Microbiology (2008), **154**, 767–780 DOI 10.1099/mic.0.2007/014167-0

RpoS induces expression of the *Vibrio anguillarum* quorum-sensing regulator VanT

Barbara Weber, Antony Croxatto,† Chang Chen‡ and Debra L. Milton

Department of Molecular Biology, Umeå University, S-901 87 Umeå, Sweden

Correspondence
Debra L. Milton
Debra.Milton@molbiol.umu.se

Received 17 October 2007
Revised 14 December 2007
Accepted 20 December 2007

In vibrios, regulation of the *Vibrio harveyi*-like LuxR transcriptional activators occurs post-transcriptionally via small regulatory RNAs (sRNAs) that destabilize the *luxR* mRNA at a low cell population, eliminating expression of LuxR. Expression of the sRNAs is modulated by the vibrio quorum-sensing phosphorelay systems. However, *vanT* mRNA, which encodes a LuxR homologue in *Vibrio anguillarum*, is abundant at low and high cell density, indicating that VanT expression may be regulated via additional mechanisms. In this study, Western analyses showed that VanT was expressed throughout growth with a peak of expression during late exponential growth. VanO induced partial destabilization of *vanT* mRNA via activation of at least one Qrr sRNA. Interestingly, the sigma factor RpoS significantly stabilized *vanT* mRNA and induced VanT expression during late exponential growth. This induction was in part due to RpoS repressing expression of Hfq, an RNA chaperone. RpoS is not part of the quorum-sensing regulatory cascade since RpoS did not regulate expression or activity of VanO, and RpoS was not regulated by VanO or VanT. VanT and RpoS were needed for survival following UV irradiation and for pigment and metalloprotease production, suggesting that RpoS works with the quorum-sensing systems to modulate expression of VanT, which regulates survival and stress responses.

INTRODUCTION

Bacteria coordinate activities as a population through a type of cell–cell communication called quorum sensing (Waters & Bassler, 2005). Small diffusible signal molecules, such as *N*-acylhomoserine lactones, are secreted by the bacteria and accumulate as the population grows. When a signal molecule reaches a threshold concentration at a critical cell density, quorum-sensing systems are activated, inducing or repressing phenotypic expression. Consequently, bacteria coordinate activities as a population, which likely provides a selective advantage in the natural environment. First characterized as a means for regulating light production in the marine bacteria *Vibrio fischeri* and *Vibrio harveyi*, quorum sensing is now recognized as a widespread mechanism for global gene regulation in many bacteria.

Vibrio anguillarum is widely distributed in the aquatic environment and is part of the normal microflora of marine fish (Austin & Austin, 1999; Urakawa & Rivera, 2006). When the health or immune system of the fish is compromised, *V. anguillarum* causes a haemorrhagic septicaemia (vibriosis) (Actis *et al.*, 1999; Austin & Austin, 1999). Production of quorum-sensing signal molecules such as *N*-acylhomoserine lactones is a common feature of both pathogenic and environmental isolates of *V. anguillarum*. Thus, quorum sensing may affect the ecology and physiology of this bacterium as well as its pathogenicity (Buch *et al.*, 2003). Moreover, the *V. harveyi*-like LuxR transcriptional activator in *V. anguillarum*, VanT, positively regulates extracellular protease activity, pigment production and biofilm formation in response to quorum-sensing signals (Croxatto *et al.*, 2002). Each of these activities may play a role in the survival of *V. anguillarum* in seawater or in the fish host.

All vibrios analysed so far contain quorum-sensing systems involving phosphorelay systems that are believed to regulate gene expression in response to cell population similarly to the quorum-sensing systems of *V. harveyi* (Waters & Bassler, 2005; Neiditch *et al.*, 2005; Timmen *et al.*, 2006; Tu & Bassler, 2007). Components of two phosphorelay quorum-sensing systems are known in *V. anguillarum* and a third is predicted. A model of these signalling systems is given in Fig. 1(a). VanM, an *N*-acylhomoserine lactone synthase, synthesizes the signal molecules *N*-hexanoyl-L-homoserine lactone (C6-HSL) and *N*-(3-hydroxyhexanoyl)-L-homoserine lactone (3-hydroxy-C6-HSL), which are sensed by VanN (Croxatto

†Present address: Center for Research on Intracellular Bacteria (CRIB), Institute for Microbiology, University of Lausanne, Switzerland.

‡Present address: Guangdong Province, South China Sea Institute of Oceanology, Xinggang Xi Lu, 164, 510301 Guangzhou, China.

Abbreviations: qRT-PCR, quantitative reverse transcriptase polymerase chain reaction; sRNA, small regulatory RNA.

The GenBank/EMBL/DDBJ accession numbers for the sequences of *rpoS* and *hfq* are EU330190 and EU330191, respectively.

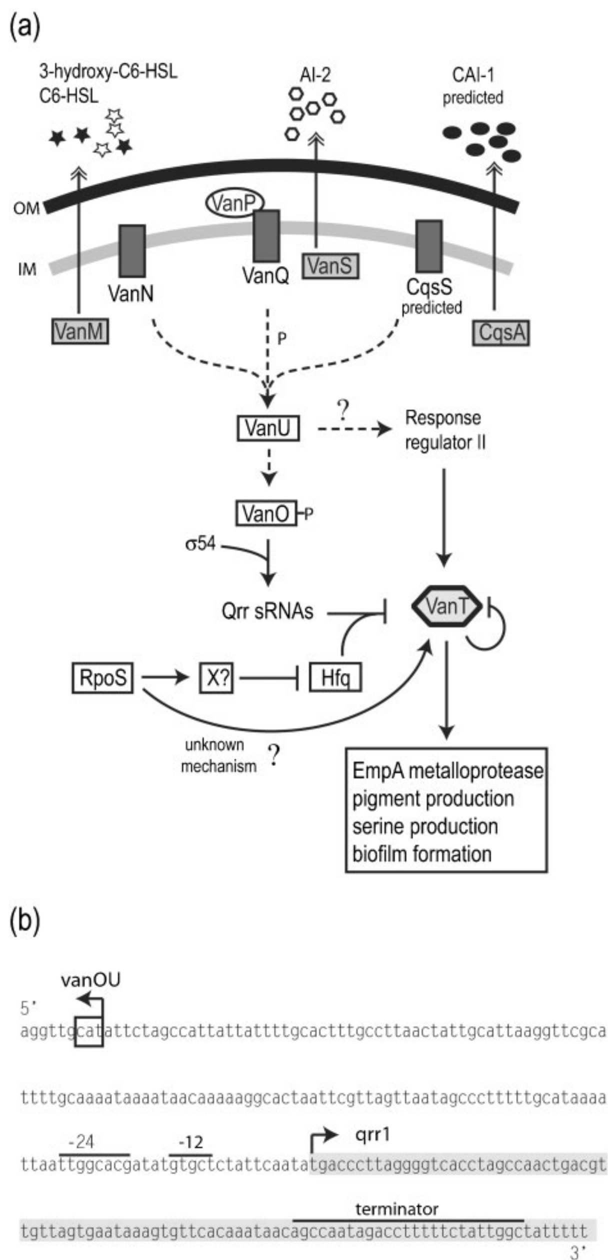

Fig. 1. (a) Model for quorum sensing in *V. anguillarum*. See the text for an explanation. Double arrowheads indicate synthesis and transport of signal molecules to the external environment. Arrows with dashed lines indicate the relay of phosphoryl groups from one protein to another. Arrows with solid lines indicate activation of gene expression and a solid line with a crossing line indicates repression of gene expression. IM, inner membrane; OM, outer membrane. (b) Genomic organization of the *qrr1* (quorum-regulatory RNA) gene. The shaded sequence indicates the predicted *qrr1* gene based on the prediction of Lenz *et al.* (2004). The rightward-pointing arrow indicates the predicted start site of *qrr1* and the terminator sequence is labelled. The predicted σ^{54} −24/−12 promoter sequences are labelled and agree with the consensus sequence YTGGCACG-N_4-TTGCW (Barrios *et al.*, 1999). The start codon for the divergently transcribed *vanOU* genes is indicated by a block and a leftward arrow.

et al., 2004). VanS likely synthesizes an AI-2 signal molecule, a furanosyl borate diester, which binds the periplasmic protein VanP. The VanP–AI-2 complex then binds to VanQ (Croxatto *et al.*, 2004; Denkin & Nelson, 2004). The CqsA/S signal system has not been characterized but is predicted to be present (Henke & Bassler, 2004). The CAI-1 signal molecule, an (*S*)-3-hydroxytridecan-4-one (Higgins *et al.*, 2007), is synthesized by CqsA and is believed to bind the receptor CqsS.

The three receptor proteins, VanN, VanQ and CqsS, are hybrid sensor kinases that channel information via a phosphorylation cascade to a single regulatory pathway. At low cell densities, in the absence of signal molecules, VanN, VanQ and CqsS act as kinases relaying phosphoryl groups to the phosphotransferase VanU, which phosphorylates the σ^{54}-dependent activator VanO. When phosphorylated, VanO activates the expression of several small regulatory RNAs (sRNAs) that, together with the RNA chaperone Hfq, destabilize mRNA encoding VanT, the master regulator. At high cell density, the signal molecules accumulate and bind their cognate sensor kinases. Signal binding represses the kinase activity of VanN, VanQ and CqsS, allowing the phosphatase activity to predominate, which leads to dephosphorylation of VanO. Consequently, VanO is inactivated, sRNAs are not transcribed, and VanT expression is induced, activating the quorum-sensing regulon.

In this study, the protein profile of VanT expression was analysed during growth of wild-type *V. anguillarum*. VanT expression could be detected at a population of 3×10^6 cells ml^{-1} and the expression peaked as the cells entered late exponential growth. Interestingly, the sigma factor RpoS was shown to play a major role in indirectly inducing VanT expression post-transcriptionally. RpoS stabilizes *vanT* mRNA by a mechanism involving Hfq. RpoS was not part of the quorum-sensing regulatory cascade since it did not regulate expression or activity of VanO and since RpoS was not regulated by the quorum-sensing systems. Finally, VanT and RpoS were needed for survival following UV irradiation and for pigment and metalloprotease production. In summary, RpoS and the quorum-sensing systems work together to modulate expression of VanT, which regulates physiological responses required for survival and stress response.

