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The Periplasmic, Group III Catalase of *Vibrio fischeri* Is Required for Normal Symbiotic Competence and Is Induced Both by Oxidative Stress and by Approach to Stationary Phase

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The catalase gene, *katA*, of the sepiolid squid symbiont *Vibrio fischeri* has been cloned and sequenced. The predicted amino acid sequence of KatA has a high degree of similarity to the recently defined group III catalases, including those found in *Haemophilus influenzae*, *Bacteroides fragilis*, and *Proteus mirabilis*. Upstream of the predicted start codon of *katA* is a sequence that closely matches the consensus sequence for promoters regulated in *Escherichia coli* by the alternative sigma factor encoded by *rpoS*. Further, the level of expression of the cloned *katA* gene in an *E. coli rpoS* mutant is much lower than in wild-type *E. coli*. Catalase activity is induced three- to fourfold both as growing *V. fischeri* cells approach stationary phase and upon the addition of a small amount of hydrogen peroxide during logarithmic growth. The catalase activity was localized in the periplasm of wild-type *V. fischeri* cells, where its role could be to detoxify hydrogen peroxide coming from the external environment. No significant catalase activity could be detected in a *katA* null mutant strain, demonstrating that KatA is the predominately expressed catalase in *V. fischeri* and indicating that *V. fischeri* carries only a single catalase gene. The catalase mutant was defective in its ability to competitively colonize the light organs of juvenile squids in coinoculation experiments with the parent strain, suggesting that the catalase enzyme plays an important role in the symbiosis between *V. fischeri* and its squid host.

The luminous marine bacterium *Vibrio fischeri* occupies a unique niche in nature: it is the only bacterial species found within the symbiotic light-emitting organ of the Hawaiian squid, *Euprymna scolopes* (7). Interestingly, not all strains of *V. fischeri* are equally capable of establishing and maintaining a colonization of the light organ (24, 31), suggesting that the symbiosis-competent strains express special factors required for growth in the squid. Such factors could include an important adhesin, enzymes for metabolizing a specific host-derived nutrient, or defenses that protect against a host-produced stress.

When *E. scolopes* juveniles hatch, their light organs are devoid of bacteria (47), but they rapidly become colonized if competent *V. fischeri* cells are present in the surrounding seawater. Within 24 h of the initiation of the association, a few infecting bacteria have multiplied to fully colonize the juvenile light organ, which can contain approximately 10^6 cells (36). These cells, however, have undergone dramatic morphological alterations, such as the loss of their flagella and a decrease in cell volume (36). These and other changes demonstrate that *V. fischeri* cells recognize and respond to the particular conditions present inside the light organ, perhaps including environmental stresses that result from their association with the eukaryotic host. While the oxidative conditions inside the light organ are only now being analyzed, host mRNA that encodes a halide peroxidase is abundant (43, 48). It has been suggested that this peroxidase, which converts hydrogen peroxide into toxic hypohalous acids (e.g., hypochlorous acid), could serve as a host defense mechanism, like the human myeloperoxidase to which it is related (48). If this hypothesis is correct, oxidative stress may be a significant condition that *V. fischeri* cells encounter in the light organ environment.

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One strategy that bacteria use to combat the oxidative stress resulting from exposure to hydrogen peroxide is to produce the enzyme catalase, which decomposes this reactive oxygen species into water and oxygen (26). The addition of hydrogen peroxide to a colony of *V. fischeri* cells results in a vigorous bubbling, a reaction which suggests that this organism does indeed possess an active catalase. In this report we describe (i) the cloning of the *V. fischeri* gene encoding this activity, which we have called *katA*, and the analysis of its predicted protein sequence; (ii) the expression pattern of the *katA* product during growth of the cells in culture; and (iii) the construction of a *katA* mutant and investigation of its ability to colonize juveniles of *E. scolopes* squid.

