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Isolation and Characterization of *rpoS* from a Pathogenic Bacterium, *Vibrio vulnificus*: Role of σ^S in Survival of Exponential-Phase Cells under Oxidative Stress

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A gene homologous to *rpoS* was cloned from a fatal human pathogen, *Vibrio vulnificus*. The functional role of *rpoS* in *V. vulnificus* was accessed by using an *rpoS* knockout mutant strain. This mutant was impaired in terms of the ability to survive under oxidative stress, nutrient starvation, UV irradiation, or acidic conditions. The increased susceptibility of the *V. vulnificus* mutant in the exponential phase to H_2O_2 was attributed to the reduced activity of hydroperoxidase I (HPI). Although σ^S synthesis was induced and HPI activity reached the maximal level in the stationary phase, the mutant in the stationary phase showed the same susceptibility to H_2O_2 as the wild-type strain in the stationary phase. In addition, HPII activity, which is known to be controlled by σ^S in *Escherichia coli*, was not detectable in *V. vulnificus* strains under the conditions tested. The mutant in the exponential phase complemented with multiple copies of either the *rpoS* or *katG* gene of *V. vulnificus* recovered both resistance to H_2O_2 and HPI activity compared with the control strain. Expression of the *katG* gene encoding HPI in *V. vulnificus* was monitored by using a *katG*::*luxAB* transcriptional fusion. The expression of this gene was significantly reduced by deletion of σ^S in both the early exponential and late stationary phases. Thus, σ^S is necessary for increased synthesis and activity of HPI, and σ^S is required for exponentially growing *V. vulnificus* to develop the ability to survive in the presence of H_2O_2.

The life cycles of pathogenic bacteria involve periods in which they exist in a nongrowing state in stressed environments. Only if they survive such conditions are they able to proliferate with high metabolic activity in the proper host environments (7, 36). Thus, these organism have evolved several mechanisms that allow them to survive under stressful conditions, such as starvation, temperature fluctuation, oxidative stress, and osmotic shock, and that enable them to resume growth once the stress is removed (27). The cellular responses to environmental stimuli have been extensively studied in many bacterial species, most notably *Escherichia coli*.

To respond properly to diverse stresses, *E. coli* requires the *rpoS* gene product, which is a second principal sigma factor (σ^S); this product induces expression of many genes and allows the organism to mediate changes in cellular physiology and structure and to adapt, resist, and survive under stress conditions (9, 16, 19). σ^S is also required for eliciting phenotypes related to virulence in many pathogenic bacteria belonging to the γ subdivision of *Proteobacteria* (21, 32, 39, 45, 50).

It is generally believed that most microorganisms that communicate with, associate with, or colonize host animals are relatively well equipped with defense mechanisms to deal with oxidative stress (6, 15, 43). *E. coli* produces at least two enzymes to overcome the presence of hydrogen peroxide and to maintain a relatively constant concentration of intracellular H_2O_2 (8); these enzymes are KatG (hydroperoxidase I [HPI]), which has both catalase and peroxidase activities, and monofunctional KatE (HPII), which has catalase activity (25). KatG, one of the members of the OxyRS regulon, is induced by direct exposure to H_2O_2 (37). In contrast, KatE is known to be regulated by σ^S, and consequently cellular expression of this enzyme increases at the onset of the stationary phase (25, 30). Open reading frames homologous to both *katG* and *katE* are present in the genomes of *Vibrio cholerae* and *Vibrio parahaemolyticus*. The presence and role of monofunctional catalases have been studied in *Vibrio fischeri* and *Vibrio rumoiensis*, but the regulation of these enzymes has not been described (11, 44, 51).

The causative agent of septicemia, *Vibrio vulnificus*, has been considered an important pathogen in humans due to its rapid pathogenic progress and its high mortality rate (10, 38). A number of studies have been performed on virulence factors of this organism, including metalloprotease (13), hemolysin (48), and siderophores (35). Several regulators, including cyclic AMP receptor protein (CRP)/LuxR (12, 33), ToxRS/CRP (1, 17), and Fur (18), have been reported to control expression of these virulence factors. While survival of this bacterial species has been studied under diverse conditions (26), the molecular mechanisms underlying its survival strategies have not been studied well.

In an effort to isolate global regulators involved in survival of

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TABLE 1. Strains, plasmids, and oligonucleotide primers used in this study

Strain, plasmid, or primer	Relevant characteristics	Reference or source
V. vulnificus strains		
ATCC 29307	Type strain	12
AR	ATCC 29307 but spontaneous rifampin resistance	This study
MO6-24/0	Clinical isolate	48
CN7	Clinical isolate	This study
CNUH94-4	Clinical isolate	This study
SC9649	Environmental isolate (seawater)	This study
SC9720	Environmental isolate (sediment)	This study
SC97126	Environmental isolate (seafood)	This study
KPR101	AR but Δ*rpoS* (allelic exchange with pKP13)	This study
E. coli strains		
DH5α	ϕ80d*lacZ*ΔM15 *recA1 endA1 gyrA96 relA1 thi-1 hsdR17*(r_K^- m_K^-) *supE44 deoR* Δ(*lacZYA-argF*)*U169*	Laboratory collection
JM109	*endA1 recA1 gyrA96 thi-1 hsdR17*(r_K^- m_K^-) *relA1 supE44* Δ(*lac-proAB*) [F′ *traD36 proAB lacI*^{q}ZΔM15]	Promega
SM10λpir	*thi-1 thr leu tonA lacY supE recA*::Rp4-2-Tc::Mu λpir; Km^r	34
AMS6	K-12 (λ^- F^- Δ*lac*)	31
ZK918	W3110 Δ*lacU169 tna-2 rpos*::*kan bolA*:*lacZ*	2
Plasmids		
pUC19	Cloning vector; *lacZ*α Ap^r	49
pINE32	pUC19 with 2.75-kb Sau3AI fragment of *V. vulnificus* DNA containing the complete coding sequence of *V. vulnificus* of *rpoS*	This study
pKP11	pINE32 with deletion of 762-bp NruI fragment (*V. vulnificus* Δ*rpoS*)	This study
pDM4	Suicide vector; *ori*R6K Cm^r	23
pKP13	pDM4 containing SmaI and XbaI fragment of pKP11	This study
pLAFR5	IncP Tc^r; derivative of pLAFR3 containing a double *cos* cassette	14
pKP14	pLAFR5 containing EcoRI and HindIII fragment of pINE32	This study
pQE30	Expression vector	Qiagen
pQE*rpoS*	pET containg 1,072-bp PCR-amplified *V. vulnificus rpoS* coding region	This study
pHK0011	pRK415 with promoterless *luxAB;* Tc^r	12
pHL-03	$katG_{vv}$::*luxAB* transcriptional fusion in pHK0011	This study
pRK415	IncP *ori;* broad-host-range vector; *oriT* of RP4; Tc^r	14
pKP51	pRK415 containing KpnI and HindIII fragment of *V. vulnificus katG*	This study
Primers		
$rpoS_{vv}$-F	5′-CGGATCCAGTATCAGCAACACAGTCACCAAAG-3′	
$rpoS_{vv}$-R	5′-GGCCAAGCTTCTATGATTCCATAGCCTTTTTTC-3′	
F2	5′-AACGCGCTAAGAGGTTATGGC-3′	
R2	5′-AGCAAGAACGGTGGATATCGC-3′	
katG-F-KpnI	5′-GTTGGTACCTCAACTACCGC-3′	
katG-R-XbaI	5′-GGATGTCTAGATTGAGGGCC-3′	
katG-R-HindIII	5′-GCAAGTCTGATAAAAGCTTCG-3′	