METHODS

Strains, plasmids and media. Bacterial strains and plasmids are described in Table 1. Plasmid transfers from *Escherichia coli* to *V. anguillarum* were done as previously described (Milton *et al.*, 1996). *E. coli* was routinely grown in Luria broth (per litre: Bacto Tryptone, 10 g; Bacto yeast extract, 5 g; and sodium chloride, 5 g) with the following antibiotic concentrations: 100 μg ml^{-1} for ampicillin and

Table 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or relevant markers	Reference or source
V. anguillarum		
NB10	Wild-type, serotype O1, clinical isolate from the Gulf of Bothnia	Norqvist *et al.* (1989)
AC10	NB10 carrying an in-frame deletion of *vanT*	Croxatto *et al.* (2002)
AC11	NB10 carrying an in-frame deletion of *vanO*	Croxatto *et al.* (2004)
AC11c	AC11 derivative in which the wild-type *vanO* gene was exchanged for the mutant allele	This study
AC12	NB10 carrying an in-frame deletion of *rpoS*	This study
AC12c	AC12 derivative in which the wild-type *rpoS* gene was exchanged for the mutant allele	This study
OTR83	NB10 carrying an in-frame deletion of *rpoN*	O'Toole *et al.* (1997)
BW11	NB10 carrying an-frame deletion of *hfq*	This study
BW11c	BW11 derivative in which the wild-type *hfq* gene was exchanged for the mutant allele	This study
Plasmids		
pBluescript	Ap^r; ColE1 origin	Stratagene
pBS-rpoS-7	Ap^r; pBluescript with a ~9.5 kb genomic fragment containing *truD* (partial), *surE*, *pcm*, *nlpD*, *rpoS* and *mutS* (partial) genes	This study
pBS-hfq	Ap^r; pBluescript with a 1.5 kb genomic fragment containing the *hfq* gene	This study
pDM4	Cm^r; suicide vector with an R6K origin (*pir* requiring) and *sacBR* of *Bacillus subtilis*	Milton *et al.* (1996)
pDM4-rpoS-AD	Cm^r; pDM4 containing a mutant allele of *rpoS*, bp 2513–2830 fused in-frame to bp 3705–3874	This study
pDM4-hfq-AD	Cm^r; pDM4 containing a mutant allele of *hfq*, bp 227–392 fused in-frame to bp 624–819 bp	This study
pDM4-rpoS-wt	Cm^r; pDM4 containing the wild-type allele of *rpoS* bp 2513–3874	This study
pDM4-vanO-wt	Cm^r; pDM4 containing the wild-type allele of *vanO* bp 635–1774	This study
pDM4-hfq-wt	Cm^r; pDM4 containing the wild-type allele of *hfq* bp 227–819	This study
pNQ705-1	Cm^r; suicide vector with an R6K origin (*pir* requiring)	McGee *et al.* (1996)
pJBA113	Ap^r; pUC18Not-$P_{A1/04/03}$-RBSII-*gfp*(ASV)-T_0-T_1, an unstable variant of *gfp*mut3*	Andersen *et al.* (1998)
pDM41	Cm^r; pNQ705-1 carrying the promoterless RBSII-*gfp*(ASV)-T_0 gene from pJBA113	This study
pDM41-vanT3-TL	Cm^r; pDM41 carrying a *vanT*::*gfp*(ASV) translational gene fusion	This study
pDM41-vanT-TC	Cm^r; pDM41 carrying a *vanT*::*gfp*(ASV) transcriptional gene fusion	This study
pDM41-rpoS-TC	Cm^r; pDM41 carrying a *rpoS*::*gfp*(ASV) transcriptional gene fusion	This study
pDM41-empA-TC	Cm^r; pDM41 carrying a *empA*::*gfp*(ASV) transcriptional gene fusion	This study
pDM41-qrr1-TC	Cm^r; pDM41 carrying a *qrr1*::*gfp*(ASV) transcriptional gene fusion	This study
pDM41-hfq-TC	Cm^r; pDM41 carrying a *hfq*::*gfp*(ASV) transcriptional gene fusion	This study

25 μg ml^{-1} for chloramphenicol. For *V. anguillarum*, Trypticase soy medium from BBL containing 1 % sodium chloride (TSB) was routinely used. For selection against *E. coli* after conjugation, the *Vibrio* selective medium TCBS agar (Difco) was used. Antibiotic concentration for *V. anguillarum* for chloramphenicol was 10 μg ml^{-1}.

Cloning the *rpoS* and *hfq* genes. An internal 477 bp DNA fragment from the *V. anguillarum rpoS* gene was amplified using PCR and degenerate primers complementary to the *Vibrio cholerae rpoS* gene VC0534 (Heidelberg *et al.*, 2000) and sequenced to confirm identity to *rpoS*. The primers used were VCrpoS-For2 (5′-GGACTAGTCCGTAACCAAAGTAGARGARTT-3′) and VCrpoS-Rev2 (5′-GGGGTACCCCAATGGTGCGTGTYTGRTTCAT-3′). The 477 bp PCR fragment was used as a probe to screen a *V. anguillarum* genomic library in the Lambda Zap II bacteriophage, and the pBluescript plasmids containing a chromosomal insert were excised from positive plaques as previously described (Milton *et al.*, 1992). One plasmid that contained the entire *rpoS* gene, pBS-rpoS-7, was chosen for sequencing.

To clone the *hfq* gene, two primers complementary to sequences flanking the *hfq* gene in *Vibrio parahaemolyticus* (Makino *et al.*, 2003) were used to amplify a 1519 bp fragment from the *V. anguillarum* chromosome. Primers used were Hfq-1 (5′-GGACTAGTGCATCAA-CAACATGTAACAA-3′), which contains a *Spe*I site at the 5′-end, and Hfq-2 (5′-CTCGAGCTCGGTTACCGACAGATGTGGGA-3′), which contains a *Sac*I site at the 5′-end. This fragment was cloned into pBluescript using *Sac*I and *Spe*I, creating pBS-hfq, and sequenced.

Mutant and complementation strains. To create a null mutation in *rpoS* and *hfq*, an in-frame deletion was made by allelic exchange as described in detail previously (Milton *et al.*, 1996). Plasmid pDM4-rpoS-AD, which carries an altered allele of *rpoS* that encodes the first 14 amino acids fused to the last 61 amino acids of RpoS, was used to create strain AC12 (Δ*rpoS*). Plasmid pDM4-hfq-AD, carrying an altered allele of *hfq* encoding the first five amino acids fused to the last five amino acids of Hfq, was used to create strain BW11 (Δ*hfq*). The in-frame deletions were confirmed by sequencing a PCR-amplified DNA fragment of the deleted chromosomal locus and by Western blot analysis.

For complementation of the Δ*rpoS*, Δ*hfq* and Δ*vanO* mutations, reverse allelic exchange was done. For each, the wild-type gene and flanking DNA were amplified by PCR and cloned into pDM4, resulting in pDM4-rpoS-wt, pDM4-hfq-wt and pDM4-vanO-wt, which were used to exchange the mutant alleles for the wild-type gene, producing strains AC12c, BW11c and AC11c, respectively. Complementation of the mutations was confirmed by Western blot analysis.

PCR conditions, DNA techniques, DNA sequencing and computer analyses. PCR was performed as previously described (McGee *et al.*, 1996; Croxatto *et al.*, 2002). Unless otherwise stated, all conditions for the various DNA techniques were as described by Sambrook *et al.* (1989). Reaction conditions for the DNA-modifying enzymes and DNA-restriction enzymes were performed as suggested by the manufacturers. Double-strand DNA sequencing was performed by

automated sequencing on an ABI Prism 377 DNA sequencer and by primer walking in two directions from known regions of DNA sequence. DNA sequence editing was done using the Genetics Computer Group Sequence Analysis software (Devereux *et al.*, 1984) of the Genetics Computer Group (University of Wisconsin). Database searches were done using the BLAST program from the National Center for Biotechnology Information. The sequence data have been submitted to the DDBJ/EMBL/GeneBank databases under accession number EU330190 for *rpoS* and EU330191 for *hfq*.

Western analysis and preparation of antisera. VanT, VanO, RpoS and Hfq were purified using the IMPACT T7 system from New England BioLabs. Genes encoding VanT, VanO, RpoS and Hfq were amplified by PCR and cloned into pTYB1 using *Nde*I and *Sap*I, which fuses the coding region for the protein splicing element intein followed by a chitin-binding tag to the 3′-end of these genes. For optimal self-splicing, an extra glycine codon was added to the 3′-end of the *vanT, rpoS* and *hfq* genes. Protein purification was performed as described by the manufacturer except for VanT cleavage from the chitin column, which was done for 48 h. Using the purified proteins, polyclonal rabbit antisera were made by AgriSera AB, Sweden. Before use in Western analyses, the antisera were affinity purified using the MicroLink Protein Coupling kit (Pierce) via the manufacturer's instructions.

For each strain, Western blot analysis was performed on protein samples taken at various time points during growth. Proteins were separated as described by Laemmli (1970) using SDS-12.5 % PAGE and transferred to a nitrocellulose membrane (Schleicher and Schuell) using a SemiPhor semidry blotter (Hoefer TE 70 series). Enhanced chemiluminescence (ECL) Western blotting was performed according to the manufacturer's instructions (Amersham Life Sciences). As no suitable loading control was found that could be used to compare all time points during growth, two approaches were taken to determine equal sample loading. A second similarly loaded 12.5 % PAGE was performed and stained with Coomassie blue. In addition, Western analysis was done using protein samples from two time points and two antisera to detect the protein of interest and the outer-membrane protein OmpU, which was used as a loading control. The intensities of the two protein bands were measured using QUANTITY ONE version 4.2.3 software (Bio-Rad) and the protein of interest was equalized to that of OmpU. The mutant to wild-type ratio was then determined.