MATERIALS AND METHODS

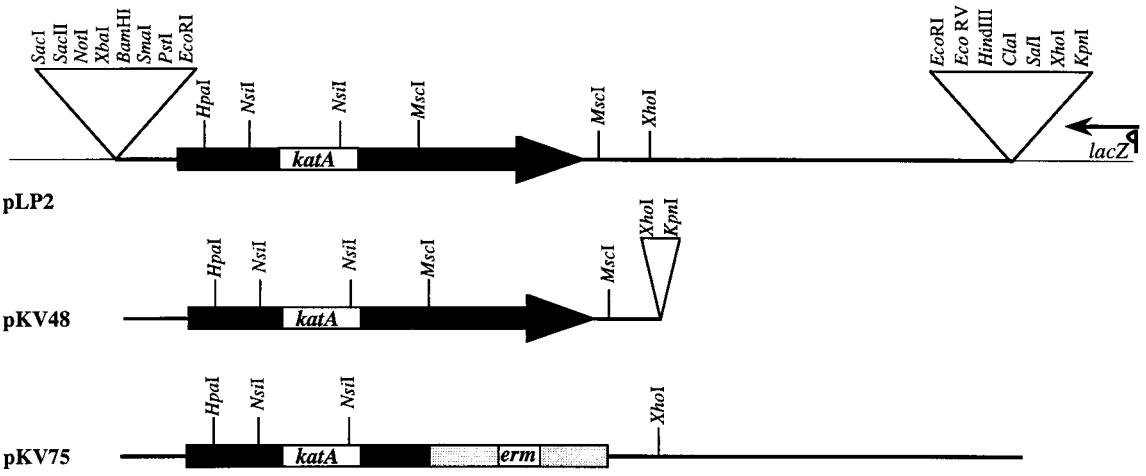
Bacterial strains, plasmids, and media. Wild-type *V. fischeri* strain ES114 (7) is a natural isolate from the squid *E. scolopes*. Strain ESR1 (20) is a rifampin-resistant derivative of ES114 and is the parent strain used in the construction of the catalase mutant. The following *Escherichia coli* strains were also used: DH5α, UM2 (*katE katG* [27]), MC1000 (parental strain for JV1012 [11]), and JV1012 (*rpoS* [44]).

LB (12) broth was used for the growth of *E. coli* strains. *V. fischeri* strains were grown either in LBS medium (16), which contains 1% tryptone, 0.5% yeast extract, 2% NaCl, and 0.3% glycerol in 50 mM Tris-HCl (pH 7.5), or in SWT medium (7), which contains 0.5% tryptone, 0.3% yeast extract, and 0.3% glycerol in 70% seawater. Agar was added to a concentration of 1.5% for solid media. Antibiotics were added when appropriate to the following final concentrations: ampicillin, 100 μg/ml; erythromycin, 150 μg/ml for *E. coli* and 5 μg/ml for *V. fischeri.*

Plasmid construction. A library of *Eco*RI-digested *V. fischeri* chromosomal DNA cloned into pBluescript KS (Stratagene Inc., La Jolla, Calif.) was used to transform *E. coli* UM2 (*katE katG*) cells. Ampicillin-resistant isolates were screened for the presence of a functional catalase clone by visual identification of bubbles when hydrogen peroxide was dropped on the individual colonies. Several such hydrogen peroxide-decomposing colonies were obtained, and the plasmids isolated from these strains all appeared to carry an *Eco*RI insert of about 4.4 kb in size. One of these plasmids, pLP2 (Fig. 1A), was saved for further study.

Plasmid pKV48 (Fig. 1A) was derived from pLP2 by the deletion of a 2.2-kb *Xho*I fragment. The following constructs (not shown) were derived from pKV48: pKV52, which was deleted for a 0.3-kb fragment of DNA from *Sma*I in the multiple cloning site to *Hpa*I; pKV53, which was deleted for the 0.75-kb fragment

A.



B.

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OxyR Consensus                                          ATAG-t---a-CTAT-------ATAG-t---A-CTAT
H.influenzae hktE OxyR                                  G--AG-T-------TT-A------T-----------T
GAATTCATTCGTAATCCTCCTGAGGGAACGACGGTTCGTGTTATTGCTCCACCTGAAGATTTTGCAGTTAAACGCCTTTCAATGAAATCTC

