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Effects of changes in membrane sodium flux on virulence gene expression in *Vibrio cholerae*

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ABSTRACT **The expression of several virulence factors of *Vibrio cholerae* is coordinately regulated by the ToxT molecule and the membrane proteins TcpP/H and ToxR/S, which are required for *toxT* transcription. To identify proteins that negatively affect *toxT* transcription, we screened transposon mutants of *V. cholerae* carrying a chromosomally integrated *toxT*::*lacZ* reporter construct for darker blue colonies on media containing 5-bromo-4-chlor-3-indolyl β-D galactoside (X-gal). Two mutants had transposon insertions in a region homologous to the *nqr* gene cluster of *Vibrio alginolyticus*, encoding a sodium-translocating NADH–ubiquinone oxidoreductase (NQR). In *V. alginolyticus*, NQR is a respiration-linked Na^+ extrusion pump generating a sodium motive force that can be used for solute import, ATP synthesis, and flagella rotation. Inhibition of NQR enzyme function in *V. cholerae* by the specific inhibitor 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) resulted in elevated *toxT*::*lacZ* activity. Increased *toxT*::*lacZ* expression in an *nqr* mutant strain compared with the parental strain was observed when the TcpP/H molecules alone were strongly expressed, suggesting that the negative effect of the NQR complex on *toxT* transcription is mediated through TcpP/H. However, the ability of the TcpP/H proteins to activate the *toxT*::*lacZ* reporter construct was greatly diminished in the presence of high NaCl concentrations in the growth medium. The flagellar motor of *V. cholerae* appears to be driven by a sodium motive force, and modulation of flagella rotation by inhibitory drugs, high media viscosity, or specific mutations resulted in increases of *toxT*::*lacZ* expression. Thus, the regulation of the main virulence factors of *V. cholerae* appears to be modulated by endogenous and exogenous sodium levels in a complex way.**

Vibrio cholerae is a Gram-negative bacterium that causes the diarrheal disease cholera. To establish infection and cause disease, *V. cholerae* must express a variety of virulence factors, including cholera toxin (CT), and colonization factors such as the toxin coregulated pilus (TCP). Expression of CT and TCP is coordinately regulated and strongly influenced by environmental stimuli (1). Transcription of the genes encoding these virulence factors is controlled by a regulatory cascade in which ToxR and TcpP control expression of ToxT, a transcriptional activator that directly controls expression of several virulence genes (2–4). ToxR and TcpP are inner membrane proteins that contain cytoplasmic DNA-binding domains. The periplasmic domains of ToxR and TcpP are thought to interact with other transmembrane regulatory proteins, ToxS and TcpH, respectively, that stimulate their activities (4–7). ToxT is a cytosine arabinonucleoside-like transcriptional activator that activates transcription of several genes, including the *ctx* and *tcp* operons, encoding CT and TCP, respectively (3).

In the present study, we isolated several *V. cholerae* transposon mutants that showed increased expression of a chromosomal *toxT*::*lacZ* reporter construct. Two of the isolated mutants had transposon insertions in a region with high homology to the *Vibrio alginolyticus nqr* gene cluster, which encodes a sodium-translocating NADH–ubiquinone oxidoreductase (NQR) (8, 9). We had previously isolated a mutant strain of *V. cholerae* with a transposon insertion in a *nqr* gene homolog by selecting for *V. cholerae* cells that produce TCP even when grown under noninducing growth conditions (4). Because overexpression of *toxT* can result in cells constitutively expressing TCP (10), this prompted further investigation of the role of the *nqr* gene homologs in the expression of virulence genes in *V. cholerae*.