V. vulnificus, we cloned the *rpoS* gene and defined its physiological role in survival of *V. vulnificus* in the presence of various stresses. These analyses showed that *V. vulnificus* in the exponential phase requires σ^S for survival in the presence of low concentrations of hydrogen peroxide. In the present study we also observed regulation of the expression and activity of a catalase involved in this response, and the results were quite different from those obtained with *E. coli*.

MATERIALS AND METHODS

Isolation of the *rpoS* gene from *V. vulnificus*. The genomic DNA of *V. vulnificus* ATCC 29307 was prepared by a standard technique (29) and then partially digested with Sau3AI and size fractionated by agarose gel electrophoresis. The DNA fragments, which ranged from 2 to 6 kb long, were pooled and ligated with the pUC19 vector which had been digested with BamHI and subsequently treated with bacterial alkaline phosphatase. The *V. vulnificus* library obtained was introduced into *E. coli* ZK918 having a deletion in its *rpoS* gene and a σ^S-dependent *bolA*::*lacZ* fusion in its chromosome (2). After transformation with the library, colonies were screened on Luria-Bertani (LB) medium supplemented with ampicillin (100 μg/ml) and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (10 μg/ml), which was blue as a result of induced expression of *bolA*::*lacZ* after addition of the RpoS homolog of *V. vulnificus*. Sequencing of the double-stranded DNA of the plasmid selected, pINE32, containing a 2,693-bp insert, was performed with an Applied Biosystems 373A automated DNA sequencer. The remaining gaps in the sequence were filled in by sequence analysis by using specifically designed internal primers that annealed to the insert region. Sequence analysis and database searches were performed by using the National Center for Biotechnology Information BLAST server.

Construction of *rpoS* knockout mutant KPR101. A 762-bp NruI fragment containing two-thirds of the *rpoS* coding sequence was deleted from pINE32. The resultant plasmid, pKP11, was digested with SmaI and XbaI, which resulted in a DNA fragment containing a region adjacent to the *rpoS* gene but not the *rpoS* gene. This DNA was cloned into suicide vector pDM4 (23), which was digested with ApaI and XbaI, yielding pKP13. pKP13 in *E. coli* SM10 λ*pir* was mobilized into strain AR, a rifampin-resistant derivative of the wild-type strain *V. vulnificus* ATCC 29307. Conjugal transfer was performed by mixing aliquots of the strains that contained about 10^8 donor cells and about 10^8 recipient cells and then incubating the preparation overnight at 37°C in close contact on a membrane filter. The cell mixture was then resuspended in LBS (LB medium containing NaCl at a final concentration of 2.5%) broth and plated onto selective plates (LBS agar plates supplemented with 4 μg of chloramphenicol per ml and 50 μg of rifampin per ml). A colony showing indications of a double homologous recombination event (resistance to 5% sucrose and sensitivity to chloramphenicol) was isolated, and deletion of its *rpoS* region was confirmed by PCR by using primers F2 and R2 (Table 1).

Determination of survival of *V. vulnificus* under various stress conditions.

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Exponential-phase cultures (optical density at 600 nm [OD_{600}] in LBS broth, 0.15 to 0.3) or stationary-phase cultures (OD_{600} in LBS broth, about 3 to 4; usually 8 to 10 h after an overnight culture was added to fresh LBS broth) were collected by centrifugation, washed with artificial seawater (ASW) (0.6 M NaCl, 0.1 M $MgSO_4$, 0.02 M $CaCl_2$, 0.02 M KCl, 50 mM Tris-HCl [pH 8.3] [28]), and resuspended in the appropriate medium at a density of approximately 10^6 to 10^7 cells/ml. During incubation under stress conditions at 30°C, aliquots were removed and spread on LBS agar plates to monitor the viability by determining the number of CFU. For survival studies under stress conditions, we used hyperosmosis (LBS medium containing 5 M NaCl), starvation (nutrient-free ASW), acidity (LBS medium titrated to pH 4.0 with HCl), UV irradiation (wavelength, 254 nm; energy, 120,000 μJ/cm^2), and hydrogen peroxide (0.088 to 10 mM H_2O_2 in ASW).

Determination of catalase activities. Cellular extracts of *E. coli* and various *V. vulnificus* strains (Table 1) were prepared in cold 50 mM potassium phosphate buffer (pH 7.0) by sonication (Vibracell; Sonics & Materials, Inc.) in ice. The amount of protein in a cell lysate was determined by the Bradford assay by using bovine serum albumin as the standard. After separation on an 8% nondenaturing polyacrylamide gel, the locations of HPI and HPII were visualized by staining the gel with a solution containing 1% $K_3Fe(CN)_6$ and 1% $FeCl_3$ (47) and compared with the locations of the corresponding enzymes of *E. coli*.

For the catalase assay, cell samples were obtained at various growth phases (OD_{600}, 0.1 to 4.2) and were resuspended in chilled catalase buffer (5 mM potassium phosphate buffer [pH 7.0], 5 mM EDTA, 10% glycerol, 25 μM phenylmethylsulfonyl fluoride) (42) and sonicated in ice. Each cellular extract was then mixed with 25 mM potassium phosphate buffer (pH 7.0) containing 5.9 mM H_2O_2, and the amount of remaining H_2O_2 in the reaction mixture was estimated by monitoring the absorbance at 240 nm at 30-s intervals for 10 min. One unit of specific activity was defined as 1 μmol of H_2O_2 degraded per min per mg of protein (42).

