TetR-Type Transcriptional Regulator VtpR Functions as a Global Regulator in *Vibrio tubiashii*.

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Received 4 May 2009/Accepted 7 October 2009

*Vibrio tubiashii*, a causative agent of severe shellfish larval disease, produces multiple extracellular proteins, including a metalloprotease (VtpA), as potential virulence factors. We previously reported that VtpA is toxic for Pacific oyster (*Crassostrea gigas*) larvae. In this study, we show that extracellular protease production by *V. tubiashii* was much reduced by elevated salt concentrations, as well as by elevated temperatures. In addition, *V. tubiashii* produced dramatically less protease in minimal salts medium supplemented with glucose or sucrose as the sole carbon source than with succinate. We identified a protein that belongs to the TetR family of transcriptional regulators, VtpR, which showed high homology with *V. cholerae* HapR. We conclude that VtpR activates VtpA production based on the following: (i) a VtpR-deficient *V. tubiashii* mutant did not produce extracellular proteases, (ii) the mutant showed reduced expression of a vtpA-lacZ fusion, and (iii) VtpR activated vtpA-lacZ in a *V. cholerae* heterologous background. Moreover, we show that VtpR activated the expression of an additional metalloprotease gene (*vthP*). The deduced VtpB sequence showed high homology with a metalloprotease, VhpA, from *V. harveyi*. Furthermore, the *vtpR* mutant strain produced reduced levels of extracellular hemolysin, which is attributed to the lower expression of the *V. tubiashii* hemolysin genes (*vthAB*). The VtpR-deficient mutant also had negative effects on bacterial motility and did not demonstrate toxicity to oyster larvae. Together, these findings establish that the *V. tubiashii* VtpR protein functions as a global regulator controlling an array of potential virulence factors.

Vibriosis is one of the most destructive larval diseases for bivalve mollusks and frequently occurs in shellfish rearing facilities. The disease is thought to be a major cause of mortalities of various shellfish larvae (9, 15, 47). Recent outbreaks of this bacterial disease have become a major threat for shellfish hatcheries in the northwest region of the United States, which has led to serious economical losses in recent years (9). It is therefore of prime importance to understand the disease mechanism and eventually prevent the significant drop in the production of shellfish larvae due to vibriosis.

The genus *Vibrio* consists of more than 30 known species, causing a variety of both human and aquatic animal diseases. One of the shellfish pathogenic *Vibrio* species, *Vibrio tubiashii*, is a recently reemerging pathogen of several species of bivalve larvae including oysters, clams, and geoducks (9, 10). Disease caused by *V. tubiashii* is characterized by a reduction in larval motility, detached vela, and necrotic soft tissue, accompanied by high mortality rates (42). Although only limited information is available about many aspects of *V. tubiashii*, including virulence factors produced, previous studies demonstrated that *V. tubiashii* strains, including RE22 and RE98, showed the highest degree of disease severity (10), with the production of high levels of extracellular metalloprotease (VtpA) and hemolysin (VthA) (7, 19, 25). We showed in a previous study that the purified VtpA protein caused significantly high toxicity to oyster larvae, strongly suggesting that the VtpA is a structural toxin to the host (18).

Moreover, mutant studies clarified that the VtpA and VthA proteins are responsible for a majority of the proteolytic and hemolytic activities, respectively, of the *V. tubiashii* culture supernatants (17, 19). In addition, we recently observed that the purified VtpA directly inactivates VthA hemolysin (17). Although virulence of *V. tubiashii* is likely multifactorial, the metalloprotease VtpA appears to be the main secreted toxin in *V. tubiashii* supernatants (18, 19).

Typically, this type of metalloprotease is tightly regulated by a TetR family of DNA-binding transcriptional regulatory proteins in a variety of *Vibrio* species (5, 22, 23, 35, 39). For example, in *V. vulnificus* a deletion of the TetR family regulator, SmcR, results in severely reduced production of the VvpE metalloprotease (22). HapR, another homolog from *V. cholerae*, positively controls production of a metalloprotease (HapA) similar to VtpA (23). In addition, it is reported that this type of transcriptional regulator is involved in the regulation of multiple virulence factors, including biofilm, exopolysaccharides, motility, and type III effectors (32), suggesting that the regulatory proteins act as global regulators in various *Vibrio* species.