CTATATTAATGGAAGGTTATCAAATGGGTGTTTCTGCTGGATTAAATCATTTATCAAGTCGTGACGGTACATTTGGATTAGATACTGAAAA

TTGTCATTTCTGTATTTAATTCTATTTTTATAAACAGTTTTATCGTGCACTTTATATAAACAAGGAATCAATCATG
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FIG. 1. (A) Partial maps of plasmids used in this study. Plasmids pLP2, pKV48, and pKV75 are derivatives of pBluescript KS (Stratagene), a portion of which, including the multiple cloning site, is denoted by the thin line. The *V. fischeri* catalase gene, *katA*, is indicated by the black box, and the erythromycin resistance gene, *erm*, is indicated by the gray box. Transcription of the *lacZ* gene is in the direction of the arrow. (B) Nucleotide sequence upstream of the *V. fischeri katA* gene from the *Eco*RI site to the putative ATG start codon. Nucleotides in the putative ribosome binding site upstream of the gene are underlined in bold, and a potential −10 promoter sequence is in boldface. Nucleotides that match the proposed OxyR consensus sequence (41, 42) are in boldface and underlined, while those that match the region upstream of the *H. influenzae hktE* gene, where an OxyR binding site has been proposed (6), are underlined. The proposed OxyR motifs are displayed above. The putative ATG start site is shown in shadowbox letters.

of DNA between the two *Msc*I sites; and pKV54, which carries a 0.4-kb deletion of DNA between the two *Nsi*I sites.

Plasmid pKV75 (Fig. 1A) was derived from pLP2 as follows. pLP2 was digested with *Msc*I, and the resulting DNA fragments were purified by gel electrophoresis. The larger fragment was extracted from the gel and purified by using a Geneclean kit (Bio101, Inc., Vista, Calif.). The 1.1-kb fragment of DNA carrying the erythromycin resistance gene was purified in a similar manner after *Sma*I and *Eco*RV digestion of pKV25 (a derivative of pUC19 [50] in which the erythromycin resistance gene from pLS3 [45] was cloned at the *Sal*I and *Pst*I sites). The two purified fragments were ligated by using T4 DNA ligase (Promega, Madison, Wis.), and the resulting mix was used to transform *E. coli* DH5α cells made competent by treatment with calcium chloride. Ampicillin- and erythromycin-resistant clones were further examined by restriction analysis to identify a clone in which the erythromycin resistance marker had replaced the C-terminal portion of *katA*, resulting in pKV75 (Fig. 1A).

Catalase activity measurements. Extracts of *V. fischeri* cells were made as follows. Cells were pelleted by a 2-min room temperature microcentrifugation and then resuspended in 0.5 ml of an extract buffer containing 5 mM potassium phosphate (pH 7.0), 5 mM EDTA, and 10% glycerol and stored on ice. The cells were lysed by a gentle sonication (four to five rounds of four to five short pulses) in a Vibra-cell microtip sonicator (Sonics and Materials, Inc., Danbury, Conn.) set at an amplitude of 50 U. Cell debris was removed by microcentrifugation at 4°C for 10 min, and the resulting extracts were kept on ice until assayed. Catalase activity was measured by using a quantitative spectrophotometric assay for the decomposition of hydrogen peroxide (4) and reported as units/milligram of protein in the extract, where 1 U equals 1 μmol of hydrogen peroxide decomposed per min. Soluble protein concentrations were determined by the method of Lowry et al. (28).

Subcellular localization of the catalase protein. Periplasmic proteins from *V. fischeri* were selectively released using a chloroform permeabilization treatment (2) as follows. Cells from 1 ml of a culture of stationary-phase *V. fischeri* grown in SWT were pelleted in a microcentrifuge, the supernatant was discarded, and the cells were resuspended in the residual fluid. Chloroform (20 μl) was added, and the cells were vortexed briefly. After a 10-min exposure, 200 μl of sterile 70% seawater was added, and the cell suspension was gently mixed, followed by a 2-min centrifugation at 4°C to pellet the cells. The upper 125 μl of supernatant were removed to a fresh tube, and aliquots were assayed both for catalase activity and for the activity of luciferase (30), a cytoplasmically located enzyme.

