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Cloning, sequencing, and functional studies of the *rpoS* gene from *Vibrio harveyi*☆

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Abstract

The *Vibrio harveyi rpoS* gene which encodes an alternative sigma factor (σ^s or σ^{38}), has been cloned and characterized. The predicted protein sequence is closely related to RpoS proteins in other bacteria with up to 86% sequence identity. A *rpoS* null mutant of *V. harveyi* was constructed and the phenotype studied. Comparison of the properties of the *V. harveyi* wild type and *rpoS* deletion mutant showed that *rpoS* affected the ability of the cells to survive only under specific types of environmental stresses. The *rpoS* null mutant had a lower survival rate compared to the wild type parental strain at high concentrations of ethanol and in the stationary phase. In contrast to other bacteria, deletion of *rpoS* in *V. harveyi* did not affect the resistance of the cells to high osmolarity or hydrogen peroxide, suggesting the existence of alternative systems in *V. harveyi* responsible for resistance to these stresses. RpoS appears not to be involved in the control of luminescence in *V. harveyi* even though it is implicated in regulation of other acyl-homoserine dependent quorum sensing systems. © 2002 Elsevier Science (USA). All rights reserved.

Keywords: Bacterial bioluminescence; *Vibrio harveyi*; Gene disruption; RpoS; Sigma 38; Stationary phase; Quorum sensing

The *rpoS* gene, which encodes the alternative sigma factor, RpoS, also known as σ^{38} or σ^s, is the central regulator of the stationary phase in *Escherichia coli* [1,2]. On entering stationary phase, the limitation in nutrients causes bacteria to differentiate and to develop stress resistance before the nutrients are exhausted. The developmental changes requiring the induction of specific genes at the onset of starvation are partially regulated by RpoS. It has been reported that RpoS in *E. coli* is involved in the survival under conditions of famine, oxidative stress, osmotic shock, and low pH [3].

Bioluminescence in marine bacteria is regulated by the process of quorum sensing in which the bacteria monitor the amount of specific small signal molecules (autoinducers) secreted by the cells into the media and then respond to the signals by the expression of genes involved in light emission [4–6]. Quorum sensing is used in both *Vibrio fischeri* and *Vibrio harveyi* as a mechanism to control light emission during cell growth. In nonluminescent bacteria, quorum sensing has been used to control a variety of physiological functions, including virulence.

N-acyl-homoserine lactones, including *N*-(3-hydroxyl-butanoyl) homoserine lactone in *V. harveyi* and *N*-oxohexanoyl homoserine lactone in *V. fischeri* [7,8] have been identified as the autoinducers in many gram-negative bacteria. In some nonluminescent bacteria, expression of *rpoS* has been related to the level of homoserine lactone derivatives. In *Pseudomonas aeruginosa*, evidence indicates that the promoter of *rpoS* is directly controlled by the regulator RhlR and its cognate autoinducer, *N*-butanoyl-L-homoserine lactone, which are part of the quorum sensing system controlling virulence in this bacterium [9]. However, other data for the same species indicate that the reverse is the case with RpoS controlling the RhlR–RhlI system involved in quorum sensing. This result is similar to that observed

☆ *Abbreviations*: LB, Luria-bertani (medium); PCR, polymerase chain reaction.

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in *Ralstonia solanacearum*. Acyl-homoserine lactone levels, which affect virulence in *R. solanacearum*, were decreased in a *rpoS* deletion mutant due to a decrease in transcription of the *sol*R and *sol*I genes, responsible for quorum sensing [10]. In contrast, RpoS in *Pseudomonas putida* strain WCS358 does not appear to be involved in the production of any of the three acyl-homoserine lactone inducers [11].