In the present study, regulation of the production of virulence factors in *V. tubiashii* is investigated. Our studies included not only the effects of environmental factors on extracellular protease production in *V. tubiashii* but also characterization and analyses of the roles of a DNA-binding transcriptional regulator, VtpR. In addition, we investigated the direct interactions between the VtpR protein and potential target genes, including the *vtpA* metalloprotease in *V. tubiashii*.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** Bacterial strains and plasmids are listed in Table 1. All bacterial strains are kept at −80°C in 20% glycerol stocks. *V. tubiashii* and *V. cholerae* strains were grown in Luria-Bertani (LB) medium.
supplemented with a final concentration of 1% sodium chloride unless specified otherwise. Escherichia coli strains were grown in LB medium. V. tubiashii was also grown in M9 medium supplemented with 1% sodium chloride and 0.5% (vol/vol) appropriate carbon sources. Although E. coli and V. cholerae strains were grown at 37°C, V. tubiashii was grown at 30°C unless specified otherwise. When required, antibiotics were supplemented as follows: ampicillin, 50 μg/ml; chloramphenicol, 20 μg/ml; and tetracycline, 5 μg/ml.

### V. tubiashii strains

All PCR and cloning reactions were conducted by using standard procedures (1). Sequences for the vtpR (accession no. GQ121130) and vtpB (accession no. GQ121132) open reading frames (ORFs) were obtained by PCR using genomic DNA of the V. tubiashii strain RE22 with primer pairs based on homologous genes from various Vibrio species. Several primer pairs per gene were designed, and PCRs of all combinations were performed under low-stringency conditions to find a pair that successfully amplified a segment of the V. tubiashii genome. These PCR products were then cloned into pCR2.1-TOPO and sequenced on homologous genes from various Vibrio species. Products obtained from inverse PCR were TA cloned into pCR 2.1-TOPO and sequenced at the Oregon State University College of Veterinary Medicine Genomics Core Facility using M13 forward and reverse primers. Sequences were verified by BLAST searches. To obtain the entire ORF for these genes, inverse PCR was performed as previously described (37) with primers designed from the V. tubiashii sequences. Products obtained from inverse PCR were TA cloned into pCR 2.1-TOPO and sequenced using the M13 primers. The Oregon State University Center for Genome Research and Biocomputing website was used for bioinformatic tools (http://biodesign.ogoregonstate.edu/). V. tubiashii chromosomal DNA from strain RE22 and the vtpR::cmR strain was prepared by using a Qiagen DNEasy Blood and Tissue kit protocol for the preparation of gram-negative bacteria. Plasmids were purified according to the Qiagen spin miniprep kit protocol.

### Construction of a V. tubiashii VtpR-deficient mutant

A plasmid containing the vtpR gene was obtained from the V. tubiashii strain RE22 library by screening pools of colonies by standard PCR using the vtpR-specific primers. The vtpR-containing plasmid, pLAFR5-vtpR, was then mutagenized by deleting the entire ORF of vtpR via the lambda red recombination system (6), using hybrid lambda red-vtpR primers and pKD3 (6) as the template. Competent cells of E. coli BW2513 carrying pKD46 (6) and pLAFR5-vtpR were electroporated with a linear PCR product containing the chloramphenicol resistance cassette flanked by the target gene sequences with the same orientation. The resulting chloramphenicol and tetracycline resistance clones were confirmed for the correct insertion by PCR, creating pLAFR5-vtpR::cmR. E. coli strain Top10 cells carrying the mutagenized plasmid were then conjugated into V. tubiashii RE22 by triparental matings with the helper E. coli strain HB101 carrying pRK23. V. tubiashii carrying pLAFR5-vtpR::cmR was grown in LB 1% NaCl medium without the vector marker (tetracycline) and subcultured overnight twice, selected for the marker (chloramphenicol) inserted into the target gene, and then tested for the loss of the vector marker (tetracycline). The allelic exchange after double homologous recombination was confirmed by PCR. For complementation experiments, pLAFR5-vtpR was introduced into the vtpR-deficient mutant by triparental matings.