Construction of transcriptional and translational *gfp* fusions. Transcriptional or translational reporter gene fusions with the gene encoding the unstable green fluorescent protein variant Gfp-ASV (Andersen *et al.*, 1998) were made using the suicide vector pDM41. The C-terminal peptide tag ASV renders the stable Gfp protein susceptible to degradation by intracellular tail-specific proteases. The half-life of Gfp-ASV was determined to be 80 min in *V. anguillarum* (data not shown). To create pDM41, the *gfp* gene, which lacks a promoter but has a ribosome-binding site (RBS), was removed from pJBA113 by *Nhe*I and *Xba*I digestion. The 850 bp fragment was purified from a 1 % agarose gel using Ultrafree-DA spin columns (Millipore) and cloned into similar restriction sites of the suicide vector pNQ705-1. For transcriptional fusions, the *Xba*I site, which cleaves upstream of the RBS, was used and for translational fusions, the *Sph*I site, which cleaves at the ATG start codon, was used. For transcriptional fusions of *vanT*, *rpoS*, *empA*, *qrr1* and *hfq*, DNA fragments containing promoters but lacking the possible RBS were amplified by PCR using the following primer sets containing either a *Sac*I or an *Xba*I site at the 5′-ends: VanT-gfp-S (5′-CTCGAGCTCATTCGTTCCTGAACC-3′) and VanT-gfp-X (5′-GCTCTAGAACTGTTGAATTGAGC-3′); RpoS-gfp-S (5′-CTCGAGCTCCCGTTGTCTATTCGG-3′) and RpoS-gfp-X (5′-GCTCTAGAGAGCTAGCAAGACAT-3′); EmpA-gfp-1 (5′-CTCGAGCTCATATGCTCAACGAAC-3′) and EmpA-gfp-2 (5′-GCTCTAGAGTTATTATTAGCATC-3′); and Rna1-gfp-S (5′-CTCGAGCTCAGCAATATGAGGTCC-3′) and Rna1-gfp-X (5′-GCTCTAGATATTGAATAGAGCAC-3′); Hfq-gfp-S (5′-CTCGAGCTCAAGCGTCAGATCACC-3′) and Hfq-gfp-X (5′-GCTCTAGAGTTGTAGTTATTTAG-3′). For a translational fusion of *vanT*, a DNA fragment containing the promoter, the RBS and codons for the first 14 amino acids was amplified by PCR using a primer set containing either a *Sac*I or a *Sph*I site at the 5′-end. The primer pair used was VanT-Sph3 (5′-GGACATGCATGCGTGATAAGCGAGTTC-3′) and VanT-gfp-S (listed above). The DNA fragments were gel purified, digested with *Sac*I/*Xba*I or *Sac*I/*Sph*I and ligated to a similarly digested pDM41, resulting in pDM41-vanT3-TL, pDM41-vanT-TC, pDM41-rpoS-TC, pDM41-empA-TC, pDM41-hfq-TC and pDM41-qrr1-TC. All constructs were sequenced to ensure that the gene fusions were made properly. Each gene fusion was integrated into the promoter region of the respective gene on the chromosome. Since the promoters were duplicated on the chromosomes, these insertions did not disrupt the respective wild-type genes. Chromosomal integrations were checked by PCR analysis.

Green fluorescent protein (Gfp) assays. *V. anguillarum* cultures carrying the *gfp* gene fusions were grown overnight at 24 °C in TSB with aeration. Overnight cultures were diluted to an OD_{600} of 0.001 in TSB and incubation was continued at 24 °C in TSB with aeration. At various time points during growth (0, 2, 4, 6, 8, 10, 12, 14, 18, 24 h), samples were taken and the OD_{600} and numbers of c.f.u. were determined. To measure fluorescence at each time point, a cell number equivalent to an OD_{600} of 0.2 was removed from each sample and, when required, the samples were diluted to an OD_{600} of 0.2 in a final volume of 2 ml. This was done since the use of too many cells quenched the fluorescence output using a Bio-Rad VersaFluor fluorometer. To minimize any effects on gene expression due to the dilution of the cells, fluorescence was measured immediately following dilution with an excitation wavelength of 490 nm and an emission wavelength at 520 nm, according to the manufacturer's instructions. The fluorescence units were then divided by an OD_{600} of 0.2 to obtain fluorescence relative to the cell number. The wild-type strain without a *gfp* gene fusion treated similarly was used as a blank before taking the measurement. All measurements were done in triplicate and averaged.

UV irradiation survival. Overnight cultures of *V. anguillarum* grown in TSB at 24 °C with aeration were diluted in the same medium to an OD_{600} of 0.001 and incubated until an OD_{600} of 0.5 (5×10^8 cells ml^{-1}) was reached. Bacteria were diluted in 4 % artificial seawater (Sigma) to a cell density of 1×10^5 cells ml^{-1}. For the zero time point, c.f.u. were measured from the culture used to inoculate each medium. Bacteria were exposed to shortwave UV light (253.7 nm) for various lengths of time and survival was measured by determining the c.f.u. in each sample. These assays were done at least three times.

RNA isolation and quantification. Overnight cultures of *V. anguillarum* grown at 24 °C with aeration were diluted in TSB to an OD_{600} of 0.001 and incubated until an OD_{600} of 1.0 was reached. Culture volumes equivalent to 1×10^8 cells ml^{-1} were mixed with 2 vols RNAprotect bacteria reagent (Qiagen) to stabilize the RNA transcripts and incubated for 5 min at room temperature. Total RNA was then isolated using the RNeasy minikit (Qiagen). RNA samples were treated with DNA-free DNase (Ambion) and RNA concentrations were then determined using the RiboGreen RNA reagent from Molecular Probes. Both were according to the manufacturers' instructions.

Real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). A one-step real-time qRT-PCR was used to measure the levels of *vanT*, *qrr1* and *hpdA* mRNA in various *V. anguillarum* strains. RNA was purified from each strain as described above and 30 ng was used in each RT-PCR reaction. An iCycler iQ

(Bio-Rad) and the iScript One-Step RT-PCR kit with SYBR Green (Bio-Rad) were used according to the manufacturer's instructions. To ensure against contaminating chromosomal DNA in the purified RNA, one reaction using purified RNA as template for each sample was done using the iQ SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions. To ensure that equal amounts of RNA were used, one reaction contained primers for 16S rRNA. RNA samples at each time point were prepared from three separate cultures. Primers used for each gene are as follows: vanT-right (5′-CTTTCGCATGCAAATCAAGA-3′) and vanT-left (5′-CCACGCAG-ATATTGCTGAAA-3′); Qrr1-RT-Fw (5′-AAAGGTCTATTGGCTGT-TATTTGTG-3′) and Qrr1-RT-Rev (5′-ACCCTTAGGGGTCAC-CTAG-3′); HpdA-RT-Fw (5′-AGCATTCCTGCGATTTATGG-3′) and HpdA-RT-Rev (5′-CGGTCATTCGTTGATTAGCA-3′); 16S-left (5′-CATGCCGCGTGTATGAAGAA-3′) and 16S-right (5′-AACAAT-TATCGTCGTAGTAAACTGC-3′). Calculations for mRNA levels were done according to the standard curve method (Larionov *et al.*, 2005), which normalizes the mRNA levels to that of the 16S mRNA. Each qRT-PCR was done using samples from three independent experiments, the results were averaged, and *P*-values were determined.

***vanT* mRNA stability assay.** Overnight cultures of *V. anguillarum* grown in TSB at 24 °C with aeration were diluted in the same medium to an OD_{600} of 0.001 and incubated to an OD_{600} of 0.2 and 1.0. To stop RNA transcription, rifampicin (Sigma-Aldrich) was added to the culture to a final concentration of 200 μg ml^{-1}. For the zero time point, a culture sample (100 μl) was taken before the addition of rifampicin. For mRNA half-life measurements, culture samples (100 μl) were taken at 1, 2, 5 and 10 min after the addition of rifampicin. Total RNA was isolated from each sample and 30 ng RNA was used in a real-time qRT-PCR as described above. For each strain, the zero time point was set to 1.0 and all following time points were normalized to the zero time point. To determine product specificity, standard curves and melting curves were analysed for multiple products. This assay was performed in triplicate.

RESULTS

Expression of VanT during growth

Previous studies suggested that the phosphorelay quorum-sensing systems in *V. anguillarum* limit the expression of *vanT* rather than induce its expression (Croxatto *et al.*, 2004). However, genes regulated by VanT are induced as the cell population enters stationary phase (Croxatto *et al.*, 2002). Thus, we wondered whether VanT protein levels were induced during growth even though it was previously shown that the mRNA levels do not vary greatly. In the wild-type, Western blot analysis (Fig. 2) showed that VanT is detected at a low cell population (OD_{600} 0.007), indicating that the sRNAs do not completely destabilize *vanT* mRNA. An induction of expression was also seen as the cell population entered stationary phase (OD_{600} 0.4–1.8), after which VanT levels decreased again. To determine if this induction is due to the quorum-sensing systems, VanT expression in a *vanO* mutant was analysed (Fig. 2). Compared to the wild-type, the *vanO* mutant showed increased levels of VanT at a low cell population (OD_{600} 0.007–0.4), indicating that the quorum-sensing systems repress VanT expression at low cell densities and the *vanO* mutation could be complemented with the wild-type gene.

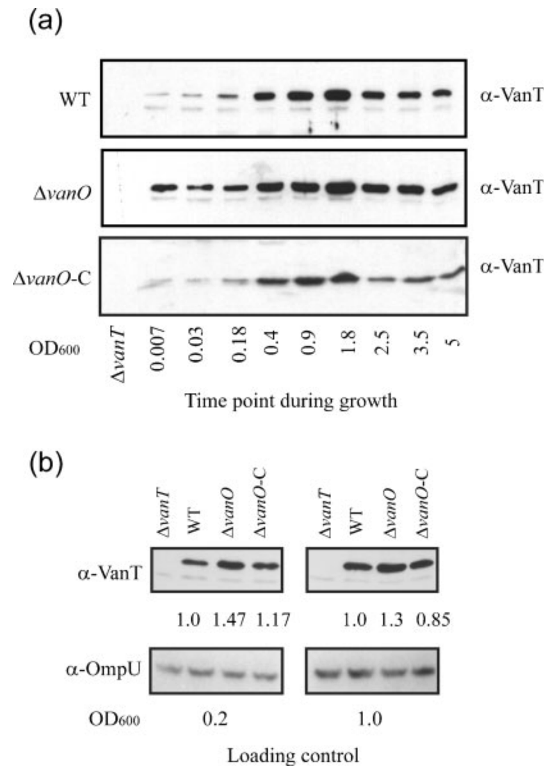

Fig. 2. Expression of VanT in the wild-type and the *vanO* mutant. (a) Culture samples of the wild-type (WT), *vanO* mutant (Δ*vanO*) and a complemented *vanO* mutant (Δ*vanO*-C) were taken at various cell densities. Proteins from equal cell numbers were separated by SDS-12.5 % PAGE and Western blot analyses were done using a VanT antiserum (α-VanT). As a negative control, the *vanT* mutant (Δ*vanT*; sampled at OD_{600} 1.0) was included. (b) OmpU was used as a loading/transfer control. Western blots were done as in (a) except that only two time points were analysed. After detection of VanT, the blot was stripped and an OmpU antiserum (α-OmpU) was applied. The intensities of the VanT and OmpU bands were measured and the intensity of VanT was equalized to that of OmpU. The mutant/wild-type ratio is given between the blots.