As a two-component system involving phosphoryl group transfer is implicated as the signal-sensing mechanism controlling luminescence in *V. harveyi* quorum sensing [4] and differs from that found in *V. fischeri* and other gram-negative bacteria [5], the relationship of RpoS to light and *N*-(3-hydroxyl-butanoyl) homoserine lactone levels as well as response to stress were investigated in *V. harveyi*. In this report, we cloned the *rpoS* gene from *V. harveyi* and studied the role played by RpoS in these functions.

Materials and methods

Bacterial strains, plasmids, and media. Both *V. harveyi* B392 and *E. coli* MM294 were from laboratory collections, and were grown at 27 and 37 °C, respectively, in LB media. Plasmid, pJQ200, was purchased from ATCC. For the purpose of bioluminescence analysis, cells were grown in 1% complex medium (10 g of NaCl, 3.7 g of Na_2HPO_4, 1.0 g of KH_2PO_4, 0.5 g of $(NH_4)_2$ HPO_4, 0.2 g of $MgSO_4 \cdot 7H_2$ O, 2 ml glycerol, 5 g of bactotryptone, and 0.5 g of yeast extract per liter). Where indicated, the concentrations of the antibiotics in the medium were: ampicillin (100 μg/ml), kanamycin (30 μg/ml), and gentamicin (25 μg/ml).

Cloning of the rpoS gene from V. harveyi. Forward and reverse primers (+ strand 5′ CGGGATCCCGGCGCGTCGCGCACTGCGT GG; CGGGATCCCGCGTGCCAGCACTTCACGCTG) were designed according to the sequence of the most conserved region of the *E. coli rpoS* gene. PCR was performed at 95 °C for 5 min, 30 cycles at 95 °C for 1 min, 1 min at 54 °C, and 40 s at 72 °C, and the reaction was completed by incubation at 72 °C for 10 min. The PCR product (149 pb) was purified by electrophoresis on a low melting agarose gel and sequenced at the Core DNA Sequencing Facility Center, Kingston, Ontario. Inverse PCR was performed by cutting 0.5*g* of *V. harveyi* genomic DNA with various four-base-cut restriction enzymes and the cut DNA ligated at different dilutions to preferentially cause lelf-ligation into circular DNA. The circular DNA was then PCR-amplified with divergent primers to obtain the flanking sequences of the initial 149 pb PCR product. Based on the flanking sequence, a 1.79 kbp DNA fragment containing the *rpoS* gene was generated by PCR.

Insertional inactivation of rpoS. The procedure of gene replacement was conducted as described previously [12]. The 1.79 kbp PCR fragment containing the *rpoS* gene was transferred into the *Hin*dIII and *Eco*RI restriction sites of pT7-5 (pTRPOS). The kanamycin (*kn*) gene (1.7 kbp) was excised with *Bam*HI and *Acc*I from pKT230 followed by blunt ending. The DNA fragment was then ligated into an *Eco*RV site in pTRPOS. The *rpoS* gene interrupted with the *kn* gene was excised with *Pvu*II *and Fsp*I and inserted into the *Sma*I sites in pJQ200 to give pJQRPOS::kn. This plasmid was subsequently transferred into *V. harveyi* by conjugation, and the colonies containing pJQRPOS::kn were selected on plates containing ampicillin, kanamycin, and gentamicin. The selected colonies were spread on plates containing ampicillin, kanamycin, and 5% sucrose to kill the bacteria harboring the plasmids, and select the cells containing the kn-interrupted rpoS gene inserted in the genome (knockout strain, BB392RPOS-). The knockout strains were then verified by Southern blots.