Construction of *katG*::*luxAB* transcriptional fusion and measurement of expression of the *katG* gene. The 504-bp DNA fragment which included nucleotide positions −318 to 115 relative to the initiation codon of *katG* (gene VV12755 in GenBank accession no. NC_004459) was amplified by PCR by using two primers, katG-F-KpnI and katG-R-XbaI (Table 1). The PCR product was digested with appropriate restriction enzymes (KpnI and XbaI) and inserted into *luxAB*-based plasmid pHK0011 (12) digested with the same enzymes. The resultant transcriptional fusion, pHL-03, was transferred into *V. vulnificus* cells via conjugative transfer. Overnight (16- to 18-h) cultures of the cells containing pHL-03, which exhibited the basal level of bioluminescence, were inoculated into fresh LBS medium containing 3 μg of tetracycline per ml, and the expression from the *katG* promoter was measured by monitoring light production in the presence of 0.006% *n*-decyl aldehyde by using luminometers (TD-20/20; Turners Designs). The specific bioluminescence was calculated by normalizing the relative light units with cell mass (OD_{600}).

Complementation of KPR101 with a broad-host-range vector containing *V. vulnificus rpoS* (pKP14) or *V. vulnificus katG* (pKP51). An EcoRI-HindIII DNA fragment that included the intact *rpoS* gene was obtained from pINE32 and cloned into the broad-host-range vector pLAFR5 (14) to obtain plasmid pKP14. The 2,569-bp DNA fragment that included the intact *katG* gene was amplified by PCR by using two primers, katG-F-KpnI and katG-R-HindIII. The PCR product was digested with appropriate restriction enzymes (KpnI and HindIII) and cloned into the broad-host-range vector pRK415 (14) to obtain plasmid pKP51. Each plasmid was introduced into the *rpoS* mutant by conjugative transfer, as described above.

Western analysis of σ^S. Two oligonucleotides, $rpoS_{vv}$-F and $rpoS_{vv}$-R (Table 1), were used to amplify a 1,032-bp DNA fragment containing the complete sequence of the *rpoS* gene from the genomic DNA of *V. vulnificus*. BamHI and HindIII sites located at both ends of the resultant *rpoS* DNA were used to clone this DNA into the pQE30 expression plasmid (Qiagen) to generate plasmid pQE*rpoS*. Recombinant σ^S was overexpressed in *E. coli* JM109 by adding isopropylthio-β-D-galactoside (Sigma) at a concentration of 1.0 mM and was purified by using an Ni^+-nitrilotriacetic acid affinity column as directed by the manufacturer (Qiagen). Polyclonal antibodies against *V. vulnificus* σ^S were produced in a rabbit by intravenous immunization with 200 μg of the recombinant *V. vulnificus* σ^S, and this initial immunization was followed by additional immunizations at 1 and 4 weeks. Ten days after the last injection, the blood of the immunized rabbit was collected, and its serum was used for Western blot analysis. Cell extracts of the *V. vulnificus* wild type and the *rpoS* mutant containing either pLAFR5 or pKP14 were prepared by sonication in TNT buffer (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.05% Tween 20) (29), and 40-μg aliquots of the extracts were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After transfer to a Hybond P membrane (Amersham), Western blot analysis was performed by serially incubating the filter with *V. vulnificus* σ^S polyclonal antibodies (1:1,000 dilution) and alkaline phosphatase-conjugated rabbit anti-rat immunoglobulin G (1:1,000 dilution; Sigma). The *V. vulnificus* σ^S band was visualized by using the nitroblue tetrazolium-BCIP (5-bromo-4-chloro-3-indolylphosphate) system (Promega).

Nucleotide sequence accession number. The *V. vulnificus rpoS* nucleotide sequence has been deposited in the GenBank database under accession number AY187681.

RESULTS AND DISCUSSION

Isolation of *V. vulnificus rpoS* by functional complementation. In *E. coli*, σ^S is known to regulate the expression of several genes involved in cellular adaptation to diverse stresses. Since the σ^S homologue of *V. vulnificus*, if there is one, may also be able to play an equivalent role in regulation of these genes, *E. coli* strain ZK918 containing the σ^S-dependent *bolA* promoter::*lacZ* fusion (2) was utilized. Upon transformation of ZK918 with the genomic library of *V. vulnificus*, the bluish transformants on LB medium supplemented with X-Gal were chosen as candidates for complementing plasmids for the *rpoS* function. One of the plasmids, plasmid pINE32 carrying a 2,693-bp insert (Table 1), was used for further investigation.

Analysis of the pINE32 insert DNA. Sequence analysis of the insert in pINE32 revealed that it coded for the proteins of *V. vulnificus* homologous to NlpD, RpoS, and MutS. Although the genetic organization of the open reading frames flanking *rpoS* is the same as that found in other bacteria, the lengths of the intergenic spaces between *nlpD* and *rpoS* and between *pcm* (the upstream gene of *nlpD*) and *nlpD* were quite different from the lengths of the intergenic spaces in *E. coli* or *Pseudomonas*. This finding suggests that the regulation of *rpoS* expression at the transcriptional level in *V. vulnificus* may be different from the regulation in other organisms which have been extensively studied (20, 30, 41). The amino acid sequence deduced from the gene homologous to *rpoS*, which codes for 343 amino acid residues corresponding to ca. 39.6 kDa with a pI of 4.92, was aligned with other known σ^S sequences and was found to exhibit overall levels of identity of 83, 79, and 70% with the sequences of *V. parahaemolyticus*, *V. cholerae*, and *E. coli*, respectively. There is complete homology in subregions 2.3 and 2.4 of σ^S, which are involved in promoter recognition (e.g., RpoD box and 14-mer) (4), and there is significant conservation in subregion 2.1, which is involved in core binding. The helix-turn-helix motif in subregion 3.1 and the −35 recognition region in subregion 4.2 showed relatively weak similarity.