However, complete derepression of VanT expression at low cell density did not appear to occur in the *vanO* mutant and we wondered if an additional regulatory factor may induce the expression of VanT as cell numbers increase.

RpoS regulates expression of VanT post-transcriptionally during entry into the stationary phase

RpoS is a good candidate as an additional regulatory factor since it has been suggested to regulate the *V. anguillarum empA* gene, which encodes an extracellular metalloprotease that is also regulated by VanT (Croxatto *et al.*, 2002; Denkin & Nelson, 2004). Moreover, in *E. coli*, RpoS is the main regulator of gene expression during stationary phase (for a review see Hengge-Aronis, 2000). The *rpoS* genetic

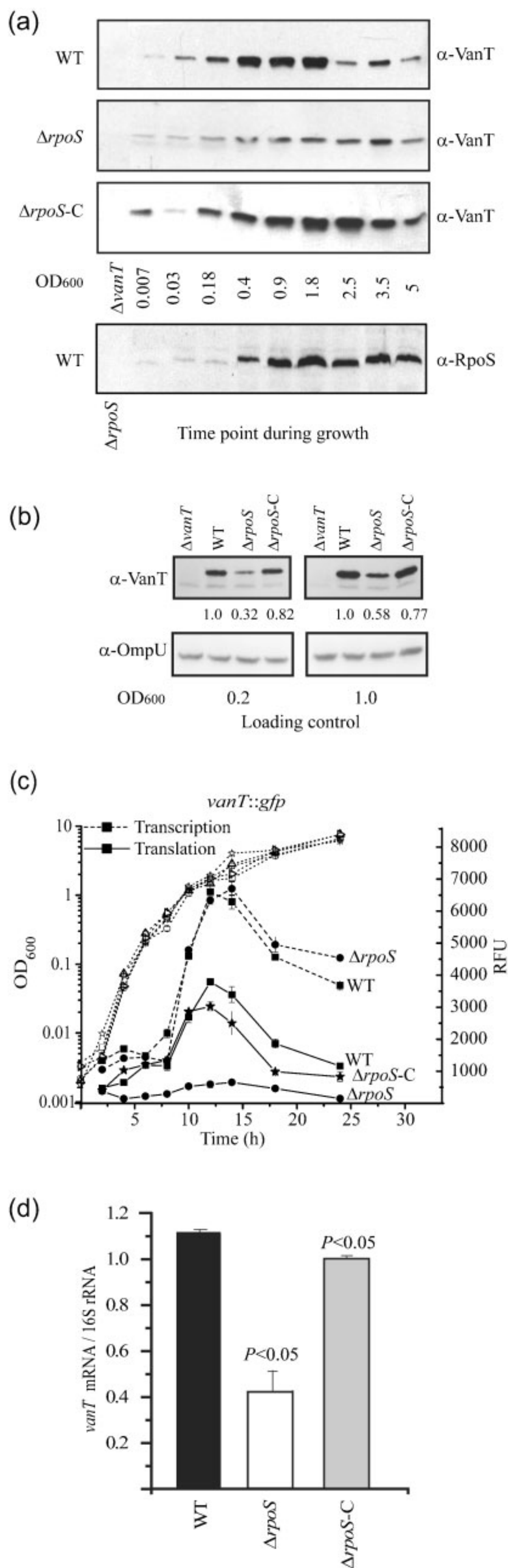

Fig. 3. RpoS regulates the expression of VanT post-transcriptionally. (a) Western blot analysis of VanT. Culture samples of the wild-type (WT), *rpoS* mutant (Δ*rpoS*) and a complemented *rpoS* mutant (Δ*rpoS*-C) were taken at various cell densities. Proteins from equal cell numbers were separated by SDS-12.5 % PAGE and Western blot analyses were done using a VanT antiserum for all three strains and an RpoS antiserum for the wild-type. As appropriate, the *vanT* mutant (Δ*vanT*) and the *rpoS* mutant (Δ*rpoS*; both sampled at OD_{600} 1.0) were included as negative controls. (b) OmpU was used as a loading/transfer control. Western blots were done as in (a) except that only two time points were analysed. After detection of VanT the blot was stripped and an OmpU antiserum was applied. The intensities of the VanT and OmpU bands were measured and the VanT intensity was equalized to that of OmpU. The mutant/wild-type ratio is given between the blots. (c) A transcriptional (dashed lines) and a translational (solid lines) *vanT*::*gfp* gene fusion were expressed from the chromosome of the wild-type (WT, black squares), the *rpoS* mutant (Δ*rpoS*, black circles) and the complemented *rpoS* mutant (Δ*rpoS*-C, black stars). Growth is indicated by dotted lines. Gfp expression was measured as fluorescence; relative fluorescence units (RFU) are fluorescence units of cells normalized to an OD_{600} of 0.2. (d) Real-time qRT-PCR of *vanT* transcripts. RNA transcripts were isolated from the wild-type (WT), *rpoS* mutant (Δ*rpoS*) and the complemented *rpoS* mutant (Δ*rpoS*-C) grown to an OD_{600} of 1.0. Real-time qRT-PCR was done as described in Methods and *vanT* mRNA was normalized to the levels of 16S RNA. *P*-values <0.05 are considered significant.

locus from *V. anguillarum* was cloned and sequenced. The RpoS amino acid sequence showed 73–86 % identity with RpoS from other *Vibrio* species in the NCBI database. A mutant (AC12) carrying an in-frame deletion fusing the first 14 amino acids to the last 61 amino acids was made and the mutation was confirmed by Western analysis (data not shown).

To determine if VanT levels are altered in an *rpoS* mutant, Western analysis of VanT expression was done (Fig. 3a, b). The *rpoS* mutant did not show the peak of VanT expression during entry into stationary phase that occurred in the wild-type. To establish whether RpoS affects the transcription or translation of *vanT*, both types of gene fusions were made to a *gfp* variant that encodes an unstable Gfp protein with a half-life of 80 min in *V. anguillarum*. Fluorescence was used as a measure of VanT expression throughout growth (Fig. 3c). For the transcriptional fusion, no difference was seen between the wild-type and the *rpoS* mutant. For the translational fusion, the induction of VanT expression seen during entry into stationary phase in the wild-type was lost in the *rpoS* mutant. In addition, using real-time qRT-PCR analysis, a decrease in *vanT* mRNA levels was seen in the *rpoS* mutant at an OD_{600} of 1.0 compared to the wild-type (Fig. 3d). For each assay, the loss of VanT expression seen in the *rpoS* mutant was restored to wild-type levels when the wild-type gene was exchanged for the mutant allele. Interestingly, the increase of VanT expression during late exponential growth

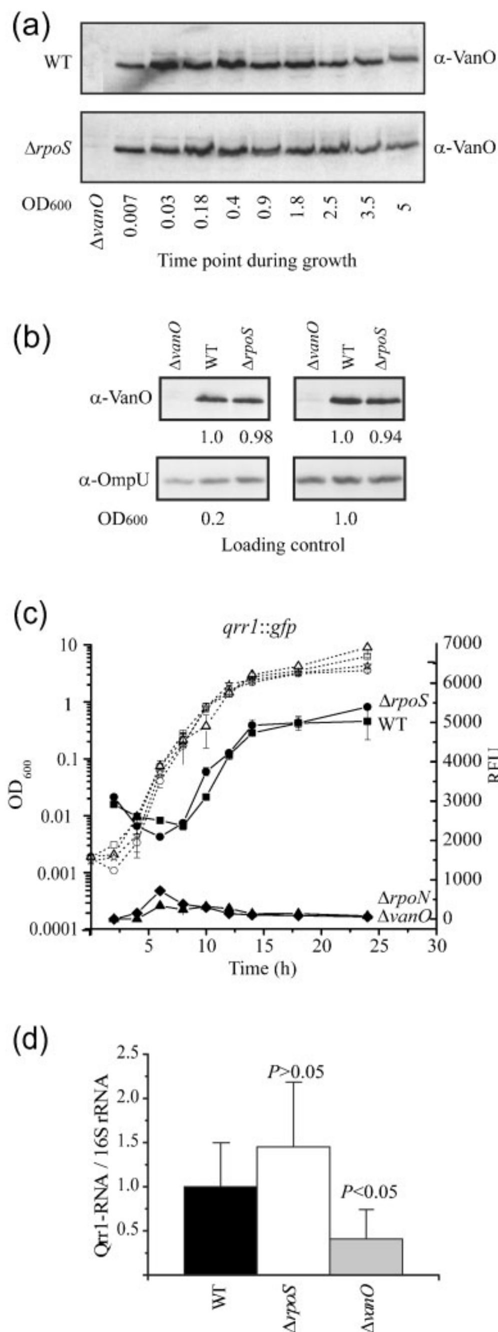

Fig. 4. RpoS regulation of *vanT* does not involve the quorum-sensing regulatory cascade. (a) Western blot analysis of VanO. Culture samples of the wild-type (WT) and the *rpoS* mutant (Δ*rpoS*) were taken at various cell densities. Proteins from equal cell numbers were separated by SDS-12.5 % PAGE and Western blot analysis was done using a VanO antiserum. The *vanO* mutant (Δ*vanO*; sampled at OD_{600} 1.0) was included as a negative control. (b) OmpU was used as a loading/transfer control. Western blots were done as in (a) except that only two time points were analysed. After detection of VanO, the blot was stripped and an OmpU antiserum was applied. The intensities of the VanO and OmpU bands were measured and the VanO intensity was equalized to that of OmpU. The mutant/wild-type ratio is given between the blots. (c) A transcriptional *qrr1* : : *gfp* gene fusion was expressed from the chromosome of the wild-type (WT, black squares), the *rpoS* mutant (Δ*rpoS*, black circles), the *rpoN* mutant (Δ*rpoN*, black diamonds) and the *vanO* mutant (Δ*vanO*, black triangles). Growth is indicated by dotted lines. Gfp expression (solid lines) was measured as fluorescence; relative fluorescence units (RFU) are fluorescence units of cells normalized to an OD_{600} of 0.2. (d) Real-time qRT-PCR of *qrr1* transcripts. RNA transcripts were isolated from the wild-type (WT), *rpoS* mutant (Δ*rpoS*) and the *vanO* mutant (Δ*vanO*) at OD_{600} 1.0. Real-time qRT-PCR was done as described in Methods. *P*-values <0.05 are considered significant.

correlates with the onset of RpoS expression during growth (Fig. 3a). Together, these data suggest that RpoS indirectly regulates VanT expression via another gene product involved in the post-transcriptional regulation of *vanT*.

