A similar mechanism seems to be employed during colonization of the mouse gut by *B. thetaiotaomicron*, which induces the presentation of the sugar fucose on host cells and then uses it as an energy source (22). However, our construction of a second galactose utilization mutant, one not defective for LPS biosynthesis (data not shown) that colonized normally suggested that this hypothesis was incorrect.

A second possible explanation is that alterations in LPS, correlated with susceptibility to stresses, prevent the mutant from colonizing to wild-type levels because it cannot withstand environmental stresses in the squid. Although relatively little is known about the environment of the light organ, the presence of defensive host cells (primitive macrophages [35]) and proteins (halide peroxidase [53]), as well as the exclusivity of the symbiosis, suggests that the bacteria likely encounter host-derived stresses during colonization.

Comprising the vast majority of the outer leaflet of the outer membrane of gram-negative bacteria, LPS plays a crucial role in maintaining membrane integrity (see reference 34 for a review). Divalent cations such as magnesium form cross-bridges between neighboring LPS molecules by binding to phosphate residues on inner core heptose components (which are often derived from UDP-glucose [see reference 37 for a review]). These interactions give strength and rigidity to the otherwise fluid outer membrane. *H. influenzae pgm* mutants, presumably defective for UDP-glucose synthesis, contain LPS species with a reduced number of sugar residues in the inner core (21). If the *pgm* mutation caused a parallel defect on the inner core of *V. fischeri* LPS, which would not be surprising given the conserved nature of LPS structure among gram-negative bacteria (16), then the strength of the outer membrane likely would be reduced. Our sensitivity assays suggest that this is indeed the case. However, it is not clear whether the moderate increase in susceptibility to agents such as polymyxin B that the *pgm* defect confers in culture can account for the 10-fold decrease in colonization ability.

A third explanation for the symbiotic defect in KV733 could be the loss or reduction of outer membrane proteins necessary for colonization. Some LPS mutants ("deep rough") that are devoid of part of the core oligosaccharide (for reviews, see references 33 and 37) show significant decreases in the amounts of the major outer membrane proteins. The *V. fischeri* outer membrane protein OmpU plays a role in symbiotic colonization (2) and may be affected by the LPS defect. Our preliminary data suggest that this is not the case; we have detected no distinct differences in the banding patterns of proteins extracted from wild-type and *pgm* mutant cells (K. Visick, unpublished observations).

In *E. coli*, UDP-glucose plays a number of roles other than LPS synthesis, including (i) protecting against thermal and osmotic stresses through the production of trehalose (7); (ii) serving as a building block for periplasmic β-D-glucans, which are implicated both in osmotic adaptation and in cell signaling (23); and (iii) regulating (negatively) σ^S, the stationary-phase sigma factor (18). Defects in any of these cellular processes could potentially result in the observed symbiotic phenotype in *V. fischeri*, and future research will have to address these possibilities.

The role of Pgm in colonization is not limited to the *Vibrio*-squid symbiosis. *V. cholerae* (32) and *H. influenzae* (21) *pgm* mutants similarly display defects in pathogenic colonization. The requirement of a metabolic enzyme in establishment of the symbiosis between *V. fischeri* and *E. scolopes* demonstrates the complexity of the association, as well as how little is known about the necessary genetic determinants. We anticipate that our characterization of the role of Pgm in this symbiotic association will provide opportunities for the comparison of symbiotic and pathogenic colonization. As we continue to search for insights on how these mutualistic partners interact, studies such as these will add to our knowledge of how symbiotic relationships—both beneficial and detrimental—are established between bacteria and animals.

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