### Construction of E. coli and V. tubiashii lacZ fusion plasmids and β-galactosidase assay

For analyzing effects of VtpR (or the homolog) on expression of the potential target genes in E. coli and V. cholerae hapR mutant backgrounds, plasmids carrying the vtpR or hapR genes from the V. tubiashii strain RE22 and V. cholerae strain 3083, respectively, were constructed. Forward and reverse primers were designed based on the published gene sequences (accession nos. GQ121130 from V. tubiashii and AF000716 from V. cholerae). The amplified DNA fragments were cloned into pBAD-TOPO and introduced into chemically competent E. coli Top10 (Invitrogen) and V. cholerae O395N1ΔZ cells. Plasmids carrying the inserts in the desired orientation (i.e., placing the gene under the control of the arabinose-inducible promoter) were identified and confirmed by sequencing. To construct the lacZ fusion plasmids, PCR-amplified DNA fragments containing ~600 bp upstream from the putative transcriptional start site of each gene were cloned into BglII-XbaI sites of pMP220 (43) to make plasmids ptpA-lacZ, ptpB-lacZ, ptpAB-lacZ, and ptpR-lacZ, which were then electroporated into E. coli strain Top10 or V. cholerae O395N1ΔZ carrying pBAD-TOPO, pBAD-vtpR, or pBAD-hapR. The presence of the pBAD expression and the appropriate lacZ fusion plasmids was confirmed by antibiotic resistance, blue/white screening, and PCR.

V. cholerae or E. coli strains were grown at 30°C in LB medium supplemented with 1% NaCl and 100 μg of ampicillin ml⁻¹ in the absence or presence of 0.02%...
Effects of environmental factors on production of extracellular protease in *V. tubiashii*. In preliminary experiments, we observed that *V. tubiashii* strain RE22 failed to grow well at temperatures higher than 34°C but showed only slightly reduced growth at 33°C, which was still comparable to that at 25 or 30°C. To test whether there is any effect of temperature on production of extracellular protease, *V. tubiashii* strain RE22 was grown at these temperatures to a similar cell density at the stationary phase (an OD_600 of ~3.2). Figure 1A shows that culture supernatants of *V. tubiashii* grown at 33°C contained significantly lower levels of proteolytic activities than those at 25 or 30°C, whereas there was not any significant difference in protease production between 25 and 30°C. These results indicated that elevated temperatures (no less than 33°C) resulted in reduced production of extracellular protease by *V. tubiashii*.

Although *V. tubiashii* strain RE22 showed healthy growth in a salt concentration of 1 or 5% in the medium, the strain grew even better with 2 to 4% NaCl concentrations. However, the strain showed relatively poor growth at salt concentrations higher than 6% (data not shown). To examine whether salinity affects the production of extracellular protease, we grew *V. tubiashii* strain RE22 in a variety of NaCl concentrations and assayed for the proteolytic activities. As shown in Fig. 1B, *V. tubiashii* produced reduced amounts of extracellular protease in 4% salt compared to 1 to 3%. In addition, a salt concentration higher than 5% caused a more severe reduction in the protease production (Fig. 1B). On the other hand, there were no statistically significant differences in the proteolytic activities produced among 1 to 3% salt conditions (Fig. 1B). Adjusting salt concentrations in the protease assay did not significantly affect the enzymatic activities (data not shown). Together, these results suggested that excessive high-salt growth conditions lead to reduced amounts of extracellular protease production.

To investigate whether sugar contents show any effect on protease production in *V. tubiashii*, we grew *V. tubiashii* strain RE22 in defined minimal medium supplemented with sucinate, glucose, or sucrose as the sole carbon source. We used minimal medium because the addition of glucose and sucrose in LB medium caused a severe reduction in bacterial growth (data not shown), with similar observations reported in *V. cholerae* (48). Figure 1C shows that culture supernatants of *V. tubiashii* contained barely detectable levels of protease in the minimal medium supplemented with glucose or sucrose compared to succinate, indicating that both glucose and sucrose cause severe reductions in protease production.