RpoS regulation of *vanT* is not via the quorum-sensing regulatory cascade

The data presented so far strongly suggest that RpoS regulates VanT expression independently of VanO and we wanted to confirm these observations. Based on other vibrio quorum-sensing systems (Lenz *et al.*, 2004), VanO is believed to be required for the expression of the sRNAs that destabilize *vanT* mRNA. However, no difference in VanO levels was seen between the wild-type and the *rpoS* mutant (Fig. 4a, b). The Western blot analysis does not determine if the activity of VanO is affected. Thus, transcription analysis of an sRNA gene was done to measure the activity of VanO directly. To identify an sRNA gene for these studies, we used the fact that the sRNA Qrr1 (quorum-regulatory RNA) in other *Vibrio* species is encoded just upstream of *luxO*; DNA flanking the *vanO* gene was analysed for *qrr1*. Similarly in *V. anguillarum*, a *qrr1* homologue with a consensus RpoN (σ^{54}) binding site in the promoter region was found to be transcribed divergently from *vanO* (Fig. 1b). A *qrr1* : : *gfp* transcriptional gene fusion was made and Gfp expression was assayed in the wild-type and in the *vanO* and *rpoN* mutants (Fig. 4c). In the wild-type, Gfp was expressed at low cell density and increased in expression during late exponential growth. In the *rpoS* mutant, Gfp expression from the *qrr1* promoter was unchanged compared to that of the wild-type; however, both VanO and RpoN were required for full expression. Real-time qRT-PCR analysis showed that *qrr1* encodes a transcript in the wild-type and that the level of *qrr1* transcripts decreased in the *vanO* mutant compared to the wild-type but not in the *rpoS* mutant (Fig. 4d). Thus, the indirect post-transcriptional regulation of *vanT* by RpoS is not due to an effect on the expression or activation of VanO and thus expression of Qrr1.

RpoS represses Hfq expression and stabilizes *vanT* mRNA

From *V. cholerae* studies, Hfq, an RNA chaperone, was shown to work together with Qrr sRNAs to destabilize

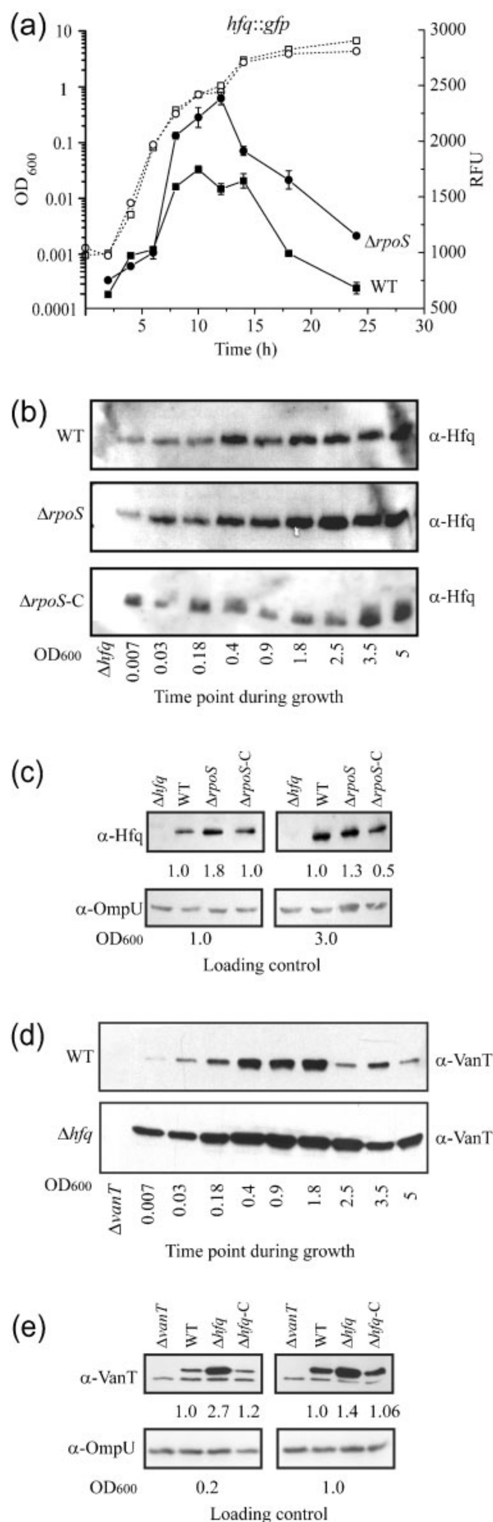

Fig. 5. RpoS negatively affects expression of Hfq and Hfq represses *vanT* expression. (a) A transcriptional *hfq* : : *gfp* gene fusion was expressed from the chromosome of the wild-type (WT; black squares) and the *rpoS* mutant (Δ*rpoS*, black circles). Growth is indicated by dotted lines. Gfp expression (solid lines) was measured as fluorescence; relative fluorescence units (RFU) are fluorescence units of cells normalized to an OD_{600} of 0.2. (b) Western blot analysis of Hfq in an *rpoS* mutant. Culture samples of the wild-type (WT), the *rpoS* mutant (Δ*rpoS*) and the complemented *rpoS* mutant (Δ*rpoS*-C) were taken at various cell densities. Proteins from equal cell numbers were separated by SDS-12.5 % PAGE and Western blot analysis was done using an Hfq antiserum. The *hfq* mutant (Δ*hfq*; sampled at OD_{600} 1.0) was included as a negative control. (c) OmpU was used as a loading/transfer control. Western blots were done as in (b) except that only two time points were analysed. After detection of Hfq, the blot was stripped and an OmpU antiserum was applied. The intensities of the Hfq and OmpU bands were measured and the intensity of Hfq was equalized to that of OmpU. The mutant/wild-type ratio is given between the blots. (d) Western blot analysis of VanT in an *hfq* mutant. Culture samples of the wild-type (WT) and the *hfq* mutant (Δ*hfq*) were analysed for VanT expression as described in (b) except that the Western blot analysis was done using a VanT antiserum and the *vanT* mutant (Δ*vanT*; sampled at OD_{600} 1.0) was included as a negative control. (e) Loading/transfer controls for (d) are as described for (c) except that a VanT antiserum was used and the complemented *hfq* mutant strain (Δ*hfq*-C) was included.

hapR mRNA (Lenz *et al.*, 2004). Thus, we wondered if RpoS induces expression of VanT by affecting the expression of Hfq. The *hfq* gene was cloned and the deduced amino acid sequence of Hfq showed 87–90 % identity with Hfq from other *Vibrio* species in the NCBI database. An *hfq* : : *gfp* transcriptional gene fusion was made and Gfp expression was assayed in the wild-type and *rpoS* mutant (Fig. 5a). In the *rpoS* mutant, Gfp expression was similar to the wild-type during early growth. At the time point when RpoS is expressed in the wild-type (see Fig. 3), an increase in Gfp expression was seen in the *rpoS* mutant and Gfp expression remained higher than in the wild-type throughout growth. Western blot analysis showed a similar increase in Hfq levels in the *rpoS* mutant compared to the wild-type, and exchange of the mutant *rpoS* gene for the wild-type gene resulted in wild-type Hfq levels (Fig. 5b, c). To determine if an *hfq* mutation affects VanT expression, an *hfq* mutant carrying an in-frame deletion fusing the first five amino acids to the last five amino acids was made. In the *hfq* mutant, VanT expression was derepressed throughout growth when compared to the wild-type (Fig. 5d, e).

Suggested roles for Hfq in gene regulation are to stabilize the sRNAs and to enhance RNA–RNA interactions (for a review see Gottesman, 2004). If an *rpoS* mutant has increased levels of Hfq, then *vanT* mRNA would be predicted to be more unstable in this strain than in the wild-type. To test this hypothesis, the stability of *vanT* mRNA was determined in the wild-type and the *rpoS, vanO* and *hfq* mutants at both a low and high cell density. Fig. 6 shows that at both cell densities *vanT* mRNA is more stable in the *hfq* mutant than in the wild-type and the *vanO* mutant and, as expected, in the *vanO* mutant *vanT* mRNA is more stable than in the wild-type at low cell densities. However, *vanT* mRNA in the *rpoS* mutant, which has

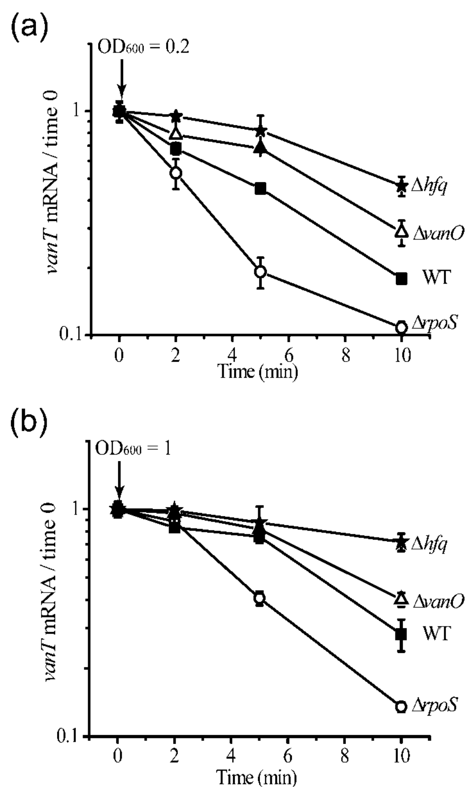

Fig. 6. Stability of *vanT* mRNA. The wild-type (black squares), the *rpoS* mutant (Δ*rpoS*, white circles), the *vanO* mutant (Δ*vanO*, white triangles) and the *hfq* mutant (Δ*hfq*, black stars) were grown to an OD_{600} of 0.2 (a) and 1.0 (b). At these time points, transcription was stopped by the addition of rifampicin (200 μg ml^{-1}) and culture samples were taken at 1, 2, 5 and 10 min. The zero time point was taken before the addition of rifampicin. Total RNA was isolated from each sample and real-time qRT-PCR was done to determine the amount of *vanT* mRNA remaining at each time point. The mRNA level at the zero time point for each sample was set at 1.0 and the mRNA remaining at each time point after rifampicin addition was normalized to the respective zero time point.

increased levels of Hfq, was much less stable than in the wild-type. These data show that Hfq and the Qrr sRNAs destabilize *vanT* mRNA and that RpoS increases the stability of *vanT* mRNA by negatively affecting the expression of Hfq, resulting in an increase of VanT expression during late exponential growth.