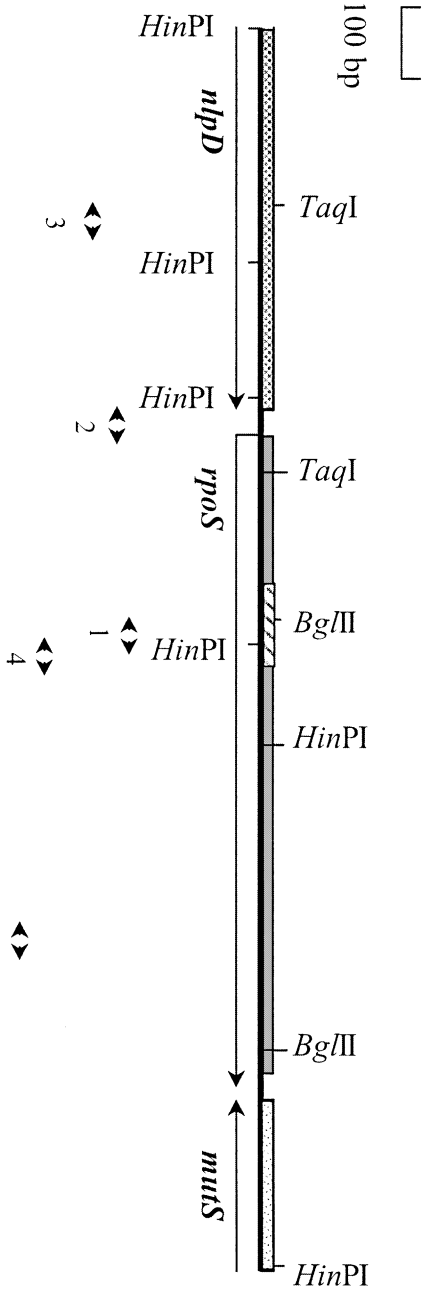

Fig. 1. Cloning of the *V. harveyi rpoS* gene and its flanking regions. A 150-bp fragment cloned by PCR using primers based on the sequence of the *E. coli rpoS* gene is given by the slashed area in the gray box representing the *rpoS* gene. The upstream sequence was cloned using inverse PCR with self-ligated *Bgl*II-restricted genomic DNA and two divergent primers (pair 1) to obtain the sequence to the upstream *Bgl*II site. Pair 2 and pair 3 primers were then used sequentially with self-ligated *Taq*I and *Hin*PI restricted DNA, respectively, to further extend the sequence to the upstream *Hin*PI site. Similarly, primer sets 4 and 5 were used sequentially with *Bgl*II and *Hin*PI restricted and then self-ligated DNA to clone the downstream sequence. The partial open reading frames predicted to code for NlpD and MutS proteins are also indicated.

Survival assays. Hydrogen peroxide (88 mM) and ethanol (18% vol/vol) was added to overnight *V. harveyi* wild type or RpoS-cultures (about 4 × 109 cells/ml) in LB media at 25 °C. At the selected time points, samples were taken and diluted before plating on LB agar for determination of viable cell numbers. For salt challenge experiments, the overnight cultures were centrifuged and suspended in LB medium containing 2.5 M NaCl. For stationary phase survival studies, the overnight cultures in LB medium were left at 25 °C and the viable cells were counted each day.

Bioluminescence. Wild type and mutant cells were grown overnight and diluted to OD660 = 0.001 in 1% complex medium. Luminescence of the growing cells was measured at the indicated cell densities with a photomultiplier tube where one light unit corresponds to 1.3 × 1010 quanta s-1 based on the light standard of Hastings and Weber [13].

Chloramphenical transferases (cat) assays. A 530-bp fragment of PCR fragment containing the rpoS promoter region was subcloned into pMGM100 [14] upstream of the cat gene. The resulting plasmid pMGMRP was then transconjugated into *V. harveyi* wild type or into autoinducer negative cells (D1). Cells were grown from a starting OD of 0.01, and harvested at two different time points, OD = 1.0 and 3.0. The protein pellets were subjected to cat assays according to established procedures [15].

Sequence data analysis. Protein database searches were performed using the Blast network service of the National Center of Biotechnology Information. Alignments of proteins were made using the CLUSTAL W (version 1.8) program.

