The Novel Sigma Factor-Like Regulator RpoQ Controls Luminescence, Chitinase Activity, and Motility in *Vibrio fischeri*

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ABSTRACT *Vibrio fischeri*, the bacterial symbiont of the Hawaiian bobtail squid, *Euprymna scolopes*, uses quorum sensing to control genes involved in bioluminescence, host colonization, and other biological processes. Previous work has shown that AinS/R-directed quorum sensing also regulates the expression of *rpoQ* (*VF_A1015*), a gene annotated as an RpoS-like sigma factor. In this study, we demonstrate using phylogenetics that RpoQ is related to, but distinct from, the stationary-phase sigma factor RpoS. Overexpression of *rpoQ* results in elevated chitinase activity but decreased motility and luminescence, three activities associated with symbiosis. The reduction in bacterial luminescence associated with the overexpression of *rpoQ* occurs both in culture and within the light-emitting organ of the squid host. This suppression of bioluminescence is due to the repression of the *luxICDABEG* promoter. Our results highlight RpoQ as a novel regulatory component, embedded in the quorum-signaling network that controls several biological processes in *V. fischeri*.

IMPORTANCE Quorum signaling is a widely occurring phenomenon that functions in diverse bacterial taxa. It is most often found associated with species that interact with animal or plant hosts, either as mutualists or pathogens, and controls the expression of genes critical to tissue colonization. We present the discovery of *rpoQ*, which encodes a new regulatory component in the quorum-signaling pathway of *Vibrio fischeri*. RpoQ is a novel protein in the RpoS family of stationary-phase sigma factors. Unlike many other regulatory proteins involved in the quorum-signaling pathways of the *Vibrionaceae*, the distribution of RpoQ appears to be restricted to only two closely related species. The role of this regulator is to enhance some quorum-signaling outputs (chitinase activity) while suppressing others (luminescence). We propose that RpoQ may be a recently evolved or acquired component in *V. fischeri* that provides this organism with an additional level of regulation to modulate its existing quorum-signaling pathway.

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The marine bacterium *Vibrio fischeri* can exist either as a free-living organism or in symbiosis with certain bioluminescent animals, including the Hawaiian bobtail squid, *Euprymna scolopes* (1). Like other bacteria, *V. fischeri* must be able to specifically adapt to different or changing environments. Two widely distributed and functionally distinct regulatory mechanisms by which bacteria react transcriptionally to changes in their environment include quorum-sensing-mediated signaling to alter transcription factor activity (2) and alternative sigma factors to modify the core transcription machinery (3).

Quorum signaling enables many species of bacteria to coordinate gene expression according to the presence of molecules called autoinducers. *V. fischeri* encodes three quorum-sensing systems, each of which depends on a specific type of autoinducer (Fig. 1). Together, these quorum-sensing systems regulate the expression of genes involved in luminescence and colonization (2, 4–8). The LuxI-LuxR system directly controls the genes responsible for light production. Briefly, at low cell density, LuxI synthesizes low levels of the autoinducer *N*-3-oxo-hexanoyl homoserine lactone (3-oxo-C6). As the culture reaches high cell density, 3-oxo-C6 accumulates to a threshold level and binds to the transcriptional regulator LuxR. The LuxR/3-oxo-C6 complex directly activates transcription of the *luxICDABEG* operon (Fig. 1), which is responsible for the bacterium's ability to produce luciferase and bioluminescence (9, 10).

Upstream of LuxI/R within this regulatory network are two other quorum-sensing systems, AinS/R and, to a lesser extent, LuxS/PQ, which each signal through the LuxU-LuxO phosphorelay (5). At low cell density, these two systems are silent, resulting in the phosphorylation of the phosphotransferase LuxU, which in turn phosphorylates the response regulator LuxO. Phosphorylated LuxO activates the expression of a noncoding RNA, *qrr1*, which destabilizes the mRNA of the master regulator LitR in an Hfq-dependent manner (11). As cell density increases, so does signaling by AinS/R and LuxS/PQ. As a result, LuxO is dephosphorylated, *litR* mRNA is stabilized and translated, and LitR positively regulates the expression of *luxR* (12). At even higher cell density, when 3-oxo-C6 accumulates and binds LuxR, *luxICDABEG* is expressed (6), resulting in light production (Fig. 1).
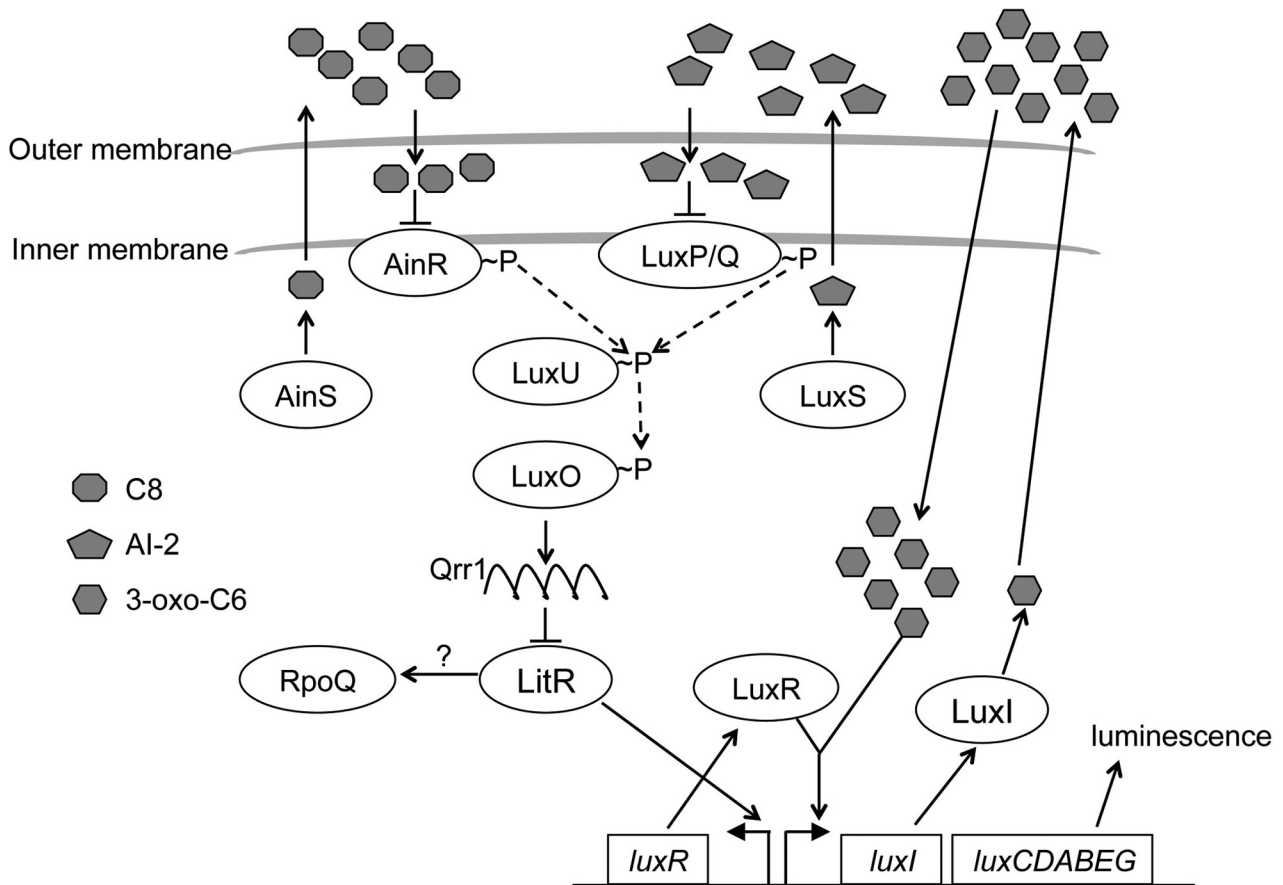
FIG 1 Model of quorum-sensing systems in *V. fischeri*. When the levels of octanoyl homoserine lactone (C8; octagons) and AI-2 (pentagons) are low, AinR and the LuxP/Q complex phosphorylate LuxU. Phosphorylated LuxU, in turn, phosphorylates LuxO, which activates the expression of the small RNA (sRNA) Qrr1. Qrr1 destabilizes the mRNA encoding LitR. Conversely, when the level of C8 and AI-2 autoinducers is high, the signaling cascade is inactivated, resulting in stable LitR production. LitR transcriptionally activates *luxR*, which encodes the transcriptional regulator LuxR. In the presence of the accumulating autoinducer 3-oxo-hexanoyl homoserine lactone (3-oxo-C6; hexagons), LuxR activates several genes, including the *luxCDABEG* locus, which is responsible for luciferase production and activity. The dashed arrows indicate phosphoryl group transfers. The question mark indicates an unknown number of steps.