Generation of an *rpoS*-deficient strain, KPR101. A major portion of the *V. vulnificus rpoS* gene in pINE32 was deleted by digestion with NruI and subsequent ligation. The *nlpD-rpoS-mutS* region with the *rpoS* gene deleted was transferred to a conjugative plasmid, pDM4, resulting in pKP13. Replacement of the wild-type *rpoS* gene located on the chromosome of *V. vulnificus* AR with this plasmid was accomplished through homologous recombination. Deletion of the *rpoS* gene in the *V. vulnificus* mutant was confirmed by PCR by using primers F2 and R2 (Table 1). A PCR analysis of a deletion of the internal region of the *rpoS* gene in the mutant revealed the expected size for the DNA fragment (754 bp); meanwhile, the intact *rpoS* gene in the wild-type produced a 1,516-bp DNA fragment

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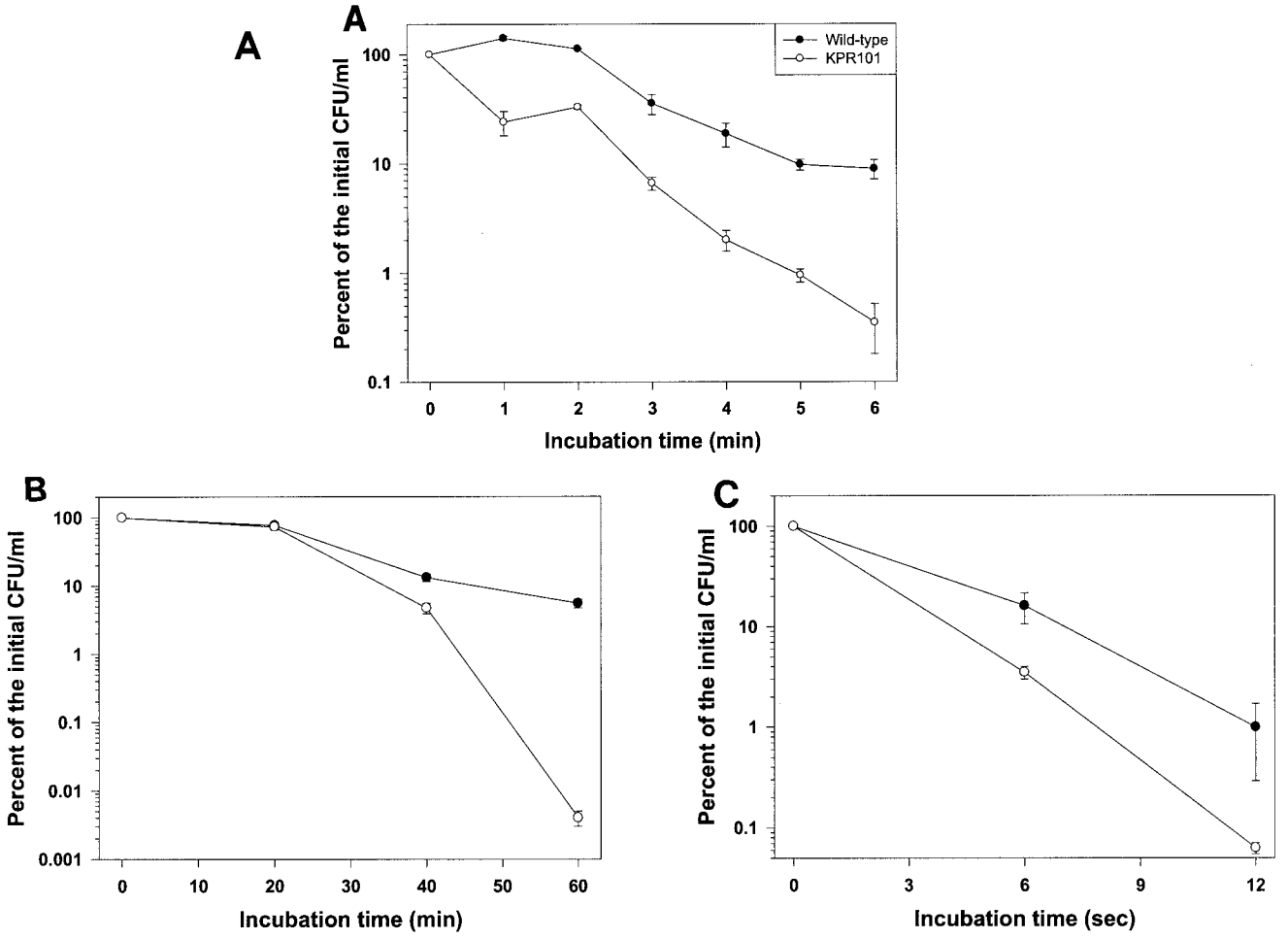

FIG. 1. Survival of exponential-phase wild-type and Δ*rpoS* mutant KPR101 cultures under stress conditions. Wild-type and KPR101 cells in the exponential phase were challenged by starvation (ASW) (A), acidic conditions (LBS medium titrated to pH 4.0) (B), and irradiation with UV light (254 nm; 120,000 μJ/cm^2) (C). At several times during exposure, aliquots of each culture were removed, and the numbers of CFU per milliliter were estimated as described in Materials and Methods. The resultant values were expressed as percentages of the initial cell density, which ranged from 10^6 to 10^7 CFU/ml.

(data not shown). The resultant mutant was designated KPR101.

Survival characteristics of KPR101. The survival of KPR101 in the exponential phase was examined when it was exposed to various stresses, including hyperosmotic conditions, starvation, an acid environment, UV irradiation, and oxidative conditions, and was compared to the survival of the wild type. In contrast to enteric bacteria (9, 19), *rpoS*-deficient *V. vulnificus* exhibited the same survival pattern in the presence of a high salt concentration (5 M NaCl) that the wild type exhibited (data not shown). The survival of the mutant, however, was severely impaired in the presence of other stresses. For example, the abilities of the mutant to survive under starvation, acidic, and UV-irradiated conditions (6 days, 1 h, and 12 s, respectively) were estimated to be 25-, 1,300-, and 16-fold less than those of the corresponding controls, respectively (Fig. 1A ~ C).

The Δ*rpoS* mutant cells in the exponential phase also showed significantly increased susceptibility to 880 μM H_2O_2; there was up to a 1,000-fold difference after 30 min of exposure (Fig. 2A). The KPR101 cells in the stationary phase showed more resistance than the cells in the exponential phase, but the susceptibility was basically similar to the susceptibility of the wild type in the presence of various concentrations of H_2O_2 up to 10 mM (Fig. 2B). This σ^S-independent increase in H_2O_2 resistance in the stationary phase is an unusual observation, since synthesis of σ^S is induced in the stationary phase in *V. vulnificus* (data not shown), and one of catalases (HPII) is known to be induced by σ^S in *E. coli* (30, 37, 40). In addition, *V. vulnificus* cells were generally more sensitive to H_2O_2 than other enteric bacteria were, because exposure to the concentrations of H_2O_2 used for *E. coli* or *V. cholerae* (e.g., ca. 10 mM for 30 min) resulted in survival of only 0.1 to 0.01% of the cells present initially (Fig. 2B). Thus, it is possible that *V. vulnificus* may have mechanisms for oxidative stress response that are distinct from those found in *E. coli*, at least under the conditions which we used.