MATERIALS AND METHODS

Strains, Plasmids, and Culture Conditions. *V. cholerae* strain O395N1 *toxT*::*lacZ* (4) was host for the tansposon mutagenesis. The transposon Tn*Mar* was introduced into *V. cholerae* on a suicide plasmid by conjugation with the *Escherichia coli* strain β2155 carrying pFD1 (11). Transposon-mutagenized *V. cholerae* cells were selected by plating dilutions of the conjugation mixture on streptomycin (100 μg/ml) and kanamycin (Kan; 50 μg/ml) containing media with 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-gal) at 20 μg/ml. Darker blue colonies were obtained by visually scoring and were tested for increased β-galactosidase activity compared with the parent strain after overnight growth in LB at 30°C. For noninducing culture conditions, the bacteria were grown at 30°C either in LB where the pH was increased to 8.5 by adding NaOH or in LB with various amounts of NaCl added. To increase media viscosity, a 10% polyvinylpyrrolidone (PVP-360) or 15% Ficoll solution was prepared in LB and dialyzed against LB. X-gal, monensin, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), and 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) were purchased from Sigma. Phenamil was purchased from Research Biochemicals (Natick, MA).

Genetic Manipulations. Chromosomal DNA was extracted from *V. cholerae* cells by using the Easy-DNA kit (Invitrogen). The genomic regions surrounding the transposon insertions were cloned into the suicide vector pCVD442 carrying the *kan*-resistance gene (pCVD442π) as described (12). Briefly, the plasmid pCVD442π was introduced into the transposon carrying *V. cholerae* strain by conjugation, selecting for ampicillin-resistant and Kan-resistant cells. This should result in the integration of the plasmid into the transposon insertion site via homologous recombination between the *kan*-resistance genes present on the plasmid and the transposon. Chromosomal DNA from these strains was prepared and digested with the restriction enzyme *Bgl*II, which does not cut in pCVD442π or in the transposon. The chromosomal digest was then diluted, ligated, and transformed into DH5αλpir *E. coli* cells. Plasmid DNA was prepared by using the Qiagen (Chatsworth, CA)

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Abbreviations: CT, cholera toxin; TCP, toxin coregulated pilus; smf, sodium motive force; NQR, NADH–ubiquinone oxidoreductase; X-gal, 5-bromo-4-chlor-3-indolyl β-D galactoside; Kan, kanamycin.

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Miniprep extraction kit and sequenced with primers specific to the transposon ends (11). The nonmotile mutants were generated by homologous recombination. The plasmid pKEK93 (13) was used to generate the *flaA*::*cat* mutation. The *motY* and *fliG* genes were amplified in PCR reactions by using specific primers and cloned into the plasmid vector pCR2.1 (Invitrogen). Internal deletions were generated by using convenient restriction sites present in the genes, and the DNA was then subcloned into pCVD442. The mutated alleles of these genes were introduced into the chromosome of the O395N1 *toxT*::*lacZ* strain following sucrose selection as described (14).

Motility Assays. Motility phenotypes were assessed for swarm diameter following inoculation into 0.3% soft agar. Bacterial cells also were assayed for swimming speed under a dark-field microscope after the addition of various drugs.

Biochemical Assays. β-Galactosidase activities were assayed as described (4, 15).

RESULTS

Identification of a Gene Cluster in *V. cholerae* with High Homology to Genes Encoding a Sodium-Translocating NQR from *V. alginolyticus*. We recently reported the construction of a *V. cholerae* strain carrying a chromosomal *toxT*::*lacZ* reporter construct (4). To identify genes involved in the negative regulation of *toxT* transcription, transposon mutagenesis of this strain was performed followed by screening for darker blue colonies on medium containing X-gal. Several mutants were isolated that exhibited increased β-galactosidase activity when compared with the parent strain. Sequencing of the DNA adjacent to the transposon insertion in these strains revealed regions with homology to both genes of known function and genes of unknown function (data not shown). Two mutant strains had transposon insertions in a chromosomal region with high homology to the *nqr* gene cluster from *V. alginolyticus* (Fig. 1). We had previously isolated a *V. cholerae* mutant with a Tn*bla* insertion in an *nqr* homolog, and this mutant displayed constitutive TCP expression (4). In *V. alginolyticus*, the *nqr* genes are part of a cluster of six genes encoding various subunits of a sodium-translocating NQR (8, 9), a respiration-driven Na^+ pump that establishes an electrochemical gradient of sodium ions across the membrane (16). By using the partial genome sequences deposited by The Institute for Genomic Research (TIGR) and various primers, we confirmed that the *nqr* gene homologs are also linked in the *V. cholerae* chromosome (Fig. 1). The *nqr* gene regions from *V. cholerae* and *V. alginolyticus* showed high sequence homology; for example, the deduced amino acid sequences of the two NqrA proteins are 86.5% identical (Fig. 1).