In addition to luminescence and squid colonization, quorum sensing regulates other biological processes in *V. fischeri*. Signaling via AinS regulates the acetate switch by inducing *acs*, which encodes acetyl coenzyme A (acetyl-CoA) synthetase, leading to a removal of previously excreted acetate and thus to a decrease in medium acidification (13). AinS, as well as LitR, was shown to also regulate motility (6). In particular, disruption of either *ainS* or *litR* results in hypermotile cells.

Alternative sigma factors provide a different mechanism for transcriptional control through the replacement of the primary sigma factor, RpoD (14). RpoS is a particularly well-characterized example of the σ^{70} family of sigma factors, which controls cellular responses to stress conditions, including the presence of reactive oxygen species, starvation, DNA damage, extreme temperature, ethanol, and hyperosmolarity (15–17). In some bacteria, RpoS is required for virulence as well as quorum-sensing regulation (18–22). In *V. fischeri*, RpoS regulates catalase activity but is not involved in the regulation of quorum-regulated genes like *acs* (13, 23). The sigma factor σ^{54}, encoded by *rpoN*, can control nitrogen metabolism, motility, bioluminescence, and host colonization in many bacteria (24–27). Similarly, RpoN contributes to the regulation of these activities and to biofilm formation in *V. fischeri* (28).

Recently, in addition to an authentic RpoS factor, RpoS-like sigma factors have been identified in several bacteria, such as *Vibrio alginolyticus* ZJ-51 and 12G01, *Vibrio splendidus* LGP32, *Vibrio* sp. MED222, and *Vibrio campbellii* BAA_1116. There are no studies about any of these RpoS-like sigma factors except for RpoX in *V. alginolyticus* ZJ-51 (29). While RpoS and RpoX are both involved in biofilm formation and stress response, they have different functions. In this study, we focus on *VF_A1015*, which is annotated as encoding an RpoS-like sigma factor and is positively regulated by *ainS* (6). This gene is located on the second chromosome of *V. fischeri* and is linked to other genes predicted to be involved in signaling. Because of its apparent regulation by quorum signaling, we have renamed *VF_A1015* as *rpoQ* (30); here we present evidence of both its position and role within the *V. fischeri* quorum-signaling pathways.

RESULTS

rpoQ encodes a novel sigma factor-like protein regulated by quorum sensing. *VF_A1015*, here designated *rpoQ*, was originally annotated as encoding an RpoS-like sigma factor (6). This annotation arose because the amino acid sequence of RpoQ is 45% identical, and is of similar length, to that of RpoS. In contrast to *rpoS*, which is present within all fully-sequenced members of the
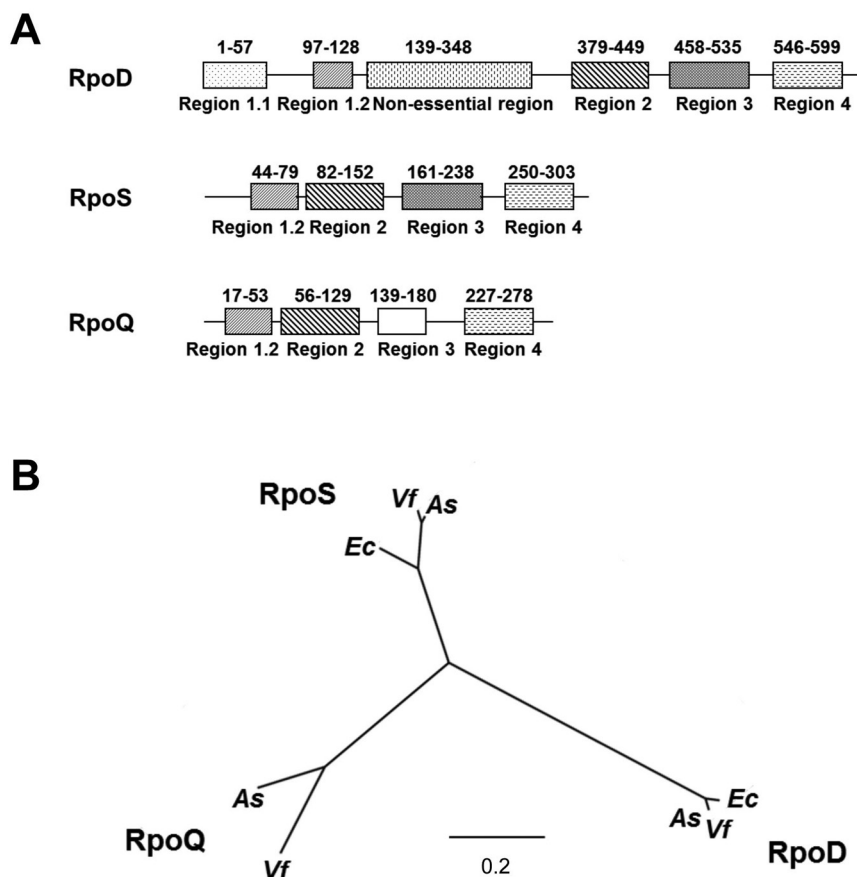
FIG 2 Structure and phylogeny of Rpo proteins. (A) Domain comparison between the *V. fischeri* RpoD, RpoS, and RpoQ proteins. Numbers above the conserved domains indicate amino acid positions. (B) Neighbor-joining phylogenetic analysis of RpoQ. Sequences spanning region 2 through region 4 were used to generate the phylogenetic tree with the PHYLIP software program. The tree was bootstrapped with 1,000 replicates, and all nodes were supported 100%. Abbreviations used are as follows: *Vf*, *Vibrio fischeri*; *As*, *Aliivibrio salmonicida*; *Ec*, *Escherichia coli*. The scale bar represents genetic distances in substitutions per residue.

Vibrionaceae, we were able to identify *rpoQ* homologues within the genomes of only two species: *V. fischeri* and *Aliivibrio* (*Vibrio*) *salmonicida* (*VSAL_II0319*). Examination using the Pfam database revealed that RpoQ contains four conserved domains: (i) σ^{70} region 1.2, (ii) σ^{70} region 2, (iii) σ^{70} region 3, and (iv) σ^{70} region 4, each of which is significant except σ^{70} region 3 (Fig. 2A). In contrast, RpoS and RpoD of *V. fischeri* each have an identifiable σ^{70} region 3 in the predicted location. Region 3 is involved in binding to the core RNA polymerase and recognition of the extended −10 promoter. Only regions 2 and 4 are highly conserved and play important roles in binding the core RNA polymerase and in recognition of the −10 and −35 promoters, respectively (31). Phylogenetic analysis of the amino acid sequences spanning from region 2 to region 4 of the RpoQ, RpoS, and RpoD homologues among two species of the *Vibrionaceae* (*V. fischeri* and *A. salmonicida*) and *Escherichia coli* indicates that RpoQ is a protein that is distinct from RpoS (Fig. 2B).

Previous results from a global transcriptional analysis (6) suggested that expression of *rpoQ* is controlled by the response regulator LuxO, which is involved in quorum sensing (Fig. 1). To validate and extend this analysis, we used quantitative reverse transcriptase PCR (qRT-PCR) to measure the expression of *rpoQ* in different mutant backgrounds of *V. fischeri*. Consistent with the previous study, the level of *rpoQ* expression is elevated about 2-fold in a Δ*luxO* mutant when compared to that of a wild-type strain (Fig. 3). In contrast to the Δ*luxO* mutant, both a Δ*litR* single mutant and a Δ*luxO* Δ*litR* double mutant exhibited 4-fold-lower levels of *rpoQ* expression than the wild type (Fig. 3), demonstrating that the regulation of *rpoQ* by LuxO operates through LitR.