Characterization of the catalase of *V. vulnificus*. The distinct susceptibility of KPR101 to H_2O_2, especially during the exponential phase, led us to examine the innate properties of hydroperoxidase(s) in wild-type *V. vulnificus*. Protein extracts of

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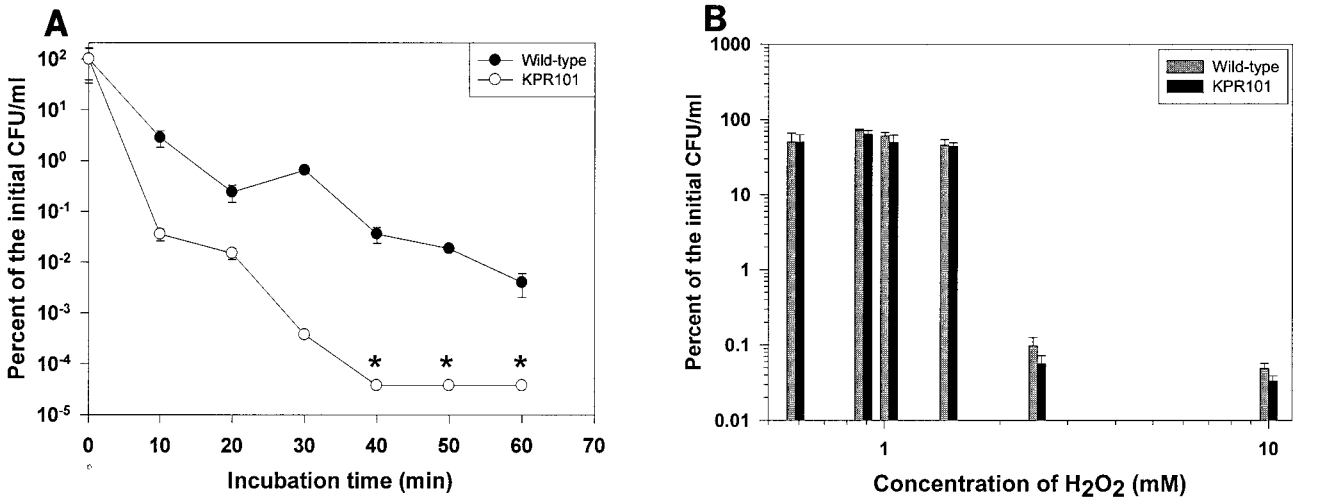

FIG. 2. Survival of the wild-type and Δ*rpoS* mutant KPR101 cells in the presence of H_2O_2. (A) Wild-type and KPR101 cells in the exponential phase were challenged with an oxidative stress (ASW containing 880 μM H_2O_2). At several times during exposure, aliquots of each culture were removed, and the numbers of CFU per milliliter were estimated. The asterisks indicate values lower than the detection limit used in this analysis. (B) Wild-type and KPR101 cells in the stationary phase (OD_{600} in LBS broth, approximately 3 to 4) were harvested and incubated in ASW containing concentrations of H_2O_2 ranging from 0.6 to 10 mM. After 30 min of exposure, the numbers of remaining culturable cells were estimated. The resultant values were expressed as percentages of the initial cell density, which was about 10^7 CFU/ml.

the type strain of *V. vulnificus* (ATCC 29307) were separated on a nondenaturing polyacrylamide gel, and the bands representing hydroperoxidase activities were compared with those of a stationary-phase *E. coli* extract. In contrast to *E. coli*, which has both KatG (HPI; a multimer composed of two 80.0-kDa subunits) and KatE (HPII; a multimer composed of four 84.2-kDa subunits), only the band corresponding to HPI was visualized in both exponential- and stationary-phase *V. vulnificus* extracts (Fig. 3A). The calculated molecular mass of the deduced KatG polypeptide of *V. vulnificus* (VV12755 in GenBank accession no. NC_004459) is estimated to be 80.4 kDa. The absence of HPII activity seemed to be common in this bacterial species, since various *V. vulnificus* strains exhibited only HPI activity, at least under the conditions which we tested (Fig. 3B).

When the *katG* gene of *V. vulnificus* was disrupted, no hydroperoxidase band appeared (data not shown), and the missing HPI band was restored when the *V. vulnificus katG* gene was supplied in a multicopy plasmid (J. H. Rhee, personal communication). The *katE* gene is present on chromosomal DNA of *V. vulnificus* (VV21473 in GenBank accession no. NC_004460), and the molecular mass of the deduced polypeptide is predicted to be 55.4 kDa. However, no apparent KatE (HPII) activity was observed under any of the conditions tested, even when we used large amounts of cellular extract (up to 200 μg of crude protein extract [data not shown]) and cellular extracts were exposed to H_2O_2 (Fig. 3A). In addition, no transcript for *katE* was detected in the total RNA (30 μg) by Northern analysis with a 1,523-bp *katE* gene probe (data not shown). Exposure of the exponential-phase cells to H_2O_2 resulted in the induced level of HPI found in the stationary-phase cells, which suggests that a redox-operated regulator, possibly OxyR, is involved in activating *katG* (46; I. Kong, A. Huelsmann, and J. D. Oliver, 102nd Gen. Meet. Am. Soc. Microbiol., poster no. K-94, 2002).

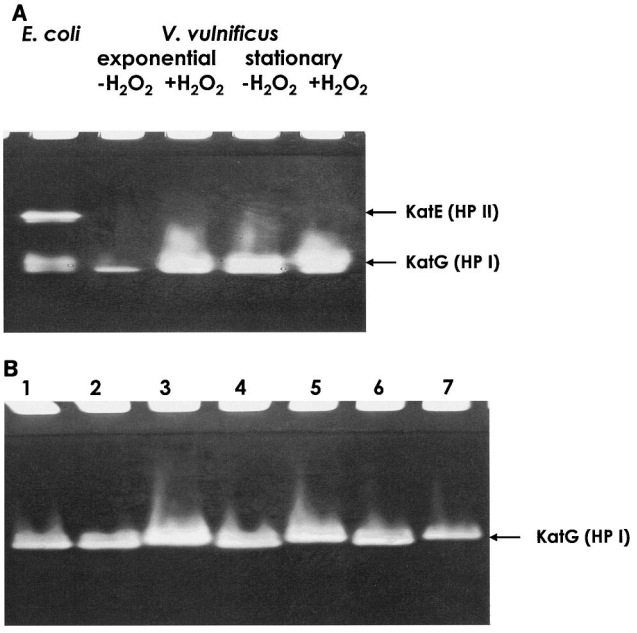

FIG. 3. Nondenaturing gels showing activity staining of catalases in bacterial crude extracts. (A) Crude extracts (120 μg) prepared from *V. vulnificus* ATCC 29307 in the exponential or stationary phase which had been treated with either 0 or 10 μM H_2O_2 for 10 min were loaded into an 8% polyacrylamide gel, and the HPI and HPII activities were examined. The typical HPI (dimer consisting of 80.0-kDa subunits) and HPII (tetramer consisting of 84.2-kDa subunits) bands found in *E. coli* AMS6 (60 μg) were visualized by staining the gel with 1% $K_3Fe(CN)_6$ and 1% $FeCl_3$, and the results were compared to the results obtained for *V. vulnificus*. (B) Crude extracts of various *V. vulnificus* strains (60 μg) were loaded into an 8% polyacrylamide gel. The strains used were ATCC 29307 (lane 1), MO6-24/0 (lane 2), clinical isolate CN7 (lane 3), clinical isolate CNUH94-4 (lane 4), environmental (seawater) isolate SC9649 (lane 5), environmental (sediment) isolate SC9720 (lane 6), and environmental (seafood) isolate SC97126 (lane 7).