Effects of Loss of NQR Activity on *toxT*::*lacZ* expression in *V. cholerae*. The *nqr* mutant strains were isolated as colonies that appeared darker blue on X-gal-containing medium compared with the parental *V. cholerae* strain, and the mutant strains produced ≈2-fold more β-galactosidase activity than the parent strain when assayed in liquid media (Fig. 2). The NQR enzyme is thought to be involved in pH and ion homeostasis in *V. alginolyticus* (16). In *V. cholerae*, high pH and low or high NaCl are known to negatively affect CT and TCP

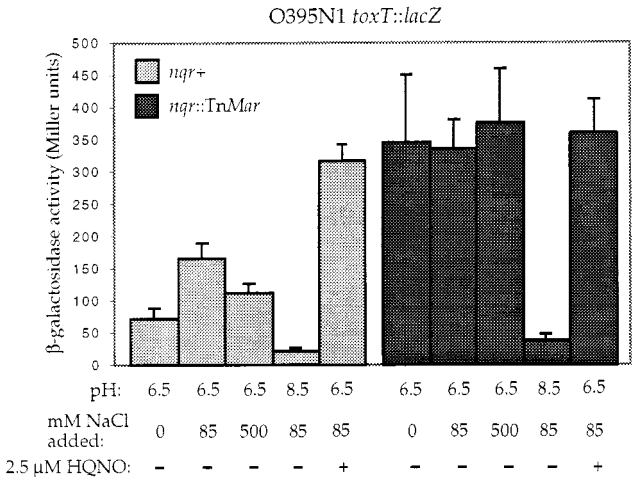

FIG. 2. Effects of growth conditions on β-galactosidase activities in wild-type and *nqr* mutant *V. cholerae* strains carrying a *toxT*::*lacZ* reporter construct. Cells were grown in LB with a starting pH of 6.5 or 8.5, in LB (pH 6.5) with low (0 mM), normal (85 mM), or high (500 mM) concentrations of NaCl, or in LB (pH 6.5) with 2.5 μM HQNO.

production, which is believed to occur via modulation of *toxT* transcription (1, 10). We assayed the *toxT*::*lacZ* expression in both the parental and *nqr* mutant strains after growth in LB with a starting pH of 6.5 or 8.5 (Fig. 2). Consistent with previous findings that the transcription of *toxT* is strongly affected by the external pH (10), β-galactosidase activity in the parental strain is much reduced when the cells are grown in media with a high starting pH. In the *nqr* mutant strains, β-galactosidase activity also was strongly reduced after growth at elevated pH (Fig. 2). In contrast, very low or high concentrations of NaCl in the growth medium resulted in reduced *toxT*::*lacZ* expression in the parental but not the *nqr* mutant strain (Fig. 2). It is interesting to note that the *nqr* mutant strain showed a slight growth defect compared with the parental strain and showed poorest growth at the low and high NaCl concentrations, suggesting an important role of this enzyme in ion homeostasis in *V. cholerae*.

HQNO was reported to be a specific inhibitor of the NQR enzyme complex from *V. alginolyticus* and blocks its activity at micromolar concentrations (17). Addition of 2.5 μM HQNO to the growth medium resulted in markedly increased β-galactosidase activities only in the parental *V. cholerae* strain (Fig. 2). These results suggest that it is the activity of the NQR enzyme rather than the absence of the protein complex that is responsible for the observed effect on *toxT* transcription.