RpoQ regulates gene expression in *V. fischeri*. To determine whether RpoQ regulates expression of other genes in *V. fischeri*, we generated a Δ*rpoQ* in-frame deletion mutant that avoids potential polar effects on the downstream gene *VF_A1014* (Fig. 4A). Previous work suggested that, like *rpoQ*, the genes *VF_A1016* and *VF_A1017*, which are predicted to encode a histidine kinase and a response regulator, respectively, are also regulated by the LuxO (6). Due to the genetic linkage between this operon and *rpoQ* (they are divergently described, with a 449-nucleotide intergenic region) (Fig. 4A), we explored the possibility that the LuxO-dependent regulation of *VF_A1016* and *VF_A1017* requires RpoQ activity. Consistent with the previous report (6), we found that the expression level of *VF_A1016* was elevated more than 2-fold in a Δ*luxO* mutant over that in the wild-type strain (Fig. 4B). In contrast, *VF_A1016* expression levels were similar to wild-type levels in a Δ*rpoQ* mutant and remained low in the Δ*luxO* Δ*rpoQ* double mutant. These data indicate that RpoQ is required for LuxO-
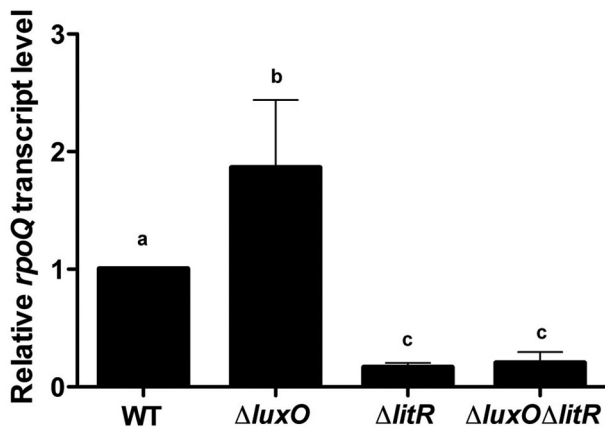
FIG 3 qRT-PCR analysis of *rpoQ* expression in *V. fischeri* strains MJM1100 (wild type [WT]), TIM305 (Δ*luxO*), TIM358 (Δ*litR*), and TIM355 (Δ*luxO*Δ*litR*). The strains were grown in LBS medium and harvested at an OD_{600} of 0.5. The transcript levels of *rpoD* were used as a normalizing control. Data are relative to wild-type levels, set at 1.0. Graphical and error bars indicate the averages and standard deviations of data from three independent experiments, respectively. Shared letters above the bars indicate no statistically significant difference ($P > 0.05$), whereas different letters indicate that there is a significant difference ($P < 0.01$) in *rpoQ* transcript levels between those strains (analysis of variance [ANOVA] and Tukey's honestly significant difference [HSD] test).

dependent changes in *VF_A1016*. Furthermore, when *rpoQ* was integrated into the Tn*7* site of the chromosome of a Δ*luxO* Δ*rpoQ* double mutant (strain CA6), the transcription level of *VF_A1016* was partially restored relative to that for the Δ*luxO* single mutant (see Fig. S1 in the supplemental material). Based on the finding that *VF_A1016* was regulated by quorum signaling, we were led to test the possibility that transcription of *VF_A1016* is reduced in the Δ*rpoQ* mutant at a higher cell density. As predicted, the transcription level of *VF_A1016* is higher (12-fold) in the wild-type strain than in the Δ*rpoQ* mutant (Fig. 4C). In addition, RT-PCR analysis indicated that *VF_A1016* and *VF_A1017* are cotranscribed (Fig. 4D). Together, these results suggest that the LuxO signaling system transcriptionally controls RpoQ, which in turn activates transcription of a two-component system. Using 5′ rapid amplification of cDNA ends (RACE), we mapped the transcriptional start sites of *rpoQ* and *VF_A1016* (Fig. 4A). Neither start site was detected in the Δ*rpoQ* mutant, supporting the idea that transcription of *rpoQ* and *VF_A1016* requires RpoQ (data not shown).

RpoQ represses luminescence in vivo and in vitro. Because quorum signaling controls both early and late colonization genes (4, 6, 8, 12), we asked whether RpoQ regulates factors involved in light-organ symbiosis. Juvenile squid colonized by the Δ*rpoQ* mutant exhibited normal symbiont population levels and luminescence over the first 48 h postcolonization (data not shown), during which the symbionts have expressed both early and late quorum-sensing responses (6). Furthermore, cocolonization experiments using the Δ*rpoQ* mutant and a wild-type strain did not reveal a competition defect in the absence of RpoQ (data not shown). Together, these results suggest that the initial steps of bacterial colonization of the host, involving known early and late quorum-regulated colonization factors, do not require RpoQ.

As an alternative approach to examine RpoQ function during colonization, we constructed a plasmid (pXCD10) that conditionally (i.e., in the presence of isopropyl-β-D-thiogalactopyranoside [IPTG]) overexpresses RpoQ, by cloning *rpoQ* downstream of the *trc* promoter (32). Such approaches, with caveats for possible artifacts, have been used successfully to manipulate target genes of other extracytoplasmic function (ECF) sigma factors (33–37). Juvenile squid colonized with wild-type *V. fischeri* harboring pXDC10 were placed in seawater, either with or without IPTG. The level of luminescence of symbionts overexpressing RpoQ was significantly lower than (i.e., <20% of) that of wild-type cells harboring the control plasmid, pTM214 (Fig. 5A). In contrast, there were no significant differences in luminescence per CFU between animals colonized with wild-type cells harboring pTM214 (vector control) with or without IPTG or wild-type cells harboring pXDC10 without IPTG; in addition, the CFU levels were similar among all animals (data not shown), indicating that overexpressing RpoQ did not affect the ability of *V. fischeri* to colonize the host.

Consistent with the results described above, the luminescence emitted by cells in culture was reduced more than 10-fold when *rpoQ* was overexpressed (Fig. 5B). In contrast, overexpression of two other sigma factors, *rpoS* and *rpoD*, did not repress luminescence. Taken together, these results provide evidence that the cells are not disturbed by these overexpression studies and show that RpoQ represses luminescence both *in vivo* and *in vitro*, as well as further demonstrating a functional distinction between RpoQ and RpoS.

RpoQ represses the luxICDABEG promoter. Because overexpression of RpoQ leads to repressed luminescence, we hypothesized that RpoQ affects transcription of the *lux* operon. To facilitate experiments that test this idea, we integrated the IPTG-inducible *rpoQ* gene into the chromosome at the Tn*7* site, resulting in strain TIM366. Overexpression of this single copy of *rpoQ* by IPTG addition also repressed luminescence but to only 31% of the extent seen when *rpoQ* is induced from the multicopy plasmid pXDC10. A two-color fluorescent reporter of *luxICDA-BEG* promoter activity (11) was used to determine whether RpoQ affects transcription of this promoter. The reporter plasmid (pTM280) was constructed by cloning the *luxICDABEG* promoter upstream of *gfp*, which encodes green fluorescent protein (GFP). This plasmid also constitutively expresses the red fluorescent protein mCherry. The *luxICDABEG* transcription level is quantitatively measured by the GFP/mCherry fluorescence ratio; this normalization accounts for potential variations in either growth rates or copy number of the reporter plasmid in different strains. We found that there was a significant difference in the level of *luxICDABEG* transcription in strain TIM366 when *rpoQ* was induced (Fig. 6). As predicted, *luxICDABEG* transcription was higher in a Δ*luxO* mutant and lower in a Δ*litR* mutant than that in the wild-type strain, independent of supplemented IPTG. In contrast, overexpression of *rpoQ* had no effect on *luxR* promoter activity (data not shown), and overexpression of VF_A1016 did not affect luminescence (see Fig. S2 in the supplemental material).