Results

Cloning and characterization of V. harveyi rpoS

Two primers designed according to the *E. coli rpoS* gene sequence were used to amplify a 150-bp sequence from *V. harveyi* genomic DNA by PCR. Inverse PCR was sequentially conducted to obtain the complete gene as well as the flanking sequences using pairs of divergent primers as outlined in Fig. 1. The slashed area shows the position of the 150 bp PCR product. An open reading frame designated as rpoS was found to extend for 981 bp.

The nucleotide sequence of the *V. harveyi rpoS* gene (accession number: AF321124) codes for a protein of 327 amino acids. The blast sequence similarity search program showed very high homology between RpoS of *V. harveyi* and RpoS of other organisms (Fig. 2) with the highest identity (86%) to RpoS from *Vibrio cholerae*.

The *rpoS* genes are reported to be flanked by the *nlpD* and *mutS* genes, which code for a novel lipoprotein (NlpD) and a DNA mismatch repair protein (MutS), respectively, in *E. coli, Yersinia enterocolitica*, and

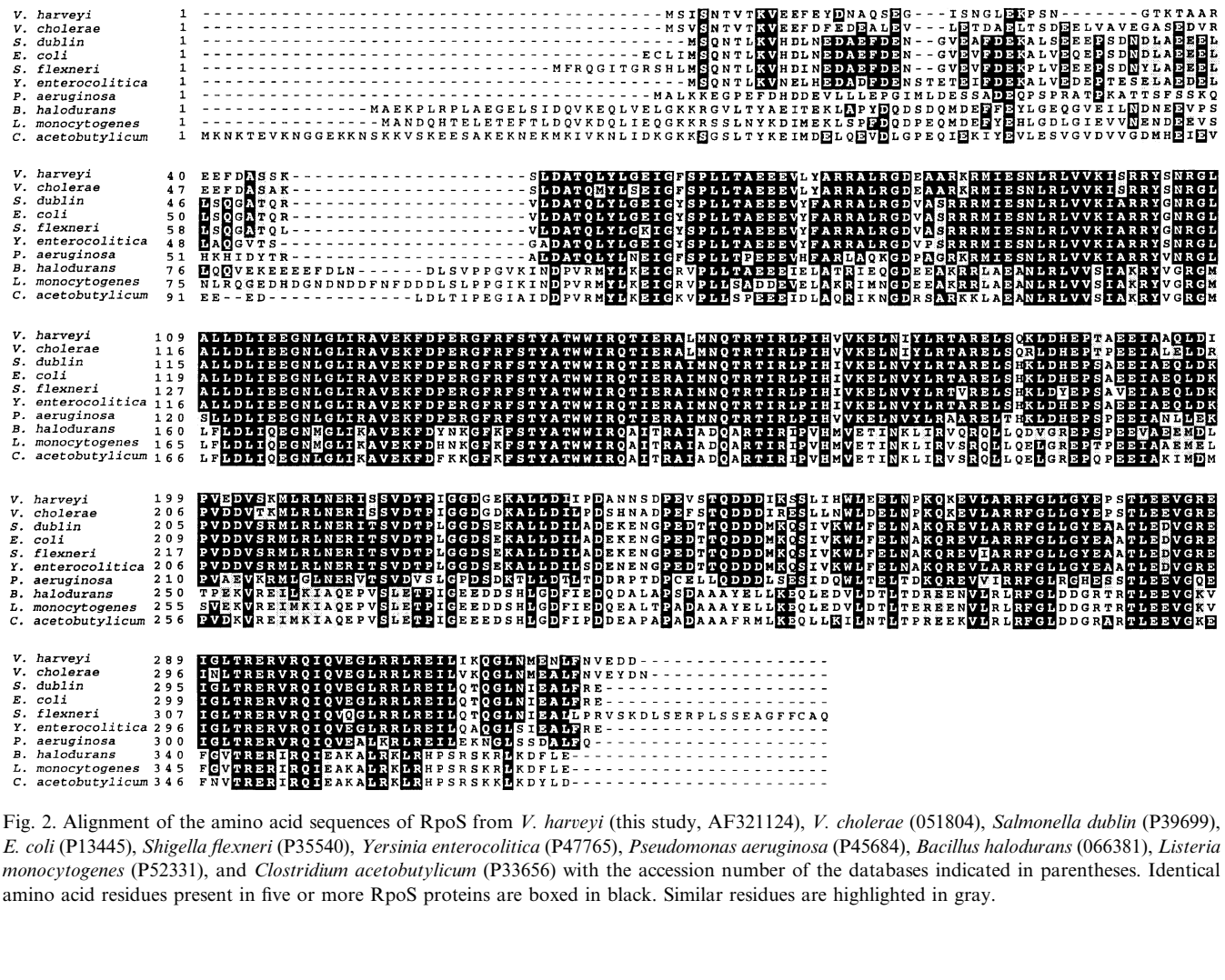

Fig. 2. Alignment of the amino acid sequences of RpoS from *V. harveyi* (this study, AF321124), *V. cholerae* (051804), *Salmonella dublin* (P39699), *E. coli* (P13445), *Shigella flexneri* (P35540), *Yersinia enterocolitica* (P47765), *Pseudomonas aeruginosa* (P45684), *Bacillus halodurans* (066381), *Listeria monocytogenes* (P52331), and *Clostridium acetobutylicum* (P33656) with the accession number of the databases indicated in parentheses. Identical amino acid residues present in five or more RpoS proteins are boxed in black. Similar residues are highlighted in gray.