Enhanced *toxT*::*lacZ* activity in a *nqr* mutant strain occurs even when only TcpP/H are expressed. To investigate whether the effects of this sodium pump on the *toxT* promoter are mediated via the ToxR/S or TcpP/H proteins, we introduced the *nqr*::Tn*Mar* transposon insertion into a Δ*toxR* Δ*tcpP* *toxT*::*lacZ* *V. cholerae* strain (4). As reported (4), the Δ*toxR* Δ*tcpP* *toxT*::*lacZ* parent strain showed very low β-galactosidase activity, and the derivative strain containing the *nqr* mutation showed similarly low β-galactosidase activity (Fig. 3*A*). Over-

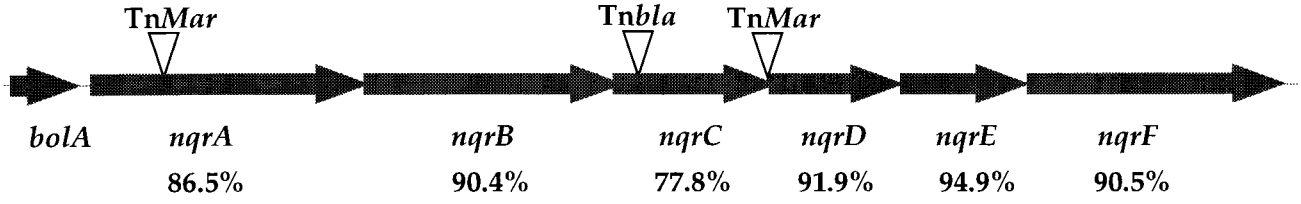

FIG. 1. Chromosomal region of the *V. cholerae nqr* gene cluster. The positions of different transposon insertions are indicated. The amino acid sequence homologies of the various Nqr proteins from *V. cholerae* and *V. alginolyticus* are shown below.

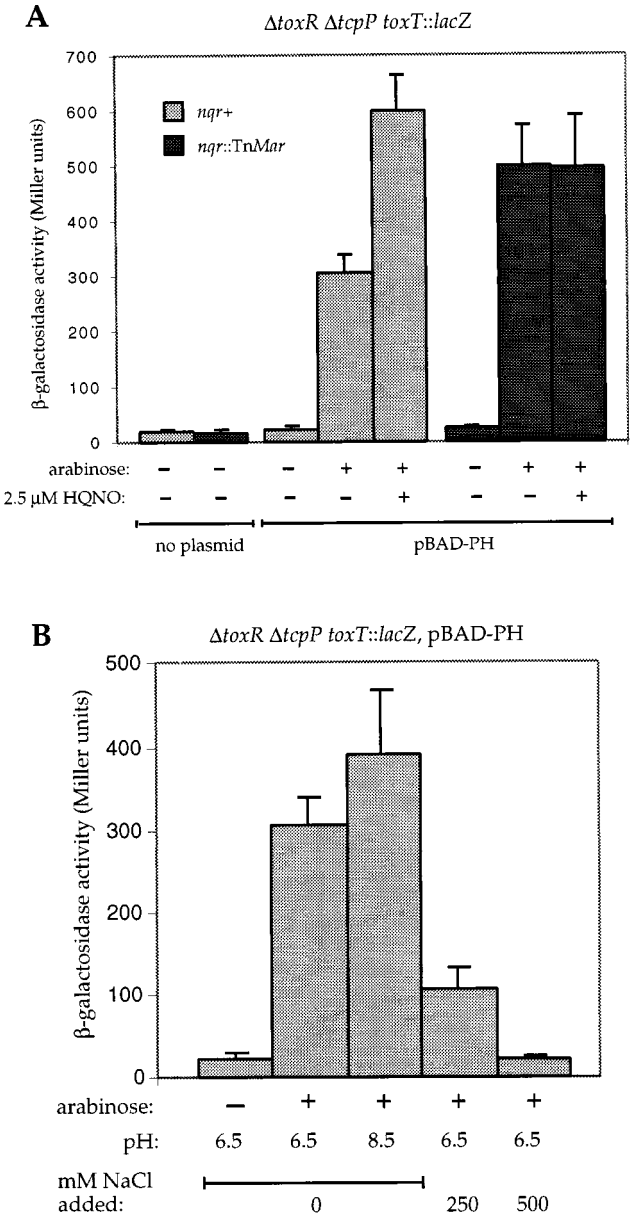

FIG. 3. Comparison of β-galactosidase activities in Δ*toxR* Δ*tcpP toxT*::*lacZ V. cholerae* strains with or without the *nqr*::Tn*Mar* mutation carrying a plasmid expressing the *tcpPH* genes from an arabinose-inducible promoter (pBAD-PH). Arabinose (0.02%) and HQNO (2.5 μM) (*A*) were added as indicated. Effects of different media pH or various concentrations of NaCl are shown (*B*).