**Sequence analyses of a TetR family transcriptional regulator, VtpR, in *V. tubiashii*.** It has been reported that metallo-protease production is controlled by the TetR family of transcriptional regulators in a variety of *Vibrio* species (5, 22, 23). The homolog from *V. tubiashii* strain RE22 was cloned by PCR, named vtpR. The full sequences of the upstream and downstream ORFs surrounding the vtpR gene were also obtained (data not shown) and showed significantly high homology with hypoxanthine phosphoribosyltransferase (*hpt*) and dihydrolipoamide dehydrogenase (*lpd*) genes, respectively. In addition, a partial ORF encoding a hypothetical carbonic anhydrase was identified downstream of the *hpt* gene (data not shown). Similar genetic arrangements are found in the genomic regions flanking the vtpR homologs in most *Vibrio* species, including *V. vulnificus*, *V. parahaemolyticus*, and *V. splendidus* (data not shown).

The coding region of the vtpR gene is 606 bp, which is predicted to encode a 201-amino-acid residue protein. The deduced VtpR protein showed high homology to several TetR family transcriptional regulators from various *Vibrio* species,
including OpaR (V. parahaemolyticus 92% identity), SmcR (V. vulnificus 86%), LuxR (V. harveyi 84%), and HapR (V. cholerae 75%) (Fig. 2).

**Mutation of VtpR results in altered production of extracellular products, reduced oyster larval toxicity, and reduced swimming motility in V. tubiashii.** To determine the effects of VtpR on extracellular protease production in V. tubiashii, we constructed a deletion mutant of the vtpR gene and assayed for proteolytic activities in culture supernatants. Figure 3A shows that culture supernatants of the vtpR::cmR strain contained virtually nondetectable levels of protease. Interestingly, the protease levels of the vtpR mutant were even less than those of the metalloprotease vtpA::cmR mutant strain (Fig. 3A). The vtpR containing plasmid fully restored production of extracellular protease in the vtpR::cmR strain (Fig. 3A). Proteolytic activity was also assessed by using zymogram gel electrophoresis (Fig. 3B). Compared to the wild type, the vtpR mutant lacked the major proteolytic bands of the approximate sizes of 35 and 28 kDa (Fig. 3B). Moreover, the mutant showed virtually no detectable proteolytic band in the gel (Fig. 3B).

To test whether culture supernatants of the VtpR-deficient mutant are toxic, we performed toxicity assays using oyster larvae. Culture supernatants of the vtpR::cmR strain caused only minimal toxicity to oyster larvae, whereas those of both the wild-type strain and the complemented mutant showed much higher larval toxicity (Fig. 3C).

To test whether hemolysin production is subject to the VtpR regulatory mechanism, we compared the hemolytic activities of culture supernatants between the wild-type and vtpR::cmR strains. As shown in Fig. 3D, at late log phase (OD_{600} = 1.8), the mutant produced much reduced amounts of hemolysin compared to the wild-type strain. Introduction of a vtpR-containing plasmid restored the hemolysin production at this growth phase (Fig. 3D). However, at stationary phase (OD_{600} = 3.2) the hemolytic activities in supernatants of the wild-type strain were diminished (Fig. 3D). Interestingly, supernatants of the vtpR mutant contained higher levels of hemolytic activities at the stationary phase (Fig. 3D). Introduction of the vtpR plasmid into the mutant reduced hemolytic activities at the stationary phase to levels comparable to the wild-type level (Fig. 3D).

Mutations in the TetR family of transcriptional regulators have been reported to affect bacterial motility, with a mutation in smcR in V. vulnificus resulting in reduced motility in soft-agar plates (20). To examine whether the bacterial motility is altered in the vtpR::cmR strain, we assayed the wild type and the mutant for motility in soft-agar medium. Compared to the wild-type strain, the vtpR mutant showed a greatly reduced swimming motility, which was restored by the vtpR-containing plasmid (Fig. 3E).

**Identification and sequence analyses of a second extracellular metalloprotease, VtpB, in V. tubiashii.** Although the V. tubiashii VtpA is responsible for the majority of proteolytic activities in the culture supernatants, residual proteolytic activities are consistently found in culture supernatants of the vtpA mutant (Fig. 3A) (18, 19), which strongly suggested that there should be another protease that is regulated by VtpR. Mass spectrophotometry analyses of V. tubiashii supernatants indicated the presence of another metalloprotease, which is similar but distinct from VtpA (data not shown).