We next asked whether the repression of the *luxICDABEG* promoter by RpoQ functioned through (i) LitR, the transcriptional regulator known to affect the activity of the *luxICDABEG* promoter, (ii) *VF_A1014*, which is the gene downstream of *rpoQ* that encodes a GGDEF- and EAL-domain-containing protein, (iii) a nearby gene encoding the response regulator VF_A1017, or (iv) ArcA, the recently discovered negative regulator of luminescence (38). We moved the inducible *rpoQ*-bearing plasmid pXDC10 into the Δ*litR* (TIM358), Δ*VF_A1014* (CA8), *VF_A1017*
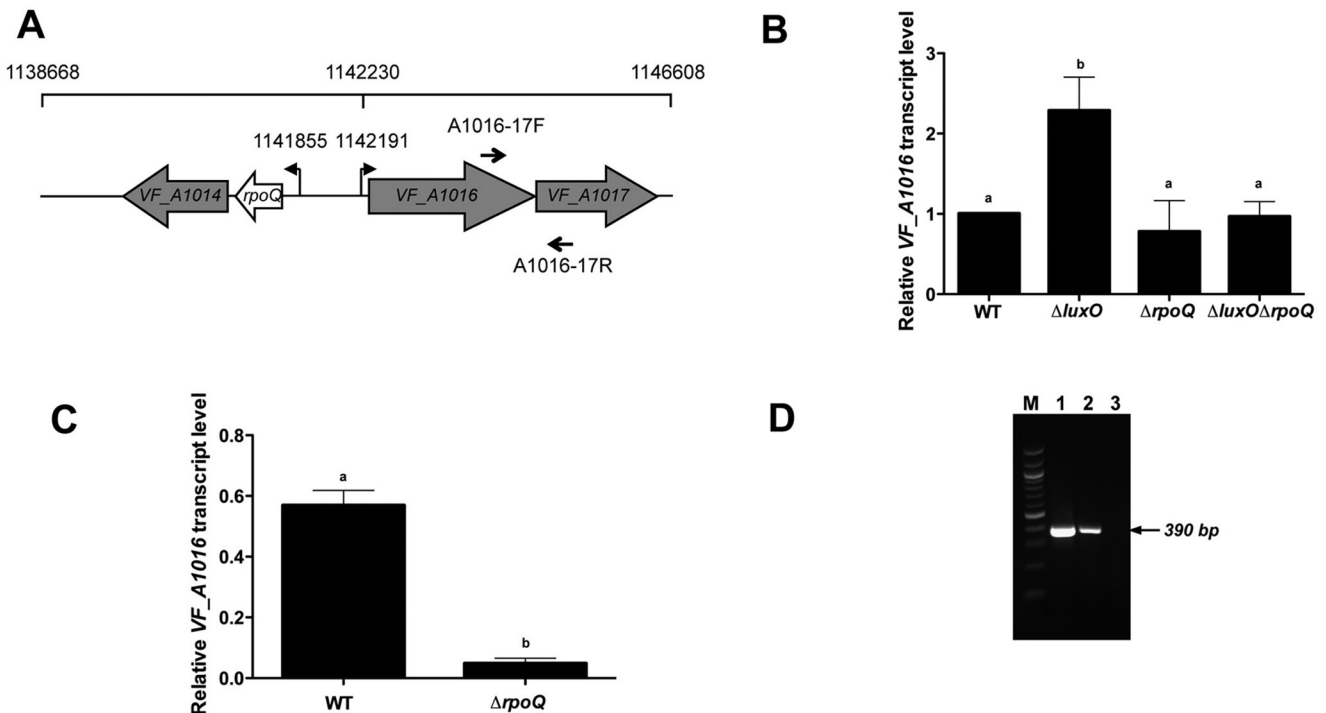
FIG 4 LuxO and RpoQ regulate transcription of the *VF_A1016-VF_A1017* operon. (A) Schematic representation of the *rpoQ* locus. Open reading frames (ORFs) are indicated as the four block arrows. *VF_A1014* is predicted to encode a GGDEF-EAL-domain-containing protein of unknown function. *VF_A1016* and *VF_A1017* are predicted to encode a two-component histidine kinase sensor and response regulator, respectively. Numbers on the scale bar indicate the location (in bp from the origin) along the second chromosome of *V. fischeri*. Transcriptional start sites of *rpoQ* and *VF_A1016* determined by 5′-RACE are shown upstream of corresponding genes. Primer sites used in RT-PCR analysis shown in panel D are indicated by arrows. (B) qRT-PCR analysis of *VF_A1016* expression in *V. fischeri* strains MJM1100 (WT), TIM305 (Δ*luxO*), CA1 (Δ*rpoQ*), and CA4 (Δ*luxO*Δ*rpoQ*). The strains were grown in LBS medium and harvested at an OD$_{600}$ of 0.5. The transcript levels of *rpoD* were used to normalize *VF_A1016* levels. Data are relative to wild-type levels, set at 1.0. Graphical and error bars indicate the averages and standard deviations of data from three independent experiments, respectively. Shared letters above the bars indicate no statistically significant difference ($P > 0.05$), whereas different letters indicate that there is a significant difference ($P < 0.05$) in *VF_A1016* transcript levels between those strains (ANOVA and Tukey's HSD test). (C) qRT-PCR analysis of *VF_A1016* expression in *V. fischeri* strains MJM1100 (WT) and CA1 (Δ*rpoQ*) at a high cell density. The strains were grown in LBS medium and harvested at an OD$_{600}$ of 3.0, which is 6 times denser than the cultures used for panel B. The transcript levels of *rpoD* were used to normalize *VF_A1016* levels. Graphical and error bars indicate the averages and standard deviations, respectively, of data from three independent experiments. Different letters indicate that there is a significant difference ($P < 0.001$) in *VF_A1016* transcript levels between the WT and the Δ*rpoQ* mutant (Student's *t* test). (D) RT-PCR analysis of *VF_A1016-VF_A1017* transcripts. Templates used are genomic DNA (lane 1), cDNA (lane 2), and RNA (lane 3). The location of the primer pair (A1016-17F/A1016-17R) is shown in panel A. Lane M contains molecular size standards (size range, in bp, from top to bottom: 1,517, 1,200, 1,000, 900, 800, 700, 600, 500/517, 400, 300, 200, and 100).

(KV1612), and Δ*arcA* (AMJ2) mutants and measured their effect on the level of culture luminescence. Overexpression of RpoQ repressed luminescence in all of the strains tested (data not shown), suggesting that the repression of luminescence by RpoQ is independent of transcriptional regulation by LitR, VF_A1014, VF_A1017, or ArcA.

Overexpression of RpoQ decreases motility. To investigate whether RpoQ is involved in the regulation of other quorum-signaling-dependent phenotypes besides luminescence, we examined the impact of overexpressing RpoQ on *V. fischeri* flagellar motility. The Δ*litR* mutant displayed hypermotile phenotypes, as previously reported (6). In contrast, when RpoQ was overexpressed in either the wild type or the Δ*litR* mutant, both were essentially nonmotile (Fig. 7), suggesting that quorum signaling regulates motility, at least in part, through an RpoQ mechanism downstream of LitR. Interestingly, overexpression of VF_A1016 had a small positive effect on wild-type motility (see Fig. S3 in the supplemental material).

RpoQ elevates chitinase activity. Quorum signaling negatively regulates chitinase activity in *Vibrio harveyi*; however, in

Chromobacterium violaceum it leads to its activation (39, 40). To test whether and how RpoQ might control chitinase activity in *V. fischeri*, we examined the spent culture supernatants of the wild type and the Δ*rpoQ* mutant carrying the vector plasmid (pTM214) and the inducible *rpoQ* allele (pXDC10), as well as the Δ*litR* mutant harboring the vector plasmid (pTM214). The supernatant of a culture of the Δ*rpoQ* mutant showed chitinase activity that was significantly lower (about 50%) than that of the wild type (Fig. 8A). In contrast, the supernatants of both the wild type and the Δ*rpoQ* mutant overexpressing RpoQ showed chitinase activity that was 2.3- to 2.8-fold-higher than that of the wild type carrying the vector. These results indicate that an increased level of RpoQ elevates chitinase activity in *V. fischeri*. Interestingly, there was no difference in chitinase activity between the wild-type strain and a Δ*litR* mutant (Fig. 8A), suggesting that the basal levels of RpoQ present even in a Δ*litR* mutant are sufficient to result in normal chitinase activity (Fig. 9). Furthermore, the supernatant of the wild type overexpressing VF_A1016 also showed chitinase activity that was 2.4-fold higher than that of the wild type carrying the vector (see Fig. S4 in the supplemental material), supporting the
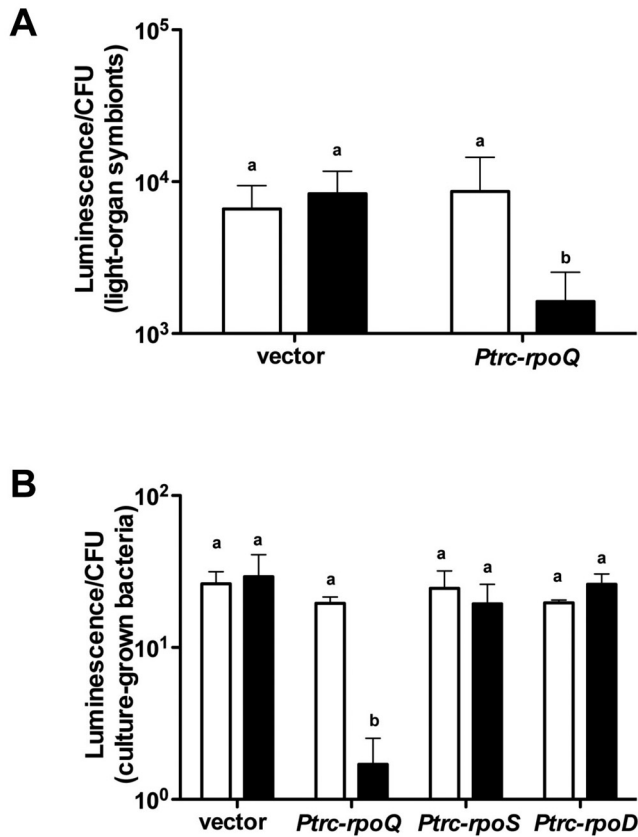
FIG 5 The impact of RpoQ on luminescence. (A) Squid luminescence (per CFU) of wild-type *V. fischeri* cells harboring either pTM214 (vector) or pXDC10 (P_{trc}-*rpoQ*) in the presence (black bars) or absence (white bars) of 1 mM IPTG. Graphical and error bars indicate the averages and standard deviations. One representative experiment performed in triplicate is analyzed. (B) Culture luminescence per CFU of wild-type MJM1100 harboring pTM214 (vector), pXDC10 (P_{trc}-*rpoQ*), pXDC35 (P_{trc}-*rpoS*), and pXDC36 (P_{trc}-*rpoD*), with (black bars) or without (white bars) IPTG addition. Graphical and error bars indicate, respectively, the averages and standard deviations, respectively, of data from three independent experiments. Shared letters above the bars indicate no statistically significant difference ($P > 0.05$), whereas different letters indicate a significant difference ($P < 0.001$ in panel A and $P < 0.05$ in panel B) between the luminescence levels (ANOVA and Tukey's HSD test).