expression of the *tcpP* and *tcpH* genes from an arabinose-dependent promoter can partially complement the *toxR* deletion for activation of the *toxT*::*lacZ* reporter construct (4) (Fig. 3*A*). If *tcpPH* are induced with the same arabinose concentrations in the strain carrying the *nqr* mutation, significantly higher β-galactosidase activities were observed compared with the parental strain (Fig. 3*A*). Furthermore, the addition of 2.5 μM HQNO to the growth media results in increased β-galactosidase activites in the parental but not in the *nqr* mutant strain (Fig. 3*A*). Together, these results indicate that the TcpP/H molecules are required for the increased *toxT* transcription in a *nqr* mutant background.

The Activity of the TcpP/H Proteins Is Sensitive to NaCl but Not to pH. Carroll *et al.* recently reported (7) that the transcription of *tcpPH* is strongly reduced at high pH and temperature. Consistent with this, expression of *tcpPH* from an independent promoter resulted in *toxT*::*lacZ* expression even under alkaline conditions (Fig. 3*B*) that result in strong repression of *toxT* transcription in a wild-type strain (Fig. 2) (10). In contrast, the β-galactosidase activity levels from the *toxT*::*lacZ* reporter achieved by overexpression of the *tcpPH* genes are dramatically reduced if the cultures are grown in the presence of elevated concentrations of NaCl (Fig. 3*B*). Together, these results indicate that the activities of the TcpP/H proteins are sensitive to high salt concentrations but not to elevated pH. Of interest, the negative effects of high NaCl concentrations on *toxT* transcription appear to be more pronounced when TcpP/H are expressed in the absence of ToxR/S than in a wild-type background. Induction of TcpP/H also leads to elevated β-galactosidase activities in an *E. coli* strain carrying a chromosomally integrated copy of the *toxT*::*lacZ* reporter construct (data not shown). Of interest, these expression levels are not negatively affected by the addition of NaCl to the growth medium (data not shown), suggesting that the TcpP/H molecules are sensitive to elevated salt concentrations only in a *V. cholerae* background.

The Flagellum of *V. cholerae* Is Energized by Sodium Ions. In several marine and halophilic bacterial species, the respiration-driven Na^+ pump, NQR, produces an electrochemical gradient of sodium ions across the membrane, which can be utilized for solute import, ATP synthesis, and flagella rotation (16). The single polar flagella of *V. alginolyticus* and *Vibrio parahaemolyticus* are energized by the translocation of sodium ions (18, 19). It was previously observed that, like in other *Vibrio* species, motility of *V. cholerae* increases with increased NaCl concentrations, indicating that Na^+ plays an important role in the motility in this organism (20). Furthermore, the partial genomic sequences of *V. cholerae* recently released by TIGR contain several homologs of genes encoding subunits specific for a Na^+-driven flagellar motor (21)—including *motX*, *motY, pomA*, and *pomB* (data not shown)—that when mutagenized result in nonmotile phenotypes (see below) (22). To determine whether the flagellum of *V. cholerae* is driven by sodium ions, we analyzed motility behavior of the *V. cholerae* wild-type strain O395 after the addition of various inhibitors. Swimming speed of *V. cholerae* was dramatically reduced after the addition of the protonophore CCCP at pH 6.5 but not at pH 8.5 (data not shown), analogous to the results obtained for *V. parahaemolyticus* (19). Furthermore, the addition of micromolar concentrations of phenamil, an amiloride compound believed to specifically block the sodium ion-conducting portion of flagellar motors (23, 24), resulted in dramatically reduced motility (data not shown). Together, these results and data recently obtained by Kojma *et al.* (25) indicate that the *V. cholerae* flagellum is energized by the translocation of sodium ions.