V. cholerae [16–18]. The same arrangement is present in the *rpoS* region of *V. harveyi* (Fig. 1). An incomplete open reading frame transcribed in the same direction was found immediately upstream of the *rpoS* gene which encoded the carboxyl terminal segment of a protein with high sequence identity to the NlpD protein. The stop codon of this predicted NlpD protein is located just 80 bp upstream of the translational start site of the *rpoS* gene. Downstream of the *rpoS* gene, a convergent open reading frame codes for the carboxyl terminal region of MutS with the stop codons of the two genes separated by 90 bp.

Gene disruption of rpoS in V. harveyi

To identify the *rpoS*-associated phenotype, a *rpoS* null allele in the chromosome of *V. harveyi* (ampicillin resistant) was constructed (Fig. 3A). A kanamycin cassette was inserted at the *Eco*RV site of the PCR-generated, 1.8 kbp *rpoS*-containing fragment which had been subcloned in the pT7 plasmid. The DNA fragment containing the *rpoS::kn* from the resulting plasmid (PTRPOS::*kn*) was then subcloned to pJQ200 (gentamycin resistant), a mobilizable suicide vector, expressing sacB, whose expression in the presence of sucrose is lethal [19] The resulting pJRPOS*::kn* plasmid was then introduced into *V. harveyi* by conjugation.

