Role of sodium bioenergetics in *Vibrio cholerae*

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Abstract

The ability of the bacterium to use sodium in bioenergetic processes appears to play a key role in both the environmental and pathogenic phases of *Vibrio cholerae*. Aquatic environments, including fresh, brackish, and coastal waters, are an important factor in the transmission of cholera and an autochthonous source. The organism is considered to be halophilic and has a strict requirement for Na\(^+\) for growth. Furthermore, expression of motility and virulence factors of *V. cholerae* is intimately linked to sodium bioenergetics and to each other. Several lines of evidence indicated that the activity of the flagellum of *V. cholerae* might have an impact on virulence gene regulation. As the *V. cholerae* flagellum is sodium-driven and the Na\(^+\)-NQR enzyme is known to create a sodium motive force across the bacterial membrane, it was recently suggested that the increased toxT expression observed in a nqr-negative strain is mediated by affecting flagella activity. It was suggested that the *V. cholerae* flagellum might respond to changes in membrane potential and the resulting changes in flagellar rotation might serve as a signal for virulence gene expression. However, we recently demonstrated that although the flagellum of *V. cholerae* is not required for the effects of ionophores on virulence gene expression, changes in the sodium chemical potential are sensed and thus alternative mechanisms, perhaps involving the TcpP/H proteins, for the detection of these conditions must exist. Analyzing the underlying mechanisms by which bacteria respond to changes in the environment, such as their ability to monitor the level of membrane potential, will probably reveal complex interplays between basic physiological processes and virulence factor expression in a variety of pathogenic species. © 2001 Published by Elsevier Science B.V.

Keywords: Cholera; Na\(^+\)-NQR; Sodium motive force; Virulence; Motility; *Vibrio cholerae*

1. Introduction

Cholera is the most severe of many diarrheal diseases that affect humans and is responsible for significant morbidity and mortality especially in children in developing countries. Cholera is a waterborne disease and the bacteria are usually transmitted via contaminated food or water. Upon ingestion, the organisms colonize the small intestine where they elaborate the potent cholera toxin. The action of this proteinaceous enterotoxin is directly responsible for the profuse diarrhea characteristic of the disease. The bacteria are shed in large numbers in the typical ‘rice water’ stool into the environment, where they can associate with various other members of the ecosystem until they are ingested again, completing the life cycle of this organism.

*Vibrio cholerae*, the causative agent of the potentially lethal disease cholera, is a Gram-negative motile bacterium with a single polar flagellum. The first
six reported cholera pandemics were caused by classical *V. cholerae* bacteria of the O1 serogroup. The seventh pandemic that still continues today began in 1961 and is caused by a new biotype of *V. cholerae* O1, called El Tor. The El Tor biotype of *V. cholerae* is known to survive better in harsh environmental conditions and has now virtually completely displaced the classical biotype throughout the world. *V. cholerae* non-O1 serogroups were not known to cause epidemics of diarrhea; however, they cause sporadic cases and small outbreaks of diarrhea and extraintestinal infections. In 1992, an epidemic of cholera caused by a non-O1 serogroup broke out. The causative agent was found to be a new serogroup of *V. cholerae*, defined as O139. Several lines of evidence suggested that *V. cholerae* O1 El Tor gave rise to O139 by acquisition of novel DNA which was inserted into and replaced part of the O antigen gene cluster of the recipient strain. Currently, the *V. cholerae* O1 and O139 serogroups coexist in the Asian continent.