Effects of Modulation of Flagella Rotation on *toxT*::*lacZ* Expression. It was previously found that motility and virulence factor expression are inversely correlated in *V. cholerae* (22). As loss of NQR activity, an enzyme complex involved in generating a sodium motive force (smf) that can be utilized by flagella rotation, resulted in increased *toxT*::*lacZ* expression, we wished to analyze whether changes in Na^+ flux through the flagellum also result in altered *toxT* transcription. The addition of phenamil, an inhibitor of sodium-driven flagella, as well as addition of monensin, an ionophore that changes the level of Na^+ chemical potential (25), resulted in a moderate increases in β-galactosidase activity in the O395N1 *toxT*::*lacZ* reporter strain (Fig. 4). Similarly, introduction of various specific mutations that produced nonmotile phenotypes resulted in slightly increased β-galactosidase activities compared with the parental strain (Fig. 4). Furthermore, increasing the media viscosity by adding 5% or 10% polyvinylpyrrolidone (Fig. 4) or 15% Ficoll (data not shown) more dramatically induced *toxT*::*lacZ* expression, reminiscent of the *laf* gene induction observed by increased media viscosity in *V. parahaemolyticus* (26).

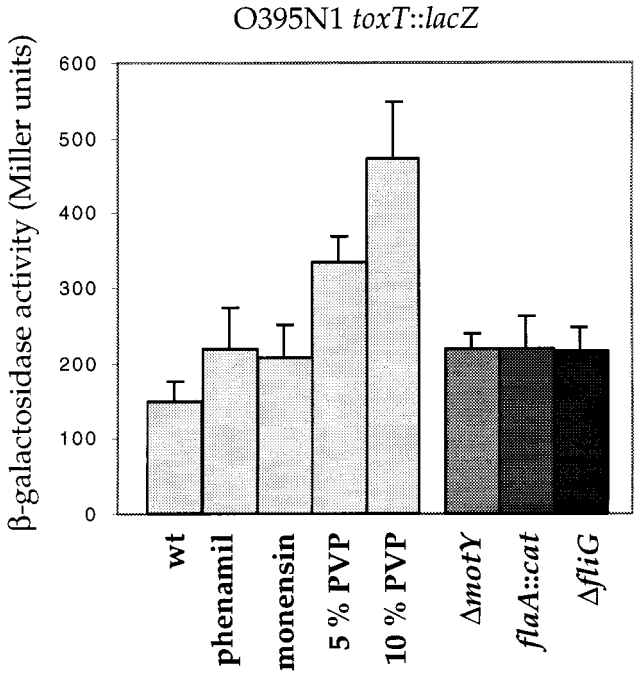

FIG. 4. Effects of modulation of flagella rotation and specific mutations affecting motility on *toxT*::*lacZ* expression. Cells were grown in LB or in LB containing 20 μM phenamil, 20 μM monensin, 5% polyvinylpyrrolidone (PVP), or 10% polyvinylpyrrolidone as indicated.

DISCUSSION

The main virulence factors of *V. cholerae*, CT and TCP, are coordinately regulated by a cascade of regulatory proteins in response to environmental conditions (1). The ToxT protein directly activates the *ctx* and *tcpA* promoters, and transcription of the *toxT* gene depends on the ToxR/S and TcpP/H proteins. Thus far, only the Cya and Crp proteins are known to negatively affect *toxT* transcription (27). To identify additional negative regulators of *toxT* transcription, we performed transposon mutagenesis of a *V. cholerae toxT*::*lacZ* reporter strain followed by a screen for darker blue colonies. Several mutants were isolated that showed increased β-galactosidase activities compared with the parental strain. Analysis of the DNA sequences adjacent to the transposon insertions revealed homologies to several genomic regions of known as well as unknown functions. Interestingly, two mutant strains had transposon insertions in genes homologous to the *nqr* gene cluster from *V. alginolyticus* (8, 9). We had previously isolated an *nqr*::Tn*bla* mutant of *V. cholerae* that showed increased TCP expression in media of elevated pH (4). Thus, we have isolated three independent mutants in the *nqr* gene cluster that resulted in elevated *toxT* transcription and/or TCP production in *V. cholerae*.