The entire ORF sequence of vtpB from V. tubiashii strain RE22 was obtained by PCR. The 2,340-bp metalloprotease gene is predicted to encode a 779-amino-acid protein. Based on the deduced amino acid sequence, the size of the protein was calculated to be ~85 kDa. As found in VtpA, as well as in other metalloproteases (33), VtpB possesses a signal peptide, an N-terminal propeptide, a mature protease, and a C-terminal propeptide (34, 45). Alignment results (data not shown) revealed that VtpB shares high homology with several metalloproteases from other marine Vibrio species, including V. campbellii, V. harveyi, and V. splendidus (ca. 88 to 90% similarity). Comparative sequence analyses of VtpB with VtpA revealed that these distinct metalloproteases share only 61% similarity, although both metalloproteases share significantly high homol-
ogy in the mature protease regions that contain the catalytically essential zinc-binding motifs (data not shown). Together, these results show that *V. tubiashii* encodes at least two secreted metalloproteases.

**Effects of VtpR on expression of metalloprotease (vtpA and vtpB), hemolysin (vthAB), and vtpR genes in heterologous hosts.** The vtpR mutant studies suggested that *V. tubiashii* VtpR positively controls the production of extracellular proteolytic and hemolytic activities (Fig. 3). To examine whether VtpR directly regulates the vtpA, vtpB, or vthAB genes, we created strains that carry a vtpR plasmid, as well as a plasmid where the lacZ gene was fused to either of these genes. We used *E. coli* strain Top10 as well as a hapR mutant strain of *V. cholerae* (O395N1/H9004Z) to host these plasmid sets. The data in Table 2 demonstrate that expression of the vtpA-lacZ fusion in Top10 was not activated in the presence of *V. cholerae* hapR or *V. tubiashii* vtpR genes. Conversely, in O395N1ΔZ, expression of the vtpA-lacZ fusion was much stronger in the presence of the hapR or vtpR gene than in the vector control (Table 2). Moreover, expression of the vtpB-lacZ fusion was considerably induced in the presence of the vtpR gene in both *E. coli* and *V. cholerae* (Table 2).

The effects of VtpR on the hemolysin gene (vthAB) expression using a vthAB-lacZ fusion were assayed. The assay showed that expression of the vthAB-lacZ fusion was not induced by the presence of the vtpR in neither background (Table 2).

Lin et al. (30) previously documented that in *V. cholerae* HapR represses its own transcription. To test any autoregulatory mechanism in *V. tubiashii* vtpR expression, VtpR was expressed in combination with a vtpR-lacZ fusion. The data presented in Table 2 show that expression of the vtpR-lacZ fusion was much reduced in the presence of the vtpR or hapR gene in both background strains.

VtpR positively controls metalloprotease and hemolysin genes, whereas it represses its own expression in *V. tubiashii*. To further investigate a role of VtpR in expression of metalloprotease and hemolysin genes in *V. tubiashii*, we compared the expression of vtpA-, vtpB-, and vthAB-lacZ fusions between the *V. tubiashii* wild-type and vtpR::cmR strains. Expression of the vtpA-lacZ and vtpB-lacZ fusions was >10-fold lower in the vtpR::cmR strain than in the wild-type strain (Fig. 4). Moreover, expression of vthAB-lacZ in the mutant was less than half of that in the wild-type strain (Fig. 4), confirming our earlier results that *V. tubiashii* VtpR activates both metalloprotease genes.

**Effects of environmental factors on expression of the vtpA and vtpR genes.** We hypothesized that the effects of environmental factors on extracellular protease production (Fig. 1) are due to reduced expression of the vtpA or vtpR genes. To examine whether expression of these genes is affected by salinity as well as certain sugars, we compared the levels of the vtpA- and vtpR-lacZ fusions in *V. tubiashii* strain RE22. Table 3 shows that the expression of vtpA-lacZ was much reduced in the medium containing 5% NaCl compared to medium containing 1% NaCl. Moreover, the presence of glucose or sucrose, but not succinate, in minimal medium caused low vtpA-
TABLE 2. Expression of vtpA-lacZ, vtpB-lacZ, vthAB-lacZ, and vtpR-lacZ fusions in E. coli strain Top10 and V. cholerae strain O395N1ΔZ in the presence of hapR or vtpR