possibility that RpoQ may elevate chitinase activity through VF_A1016.

Previous studies predicted that there are 7 chitinase genes encoded by *V. fischeri* ES114: *VF_0655*, *VF_0986*, *VF_1059*, *VF_1146*, *VF_1390*, *VF_1598*, and *VF_A0715* (41). To test which of these chitinase genes is regulated by RpoQ, qRT-PCR was carried out using the wild-type strain carrying either the empty vector plasmid (pTM214) or the inducible *rpoQ* allele (pXDC10) in the present of IPTG. The transcription levels of four of the genes (*VF_0986*, *VF_0655*, *VF_1598*, and *VF_1390*) were significant higher when RpoQ was overexpressed (Fig. 8B). In contrast, the transcription levels of *VF_A0715*, *VF_1146*, and *VF_1059* were independent of RpoQ. These data reveal that RpoQ activates the transcription of some chitinase genes, resulting in elevated levels of secreted exochitinase activity.

DISCUSSION

In this study, we have shown that RpoQ is a novel component that functions downstream of the core LuxO-LitR quorum-signaling
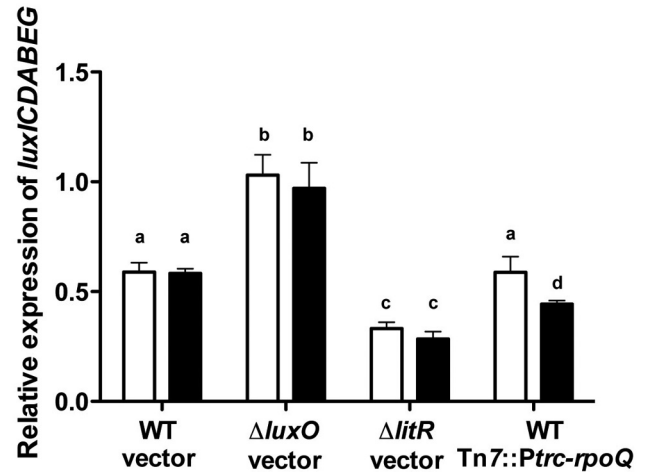

FIG 6 Transcriptional activity of *luxICDABEG*. Relative expression levels of *luxICDABEG* (GFP/mCherry) for strains TIM313 (WT vector), TIM315 (Δ*luxO* vector), CA19 (Δ*litR* vector), and TIM366 (WT Tn*7*::P_{trc}-*rpoQ*) either with (black bars) or without (white bars) the addition of 1 mM IPTG. Each strain carried the two-color fluorescent reporter plasmid pTM280 (P_{luxI}-*gfp* P_{tetA}-*mCherry*). Graphical and error bars indicate the averages and standard deviations of data from three independent experiments, respectively. Shared letters above the bars indicate no statistically significant difference ($P > 0.05$), whereas different letters indicate a significant difference ($P < 0.05$) between *luxICDABEG* transcript levels (ANOVA and Tukey's HSD test).

system of *V. fischeri* (Fig. 1). The domain structure and phylogenic similarities between RpoQ and RpoS (Fig. 2) suggest that the former could have been evolutionarily derived from the latter, per-

FIG 7 The motility of wild-type *V. fischeri* cells harboring either pTM214 (WT vector) or pXDC10 (WT P_{trc}-*rpoQ*), compared to strain TIM358 harboring either pTM214 (Δ*litR* vector) or pXDC10 (Δ*litR* P_{trc}-*rpoQ*). Relative rates of motility were determined in minimal medium containing 1 mM IPTG and solidified with 0.25% agar. One representative experiment of three is shown. Growth rates of the strains were comparable in the minimal medium (data not shown).

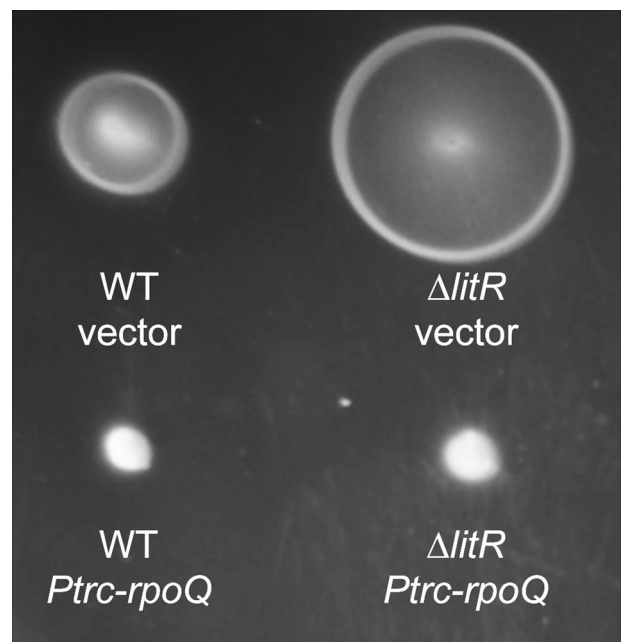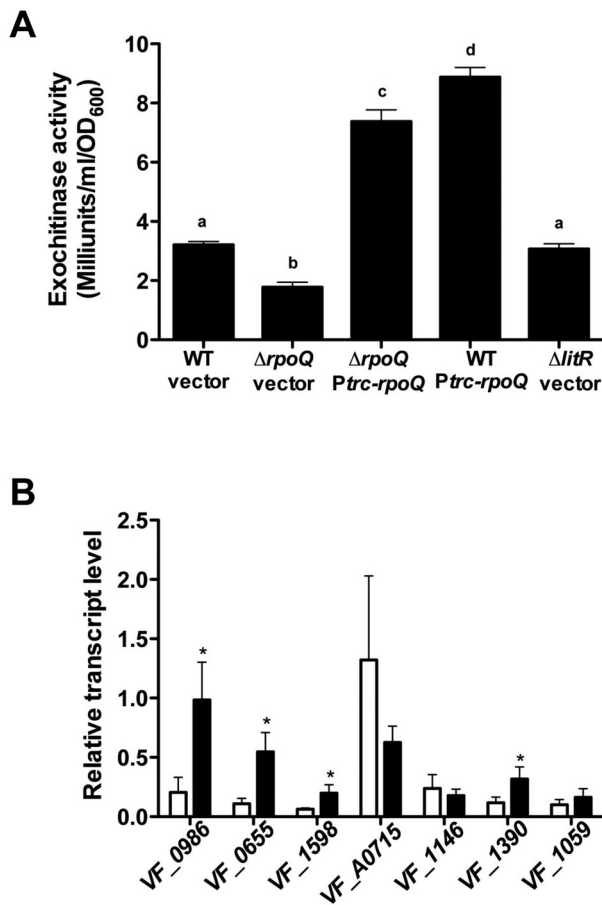FIG 8 Regulation of chitinase genes by RpoQ. (A) Exochitinase activity of wild-type *V. fischeri* cells harboring either pTM214 (WT vector) or pXDC10 (WT P_{trc}-*rpoQ*), strain CA1 harboring either pTM214 (Δ*rpoQ* vector) or pXDC10 (Δ*rpoQ* P_{trc}-*rpoQ*), and TIM358 harboring pTM214 (Δ*litR* vector). Secreted chitinase activity was determined in the cell-free supernatant. Graphical and error bars indicate, respectively, the averages and standard deviations of data from three independent experiments. Shared letters above the bars indicate no statistically significant difference ($P > 0.05$), whereas different letters indicate a significant difference ($P < 0.05$) in exochitinase activity between those strains (ANOVA and Tukey's HSD test). (B) Wild-type cells carrying the empty vector plasmid (pTM214; white bars) or the inducible *rpoQ* allele (pXDC10; black bars) were grown in LBS medium supplemented with 1 mM IPTG and harvested at an OD_{600} of 1.4 to 1.5. VF_0986, chitodextrinase; VF_0655, endochitinase; VF_1598, exochitinase; VF_A0715, chitodextrinase precursor; VF_1146, chitodextrinase precursor; VF_1390, chitinase; VF_1059, chitinase. Graphical and error bars indicate, respectively, the averages and standard deviations of data from four independent experiments. The asterisks indicate a significant difference in transcription levels between each set of paired strains.
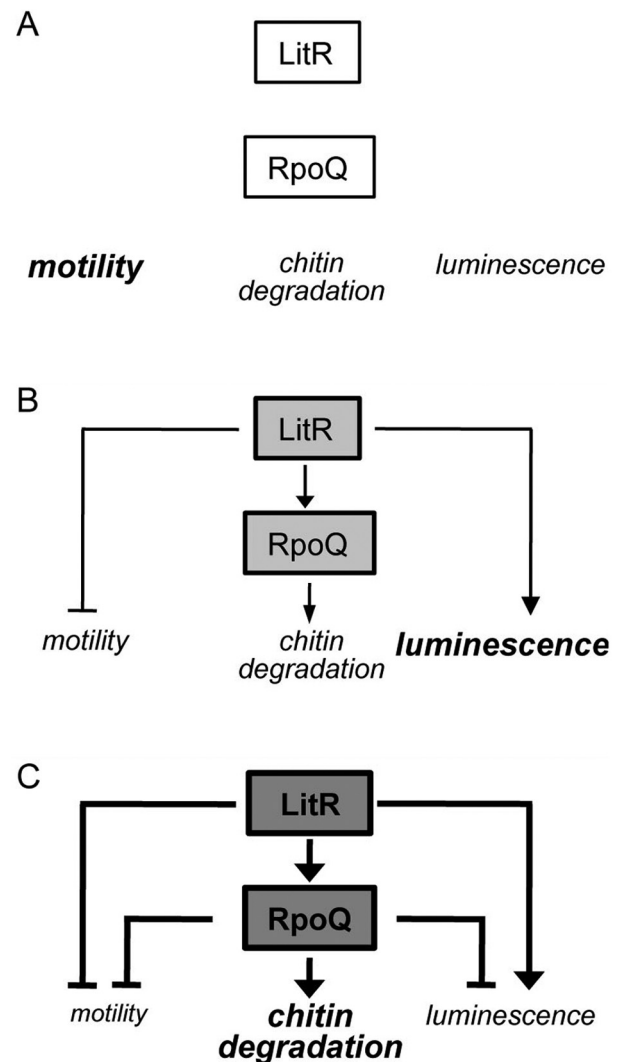