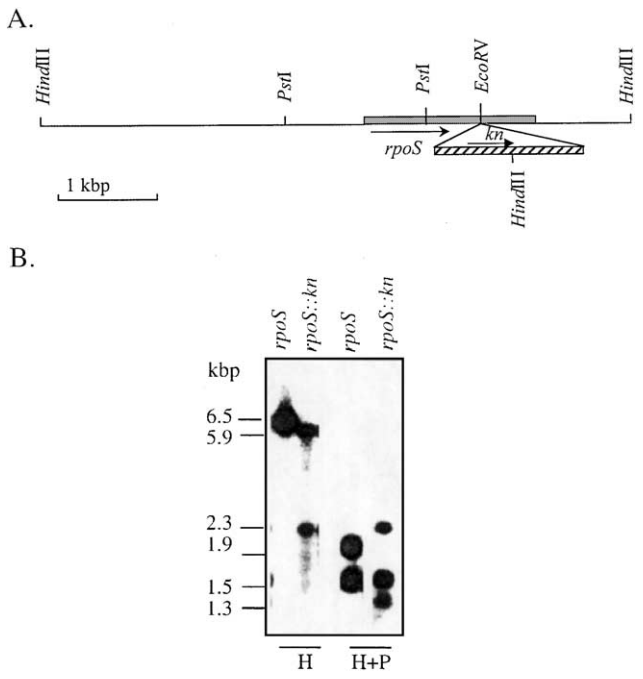

Fig. 3. Generation and confirmation of the *rpoS* insertion mutation. (A) A physical map of the *rpoS* (solid bar) region of the chromosome showing the *Eco*RV site where the kanamycin gene (*kn*) was inserted. The *Hin*dIII and *Pst*I sites for Southern analysis are also indicated. (B) Southern analysis of *V. harveyi* wild type and *rpoS* mutant genomic DNA (*rpoS* and *rpos::kn*, respectively) digested with *Hin*dIII (H), and the combination of *Hin*dIII and *Pst*I (H + P).

The transconjugates harboring the pJRPO S::kn conferring ampicillin, gentamycin, and kanamycin resistance were selected. These transconjugates were then spread on LB plates containing ampicillin, kanamycin, and 5% sucrose to select double recombinants which were verified by their gentamycin sensitivity as well as by Southern analysis to confirm that *rpoS* was interrupted by the kanamycin cassette (Fig. 3B).

Response of the rpoS-deletion mutant to environmental stress

The *rpoS* null mutants produced in other bacteria have been shown to be more susceptible to environmental stress than the corresponding parental strains [3,11,18,20–24]. We therefore examined the effect of the *rpoS* disruption in *V. harveyi* on survival by determining the viable plate counts after exposure to various stress conditions. As shown in Fig. 4, like the *rpoS* null mutants of most bacteria studied, the *rpoS* null mutant of *V. harveyi* is much less resistant to an environment of high ethanol concentration (Fig. 4A) or being in the stationary phase (Fig. 4B). When challenged with hyperosmolarity (2.5 M NaCl), the viable plate counts of both the wild type and the mutant cells declined rapidly, but at the same rate (Fig. 4C). This result is surprising as the *rpo*S null mutants of other bacteria studied were much less resistant to salt than the wild type cells. For example, a 10–100-fold greater loss of the *rpoS* mutant cells was observed for *E. coli* and *Salmonella* spp. compared to the wild type cells at high salt concentrations [3,25].

Survival under conditions of high oxidative stress was compared for the *rpoS* null mutant and wild type cells by adding H_2O_2 to the medium (Fig. 4D). The wild type cells showed complete resistance to 88 mM H_2O_2 over the five hours period examined. This is not surprising for the wild type *V. harveyi* cells since the marine habitat in shallow water requires that it be resistant to H_2O_2 generated by the effects of UV radiation on water. However, the same resistance to H_2O_2 was observed in the *rpoS* null mutant, an unexpected result, since in most other bacteria studied, more than 99% of *rpoS* null mutants was killed after few minutes of exposure to a lower level of H_2O_2 [3,18,20]. The results indicate that there are additional and probably unique systems in *V. harveyi* involved in regulating oxidative stress resistance which could come to rescue the *rpoS* null mutant exposed to these particular conditions. Indeed, although rpoS null mutants of a few bacteria (*R. solanacearum* [10], *Y. enterocolitica* [21], *P. putida* [11]) can retain reasonable resistance to oxidative stress compared to the resistance of the wild type cells, few bacteria if any have *rpoS* null mutants that have the same resistance to salt as the corresponding wild type cells.