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TABLE 2. Catalase activities of the wild type and Δ*rpoS* mutant KPR101 in various growth phases

OD_{600}	Catalase sp act[a]		Ratio (wild type/KPR101)
	Wild type	KPR101	
0.1	2.25 ± 0.19	1.33 ± 0.12	1.7
0.3	0.86 ± 0.10	0.51 ± 0.10	1.7
0.6	0.71 ± 0.07	0.41 ± 0.05	1.7
1.3	0.60 ± 0.14	0.32 ± 0.05	1.9
2.2	0.98 ± 0.06	0.68 ± 0.19	1.4
3.2	2.85 ± 0.07	2.43 ± 0.05	1.2
3.9	7.60 ± 0.07	3.56 ± 0.70	2.1
4.2	6.39 ± 0.55	2.60 ± 0.70	2.4

[a] Wild-type and KPR101 cells grown in LBS broth were harvested at different cell densities, and the crude extracts were processed in order to estimate the rates of H_2O_2 degradation by a spectrophotometric assay, as described in Materials and Methods. The concentrations of H_2O_2 were determined by using an extinction coefficient of 43.6 mM (3). The catalase specific activities were obtained from two independent experiments and were calculated by using the following equation (42): (1,000 × average ΔOD_{240}/min)/[(43.6 × milligrams of enzyme)/milliliters of reaction mixture].

Determination of HPI activity in the wild-type strain and KPR101. To identify the possible role of σ^S in regulation of the activity of KatG, HPI activities were monitored along the growth curve (Table 2). The HPI catalase specific activity of the wild-type strain was about 2 U during the early exponential phase (for the first 2 h of growth) and gradually decreased during the mid-exponential phase. Then it reached a maximal level, approximately 6 to 8 U, after the cells entered the late stationary phase. The activity of HPI in *V. vulnificus* cells during the whole growth period, except for an initiation period during the stationary phase (OD_{600}, 2.2 and 3.2 [Table 2]), was regulated by the presence of σ^S, since the *rpoS* mutant contained about twofold less HPI than the wild-type strain contained. Thus, the induction of HPI at the onset of the stationary phase may depend on other regulators.

Similarly, the HPI catalase specific activity in KPR101 was 2 to 3 U at the onset of the stationary phase, but it did not reach the maximal level observed in the wild type (Table 2). However, the twofold reduction in HPI activity in late-stationary-phase KPR101 compared to the activity in the wild type did not result in a difference in survival in the presence of H_2O_2 (Fig. 2B), because this amount of HPI activity (i.e., more than 2 to 3 U) might provide as much resistance to the concentrations of H_2O_2 used (up to 10 mM) as the amount in the wild type provides.

The medium used to grow KPR101 contained less HPI activity than the medium used to grow the wild type contained. No activity was detected in a cell-free medium (glucose-based ASW) which was used to grow KPR101 at the exponential phase, whereas significant HPI activity (0.38 ± 0.05 U) was