Construction of the catalase mutant. A catalase mutant of *V. fischeri* was constructed by marker exchange using pKV75, a plasmid in which about 750 nucleotides between the two *Msc*I sites in the *katA* gene are replaced by the gene for erythromycin resistance (Fig. 1A). This plasmid was introduced sequentially into a *dam* mutant strain of *E. coli*, and then into *V. fischeri* strain ESR1, by electroporation (45). Erythromycin-resistant colonies of *V. fischeri* were purified by several passages on SWT agar, and each of these was tested for the inability to decompose hydrogen peroxide. Several colonies that produced no bubbles upon the addition of hydrogen peroxide were designated as presumptive catalase mutants. Southern blot analysis of one such strain (KV433) and its parent (ESR1), using the ^{32}P-radiolabeled *katA* gene as a probe, revealed that the internal *Msc*I restriction fragment that is present in ESR1 is missing in KV433. The predicted insertion was further confirmed when the probe hybridized to an *Eco*RI fragment of KV433 DNA that was slightly larger than the hybridizing fragment of the parent strain (data not shown).

Colonization of the juvenile squid light organ. To determine the effect of the *katA* mutation on colonization of juvenile *E. scolopes*, animals were inoculated with either KV433 or ESR1. The development of the symbiotic infection was monitored by using the onset of luminescence of the squids as an indicator, as previously described (35). After 45 and 72 h postinoculation, individual squids were rinsed in sterile seawater and homogenized. Dilutions of the homogenates were spread on SWT agar, and the *V. fischeri* colonies that arose were counted to estimate the extent of colonization. Mixed culture experiments were performed with a 1:1 ratio of KV433 to ESR1 cells. The juvenile squid in these experiments were exposed for a short amount of time (3 to 3.5 h) with a low inoculum of cells (about 600 to 800 CFU/ml of seawater) and then removed and placed in symbiont-free seawater for the remainder of the experiment. Squid homogenates were diluted, and aliquots were spread on SWT agar plates and

erythromycin-containing LBS plates to determine the level of colonization by KV433 (erythromycin resistant) and ESR1 (erythromycin sensitive).

Sequence analysis. The *V. fischeri katA* gene was sequenced by using a dye terminator cycle sequencing system at the Biotechnology-Molecular Biology Instrumentation Facility of the University of Hawaii, Manoa. Subclones of the pLP2 plasmid were constructed (not shown), and sequencing of both strands was performed with primers complementary to the vector DNA. A BLAST search (1, 18) was used to compare the *V. fischeri* KatA deduced amino acid sequence to those of catalases from other organisms.

Nucleotide sequence accession number. The GenBank nucleotide accession number for the *V. fischeri katA* gene sequence is AF011784.

RESULTS

Isolation of the *katA* gene of *V. fischeri*. A plasmid, pLP2 (Fig. 1A), was identified from a plasmid library carrying *V. fischeri* genomic DNA based on its ability to complement a catalase-negative (*katE katG* double mutant) strain of *E. coli*. Restriction digests and subcloning experiments localized the catalase activity to within a 2.2-kb region of the insert DNA (pKV48 [Fig. 1A]) that retained the ability to complement the *E. coli* mutant. A deletion of DNA between either the two *Msc*I sites or the two *Nsi*I sites, however, resulted in a loss of catalase activity. In addition, a 0.3-kb deletion of DNA between the *Eco*RI and *Hpa*I sites also resulted in a loss of catalase activity (not shown). Taken together, these data suggested that the *V. fischeri* catalase gene extends most of the length of the DNA located between the *Eco*RI and *Xho*I restriction sites in pLP2 (Fig. 1A).

Sequence analysis of the *V. fischeri katA* gene. Both strands of a 1,913-base region of pLP2, including the presumed location of the catalase-encoding gene, were sequenced (Fig. 1B and data not shown). An open reading frame 482 codons in length, which could potentially encode a protein with a molecular mass of 54,830 Da, was located in this region. A potential ribosome binding site (38) (AGGA) was found 9 bases upstream of the putative start codon (Fig. 1B). Further upstream, at position −161 relative to the presumed translational start, is a sequence (CTATAAT) that is a reasonable match to the consensus −10 sequence recognized by *E. coli* RNA polymerase carrying the housekeeping sigma subunit, σ^{70} (21). However, there was no such match for the corresponding −35 region that would be expected for a σ^{70}-regulated promoter. Promoters recognized by the alternative sigma factor σ^{S} (encoded by *rpoS*), a key positive regulator of genes induced by stress and at stationary phase in many bacteria (22), have a −10 consensus sequence similar to σ^{70} promoters but often lack identifiable −35 regions (17, 49). The sequence features upstream of the *katA* gene suggest that it could be regulated by such an alternative sigma factor.