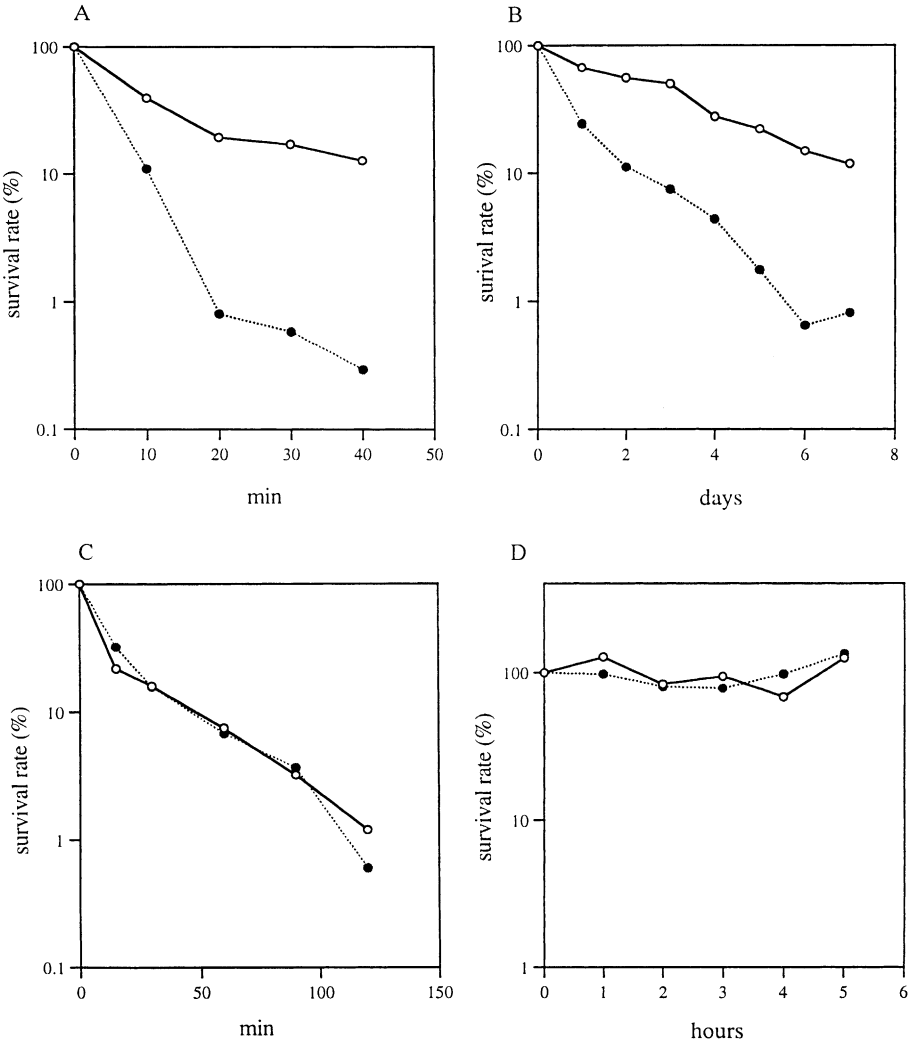

Fig. 4. Comparison of the stress response of *V. harveyi* wild type and the *rpoS* null mutant to different conditions of stress. *V. harveyi* wild type (open circles) and *rpoS* null mutant (closed circles) were exposed to various stress conditions and the survival rate determined as percentage of viable cell counts at different times. One hundred percent survival corresponds to the viable cell count determined prior to exposure to the indicated stress. Survival on exposure to (A) 18% ethanol, (B) stationary phase, (C) 2.5 M NaCl, and (D) 88 mM H_2O_2.

Luminescence and autoinduction are not affected by rpoS

Density-dependent luminescence was investigated for both the *V. harveyi* wild type and the *rpoS* mutant cells. Since the *rpoS* mutant cells showed the same luminescence–induction pattern as the wild type cells (Fig. 5), it appears that RpoS is not involved in regulating the production of the *N*-(3-hydroxyl-butanoyl) homoserine lactone autoinducers contrasting with results shown for *R. solanacearum*, which shows reduced acyl-HSL production in the *rpoS* deletion mutant compared to the parent strain [10].