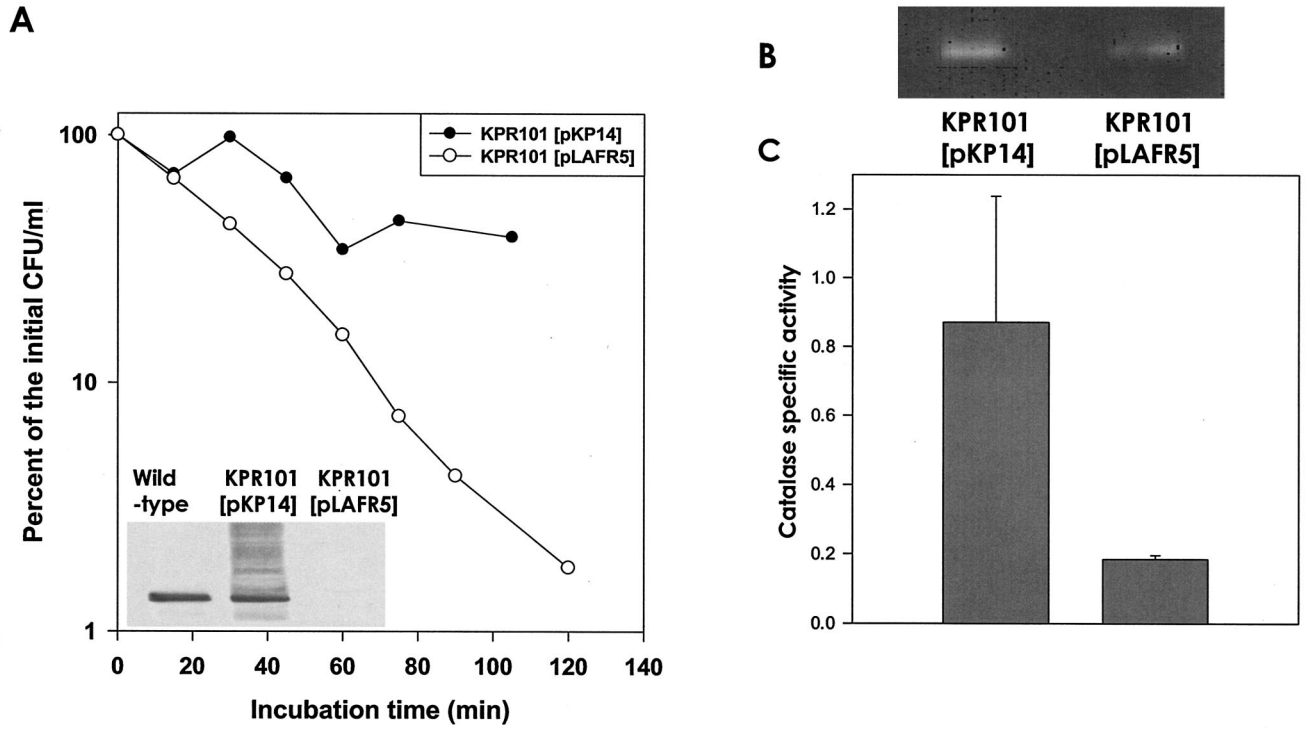

FIG. 4. Complementation of Δ*rpoS* mutant KPR101 with intact *V. vulnificus rpoS*. (A) Complementation of KPR101 with the *rpoS* gene (in the broad-host-range pLAFR5 vector containing the *V. vulnificus rpoS* gene or pKP14) resulted in synthesis of σ^S, as determined by Western blotting (inset). Exponential-phase KPR101 containing pKP14 or pLAFR5 was grown in ASW supplemented with tetracycline and exposed to 880 μM H_2O_2. At several times during exposure, aliquots of the culture were removed, and the numbers of CFU per milliliter were estimated as described in Materials and Methods. The resultant values were expressed as percentages of the initial cell density, which was approximately 10^6 CFU/ml. (B and C) The HPI activity in a crude extract from an exponential-phase KPR101 culture containing pKP14 was compared to the HPI activity of KPR101 containing pLAFR5 by either the catalase staining method with a nondenaturing gel (B) or the H_2O_2 degradation assay with a spectrophotometer (C).

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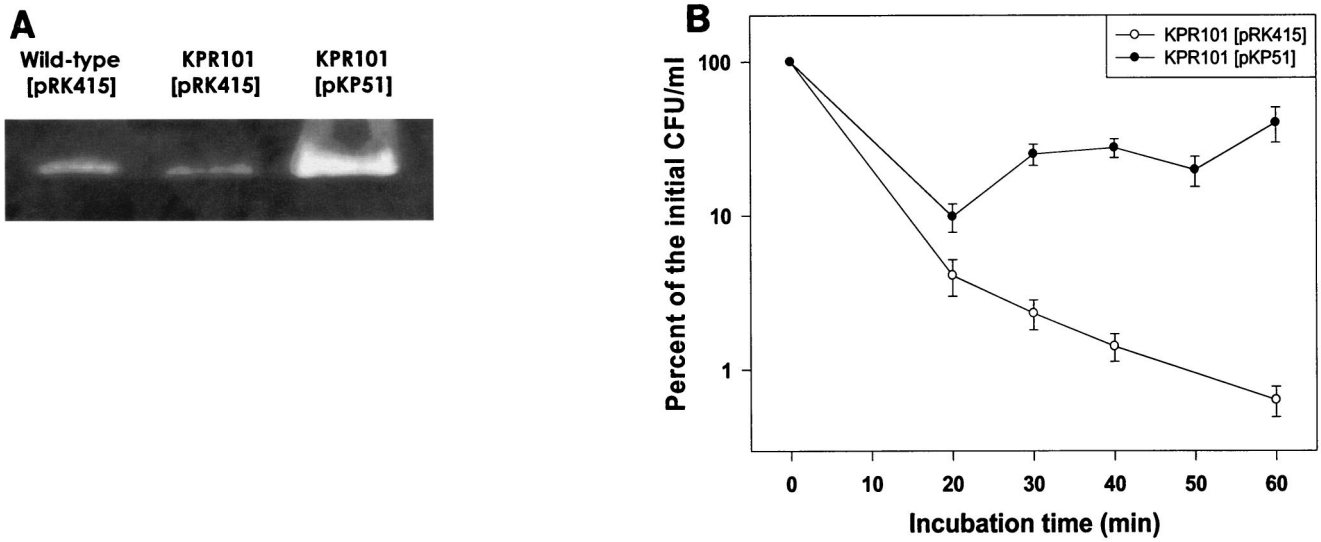

FIG. 5. Complementation of the Δ*rpoS* mutant KPR101 with *V. vulnificus katG*. (A) Complementation of KPR101 with the *katG* gene (in the broad-host-range vector pRK415 containing the *V. vulnificus katG* gene or pKP51) resulted in increased activity of HPI, as determined by the catalase staining method with a nondenaturing gel. The HPI activity in the crude extract of an exponential-phase KPR101 culture containing pKP51 was compared to the HPI activities of KPR101 and the wild type containing pRK415. (B) An exponential-phase KPR101 culture containing pKP51 or pRK415 in LBS medium was grown in LBS broth supplemented with tetracycline and exposed to 880 μM H_2O_2. At several times during exposure, aliquots of the culture were removed, and the numbers of CFU per milliliter were estimated as described in Materials and Methods. The resultant values were expressed as percentages of the initial cell density, which was approximately 10^7 CFU/ml.

found in the spent medium used for the wild type at the exponential phase. In the medium used to culture KPR101 at the stationary phase, the extracellular HPI activity (0.97 ± 0.06 U) was about 70% of the activity of the wild type (1.32 ± 0.08 U). This suggests that the smaller amount of KatG activity in KPR101 than in the wild type was not due to increased excretion of this enzyme.

Complementation of KPR101 with a broad-host-range vector containing the *V. vulnificus rpoS* gene, pKP14. After pKP14, a pLAFR5-based plasmid containing the *V. vulnificus rpoS* gene, was added to KPR101, the expression of *V. vulnificus* σ^S in this strain was confirmed by Western blotting by using *V. vulnificus* σ^S-specific polyclonal antibody, which clearly showed the presence of an ~39-kDa *V. vulnificus* σ^S (Fig. 4A, inset). The exponential-phase KPR101 culture, containing pKP14 grown in glucose-based ASW supplemented with tetracycline, was exposed to 880 μM H_2O_2, and changes in the number of CFU were monitored for 2 h. This strain was found to be resistant to H_2O_2, whereas KPR101 containing pLAFR5 was found to be sensitive to the same concentration of H_2O_2 (Fig. 4A).

When the *V. vulnificus rpoS* gene was present in *trans*, apparently increased HPI activity was observed by the gel staining assay (Fig. 4B), and the increase was estimated to be about fourfold as determined by an H_2O_2 degradation kinetic study (Fig. 4C). These results show that in the exponential phase the *rpoS* mutant complemented with the *V. vulnificus rpoS* gene exhibited both resistance to H_2O_2 and HPI activity.