2. Environmental phase

The history of cholera reveals a remarkably strong association with the sea. Virtually all of the great pandemics followed coastlines of the world oceans. Initial cases characteristically occur along coastal waters and outbreaks were commonly ascribed to ships arriving from cholera-epidemic regions and more recently to discharge of ballast water from ships. The occurrence of the disease is typically seasonal and correlates with tidal estuaries and riverine systems. Zooplankton blooms quickly follow phytoplankton blooms, leading to conditions associated with cholera outbreaks. An association of *V. cholerae* with plankton has been demonstrated and is influenced and probably controlled by the physical and chemical characteristics of the environment [1]. The end of outbreaks in some endemic regions usually coincides with the beginning of the monsoons, when the salinity of the water decreases [2,3]. The autochthonous nature of *V. cholerae* in the aquatic environment has great significance with respect to function in the natural cycles of aquatic ecosystems.

*V. cholerae* is a normal inhabitant of aquatic environments as one of the free-living bacterial species found in estuarine areas [4]. Salinities favorable for growth of *V. cholerae* are found primarily in inland coastal areas and estuaries but the bacterium thrives in seawater as well. The estuarine environment is an ideal setting for the survival and persistence of *V. cholerae* [5]. The natural niche of this organism might include association with plants, cyanobacteria, and algae as part of the phytoplankton. Many *V. cholerae* species are associated with mollusks and chitinous zooplankton, such as copepods, and also can survive on fish or shellfish [3]. Resistance and survival in saline aquatic habitats may play a key role in the persistence of cholera and the emergence of new epidemics. Indeed, it was hypothesized that the sodium cycle is essential for the persistence of *V. cholerae* in the environment as induction of this type of energy coupling may increase the resistance of the bacteria to various environmental factors [2]. Although most environmental strains of *V. cholerae* are non-pathogenic and in general, *V. cholerae* non-O1 strains survive better than O1 under varying environmental conditions, there is a close relationship between the titers of *V. cholerae* O1 and the temperature and salinity of estuary water [2,3]. Furthermore, serogroup conversion of *V. cholerae* non-O1 to *V. cholerae* O1 was affected by the growth state of the cells, temperature, and salinity [6].

*V. cholerae* is a halotolerant microorganism whose growth is stimulated by sodium and it survives under a wide range of conditions of salinity and pH. *V. cholerae* strains are mostly isolated from environmental sites with NaCl concentrations between 0.2 and 2.0% [4], and the bacteria survive in vitro in 0.25–3.0% salt; the optimal salinity is considered to be 2.0% [7]. Indeed, *V. cholerae* has a strict requirement for Na\(^+\) for its growth. The optimal pH for survival ranges from 7.0 to 9.0 depending on the salinity [7]. Pathogenic *V. cholerae* strains grow in water with low salinity if the water temperature is relatively high and organic nutrients are present in high concentrations and survival of *V. cholerae* in seawater for more than 50 days was demonstrated. *V. cholerae* can survive under unfavorable environmental conditions in a dormant, viable but non-culturable, state [4]. Presenting a spore-like stage, dormant cells can survive changes in temperature, salinity or availability of organic matter and can remain infectious.
2.1. Sodium motive force generation (SMF)

In vivo, *V. cholerae* must survive a large range of conditions, including passage through the acidity of the stomach, in order to colonize the small intestine. All organisms survive variations in pH and salinity by employing homeostatic mechanisms. Some organisms that thrive in a high sodium environment with moderately alkaline pH can use sodium instead of protons as a chemiosmotic ion. At alkaline pH the bacterial cytosol is more acidic than the external medium. This makes it difficult to build up a significant $\Delta\mu_{\text{H}^+}$. On the other hand, the high sodium concentration outside the cells makes it relatively easy to build up a large $\Delta\mu_{\text{Na}^+}$ (the sum of $\Delta p_{\text{Na}^+}$ and $\Delta \Psi$). Consistent with this, the sodium motive force (SMF) has been shown to drive several transporters and the movement of one type of flagellum in these organisms [8,9]. In *V. cholerae*, SMF is believed to be generated by different mechanisms depending on the outside pH, including by use of antiporters at neutral pH and direct sodium extrusion via Na$^+$-translocating NADH:ubiquinone oxidoreductase (Na$^+$-NQR) at alkaline conditions (Fig. 1).