The NQR enzyme has been extensively studied in *V. alginolyticus* and is a respiration-linked Na^+ pump (a Na^+-dependent NQR) (16) establishing an electrochemical gradient of sodium ions across the membrane, resulting in a smf. Several bacterial species can use a smf for solute transport, ATP synthesis, and flagella rotation. This alternative energy coupling of sodium ions rather than protons enables the bacteria to maintain a cytoplasmic pH near neutrality in an alkaline environment. At alkaline pH, a strong reduction in *toxT* transcription is observed in both the wild-type and to a somewhat lesser extent in the *nqr* mutant strain, suggesting that the NQR enzyme is not the primary factor involved in the transcriptional repression of *toxT* and ToxT-regulated virulence genes in *V. cholerae* in response to alkaline conditions. In contrast, very low or high NaCl concentrations resulted in decreased *toxT*::*lacZ* expression in the parental but not in the *nqr* mutant strain, suggesting that the NQR enzyme may play a role in the response of virulence factor expression to changes in NaCl concentration.

The ability of TcpP/H to activate the *toxT*::*lacZ* fusion is dramatically reduced at high NaCl levels in *V. cholerae*. This suggests that TcpP/H may directly sense elevated Na^+ ion concentrations or some other signal associated with high osmotic stress (e.g., turgor pressure or perhaps the conformation of other membrane proteins that undergo osmotically triggered structural changes). If so, it would not be surprising that loss of the NQR activity (by mutation or HQNO intoxication) causes elevated *toxT*::*lacZ* activity, because the effect of the NQR complex is to pump out Na^+ ions. However, TcpP/H mediated activation of *toxT*::*lacZ* does not respond to elevated Na^+ ion concentrations in the *E. coli* heterologous background. Thus, the negative signal that TcpP/H sense as a result of high Na^+ concentrations may depend on another *V. cholerae*-specific product or physiological state. For example, another protein that negatively modulates TcpP/H activity may be induced by growth under elevated levels of NaCl. Because TcpP/H are putative membrane proteins, they may sense the activation state of the NQR complex directly through protein–protein interactions in the membrane. Alternatively, TcpP/H may sense the level of sodium gradient rather than high Na^+ concentrations *per se*.

Motility is an important virulence factor in a variety of pathogenic bacteria and, in some cases, is inversely regulated with other virulence factors (28). Motility in *V. cholerae* is known to be negatively regulated by the ToxR regulon. At least two ToxR-regulated genes on the TCP–ACF island, *tcpI* and *acfB*, encode proteins with high homology to methyl-accepting chemotaxis proteins, suggesting they are chemoreceptors, and mutations in these two genes negatively affect motility of *V. cholerae* as assayed by swarm plate assays (29, 30). Furthermore, *toxR* mutant strains display a hypermotile phenotype (22). Conversely, some nonmotile mutants showed constitutive expression of CT and TCP at alkaline conditions, whereas some hypermotile mutants expressed no CT and TCP under normally inducing conditions (22). It may be that the effects of the *nqr* mutation on *toxT* transcription are mediated indirectly via motility. Unlike *E. coli*, the single polar flagella of several *Vibrio* species are energized by sodium ions rather than protons. Inhibition of motility by phenamil or monensin as shown here strongly suggests that the flagellar motor of *V. cholerae* is also energized by the smf via translocation of sodium ions. Because the activity of the NQR enzyme complex is believed to generate a smf that can energize flagella rotation, perhaps a lack of the NQR activity reduces smf, which in turn slows flagella. TcpP/H or another regulatory factor might sense flagellar rotation rates directly via a mechanosensory mechanism or by sensing sodium flux through the flagellar motor. Consistent with this idea, we found that inhibiton of flagellar rotation by the addition of phenamil (a known inhibitor of sodium-driven flagellar motors) or monensin (an ionophore that changes the level of Na^+ chemical potential) or introduction of mutations resulting in nonmotile phenotypes lead to moderate increases in *toxT*::*lacZ* expression and CT production under *in vitro* expression conditions. Furthermore, increasing the media viscosity resulted in an even more dramatic induction of virulence factor expression. Viscosity is thought to increase laf gene expression in *V. parahaemolyticus* by a signaling process that involves the sensing of flagellar rotation speed (26), and perhaps a similar mechanism explains the relationship between virulence gene expression and flagellar function in *V. cholerae*. During infection, *V. cholerae* encounters a high-viscosity environment in the mucus lining of the gut. Sensing of the changes in viscosity may be one of the