FIG 9 A model of RpoQ regulation of quorum-sensing-dependent phenotypes in *V. fischeri*. (A) At low cell density (no quorum signaling), LitR has little effect on basal levels of motility (high) and luminescence (low). (B) At moderate cell density, LitR signaling represses motility and induces luminescence as previously described (5); LitR also activates transcription of RpoQ. However, while this level of RpoQ remains insufficient to affect motility and luminescence, it does lead to increased chitinase activity. (C) At high cell density, LitR signaling increases enough to induce RpoQ to a higher level, leading to a strong repression of both motility and luminescence, as well as an induction of chitinase activity. Lines with arrowheads indicate positive regulation, while those with a bar at the end indicate negative regulation; the lines may represent a pathway with several steps of regulation.

haps after the divergence between the clade containing *V. fischeri* and *A. salmonicida* and the rest of the *Vibrionaceae* (42). Interestingly, *rpoQ* and its *A. salmonicida* homologue, *VSAL_II0319*, are found on the smaller, second chromosomes of *V. fischeri* and *A. salmonicida*, respectively, where many paralogs and nonessential genes are also located (43). Such a position would be consistent with our hypothesis that *rpoQ* is a recently duplicated gene and diverged in the *Vibrionaceae* (44, 45).

RpoS has been shown to participate in bacterial quorum sensing; for instance, in *Vibrio anguillarum*, RpoS induces expression of the LitR homologue, VanT, in a manner independent of the LuxO homologue, VanO (21). Because of the role of VanT in inducing pigment and metalloprotease production, these authors suggest that *V. anguillarum* RpoS works with the quorum-signaling system to regulate survival and stress responses. Similarly, in *Vibrio cholerae*, RpoS can upregulate the expression of its LitR homologue, HapR (19, 46). However, in this species, HapR in turn induces the expression of RpoS, which reinforces the stress response (47). In contrast, RpoS does not affect quorum-sensing regulation in *V. harveyi* (15). Yet another pattern is seen within *Pseudomonas aeruginosa*, in which quorum sensing regulates the transcription of *rpoS*. RpoS, in turn, induces expression of *lasR*

and *rhlR*, which encode homologues of the *V. fischeri* activator LuxR (20). In addition, RpoS is involved in the repression of *rhlI*, which encodes one of the autoinducer synthases in *P. aeruginosa* (21). Thus, in *P. aeruginosa*, whose quorum-signaling pathway has no LuxU-LuxO phosphorelay nor LitR master regulator homologs, the impact of RpoS functions instead through the LuxR and LuxI homologs. This mode of regulation contrasts with that of *V. fischeri* RpoQ, which has no effect on *luxR* expression. As described above, other than in *V. fischeri*, we can identify an *rpoQ* homolog only within the genome of the closely related *A. salmonicida*. Because homologs of other quorum-sensing regulators can also be found in this species (11, 48), we hypothesize that RpoQ is a component of quorum signaling in *A. salmonicida* as well.

Numerous studies have shown that quorum sensing controls functions important for host interaction, such as luminescence, motility, and chitin utilization (4, 6, 8, 40). Our results revealed that RpoQ is involved in regulation of each of those functions (Fig. 9). The *lux* regulon is responsible for the production of bioluminescence and has served as a model for the genetic regulation underlying quorum sensing (10, 49, 50). Several genetic elements (e.g., those encoding ArcA and LexA) have been reported to negatively affect luminescence by repressing the expression of the *luxICDABEG* operon (38, 51, 52). Our finding that overexpression of RpoQ leads to repressed luminescence suggests that RpoQ is also a source of negative regulation in the circuit controlling *lux* expression.

In addition to luminescence, the quorum-sensing system also regulates motility in several different bacteria (53). In *V. fischeri*, the AinS-LitR pathway has been shown to be involved in the repression of motility (6, 12). Similarly, overexpression of RpoQ resulted in a significant motility defect (Fig. 7), suggesting that RpoQ may regulate the disappearance of flagellation at the extremely high cell density found within the light organ (54), thereby not only conserving nutrients and energy but also allowing the cells to pack more tightly.

Recently, transcriptional evidence has suggested that the *V. fischeri* population in the mature squid light-organ symbiosis ferments chitin at night, resulting in the production of formic and acetic acids (55). This hypothesis has been supported by the discovery that host blood cells carry particulate chitin to the tissue surrounding the symbionts (56). Essentially all *Vibrio* species encode numerous chitinases (41), and the elevated chitinase activity associated with high RpoQ levels may allow *V. fischeri* to more effectively use host-derived chitin as a nutrient.

How might LitR and RpoQ work together to regulate these three quorum-sensing-dependent phenotypes? At low LitR levels (e.g., in uninduced cells or the Δ*litR* mutant), cells produce low luminescence and are highly motile (Fig. 9A). However, low LitR has no effect on chitinase activity. As quorum signaling leads to higher levels of LitR, its increased activity results in higher luminescence and lower motility (Fig. 9B). Meanwhile, at this early stage of induction, LitR activates transcription of *rpoQ*. However, while inducing chitinase activity, RpoQ levels remain insufficient to have an effect on motility and luminescence. At very high LitR levels, RpoQ is highly induced and begins to have a dominant effect on other downstream genes, reversing luminescence output and increasing the loss of motility, as well as further activating chitinase activity (Fig. 9C). This model is also supported by the observation that luminescence (see Fig. S2 in the supplemental

material) and motility (see Fig. S3) but not chitinase activity (see Fig. S4) are directly regulated by LitR.