The putative promoter region of the *katA* gene may also contain a binding site for OxyR, a conserved activator of transcription of catalases and other genes required for defense against oxidative stress (40). Although this potential binding site matches only poorly the proposed *E. coli* OxyR binding site consensus sequence (Fig. 1B) (41, 42), there is a higher similarity of the *V. fischeri* upstream sequence to the nucleotides in a proposed OxyR binding site upstream of the catalase gene in *Haemophilus influenzae* (Fig. 1B) (6). A hypothetical *V. fischeri* OxyR protein might, therefore, regulate the activation of *katA* in response to environmental cues.

The *V. fischeri* catalase sequence shows high identities to catalases of many other bacteria, including *H. influenzae* (78% [6]), *Bacteroides fragilis* (74% [34]), *Proteus mirabilis* (71% [10]), *Bordetella pertussis* (73%), and *Bacillus subtilis* (54%). In addition, *katA* has significant similarity to human (60%), bovine (59%), and other mammalian catalases. These similarities suggest that the *V. fischeri katA* gene is a "typical monofunctional" catalase as defined by Loewen (26) and, specifically, that it can be classified with the very recently identified group III phylogenetic class of bacterial catalases described by Klotz et al. (23). The amino acid sequence of the *V. fischeri* catalase also shows a lower sequence identity (46%) to the *E. coli* catalase, HPII (46), that is induced at stationary phase.

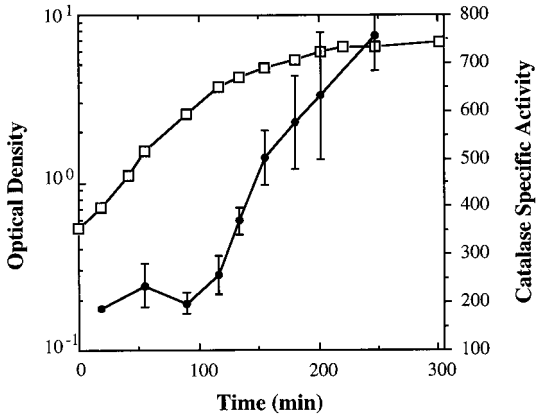

FIG. 2. Catalase specific activity during the growth of *V. fischeri* ES114. The average optical density of the cultures is indicated by open squares; catalase specific activity, reported in units/milligram of protein, is designated by black circles. The data are averages of activity assays performed on extracts of triplicate cultures. The error bars are equal to 1 standard deviation.

Regulation of expression of the catalase enzyme in response to entry into stationary phase and to oxidative stress. Catalase activity in cell extracts of wild-type *V. fischeri* strain ES114 was measured at different times during its growth in a rich medium. While significant catalase activity could be detected during all phases of growth, the amount of catalase was induced more than threefold in cells that were beginning to enter stationary phase (Fig. 2). Similar results were obtained for *V. fischeri* ESR1 (not shown). The level of activity of the *V. fischeri* catalase enzyme in extracts of cells in stationary phase, approximately 800 U/mg of protein, is about 10-fold higher than that observed in extracts of an overnight culture of *E. coli* (data not shown) and may reflect different levels of catalase expression in the two organisms.

The induction of catalase activity in *V. fischeri* cells entering stationary phase suggested that the gene product of an *rpoS* homolog might be involved in the regulation of *katA*. Because *V. fischeri rpoS* mutants have not yet been isolated or constructed, this hypothesis was addressed by an alternative method. The *katA*-encoding plasmid pKV48 (Fig. 1A) was introduced into both an *E. coli rpoS* mutant and its wild-type parent. When catalase specific activity was assayed in cell extracts after overnight growth, high levels were observed in the wild-type strain carrying pKV48 (Table 1). These levels were over 15-fold higher than the activity of the endogenous *E. coli* catalases observed in a control strain transformed with the vector alone. In contrast, when pKV48 was present in the *rpoS* mutant, this high level of catalase activity was not observed (Table 1). These data suggest that in *E. coli*, transcription of the *katA* gene may be directed almost exclusively by RNA polymerase containing σ^{S}. This finding, taken together with the temporal pattern of catalase expression in *V. fischeri*, lends support to the model that the *katA* gene is transcribed under the control of an *rpoS* homolog in *V. fischeri.*

Catalases from a number of other organisms, including the highly similar group III enzyme from *H. influenzae*, are induced upon exposure to low levels of hydrogen peroxide. In *E. coli*,