VanO and VanT do not affect expression of RpoS

Quorum-sensing signalling in vibrios has been predicted to affect the levels of RpoS in the cell (McDougald & Kjelleberg, 2006). To determine if the vibrio quorum-sensing systems regulate expression of RpoS, Western blot analysis using an antiserum against RpoS was done in the wild-type and the *vanO* and *vanT* mutants. In addition, an *rpoS*::*gfp* transcriptional fusion was assayed in the same strains. No difference in RpoS expression was seen in either the *vanO* or the *vanT* mutant compared to the wild-type (Fig. 7), indicating that the quorum-sensing systems do not regulate RpoS expression.

VanT and RpoS regulate similar cellular functions

Studies in *Vibrio vulnificus* and *V. cholerae* showed that the vibrio quorum-sensing systems may play a role in bacterial physiology by regulating starvation adaptation and oxidative stress responses (McDougald *et al.*, 2001, 2002; Joelsson *et al.*, 2007). RpoS is a major transcriptional regulator essential for adaptation of bacteria to many stress responses during growth (for a review see Hengge-Aronis, 2000). In *V. anguillarum*, VanT may be part of the RpoS stress response. To test if VanT and RpoS are involved in stress response, the survival of bacterial cells from the wild-type and the *rpoS* and *vanT* mutants that were entering stationary phase was measured after exposure to UV irradiation for 45 s. Resistance to UV irradiation, which damages DNA or induces the production of reactive oxygen species, is a likely stress response for many marine vibrios that are exposed to sunlight. Cells from both mutants showed a 100-fold decrease in survival compared to the wild-type and this phenotype could be complemented in the *rpoS* mutant with the wild-type *rpoS* gene (Fig. 8a).

Previously, we showed that VanT regulates gene expression during entry into stationary phase. Two genes identified were *hpdA*, which encodes a 4-hydroxyphenylpyruvate dioxygenase that is involved in pigment production, and *empA*, which encodes an extracellular metalloprotease (Croxatto *et al.*, 2002). We asked whether RpoS regulates these genes as well. Using real-time qRT-PCR, a decrease in the *hpdA* mRNA was seen in the *vanT* and *rpoS* mutants as compared to the wild-type (Fig. 8b). Expression of EmpA, which was measured using an *empA*::*gfp* transcriptional fusion, required both RpoS and VanT as no or very little Gfp expression was detected in either of the two mutants (Fig. 8c). Expression of both genes returned to approximately wild-type levels in the *rpoS* mutant when the mutation was complemented with the wild-type *rpoS* gene. These data show that RpoS and VanT regulate similar physiological responses.

DISCUSSION

For *Vibrio* species studied so far, vibrio-specific phosphorelay quorum-sensing systems are a main regulatory pathway for modulating expression of *V. harveyi* LuxR-type transcriptional activators (reviewed by Milton, 2006; McDougald & Kjelleberg, 2006). These quorum-sensing systems tightly repress expression of LuxR-type activators at low cell density. As the bacterial population grows, signal molecules accumulate and repress the phosphorylation relay, resulting in derepressed expression of the LuxR homologues. Previously, we showed that at low cell density,

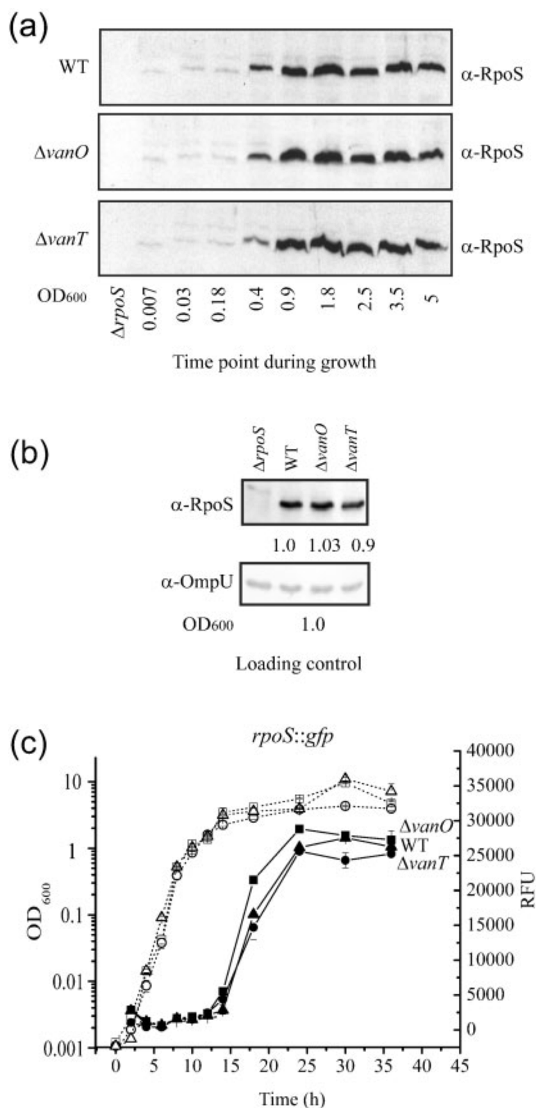

Fig. 7. VanO and VanT do not affect expression of *rpoS*. (a) Western blot analyses of RpoS were done using the wild-type (WT), the *vanO* mutant (Δ*vanO*) and the *vanT* mutant (Δ*vanT*). Culture samples were taken at various cell densities. Proteins from equal cell numbers were separated by SDS-12.5 % PAGE and Western blot analyses were done using an RpoS antiserum. The *rpoS* mutant (Δ*rpoS*; sampled at OD_{600} 1.0) was included as a negative control. (b) OmpU was used as a loading/transfer control for the Western blots. Western blots were done as in (a) except that only one time point was analysed. After detection of RpoS, the blot was stripped and an OmpU antiserum was applied. The intensities of the RpoS and OmpU bands were measured and the intensity of RpoS was equalized to that of OmpU. The mutant/wild-type ratio is given between the blots. (c) A transcriptional *rpoS*::*gfp* gene fusion was expressed from the chromosome of the wild-type (WT, black squares), the *vanT* mutant (Δ*vanT*, black circles) and the *vanO* mutant (Δ*vanO*, black triangles). Growth is indicated by dotted lines. Gfp expression (solid lines) was measured as fluorescence; relative fluorescence units (RFU) are fluorescence units of cells normalized to an OD_{600} of 0.2.

similar quorum-sensing systems in *V. anguillarum* do not completely repress the mRNA levels of *vanT*, which encodes a LuxR-type transcriptional activator (Croxatto *et al.*, 2004). This observation led to the suggestion that these regulatory systems limit rather than tightly repress the expression of VanT.

In the present study, we further characterized the expression of VanT in *V. anguillarum*. As predicted from other vibrio systems, VanO was shown to be required for expression of the sRNA Qrr1 and thus likely activates expression of other *qrr* genes seen in other *Vibrio* species but not yet identified in *V. anguillarum*. Previously, *vanT* mRNA was shown to be abundant in the wild-type; however, a *vanO* mutant showed a twofold increase in *vanT* mRNA, suggesting that repression of VanT expression does occur via the quorum-sensing regulation (Croxatto *et al.*, 2004). Here, *vanT* mRNA was shown to be more stable in a *vanO* mutant and significantly more stable in an *hfq* mutant than in the wild-type. These data suggest that *vanT* mRNA destabilization occurs via the sRNAs induced by VanO, that sRNAs work with the RNA chaperone Hfq to destabilize *vanT* mRNA, and that additional unidentified Qrr sRNAs are also likely involved as is the case for *V. harveyi* and *V. cholerae* (Lenz *et al.*, 2004; Tu & Bassler, 2007). VanO has less of an effect on destabilizing *vanT* mRNA than Hfq since it may not be absolutely required for expression of the *qrr* sRNA genes. A low level of *qrr1* expression was seen in the absence of VanO (Fig. 4). Thus, *V. anguillarum* utilizes the sRNAs induced by VanO to destabilize *vanT* mRNA; however, repression of VanT expression is not as tight as in other vibrios.

In *V. anguillarum*, the Qrr sRNAs may be less effective at destabilizing *vanT* mRNA than Qrr sRNAs in other *Vibrio* species. One possible reason is that the affinity of the Qrr sRNAs for *vanT* mRNA is less than that in other vibrios. Moreover, other target mRNAs may exist for the Qrr1 sRNA that are not yet identified and that may be better targets for Qrr1 compared to *vanT* mRNA. Recently, *V. cholerae* was shown to have additional target mRNAs other than *hapR* mRNA that are regulated by the Qrr sRNAs (Hammer & Bassler, 2007). A second explanation may be that the Qrr sRNAs may compete with other sRNAs or RNA-binding proteins for binding to *vanT* mRNA. One speculation is that since VanU was shown to activate instead of repress VanT expression, VanU may play a pivotal role in VanT expression by creating a balance between repression and activation (Croxatto *et al.*, 2004). To do this, VanU may activate a second response regulator not yet identified that induces the expression of additional sRNAs or an RNA-binding protein that can interfere with Qrr sRNA binding.