Complementation of KPR101 with a broad-host-range vector containing the *V. vulnificus katG* gene, pKP51. If the effect of an *rpoS* mutation on increased sensitivity to H_2O_2 was mainly due to a lower level of HPI, KPR101 should become resistant to H_2O_2 if multiple copies of the *katG* gene are provided. Thus, KPR101 was complemented with pKP51, a pRK415-based plasmid containing the *V. vulnificus katG* gene. In the exponential phase KPR101 containing pKP51 grown in LBS medium supplemented with tetracycline exhibited much greater HPI activity (Fig. 5A). This strain also exhibited greater resistance to 880 μM H_2O_2 than KPR101 containing pRK415 exhibited (Fig. 5B). However, it is possible that the other gene products regulated by σ^S play an important role in survival in the presence of higher concentrations of H_2O_2 than we tested. Currently, the proteins which are induced in the wild type in the exponential phase by exposure to H_2O_2 but are not induced in KPR101 in the exponential phase under the same conditions are being investigated by proteome analyses by using two-dimensional matrix-assisted laser desorption ionization—time of flight mass spectrometry.

Determination of *katG* expression in the wild type and KPR101. Since the presence of σ^S apparently increases HPI activity, its role in controlling HPI was analyzed further by monitoring expression of the *katG* gene in the wild type and KPR101. To do this, a *luxAB*-based transcriptional fusion, pHL-03 containing the promoter region of the *katG* gene, was constructed and transferred to both strains. In the wild-type cells, induction of the *katG* fusion was apparently initiated during the early exponential phase (during the first 1 to 2 h of incubation), and the level decreased to the basal level during the mid-exponential phase. A high level of expression during the initial incubation period was also observed in *E. coli* (8), and the HPI activity was also high at this stage of growth (Table 2). Then induction occurred again at the onset of the stationary phase, and the level of expression reached the maximal level (about 10-fold greater than the basal level) when the cells entered the late stationary phase (Fig. 6). This expression

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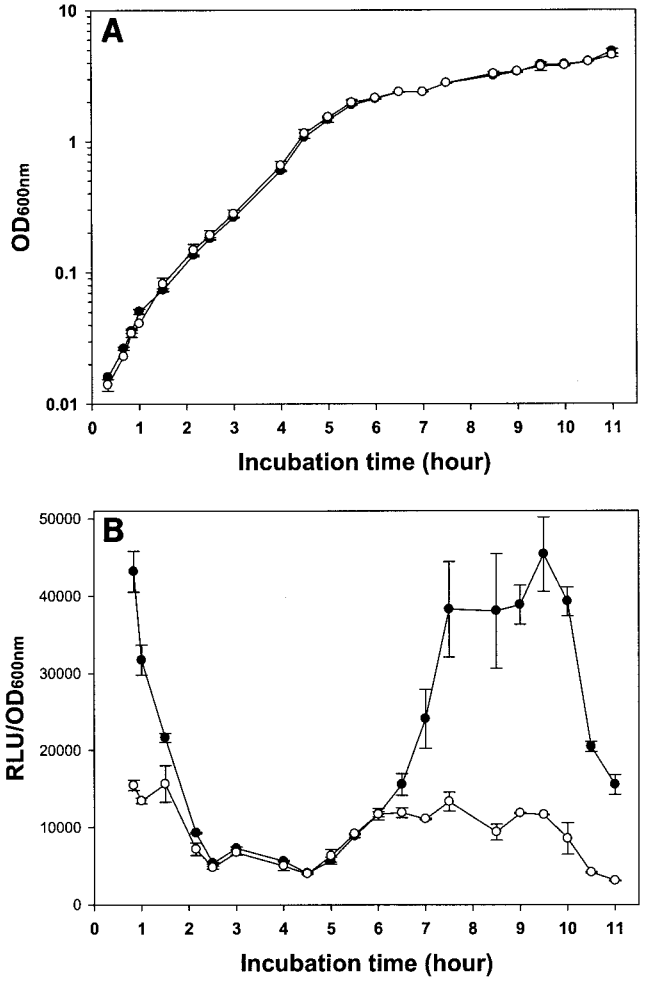

FIG. 6. Expression of the *katG* gene determined by using a *katG*::*luxAB* transcriptional fusion (pHL-03). Wild-type (●) and KPR101 (○) cells containing the *katG*::*luxAB* fusion were freshly grown in LBS broth supplemented with 3 μg tetracycline per ml by inoculating overnight cultures, whose levels of bioluminescence were the basal levels. Aliquots were removed, and the cell masses (OD_{600}) (A) and bioluminescence (relative light units [RLU]) (B) were estimated. The luciferase activities were expressed as normalized values obtained by dividing the number of RLU by the OD_{600} of each sample. The activities of three independent experiments were averaged, and the error bars indicate the standard deviations.

profile essentially parallels the pattern of catalase activity measured directly (Table 2).

The *katG* expression in KPR101 followed the same pattern as the *katG* expression in the wild type, but the degrees of induction in both the early exponential and late stationary phases were significantly reduced (Fig. 6). This pattern of *V. vulnificus katG* expression is quite different from the *katG* expression in *E. coli*. In *E. coli*, the maximal *katG* expression was only slightly reduced in an *rpoS* mutant (8), the maximal induction of *katG* occurred during the late exponential phase (22), and the duration of induced *katG* expression was not prolonged during the late stationary phase (8). Interestingly, an HPII-deficient *E. coli* mutant has been reported to exhibit higher HPI activity than the parental strain exhibits (42).

While no difference in *katG* expression was observed during the mid-exponential phase (Fig. 6), the estimated HPI activities in KPR101 were about 50% of those in the wild type at the same growth stage (Table 2). This difference in activity might result from different expression during the early exponential phase. Otherwise, σ^S might not directly regulate KatG at the transcriptional level, at least during the mid-exponential phase. Instead, some factors regulated by σ^S are involved in increased (or sustained) activity of HPI. It is necessary to identify these factors in order to elucidate the regulatory pathways for catalase via σ^S in exponential-phase *V. vulnificus*. The experiments in which we examined the effect of H_2O_2 on synthesis of σ^S revealed a slightly increased amount of σ^S in the exponential-phase cells exposed to nonlethal concentrations of H_2O_2 (data not shown). However, additional studies are necessary to clarify whether the slightly increased amount of σ^S upregulates the synthesis and/or activity of KatG during the exponential phase.

Several research groups have emphasized the importance of σ^S in bacterial survival in the stationary phase and in resistance to H_2O_2 via regulation of HPII. Although it has been reported that in the stationary phase HPI is partially induced by σ^S in *E. coli* (8, 24) and in the exponential phase some virulence genes are regulated by σ^S in *Salmonella dublin* (5), exponential-phase induction of HPI by σ^S has not been examined previously. In the present study, however, we found that in *V. vulnificus* this global regulator plays a role in the response to oxidative stress during the exponential phase by increasing the amount of HPI with no involvement of HPII. Investigation of the other roles of σ^S in hierarchical regulatory cascades and the expression of *rpoS* in the presence of specific stresses is in progress.

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