| Strain     | Bacterial construct | Mean β-galactosidase activity (Miller unit) ± SD
|------------|---------------------|-----------------------------------------------
| Top10      | pBAD + pvtpA-lacZ   | 91 ± 4                                        |
|            | pBAD-hapR + pvtpA-lacZ | 61 ± 8                                        |
|            | pBAD-vtpR + pvtpA-lacZ | 69 ± 18                                       |
| O395N1ΔZ  | pBAD + pvtpa-lacZ   | 209 ± 76                                      |
|            | pBAD-hapR + pvtpa-lacZ | 1,670 ± 152                                   |
|            | pBAD-vtpR + pvtpa-lacZ | 1,710 ± 280                                   |
| Top10      | pBAD + pvtpB-lacZ   | 39 ± 2                                        |
|            | pBAD-hapR + pvtpB-lacZ | 1,029 ± 82                                   |
|            | pBAD-vtpR + pvtpB-lacZ | 1,055 ± 124                                   |
| O395N1ΔZ  | pBAD + pvtpB-lacZ   | 120 ± 53                                      |
|            | pBAD-hapR + pvtpB-lacZ | 953 ± 89                                      |
|            | pBAD-vtpR + pvtpB-lacZ | 943 ± 130                                     |
| Top10      | pBAD + pvthAB-lacZ  | 45 ± 15                                       |
|            | pBAD-hapR + pvthAB-lacZ | 42 ± 23                                       |
|            | pBAD-vtpR + pvthAB-lacZ | 39 ± 24                                       |
| O395N1ΔZ  | pBAD + pvthAB-lacZ  | 52 ± 24                                       |
|            | pBAD-hapR + pvthAB-lacZ | 59 ± 8                                        |
|            | pBAD-vtpR + pvthAB-lacZ | 60 ± 21                                       |
| Top10      | pBAD + pvtpR-lacZ   | 6,754 ± 1,435                                 |
|            | pBAD-hapR + pvtpR-lacZ | 1,764 ± 365                                  |
|            | pBAD-vtpR + pvtpR-lacZ | 1,677 ± 436                                   |
| O395N1ΔZ  | pBAD + pvtpR-lacZ   | 8,920 ± 1,204                                 |
|            | pBAD-hapR + pvtpR-lacZ | 2,239 ± 261                                  |
|            | pBAD-vtpR + pvtpR-lacZ | 2,201 ± 497                                   |

The values are means for three determinations.

**DISCUSSION**

We have observed that a secreted metalloprotease, VtpA, was shown to be a major toxic factor produced by *V. tubiashii* (19). Similar metalloproteases from other marine *Vibrio* species were also found to cause oyster larval mortalities (18). Due to the economic importance of vibriosis in shellfish hatcheries, it is of interest to investigate effects of environmental conditions on metalloprotease production in *V. tubiashii*. In this report, we showed that extracellular proteolytic activities are reduced at elevated temperatures, salinity, and the presence of sugars such as glucose and sucrose in *V. tubiashii*. This led us to further investigate the genetic mechanisms of metalloprotease regulation in this organism.

It has been well established that the TetR family of transcriptional regulators positively controls protease production in a variety of *Vibrio* species, including *V. anguillarum* (VanT), *V. cholerae* (HapR), *V. harveyi* (LuxR), and *V. vulnificus* (VvpR/SmcR) (5, 22, 23, 35, 39). Here, we report a *V. tubiashii* gene, *vtpR*, encoding a homolog of the TetR family transcriptional regulators. Our sequence analyses revealed that the VtpR protein showed significantly high homology with the TetR family of regulatory proteins from several *Vibrio* species including SmcR, LuxR, and HapR. The extensive study (8) revealed that HapR from *V. cholerae* possesses typical DNA-binding helix-turn-helix (HTH) motifs in the N-terminal region. We also observed the putative HTH motifs in the N-terminal region of VtpR, highly similar to other homologs from several other *Vibrio* species. Therefore, we anticipated that this protein is involved in regulation of the *vtpA* metalloprotease gene in *V. tubiashii*.