TABLE 1. Catalase specific activities of *E. coli* strains carrying the *V. fischeri katA* gene and of *V. fischeri* cells challenged with hydrogen peroxide

Expt	Avg catalase sp act ± 1 SD (U/mg of protein)[a]
1[b]	
E. coli MC1000/pKV48	756 ± 210
E. coli MC1000/pBS	23 ± 12
E. coli JV1012/pKV48	64 ± 5
E. coli JV1012/pBS	38 ± 8
2[c]	
$-H_2O_2$	197 ± 34
$+H_2O_2$	709 ± 32

[a] Average of activity assays performed on extracts of triplicate cultures.
[b] Stationary-phase cultures of *E. coli* MC1000 and its isogenic *rpoS* mutant derivative JV1012, carrying either plasmid pKV48, which encodes the *V. fischeri katA* gene, or the control vector pBluescript (pBS) were assayed for catalase activity.
[c] Exponentially growing cultures of *V. fischeri* ES114 were exposed to 60 μM hydrogen peroxide for 30 min and then assayed for the ability to decompose hydrogen peroxide. Extracts were made from cells either exposed or not exposed to hydrogen peroxide.

however, the catalase (HPI) that is induced in response to this reactive oxygen species is distinct from the catalase (HPII) that is induced at stationary phase; the former requires OxyR for induction, while the latter is dependent on σ^S. We therefore asked whether wild-type *V. fischeri* would induce the expression of its catalase in response to the presence of hydrogen peroxide. Exposing a culture of *V. fischeri* ES114 cells to 60 μM hydrogen peroxide for 30 min early in logarithmic growth resulted in a 3.5-fold increase in the level of catalase specific activity (Table 1), e.g., up to the level seen in stationary phase cells (Fig. 2). Thus, *V. fischeri* cells can respond to the presence of hydrogen peroxide in their external environment by inducing the expression of catalase activity. This induction most likely occurs at the transcriptional level and may be dependent on OxyR acting at the putative OxyR binding site described above (Fig. 1B).

Subcellular localization of the catalase protein. Although many of the well-characterized bacterial catalases are typically present in the cytoplasm, some, such as the KatA catalase from *B. subtilis* (8, 29), are exported. Because the *V. fischeri* enzyme has a high degree of identity with the *B. subtilis* catalase, and because a secreted catalase might be of particular value if the light organ environment were a significant source of reactive oxygen species, we investigated whether the catalase protein from *V. fischeri* was exported. While there was no evidence of catalase activity in the cell-free supernatant from an overnight culture of *V. fischeri*, permeabilization of cells with chloroform to specifically release periplasmic proteins yielded 100% of the catalase activity but less than 5% of the cytoplasmic luciferase activity. We conclude that the catalase enzyme is localized in the periplasm of *V. fischeri.*

Characterization of a *V. fischeri katA* mutant. To determine whether *V. fischeri* has multiple catalase genes and to investigate the physiological role of KatA, we constructed a *katA* null mutant of *V. fischeri* in which the C-terminal portion of the gene was replaced by an erythromycin resistance marker. While high levels of catalase activity were present in the parent strain after overnight growth in a rich medium, no significant activity (<3% of wild-type activity) was detectable in the *katA* mutant at any time in the growth cycle. Thus, *V. fischeri* cells apparently synthesize one catalase, and this enzyme is responsible for the high specific activity and efficient decomposition of hydrogen peroxide displayed by wild-type cells.

The susceptibility of the *katA* mutant and its parent to external hydrogen peroxide was assayed to determine whether KatA has a physiological role in protecting *V. fischeri* from oxidative stress. When the cells were grown to early stationary phase and exposed to 20 mM hydrogen peroxide, the catalase mutant was readily killed (<0.001% survival), whereas its parent survived well (>50%). In spite of this greatly enhanced sensitivity, there was no detectable difference between the aerobic growth rates or yields of the *katA* mutant and its parent in a rich medium.