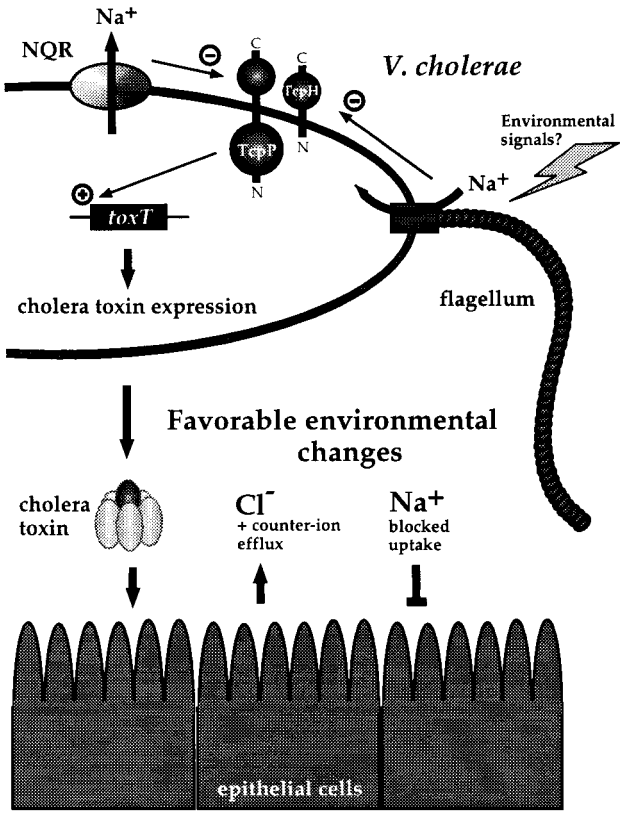

FIG. 5. Model of the interactions of some of the molecules affected by changes in membrane Na^+ flux. See text for detailed explanation.

signals that converts this organism from its environmental to its pathogenic phase.

Further experiments will be necessary to elucidate how changes in membrane sodium flux and motility affect virulence gene expression in *V. cholerae*. It is tempting to speculate that many signals affecting the ToxR regulon may do so by altering motility, smf, or Na^+ flux (Fig. 5). In agreement with this model are the observations that some of the conditions that negatively affect the ToxR regulon, such as high pH and bile, result in hypermotility (20, 31). Alternatively, it is possible that the effects of the *nqr* mutation on *toxT* transcription are mediated indirectly by affecting the ATP levels and hence cAMP levels in the cell. This could lead to altered states of the CRP protein, which is known to negatively affect *toxT* transcription (27). However, although we obtained three independent *nqr* mutants and mutations in several other genes, including *hns*, *fumA*, and *glmS*, that resulted in significantly raised *toxT* transcription, none of these were in either *crp* or *cya*, suggesting that the effects of these mutations may be more prominent than the latter two.

The data presented here strongly suggest that the expression of the main virulence factors of *V. cholerae* appears to be intimately connected to the sodium energetics in this halophilic organism. Sodium regulation probably plays a role in both of the major environments of *V. cholerae*, the intestine and water sources. It has been argued that one of cholera toxin's functions is to generate a high-Na^+ environment for *V. cholerae* in the lumen of the intestine (32). It is clear that the toxin causes electrolyte levels in the intestinal lumen to increase, and perhaps this milieu is a more favorable environment for the intraintestinal growth of *V. cholerae* (Fig. 5). This might lead to a negative-feedback mechanism, because elevated extracellular NaCl concentrations result in reduced cholera toxin production. Furthermore, it has been hypothesized that the sodium cycle of energy plays a role in the persistence of *V. cholerae* in the environment, as induction of this type of energy coupling may increase the resistance of bacteria to various environmental factors (33). It is conceivable that changes in the sodium cycle of energy are the primary signals that this bacterial species uses to sense whether it is in the extrahost environment or the human gut.

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