At this point in our analyses, we do not know whether RpoQ regulates using a sigma-like activity. Nevertheless, the results of a domain comparison suggest that RpoQ is a group 2 sigma factor (Fig. 2A), which generally play a role in upregulating transcription initiation at target promoters (31). Therefore, while it is possible that RpoQ directly binds to the promoter regions of the *luxICDABEG* operon or motility-related genes, thereby repressing transcription, we favor the hypothesis that RpoQ upregulates transcription of an unknown factor(s) that then represses transcription of the target genes identified here.

In summary, the novel *V. fischeri* sigma-like factor RpoQ is similar to but distinct from the alternative sigma factor, RpoS, and is regulated by the quorum-sensing system. RpoQ is involved in the regulation of several quorum-sensing-dependent phenotypes at high cell density (Fig. 9), suggesting that it acts as a global regulator of symbiotic maintenance in the host, enhancing some of those phenotypes while attenuating others.

MATERIALS AND METHODS

Bacterial strains and media. Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown at 37°C in LB medium (1% [wt/vol] tryptone, 0.5% [wt/vol] yeast extract, and 1% [wt/vol] NaCl). Liquid cultures of *V. fischeri* were grown at 28°C with aeration either in LBS medium (1% [wt/vol] tryptone, 0.5% [wt/vol] yeast extract, 2% [wt/vol] NaCl, 50 mM Tris-HCl [pH 7.5]) or in SWT medium (57). When necessary, antibiotics were used at the following concentrations: 25 μg ml^{-1} chloramphenicol (Cam) and 50 μg ml^{-1} kanamycin (Kan) for *E. coli* or 2.5 μg ml^{-1} Cam and 5 μg ml^{-1} erythromycin (Erm) for *V. fischeri*. Stock solutions of the *N*-3-oxo-hexanoyl homoserine lactone autoinducer (3-oxo-C6) were made in dimethyl sulfoxide.

Bioinformatics analysis. Homologues of *V. fischeri* RpoQ (VF_A1015) were identified in *A. salmonicida* strain LFI1238 (VSA-L_II0319) by BLAST analysis (58). Homologues of *V. fischeri* RpoD (VF_2254) and RpoS (VF_2067) were identified in *E. coli* strain MG1655 and *A. salmonicida* strain LFI1238 by BLAST. The conserved domains of these sigma factors were found using the Pfam database (59). Amino acid sequences were aligned using the ClustalX 2.0.11 software program (60) and used to generate a neighbor-joining tree using the PHYLIP program (61).

Plasmid and strain construction. All the restriction enzymes used were from NEB (New England Biolabs, Ipswich, MA). *V. fischeri* mutants CA1 (Δ*rpoQ*), CA4 (Δ*luxO* Δ*rpoQ*), and CA8 (Δ*VF_A1014*) were constructed using allelic exchange based on pEVS79 (62). To construct the plasmid pSVS112, which contains the Δ*rpoQ* allele as an in-frame deletion of the sequence encoding amino acids 10 to 288, the homologous regions upstream and downstream of *rpoQ* were amplified by PCR using the primer pairs rpoQF1/rpoQR1 and rpoQF2/rpoQR2 (Table 2), digested by XhoI/SalI and SalI/SpeI, and ligated into the Cam resistance-encoding vector pEVS79 (62), which had been digested with XhoI/SpeI. To construct the plasmid pXDC1, which contains the Δ*VF_A1014* allele as an in-frame deletion of the sequence encoding amino acids 34 to 421, the homologous regions upstream and downstream of *VF_A1014* were amplified by PCR using the primer pairs VF_A1014F1/VF_A1014R1 and VF_A1014F2/VF_A1014R2 (Table 2), digested by XhoI/EcoRI and EcoRI/BamHI, and ligated into pEVS79, which had been digested with XhoI/BamHI. Each of these allelic-exchange vectors was then moved into *V. fischeri* by triparental mating using the conjugal helper plasmid pEVS104 (62). Conjugants were selected on LBS agar medium containing Cam, after which the appearance of a second recombination event was screened for on LBS agar medium containing no antibiotic. Colonies were purified, and their sequence was confirmed by PCR using the primers rpoQF3/rpoQR3 and rpoQF4/rpoQR4 or VF_A1014F3/VF_A1014R3

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Relevant genotype	Source or reference(s)
Strain		
MJM1100	Sequenced wild-type *V. fischeri* ES114	30, 43
TIM305	ES114 Δ*luxO*	11
TIM355	ES114 Δ*luxO* Δ*litR*	11
TIM358	ES114 Δ*litR*	11
TIM313	ES114 Tn*7*::pEVS107 (*erm*)	11
TIM315	ES114 Δ*luxO* Tn*7*::pEVS107 (*erm*)	11
TIM366	ES114 Tn*7*::pTM319 (*Ptrc-rpoQ*)	This study
CA1	ES114 Δ*rpoQ*	This study
CA4	ES114 Δ*luxO* Δ*rpoQ*	This study
CA6	ES114 Δ*luxO* Δ*rpoQ* Tn*7*::*rpoQ*	This study
CA8	ES114 Δ*VFA1014*	This study
CA19	ES114 Δ*litR* Tn*7*::pEVS107 (*erm*)	This study
KV1612	ES114 *VFA1017::kan*	66
AMJ2	ES114 Δ*arcA*	38
Plasmid		
pEVS79	pBC SK(+) *oriT cat*	62
pEVS104	R6KoriRP4 *oriT trb tra kan*	62
pEVS107	R6Kori *oriT* mini-Tn*7 mob erm kan*	64
pVSV105	R6Kori *ori*(pES213) RP4 *oriT cat*	67
pUX-BF13	R6Kori *tns bla*	65
pSVS112	pEVS79 Δ*rpoQ*	This study
pTM214	pVSV105 P_{trc}-*mCherry*	63
pTM280	pVSV105 P_{luxI}-*gfp* + *PtetA-mCherry*	11
pTM319	pEVS107 *lacI*^{q}P_{trc}-*rpoQ*	This study
pXDC1	pEVS79 Δ*VFA1014*	This study
pXDC10	pTM214 P_{trc}-*rpoQ* Δ*mCherry*	This study
pXDC23	pTM214 P_{trc}-*VF_A1016* Δ*mCherry*	This study
pXDC35	pTM214 P_{trc}-*rpoS* Δ*mCherry*	This study
pXDC36	pTM214 P_{trc}-*rpoD* Δ*mCherry*	This study

and VF_A1014F4/VF_A1014R4. Verification that the expected mutations were fixed in the chromosome was obtained by sequencing.

To construct the plasmid pXDC10, which is an *rpoQ* overexpression vector, *rpoQ* was amplified by PCR using the primer pair RpoQ-eF/RpoQ-eR (Table 2), digested with SacI/EcoRV, and ligated into the vector pTM214 (58) after digestion with SacI/XmnI. To construct the plasmid pXDC35, which is an *rpoS* overexpression vector, *rpoS* was amplified by PCR using primer pair RpoS-eF/RpoS-eR (Table 2), digested with KpnI, and ligated into the vector pTM214 (63) after digestion with KpnI/XmnI. To construct the plasmid pXDC36, which is an *rpoD* overexpression vector, *rpoD* was amplified by PCR using primer pair RpoD-eF/RpoD-eR (Table 2), digested by KpnI/SalI, and ligated into the vector pTM214 (63). All constructs were verified by sequencing and then conjugated into the wild-type strain MJM1100 using pEVS104 (62). Conjugates were selected on LBS agar medium containing Cam.

To generate the plasmid pTM319, pXDC10 was digested by NotI and made blunt by T4 DNA polymerase. The resulting product was cut by SpeI and ligated into pEVS107 (64) after digestion with KpnI, making the end blunt with T4 DNA polymerase and cutting by SpeI. Integration of pTM319 into the Tn*7* site of *V. fischeri* was performed using the helper plasmids pEVS104 (62) and pUX-BF13 (65). Conjugants were selected on LBS agar medium containing Erm.

Quantitative qRT-PCR measurements. Total RNA from *V. fischeri* cultures was isolated using a QuickExtract RNA extraction kit and DNase I (Epicentre Biotechnologies, Madison, WI). RNA served as the template for cDNA synthesis with random hexamer primers and avian myeloblastosis virus (AMV) reverse transcriptase (Promega, Madison, WI). Quantitative reverse transcriptase PCR (qRT-PCR) measurements were performed using an iCycler thermal cycler (Bio-Rad Laboratories, Hercules, CA), as described previously (11). Amplification was performed at 95°C for 5 min, followed by 40 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 30 s. The primer sequences used (CrpoDF/CrpoDR, CrpoQF/CrpoQR,

and CVF_A1016F/CVF_A1016R) are listed in Table 2. The C_T values of the genes are between 20 and 30.