Survival of the *katA* mutant during colonization of the squid light organ. To determine whether the ability of KatA to protect against hydrogen peroxide killing in culture enhanced the survival of *V. fischeri* in the light organ environment, the effectiveness of colonization by the *katA* mutant was compared to that of its parent. The course of colonization by these two strains was monitored by observing the luminescence levels of infected juvenile *E. scolopes* squid. When presented by itself, the *katA* mutant was not defective in initiating a colonization of the light organ of *E. scolopes*, and at both 2 and 3 days postinoculation, the extent of colonization (as determined by CFU per light organ) was indistinguishable between the two strains (data not shown). However, the *katA* mutant was clearly defective in its ability to compete in a mixed infection with its catalase-positive parent strain (Fig. 3). When the two strains were mixed in equal ratios and used to coinoculate juveniles of *E. scolopes*, the ratios of the two strains in the colonized animal at 17 h postinoculation were skewed in favor of the wild-type parent. This bias was observed in each of the several times the experiment was performed. These data suggest that although a catalase mutant is able to establish a symbiotic colonization, it carries a deficiency that decreases the overall fitness of the strain. The presence of a functional catalase enzyme is likely therefore to be critical to the ability of *V. fischeri* to achieve a successful colonization in nature.

DISCUSSION

Despite the fact that all animals typically have long-term, cooperative associations with microbial symbionts, little is known about such benign interactions between bacteria and

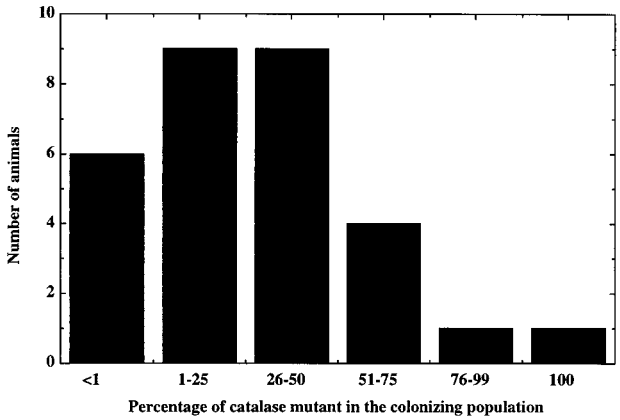

FIG. 3. Colonization of juveniles of the squid *E. scolopes* by the *katA* mutant and its parent. Juveniles of *E. scolopes* were exposed to a 1:1 mixture of the *katA* mutant and its wild-type parent. The percentage of the *katA* mutant strain in the population of *V. fischeri* cells colonizing the light organ was determined for each animal at 17 h postinoculation.

their animal hosts (13). We believe that the lessons learned from the study of one specific host-symbiont pair, the squid *E. scolopes* and the bioluminescent bacterium *V. fischeri*, will advance our understanding of the mechanisms by which many diverse bacteria, including pathogens, interact with their hosts. In this paper, we report the characterization of a stationary-phase catalase that is important in protecting *V. fischeri* from oxidative stress and that is required for the organism to successfully compete against other bacteria for the symbiotic colonization of juvenile squid in coinoculation experiments.

Our characterization of the *V. fischeri katA* gene and its product yielded a number of interesting results: (i) wild-type cells express a high catalase specific activity that is further induced upon stationary phase growth in culture, (ii) the presence of low levels of hydrogen peroxide in the culture medium induces log-phase cells to express a similarly high level of catalase specific activity, and (iii) the enzyme appears to be entirely localized to the periplasm. These and other data suggested that *V. fischeri* cells have developed a powerful system of protection against the potential oxidative stress posed by hydrogen peroxide in their environment.

The light organ of *E. scolopes* has an abundance of mRNA encoding a squid halide peroxidase activity that catalyzes the conversion of hydrogen peroxide into the bactericidal compound hypochlorous acid (43, 48). This enzyme is similar to the mammalian defense protein, myeloperoxidase, and although the role of the halide peroxidase in the light organ has not yet been demonstrated, these data support a hypothesis in which oxidatively stressful conditions exist in the light organ environment (48). This hypothesis, in light of our results regarding the activity and location of the catalase enzyme, can be extended to predict that the catalase enzyme would be a requirement for successful symbiotic colonization by *V. fischeri.*

Curiously, when presented as the only bacterial strain in the inoculation, a catalase mutant displayed no observable defect in the ability to colonize juveniles of *E. scolopes*. However, when competition assays were performed to determine whether the catalase mutant could compete in mixed culture against its catalase-positive parent strain, it was found that the catalase mutant was deficient in its ability to compete for colonization. Thus, in the natural environment of the ocean, a catalase mutation would confer a serious fitness defect upon a strain competing against wild-type bacteria co-occurring in the seawater. This type of phenotype, in which a mutant that is competent for colonization when presented alone but that is unable to successfully compete when present with a wild-type parent strain, has been reported in the *Rhizobium*-root nodule association as well (3, 19). It is likely that such subtle, competition-dependent phenotypes, while easily missed in the laboratory, may have dramatic consequences for bacteria existing in the natural environment.