This putative balance between repression and activation of VanT expression may easily be influenced by other elements that affect the sRNAs or the response regulator, or possibly by other regulatory elements that act indepen-

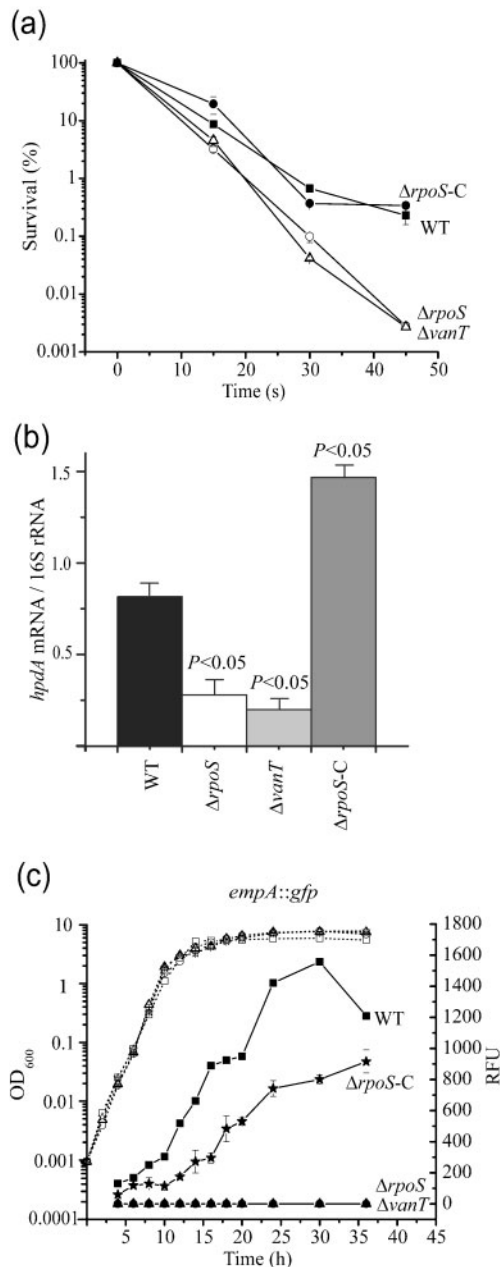

Fig. 8. VanT and RpoS regulate similar cellular functions. (a) UV irradiation survival. Cultures of the wild-type (WT, black squares), the *rpoS* mutant (Δ*rpoS*, open circles), the complemented *rpoS* mutant (Δ*rpoS*-C, black circles) and the *vanT* mutant (Δ*vanT*, open triangles) that were entering stationary phase were irradiated with UV light for various times. Aliquots (10^5 cells ml^{-1}) of each culture were removed at various time points during exposure and c.f.u. counts were determined. The results are given as percentage survival of the initial cell density at time zero. (b) Real-time qRT-PCR of *hpdA* transcripts. RNA transcripts were isolated from the wild-type (WT), *rpoS* mutant (Δ*rpoS*), the complemented *rpoS* mutant (Δ*rpoS*-C) and the *vanT* mutant (Δ*vanT*) at an OD_{600} of 1.0. Real-time qRT-PCR was done as described in Methods. *P*-values <0.05 are considered significant. (c) A transcriptional *empA* : : *gfp* gene fusion was expressed from the chromosome of the wild-type (WT, black squares), the *rpoS* mutant (Δ*rpoS*, black triangles), the complemented *rpoS* mutant (Δ*rpoS*-C, black stars) and as a control, the *vanT* mutant (Δ*vanT*, black circles). Growth is indicated by dotted lines. Gfp expression (solid lines) was measured as fluorescence; relative fluorescence units (RFU) are fluorescence units of cells normalized to an OD_{600} of 0.2.

dently of the quorum-sensing phosphorelay mechanism. As shown in this study, RpoS induced post-transcriptionally the expression of VanT at high cell density by negatively affecting Hfq expression. Consequently, *vanT* mRNA was less stable in an *rpoS* mutant than in the wild-type (Fig. 6). It is also likely that RpoS may affect *vanT* expression by other mechanisms not yet characterized. Interestingly, VanO and VanT did not regulate RpoS and RpoS did not regulate VanO, suggesting that RpoS works independently of the quorum-sensing system to regulate VanT. A model for regulation of *vanT* expression is given in Fig. 9. The role of RpoS in quorum sensing in vibrios differs. In *V. harveyi*, RpoS is not involved in regulation of luminescence (Lin *et al.*, 2002). In *V. vulnificus*, RpoS does not affect the expression of SmcR, a LuxR homologue, and vice versa (Jeong *et al.*, 2003). In *V. cholerae*, RpoS represses LuxO expression during exponential growth, which enhances derepression of HapR expression via the quorum-sensing regulatory systems (Yildiz *et al.*, 2004; Nielsen *et al.*, 2006). In addition, HapR enhances expression of RpoS, suggesting a possible autoregulation loop in this bacterium (Joelsson *et al.*, 2007).

For many bacteria, quorum-sensing regulation is integrated within other global regulatory networks. Signal molecule accumulation is not always sufficient for induction of quorum-sensing-regulated genes. Additional regulatory factors are required that allow the bacteria a second check before inducing a large number of genes that will be energetically costly (Schuster & Greenberg, 2006). A well-studied example of this type of integration is the relationship between RpoS, the global regulator for bacterial adaptation to stress responses (for a review see Hengge-Aronis, 2000), and quorum-sensing regulation in *Pseudomonas aeruginosa* (Schuster *et al.*, 2004). Transcriptome analyses showed that RpoS regulates, directly or indirectly, more than 40 % of all quorum-sensing-regulated genes. Moreover, the quorum-sensing transcriptional regulators LasR and RhlR weakly induce expression of RpoS and vice versa. Schuster *et al.* (2004) proposed a model for the interaction between these two global regulatory networks by grouping the regulated genes into classes. Some genes are directly regulated by RpoS but indirectly regulated by quorum sensing, which weakly regulates *rpoS*. Other genes are directly regulated by quorum sensing and indirectly regulated by RpoS, which weakly regulates *lasR* and *rhlR*. However, some genes are directly regulated by both RpoS and quorum sensing. Although not as well studied as in *P. aeruginosa*, the integration of RpoS regulation with quorum-sensing

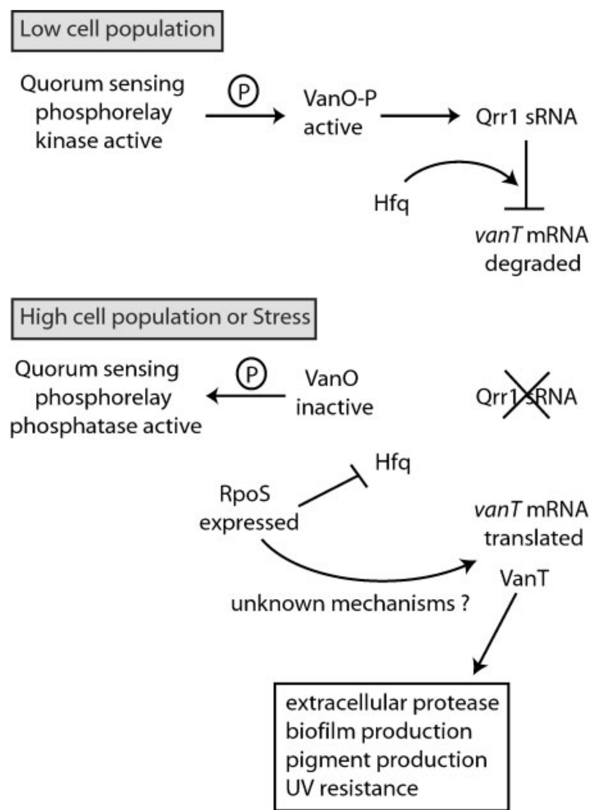

Fig. 9. Model for regulation of VanT expression. At a low cell population, quorum-sensing signal molecules are present in very low concentrations if at all. VanO, which induces expression of at least one sRNA Qrr1, is phosphorylated and activated by the quorum-sensing phosphorelay. Small RNAs together with the RNA chaperone Hfq aid degradation of *vanT* mRNA, greatly reducing the expression of VanT and all genes activated by VanT. At a high cell population, two independent regulatory systems activate *vanT* expression. First, quorum-sensing signal molecules enable a phosphatase activity to predominate in the phosphorelay, which leads to dephosphorylation and inactivation of VanO. Consequently, sRNAs are not transcribed and VanT expression is induced. Second, RpoS induces VanT expression post-transcriptionally by inhibiting expression of Hfq and possibly via other mechanisms not yet identified. Since RpoS is a main regulator of gene expression during stress response, we suggest that stress response will also induce VanT expression.

regulation is seen with other bacteria as well, for instance *Burkholderia cepacia* (Aguilar *et al.*, 2003), *Ralstonia solanacearum* (Flavier *et al.*, 1998) and *Erwinia carotovora* (Mukherjee *et al.*, 2000).

An interesting observation in this study is that Qrr1 expression in *V. anguillarum* was activated throughout growth and the expression increased during late exponential growth instead of decreasing as was seen for the *qrr* genes in *V. harveyi* (Tu & Bassler, 2007). Continual expression of Qrr1 during growth is likely due to VanO since Qrr1 expression required VanO throughout growth. If this is true, it opens up the possibility that VanO is phosphorylated and active at high cell density as well as at low cell density, possibly by a mechanism other than the quorum-sensing systems. Alternatively a more complex regulatory mechanism is likely required for the increase in Qrr1 expression during late exponential growth that involves VanO and possibly another unidentified transcriptional regulator. Further studies are required to determine why Qrr1 expression increases as VanT expression increases and as the bacterial cell population increases.

Taken together these data suggest that VanT expression appears to respond both to the cell population via quorum-sensing regulation and to stress responses via RpoS regulation. In *E. coli* and *P. aeruginosa* (Hengge-Aronis, 2000; Schuster *et al.*, 2004) the RpoS induction is suggested to occur during stress responses, such as those to heat, osmotic and oxidative stress, irrespective of the growth phase. VanT and RpoS regulate similar cellular functions, suggesting that VanT is part of the RpoS regulon in *V. anguillarum*. VanT function can thus be linked to the bacterial stress response and general physiology. Vibrio-specific quorum-sensing systems have previously been suggested to play a role in physiology by regulating starvation adaptation and stress responses (McDougald *et al.*, 2001, 2002). *V. anguillarum* is widely distributed in the aquatic environment from fresh to deep-sea waters (Urakawa & Rivera, 2006) and forms biofilms on abiotic surfaces in seawater and on fish skin tissue (Tait *et al.*, 2005; Croxatto *et al.*, 2007). Quorum sensing is likely required to coordinate and to perform numerous physiological activities needed for the bacteria to survive as a population in the highly variable aquatic environment.

ACKNOWLEDGEMENTS

This work was performed within the Umeå Centre for Microbial Research (UCMR). We would like to thank Andrea Hardman for technical assistance during the cloning of *rpoS* and Udo Bläsi for antiserum to Hfq. This work was supported by grants from the Swedish Council for Environment, Agricultural Sciences and Spatial Planning, from the Carl Tryggers Foundation, Sweden, from the Swedish Research Council and from The Wenner-Gren Foundation, which are gratefully acknowledged.

REFERENCES

Actis, L. A., Tolmasky, M. E. & Crosa, J. H. (1999). Vibriosis. In *Fish Diseases and Disorders*, vol. 3, *Viral, Bacterial and Fungal Infections*, pp. 523–557. Edited by P. T. K. Woo & E. W. Bruno. Wallingford: CABI.