The rpoS gene is not controlled by the autoinducer, N-(3-hydroxybutanoyl)-homoserine lactone

The promoter region of rpoS gene was fused to a cat reporter gene [14]. The resulting plasmid was then transconjugated into *V. harveyi* and D1 mutant cells and the cat activities were measured. The D1 mutant is defective in producing *N*-(3-hydroxybutanoyl)-homoserine lactone (HBHL) [26]. The cat activity in both strains is identical suggesting that rpoS is not under the control of HBHL (Table 1). This conclusion is the same as that reached in *P. aeruginosa* by Whiteley et al. [27] who show that rpoS is not affected by the acyl-homoserine lactone-dependent quorum sensing system.

Discussion

In this work, the *rpoS* gene of *V. harveyi* was cloned and shown to code for a protein with high sequence homology to RpoS in other bacterial species. The presence of the *nlpD* gene immediately upstream and a

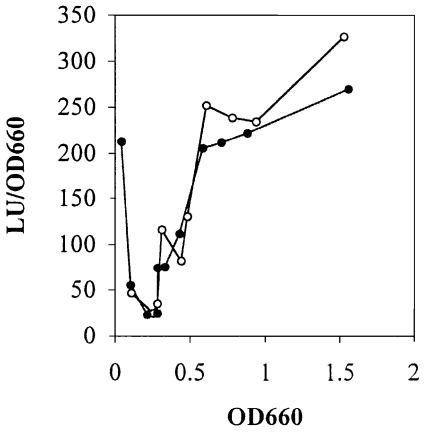

Fig. 5. Effect of deletion of *rpoS* in the *V. harveyi* genome on light induction. Cultures of *V. harveyi* wild type (open circles) and *rpoS:: kn* mutant (solid circles) were grown overnight in 1% complex medium. The overnight cultures were diluted 500-fold in fresh 1% complex medium and light intensity (LU) was measured with time as a function of cell density (OD_{660}).

Table 1
The *rpoS* promoter is not under the control of *N*-(3-hydroxybutanoyl) homoserine lactose (HBHL)

V. harveyi strain[a]	Genotype	*rpoS* promoter–cat activity (U/mg)[b]
Wild type BB392	Wild type	800 ± 23
D1 mutant	HBHL	980 ± 110

[a] Each strain contains pMGMRP (*rpoS* promoter–cat fusion in pMGM100) [25].

[b] Values shown are means $\pm$ SD of three independent experiments. One unit corresponds to one nanomole of chloramphenicol acetylated per minute at 37 °C under optimal assay conditions.

mutS gene immediately downstream confirms the identity of the *rpoS* gene, as this gene arrangement is conserved in several bacteria.

Although the *rpoS* gene was found to be important for survival of *V. harveyi* in response to 18% ethanol as well as in the stationary phase, the inactivation of the *rpoS* gene did not affect the survival of *V. harveyi* on exposure to high concentrations of NaCl or H_2O_2. These observations were surprising as the resistance to high osmolarity and oxidative stress has been a hallmark for recognition of the *rpoS* gene in other bacteria. The results indicate that there are additional and probably unique systems in *V. harveyi* involved in regulating the osmotic and oxidative stress resistance which could come to rescue the *rpoS* null mutant exposed to these particular conditions.

Cell-density-dependent luminescence does not appear to be affected by the deletion of *rpoS* gene suggesting that RpoS is not involved in regulating the quorum-sensing system in *V. harveyi*. The results also show that the *rpoS* gene is not regulated by HBHL as expression of the *rpoS* promoter::*cat* fusion gave the same activity in *V. harveyi* wild type and D1 mutant cells. Based on the data from this study, *RpoS* appears not to be involved in the two-component quorum-sensing system of *V. harveyi* even though it is implicated in regulation of other acyl-homoserine lactone-dependent quorum-sensing system. Moreover, RpoS for *V. harveyi* appears to be involved in response to only certain stress conditions and suggests that alternative systems must be available to protect against hyperosmolarity and oxidative stress in this marine bacteria.

Acknowledgment

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