Our data confirm a requirement for the bacterium to protect itself from oxidative stress in the light organ but suggest that the bacterium does not rely solely on the catalase enzyme for protection from stress factors in the light organ environment. Similarly, it is known from work performed with *E. coli* that the *rpoS* gene is important for the resistance of this organism to hypochlorous acid, although interestingly, the catalase genes *katE* and *katG* are not the major contributors to this resistance (15). Other contributors may include the superoxide-induced gene regulators, SoxRS, which are also activated by hypochlorous acid (14). Similarly, cholera toxin, produced by *Vibrio cholerae*, is an ADP-ribosyltransferase enzyme that has been shown to inhibit the superoxide (and ultimately hydrogen peroxide)-generating respiratory burst (37). Two ADP-ribosylating activities have recently been found in *V. fischeri* (32, 33), and these exported enzymes may also be involved in reducing the oxidative stress environment of the light organ. Clearly, many avenues exist for bacteria to combat oxidative stress, and the production of catalase is just one of these.

Due to the periplasmic location of the catalase enzyme in *V. fischeri*, and data regarding its activity in culture, one might predict that in mixed-culture experiments, the wild-type strain could protect the catalase mutant strain by removing hydrogen peroxide in the general vicinity of both of the cell types. Our colonization data suggest that this is not the case. What then is the mechanism underlying the catalase mutant's competitive defect even though, when not forced to compete with the wild type, it can colonize and persist normally for several days? We envision two hypotheses that address this question, both of which arise from the population dynamics that characterize the daily pattern of symbiont growth within the squid light organ. Each morning about 90% of the symbiont cells are expelled from the light organ crypts (25), and the remaining 10% go on to fully recolonize the crypt spaces by nightfall. In our first hypothesis, as a result of its greater susceptibility to a constant, low level of oxidative stress during this period of regrowth, the catalase mutant would experience a higher level of attrition than the wild-type parent, and its relative abundance in the recovering population could ultimately decrease. Our second hypothesis is based on recent evidence suggesting that the host peroxidase may be most highly expressed during a specific period each day (39). Thus, the catalase mutant may experience an episodic exposure to oxidative stress that diminishes its numbers relative to the wild type. Subsequent proliferation of the surviving symbionts in a mixed-infection organ would thereby result in a predominance of wild type over the catalase mutant. Under either of these scenarios, the catalase mutant, when occupying the light organ by itself, could ultimately recolonize to the same level as the wild type; however, we would predict that this recolonization should take somewhat longer as a result of either the mutant's diminished growth rate or its lower abundance due to its reduced resistance to an ambient oxidative stress.

An understanding of these events will require a description of the kinetics of recolonization (i.e., how rapidly the population multiplies and over what portion of the day). Unfortunately, these kinetics remain experimentally difficult to determine due to the wide variation in colonization levels among individual animals (36). In addition, it is not known whether the bacteria achieve a stationary phase of growth for any significant period of time in the light organ, thus making it difficult to assign a significance to the stationary-phase regulation of *katA*. We expect that further studies of the pattern of growth and recolonization by the catalase mutant after the expulsion event, as well as studies of the expression pattern of this stationary-phase-regulated catalase gene in the light organ, will begin to answer some of these questions.

The ability of a cell to use catalase to protect itself from host-imposed oxidative stress has been shown not to be a requirement for tissue colonization by several species of bacterial pathogens (5, 9). However, in these studies, mixed-inoculation experiments were not performed. A thorough investigation of mutant phenotypes may require the examination of strains presented to the host in mixtures, a situation that not only provides an experimental test of subtle phenotypes but also more closely mimics the natural environment. Studies such as this one, investigating the symbiotic association between a marine bacterium and its squid host, may thus provide insight into other naturally occurring microbe-animal interactions.

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