Aguilar, C., Bertani, I. & Venturi, V. (2003). Quorum-sensing system and stationary-phase sigma factor (*rpoS*) of the onion pathogen *Burkholderia cepacia* genomovar I type strain, ATCC-25416. *Appl Environ Microbiol* **69**, 1739–1747.

Andersen, J. B., Sternberg, C., Poulsen, L. K., Bjørn, S. P., Givskov, M. & Molin, S. (1998). New unstable variants of green fluorescent protein for studies of transient gene expression in bacteria. *Appl Environ Microbiol* **64**, 2240–2246.

Austin, B. & Austin, D. A. (1999). Pathogenicity. In *Bacterial Fish Pathogens: Disease of Farmed and Wild Fish*, pp. 272–275. Chichester: Springer-Praxis.

Barrios, H., Valderrama, B. & Morett, E. (1999). Compilation and analysis of σ^{54}-dependent promoter sequences. *Nucleic Acids Res* **27**, 4305–4313.

Buch, C., Sigh, J., Nielsen, J., Larsen, J. L. & Gram, L. (2003). Production of acylated homoserine lactones by different serotypes of *Vibrio anguillarum* both in culture and during infection of rainbow trout. *Syst Appl Microbiol* **26**, 338–349.

Croxatto, A., Chalker, V. J., Lauritz, J., Jass, J., Hardman, A., Williams, P., Cámara, M. & Milton, D. L. (2002). VanT, a homologue of *Vibrio harveyi* LuxR, regulates serine, metalloprotease, pigment, and biofilm production in *Vibrio anguillarum. J Bacteriol* **184**, 1617–1629.

Croxatto, A., Pride, J., Hardman, A., Williams, P., Cámara, M. & Milton, D. L. (2004). A distinctive dual-channel quorum-sensing system operates in *Vibrio anguillarum. Mol Microbiol* **52**, 1677–1689.

Croxatto, A., Lauritz, J., Chen, C. & Milton, D. L. (2007). *Vibrio anguillarum* colonization of rainbow trout integument requires a DNA locus involved in exopolysaccharide transport and biosynthesis. *Environ Microbiol* **9**, 370–382.

Denkin, S. M. & Nelson, D. R. (2004). Regulation of *Vibrio anguillarum empA* metalloprotease expression and its role in virulence. *Appl Environ Microbiol* **70**, 4193–4202.

Devereux, J., Haeberli, P. & Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res* **12**, 387–395.

Flavier, A. B., Schell, M. A. & Denny, T. P. (1998). An RpoS (σ^S) homologue regulates acylhomoserine lactone-dependent autoinduction in *Ralstonia solanacearum. Mol Microbiol* **28**, 475–486.

Gottesman, S. (2004). The small RNA regulators of *Escherichia coli*: roles and mechanisms. *Annu Rev Microbiol* **58**, 303–328.

Hammer, B. K. & Bassler, B. L. (2007). Regulatory small RNAs circumvent the conventional quorum sensing pathway in pandemic *Vibrio cholerae. Proc Natl Acad Sci U S A* **104**, 11145–11149.

Heidelberg, J. F., Eisen, J. A., Nelson, W. C., Clayton, R. A., Gwinn, M. L., Dodson, R. J., Haft, D. H., Hickey, E. K., Peterson, J. D. & other authors (2000). DNA sequence of both chromosomes of the cholera pathogen *Vibrio cholerae. Nature* **406**, 477–484.

Hengge-Aronis, R. (2000). The general stress response in *Escherichia coli*. In *Bacterial Stress Responses*, pp. 161–178. Edited by G. Storze & R. Hengge-Aronis. Washington, DC: American Society of Microbiology.

Henke, J. M. & Bassler, B. L. (2004). Three parallel quorum-sensing systems regulate gene expression in *Vibrio harveyi. J Bacteriol* **186**, 6902–6914.

Higgins, D. A., Pomianek, M. E., Kraml, C. M., Taylor, R. K., Semmelhack, M. F. & Bassler, B. L. (2007). The major *Vibrio cholerae* autoinducer and its role in virulence factor production. *Nature* **450**, 883–886.

Jeong, H. S., Lee, M. H., Lee, K.-H., Park, S.-J. & Choi, S. H. (2003). SmcR and cyclic AMP receptor protein coactivate *Vibrio vulnificus vvpE* encoding elastase through the RpoS-dependent promoter in a synergistic manner. *J Biol Chem* **278**, 45072–45081.

Joelsson, A., Kan, B. & Zhu, J. (2007). Quorum sensing enhances stress response in *Vibrio cholerae. Appl Environ Microbiol* **73**, 3742–3746.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Larionov, A., Krause, A. & Miller, W. (2005). A standard curve based method for relative real time PCR data processing. *BMC Bioinformatics* **6**, 62.

Lenz, D. H., Mok, K. C., Lilley, B. N., Kulkarni, R. V., Wingreen, N. S. & Bassler, B. L. (2004). The small RNA chaperone Hfq and multiple small RNAs control quorum sensing in *Vibrio harveyi* and *Vibrio cholerae. Cell* **118**, 69–82.

Lin, Y. H., Miyamoto, C. & Meighen, E. A. (2002). Cloning, sequencing, and functional studies of the *rpoS* gene from *Vibrio harveyi. Biochem Biophys Res Commun* **293**, 456–462.

Makino, K., Oshima, K., Kurokawa, K., Yokoyama, K., Uda, T., Tagomori, K., Iijima, Y., Najima, M., Nakano, M. & other authors (2003). Genome sequence of *Vibrio parahaemolyticus*: a pathogenic mechanism distinct from that of *V. cholerae. Lancet* **361**, 743–749.

McDougald, D. & Kjelleberg, S. (2006). Adaptive responses of vibrios. In *The Biology of Vibrios*, pp. 133–155. Edited by F. L. Thompson, B. Austin & J. Swings. Washington, DC: American Society for Microbiology.

McDougald, D., Rice, S. A. & Kjelleberg, S. (2001). SmcR-dependent regulation of adaptive phenotypes in *Vibrio vulnificus. J Bacteriol* **183**, 758–762.

McDougald, D., Gong, L., Srinivasan, S., Hild, E., Thompson, L., Takayama, K., Rice, S. A. & Kjelleberg, S. (2002). Defences against oxidative stress during starvation in bacteria. *Antonie Van Leeuwenhoek* **81**, 3–13.

McGee, K., Hörstedt, P. & Milton, D. L. (1996). Identification and characterization of additional flagellin genes from *Vibrio anguillarum. J Bacteriol* **178**, 5188–5198.

Milton, D. L. (2006). Quorum sensing in vibrios: complexity for diversification. *Int J Med Microbiol* **296**, 61–71.

Milton, D. L., Norqvist, A. & Wolf-Watz, H. (1992). Cloning of a metalloprotease gene involved in the virulence mechanism of *Vibrio anguillarum. J Bacteriol* **174**, 7235–7244.

Milton, D. L., O'Toole, R., Hörstedt, P. & Wolf-Watz, H. (1996). Flagellin A is essential for the virulence of *Vibrio anguillarum. J Bacteriol* **178**, 1310–1319.

Mukherjee, A., Cui, Y., Ma, W., Liu, Y. & Chatterjee, A. K. (2000). *hexA* of *Erwinia carotovora* ssp. *carotovora* strain Ecc71 negatively regulates production of RpoS and *rsmB* RNA, a global regulator of extracellular proteins, plant virulence and the quorum-sensing signal, *N*-(3-oxohexanoyl)-L-homoserine lactone. *Environ Microbiol* **2**, 203–215.

Neiditch, M. B., Federle, M. J., Miller, S. T., Bassler, B. L. & Hughson, F. M. (2005). Regulation of LuxPQ receptor activity by the quorum-sensing signal autoinducer-2. *Mol Cell* **18**, 507–518.

Nielsen, A. T., Dolganov, N. A., Otto, G., Miller, M. C., Wu, C. Y. & Schoolnik, G. K. (2006). RpoS controls the *Vibrio cholerae* mucosal escape response. *PLoS Pathog* **2**, e109.

Norqvist, A., Hagström, Å. & Wolf-Watz, H. (1989). Protection of rainbow trout against vibriosis and furunculosis by the use of attenuated strains of *Vibrio anguillarum. Appl Environ Microbiol* **55**, 1400–1405.

O'Toole, R., Milton, D. L., Hörstedt, P. & Wolf-Watz, H. (1997). RpoN of the fish pathogen *Vibrio* (*Listonella*) *anguillarum* is essential for flagellum production and virulence by the water-borne but not intraperitoneal route of inoculation. *Microbiology* **143**, 3849–3859.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Schuster, M. & Greenberg, E. P. (2006). A network of networks: quorum-sensing gene regulation in *Pseudomonas aeruginosa. Int J Med Microbiol* **296**, 73–81.

Schuster, M., Hawkins, A. C., Harwood, C. S. & Greenberg, E. P. (2004). The *Pseudomonas aeruginosa* RpoS regulon and its relationship to quorum sensing. *Mol Microbiol* **51**, 973–985.

Tait, K., Joint, I., Daykin, M., Milton, D. L., Williams, P. & Cámara, M. (2005). Disruption of quorum-sensing in seawater abolishes attraction of zoospores of the green alga *Ulva* to bacterial biofilms. *Environ Microbiol* **7**, 229–240.

Timmen, M., Bassler, B. L. & Jung, K. (2006). AI-1 influences the kinase activity but not the phosphatase activity of LuxN of *Vibrio harveyi*. *J Biol Chem* **281**, 24398–24404.

Tu, K. C. & Bassler, B. L. (2007). Multiple small RNAs act additively to integrate sensory information and control quorum sensing in *Vibrio harveyi*. *Genes Dev* **21**, 221–233.

Urakawa, H. & Rivera, I. N. G. (2006). Aquatic environment. In *The Biology of Vibrios*, pp. 175–189. Edited by F. L. Thompson, B. Austin & J. Swings. Washington, DC: American Society for Microbiology.

Waters, C. M. & Bassler, B. L. (2005). Quorum sensing: cell-to-cell communication in bacteria. *Annu Rev Cell Dev Biol* **21**, 319–346.

Yildiz, F. H., Liu, X. S., Heydorn, A. & Schoolnik, G. K. (2004). Molecular analysis of rugosity in a *Vibrio cholerae* O1 El Tor phase variant. *Mol Microbiol* **53**, 497–515.

Edited by: P. Cornelis