VtpR positively affected production of the extracellular hemolysin, VthA. It has been reported that production of an extracellular hemolysin (VvhA) is negatively regulated by SmcR in *V. vulnificus* (38). Unlike *V. vulnificus*, the *V. tubiashii* vtpR-deficient mutant expressed lower levels of the hemolysin genes *vthAB* at the late log phase of growth compared to the wild-type strain. These results suggested that VtpR positively controls hemolysin production.

Our recent observations (17, 19) revealed that the hemolytic activities of the *V. tubiashii* wild-type strain severely deteriorated at the stationary phase due to the high levels of protease production. Interestingly, hemolysin production by the *vtpR*::cmR strain was increased at the stationary phase, probably due to further investigation of the genetic mechanisms of metalloprotease regulation in this organism.

FIG. 4. Comparison between *V. tubiashii* strain RE22 and the VtpR-deficient mutant in expression of vtpA, vtpB, and vthAB genes. Bacteria were grown in LB medium supplemented with 1% NaCl at 30°C, and samples were harvested at the late log (for vthAB-lacZ) and stationary phases and analyzed for β-galactosidase activities. The error bars of the Miller units indicate standard deviations (n = 3).

TABLE 3. Effects of NaCl concentrations and carbon sources on expression of vtpA-lacZ and vtpR-lacZ fusions in *V. tubiashii* strain RE22

| Bacterial construct | Growth condition | Mean β-galactosidase activity (Miller unit) ± SD
<table>
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<tr>
<td>RE22(pvtpA-lacZ)</td>
<td>1% NaCl</td>
<td>3,248 ± 199</td>
</tr>
<tr>
<td></td>
<td>3% NaCl</td>
<td>1,300 ± 68</td>
</tr>
<tr>
<td></td>
<td>5% NaCl</td>
<td>273 ± 49</td>
</tr>
<tr>
<td>RE22(pvtpR-lacZ)</td>
<td>1% NaCl</td>
<td>6,826 ± 1,071</td>
</tr>
<tr>
<td></td>
<td>3% NaCl</td>
<td>2,994 ± 399</td>
</tr>
<tr>
<td></td>
<td>5% NaCl</td>
<td>1,206 ± 320</td>
</tr>
<tr>
<td>RE22(pvtpA-lacZ)</td>
<td>Succinate</td>
<td>1,401 ± 276</td>
</tr>
<tr>
<td></td>
<td>Glucose</td>
<td>334 ± 31</td>
</tr>
<tr>
<td></td>
<td>Sucrose</td>
<td>279 ± 58</td>
</tr>
<tr>
<td>RE22(pvtpR-lacZ)</td>
<td>Succinate</td>
<td>5,146 ± 812</td>
</tr>
<tr>
<td></td>
<td>Glucose</td>
<td>1,564 ± 378</td>
</tr>
<tr>
<td></td>
<td>Sucrose</td>
<td>1,594 ± 354</td>
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The values are means for three determinations.
to the absence of inhibitory activities of the VtpA metalloprotease (17). Also, other regulatory proteins, including CRP (for cyclic AMP receptor protein) and ToxR (2), in *V. tubiashii* might be involved in the activation of *vthAB* transcription, as documented that in *V. vulnificus* expression of the *vthAB* operon is directly activated by CRP (4) as well as ToxR (27).

Although motility in the *V. cholerae hapR* mutant was comparable to that of the wild-type strain (49), in *V. vulnificus* an *smcR* mutant led to reduced motility (20). Our data demonstrated that the VtpR protein positively controls swimming motility. Thus, it appears that VtpR in *V. tubiashii* controls the bacterial motility similar to SmcR in *V. vulnificus*.

In the present study, we found an additional putative metalloprotease gene, *vtpB* in *V. tubiashii*, which appeared to be homologous to that from multiple *Vibrio* species. Although the VtpB protein contains some of features typically found in metalloproteases, VtpB is clearly distinct from the VtpA-type of metalloproteases in terms of size as well as amino acid sequence. It is interesting that *V. splendidus*, *V. campbellii*, and *V. harveyi*, all of which are known shellfish pathogens (14, 16, 44), carry homologs of VtpB, whereas other pathogenic *Vibrio* species do not. This correlation implies that the VtpB type of metalloproteases might play a role in shellfish diseases.