RT-PCR analyses. Total RNA from *V. fischeri* cultures was isolated as described above, and RT-PCR was performed using the Access RT-PCR system (Promega, Madison, WI). Wild-type genomic DNA was used as the positive control, while an RNA sample with no reverse transcriptase added was the negative control. Primer pairs A1016-17F/A1016-17R (Table 2) were used to amplify the intergenic regions between *VF_A1016* and *VF_A1017*.

5′ RACE. Six micrograms of total RNA was extracted from Δ*luxO* (TIM305) and Δ*luxO* Δ*rpoQ* (CA4) cultures grown to an optical density at 600 nm (OD$_{600}$) of 0.9 and subjected to dephosphorylation by tobacco acid pyrophosphatase (TAP) (Epicentre Biotechnologies, Madison, WI) for 30 min at 37°C. The RNA oligoRNA linker was ligated to total RNA using T4 RNA ligase (New England Biolabs, Inc., Ipswich, MA) according to the manufacturer's instructions. cDNA was synthesized using AMV RT (Promega Corp., Madison, WI) according to the manufacturer's instructions with the *rpoQ*-specific primer rpoQ-RACE and the *VF_A1016*-specific primer VF_A1016-RACE, respectively. PCR amplification was performed using the nested primers RACE-adapter and either rpoQ-RACE-nested or VF_A1016-RACE-nested. The single band that was present within the reaction containing TAP but absent from the TAP-minus control reaction was subcloned using the Topo TA cloning kit (Invitrogen) and sequenced as recommended by the manufacturer.

Culture luminescence assays. Luminescence assays were performed using cultures of *V. fischeri* as described previously (11) with the following modification. Overnight cultures were diluted 1:100 in LBS medium either with or without 1 mM IPTG and grown at 28°C for 2 h. Cultures were then diluted 1:10 in medium containing 120 nM of the autoinducer 3-oxo-C6. After 3 h, luminescence and CFU were measured and reported in relative luminescence units (RLU), where 1 RLU corresponds to 1.9×10^4 quanta s^{-1}.

TABLE 2 Primers used in this study

Primer	Sequence (5′–3′)[a]
A1016-17F	GAGATCCGGAAAGTAGAAAAAAACC
A1016-17R	GAAGTCGATGAAAAGTAATGCGTAT
CrpoDF	GAATACCGCCGTGGTTACAAA
CrpoDR	TTGACGCGAGATACGATTCAGT
CrpoQF	TCCTTGCTGAAACATCCCC
CrpoQR	AAGTTAGATATGAGAGTCTGCGAAG
CVF_A1016F	CACGAAATAAGAACACCACTCAATG
CVF_A1016R	TATCGTTAATCAAAACAAGCAAGGTT
RpoD-eF	GG**GGTACC**ATGGATCAAAATCCGCAGTCACAGC
RpoD-eR	GC**GTCGAC**TTATTCGTCTAAGAAGCTACGTAAT
RpoQ-eF	C**GAGCTC**GCTAGGTACAAGGATATGTTATGG
RpoQ-eR	GC**GATATC**TCAACCCAGTGCTATTTCTAAATCC
RpoS-eF	GG**GGTACC**ATGAGTAAAAGCAATGCAGTAACTA
RpoS-eR	TTAGTATTCTTCAACTGCAAATAAT
rpoQF1	CC**CTCGAG**AACGCCAGACATACCAATAATACC
rpoQR1	GC**GTCGAC**TCTCATATCTAACTTTGCATAAGC
rpoQF2	GC**GTCGAC**TTAGAAATAGCACTGGGTTGATAT
rpoQR2	GG**ACTAGT**ATTTCGCACTTTTCATACCCTTTA
rpoQF3	ACAGCACCAAGATCTAAGGCACGA
rpoQR3	CACTTCACGTACATTGCTTGGCTG
rpoQF4	GTATTGCCGCTTGTTAGTCT
rpoQR4	ACAACCTCAACATATCCATGACA
RNA-linker	AUAUGCGCGAAUUCCUGUAGAACGAACACUAGAAGAAA
RACE-adapter	GCGCGAATTCCTGTAGA
rpoQ-RACE	CTGATAGCGATTTATTTCCTTTAA
rpoQ-RACE-nested	GTTCTTCGCAGACTCTCATATCTA
VF_A1016-RACE	GAGAGATAAGAATTAAGGGAACAA
VF_A1016-RACE-nested	GACCAAAACTCTTGATTTGATAGA
VF_A1014F1	CG**CTCGAG**ACGGTATTGCCGCTTGTTAGTCTTT
VF_A1014R1	CG**GAATTC**ATTATTCTTAACAAGATCATA
VF_A1014F2	CG**GAATTC**GGTACAGGGTACTCCTCTTTAT
VF_A1014R2	CG**GGATCC**AATTAGCCTGTAATCGTTGGCTTT
VF_A1014F3	GTTATTTAATGTGGCGGCCTATGAC
VF_A1014R3	AACGCTTTCGCGACTCTAACAAGCA
VF_A1014F4	TGGATTTAGAAATAGCACTGGGTTG
VF_A1014R4	ATGTGCAAGATAAATCGTCGATGCC

[a] Nucleotides in bold represent restriction enzyme sites added to the 5′ region of the primer.

Squid colonization and luminescence experiments. Overnight cultures of *V. fischeri* were diluted 1:100 in fresh LBS medium and grown aerobically at 28°C to an OD_{600} of 1.0. Cultures were diluted to between 2,000 and 5,000 CFU/ml in filter-sterilized artificial seawater (Instant Ocean; IO) (Spectrum Brands Inc., Atlanta, GA) containing newly hatched squid. At 24 h postinoculation, sets of animals were placed in fresh IO either with or without 1 mM IPTG. At 48 h postinoculation, the luminescence of each animal was measured using a TD 20/20 luminometer (Turner Design, Sunnyvale, CA). To measure the RLU produced by symbiotic bacteria, animals were homogenized and the homogenate was measured for luminescence; the homogenate was also serially diluted and spread on LBS agar medium to determine CFU levels.

Fluorescence promoter-reporter assay. *V. fischeri* cells were grown as described in the culture luminescence assay. Cultures were cooled quickly using an ice-slurry mix before 1-ml samples were harvested by centrifugation at 4°C for 2 min at 8,000 × *g*. Cell pellets were resuspended in 1 ml of defined seawater minimal medium (50 mM $MgSO_4$, 10 mM $CaCl_2$, 300 mM NaCl, 10 mM KCl, 0.01 mM $FeSO_4$, 0.33 mM K_2HPO_4, 18.5 mM NH_4Cl, 50 mM Tris-HCl [pH 7.5]) without a carbon source. Samples of 100 μl (each) in volume were placed in a microtiter plate well, and the OD_{600}, as well as the levels of GFP and mCherry fluorescence, were measured using a Tecan Genios Pro plate reader (Tecan Group, Männedorf, Switzerland). A 485-nm excitation and 535-nm emission filter set was used for GFP measurements, and a 535-nm excitation and 612-nm emission filter set was used for mCherry measurements. A control nonfluorescent strain of *V. fischeri* was used to subtract any autofluorescence.

Motility assay. Strains of *V. fischeri* were grown in SWT medium to an OD_{600} of approximately 0.3. A 3-μl culture sample was then stabbed into a defined seawater minimal medium (described above) supplemented with 0.5 g *N*-acetylglucosamine per liter and containing 0.25% agar. After 10 h, the diameters of the rings of migrating cells were measured.

Chitinase activity. Exochitinase activity was measured using the chromogenic artificial substrate 4-nitrophenyl N,N′-diacetyl-β-D-chitobioside (Sigma-Aldrich Corp., St. Louis, MO). Briefly, *V. fischeri* strains were grown in LBS medium supplemented with 1 mM IPTG overnight at 28°C. The OD_{600} of the cultures was determined, and cell-free supernatants were collected by centrifugation. The substrate was then added to the supernatants and, after incubation for 30 min, the OD_{420} was determined and used to estimate the specific exochitinase activity (mU/ml/OD_{600}) according to the manufacturer's instructions.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00285-11/-/DCSupplemental.

Figure S1, TIF file, 0.1 MB.
Figure S2, TIF file, 0.1 MB.
Figure S3, TIF file, 0.4 MB.
Figure S4, TIF file, 0.1 MB.

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