We observed that VtpR activated the *vtpA* gene when expressed in *V. cholerae* but not in *E. coli*. This difference might be due to additional factors present in *V. cholerae* that are absent in *E. coli*. Jeong et al. (22) reported that in *V. vulnificus*, CRP, IHF (for integration host factor), and RpoS were required for activation of a homolog of *vtpA*, *vvpE*. It is also documented that in *V. cholerae*, not only CRP and RpoS but also H-NS (The histone-like nucleoid structuring protein), as well as a yet-unidentified factor, is necessary to express the *V. cholerae* metalloprotease gene, *hapA* (41). Therefore, it is possible that these factors are involved in transcriptional regulation of the *vtpA* gene in *V. tubiashii*. On the other hand, VtpR activated the *vtpB* gene in the *V. cholerae* as well as *E. coli* backgrounds, indicating not only that the regulatory mechanism of VtpB is distinct from that of VtpA but that activation of the *vtpB* gene does not require other transcription factors than VtpR.

Expression data in *V. tubiashii* background further cemented the evidence that VtpR functions as an activator of both *vtpA* and *vtpB*. The reporter expression of both genes was severely reduced in the *vtpR::cmR* strain. These results, together with the β-galactosidase assays in *E. coli* and *V. cholerae*, strongly suggested that VtpR is responsible for expression of these genes in *V. tubiashii*. To our knowledge, this is the first report to establish that a single TetR family transcriptional regulator activates distinct secreted metalloproteases in *Vibrio* species.

Mutation of the *vtpR* gene resulted in much-reduced expression of the *vthAB* hemolysin genes in *V. tubiashii*, indicating that the VtpR protein positively controls transcript levels of *vthAB*, which correlates with our observations that the mutant produces much reduced levels of extracellular hemolysin compared to the wild-type strain. The reduction of the *vthAB-lacZ* expression in the mutant was not as dramatic as that of *vtpA-* or *vtpB-lacZ*, again implying additional factors that activate the *vthAB* gene expression.

The presence of VtpR severely diminished expression of the *vtpR-lacZ* fusion, indicating that VtpR interacts with its own promoter as an autorepressor. In *V. cholerae*, HapR represses transcription of the *hapR* gene by direct binding (30). Therefore, it is likely that the much-reduced expression of the *vtpR-lacZ* fusion by VtpR and HapR was due to direct binding. In general, these types of transcriptional regulators frequently affect large number of genes both positively and negatively (26, 36, 46). Thus, we expect VtpR to control a large variety of genes in *V. tubiashii*.

The severe reduction in protease production by the presence of glucose or sucrose correlated well with reduced production of *vtpA* in *V. tubiashii*. This was not surprising since these carbon sources are known to act as inhibitors of the *vvpE* and *hapA* genes in *V. vulnificus* (21) and *V. cholerae* (3, 39, 40), respectively. Therefore, it is likely that *V. tubiashii* possesses similar sugar-sensitive regulatory mechanisms. Moreover, CRP has been reported to positively regulate transcription of the *hapR* gene in *V. cholerae* (12, 28, 29). In the present study we observed strongly repressed expression of *vtpR-lacZ* fusion in the presence of these carbon sources. It is tempting to speculate that protease production is hierarchically controlled by CRP via VtpR in *V. tubiashii*.

In summary, we have demonstrated that in *V. tubiashii* the TetR-type transcriptional regulator, VtpR, positively controls production of multiple extracellular proteins, including the metalloproteases and hemolysins, as well as swimming motility (Fig. 5). Our observations suggested that while VtpR is directly involved in activation of at least two distinct metalloprotease genes, it positively affects a hemolysin gene. Our results also revealed that VtpR acts as an autorepressor. Furthermore, transcription of the *vtpR* gene is attenuated by elevated environmental conditions, which might, in turn, contribute to lower expression of the metalloprotease gene, leading to loss of proteolytic activities of the culture supernatants. Thus, VtpR appears to be a global regulator of various phenotypes and regulates potential virulence factors in *V. tubiashii*.

**ACKNOWLEDGMENTS**

We thank R. A. Elston for providing the *V. tubiashii* strain. We thank Erin J. Lind and Dima N. Gharaibeh for excellent technical assistance.
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This study was partially funded by grants from Oregon Sea Grant, USDA/CRESES Animal Health and Diseases, and the Agricultural Research Foundation.

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