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**Fig. 1. Proposed *V. cholerae* energy-coupling system.** The picture shows the membrane protein complexes involved in sodium and proton translocation in *V. cholerae*. This figure was constructed based on the information obtained from the *V. cholerae* genome from the web site of the Institute for Genomic Research (TIGR): http://www.tigr.org/tigr-scripts/CMR2/GenomePage3.spl?database=gvc. The aerobic respiratory chain is depicted starting with Na$^+$-NQR as the gateway for electrons, which are transferred to ubiquinone-8 (Barquera and Gennis, unpublished results). A $b_{c_1}$ complex is then reduced by ubiquinol and then electrons are moved to one of the multiple cytochrome $c$s and hence to cytochrome $c$ oxidase, which is homologous to known $cbb_3$-type oxidases. In fact preliminary biochemical and genetic data (Christian, Barquera and Gennis) indicate the presence of a $cbb_3$-type cytochrome $c$ oxidase. For simplicity other oxidases were not included; at least two gene clusters apparently encoding cytochrome $b$-type oxidases are present. Other enzyme complexes shown include the sodium-dependent flagellum, the sodium-dependent amino acid uptake system (i.e., sodium-alanine, sodium-glutamate and sodium-proline symporters), the Na$^+$/H$^+$ antiporter(s), and the H$^+$-ATPase.
2.2. Na\textsuperscript{+}-NQR

Na\textsuperscript{+}-NQR is a unique redox-driven sodium pump which is found in the respiratory chains of a number of marine and pathogenic bacteria [10–12]. This enzyme is of special interest, for two reasons. First, it is an important part of the sodium cycle, a critical aspect of bacterial physiology. Secondly, Na\textsuperscript{+}-NQR offers new possibilities for the mechanistic study of redox-driven ion pumps. Most such pumps translocate protons, and this limits the experimental approaches that can be used in aqueous solution, whereas sodium is much more easily manipulated and traced. In those organisms that express Na\textsuperscript{+}-NQR, this enzyme is the gateway for electrons into the respiratory chain. Na\textsuperscript{+}-NQR accepts reducing equivalents from NADH and donates them to the quinone pool (Fig. 2) [13,14]. The energy from this redox reaction is used to pump sodium from the inner to the outer side of the membrane building a SMF, which is used for metabolic work [8,9]. Almost all of the organisms in which Na\textsuperscript{+}-NQR has been found are either marine or blood-borne bacteria [10,12].

The DNA sequence of the Na\textsuperscript{+}-NQR operon is now known for a number of organisms including V. alginolyticus, V. harveyi, V. cholerae, Haemophilus influenzae, Neisseria gonorrhoeae, and N. meningitidis, Yersinia pestis, Shewanella putrefaciens, Pseudomonas aeruginosa and Porphyromonas gingivalis. The enzyme does not share any structural or sequence homology with the proton translocating NADH quinone oxidoreductase or NDH2s. Instead it appears to make up a unique family of bacterial sodium motive NADH quinone reductases. In all these organisms the Na\textsuperscript{+}-NQR operon is composed of six structural genes, which are arranged in the same order, designed A–F (or 1–6) (Table 1). Three of the subunits, A, F and C, are relatively hydrophobic, with one or two transmembrane helices. The other three, B, D and E, are very hydrophobic with several transmembrane helices predicted in each. Subunit F con-
tains binding motifs for substrate (NADH), FAD and an FeS center and may constitute the dehydrogenase portion of the enzyme [15,16]. Computer predictions indicate that this subunit has one or two transmembrane helices followed by a relatively large hydrophilic domain where the NADH binding motif is found. One possible objection to two transmembrane helices is that the second putative helix includes an FeS binding motif. This may not be a problem because, as pointed out by Rich et al. [16], the FeS center would be located at the edge of the membrane rather than in the interior. In any case, the topology of the subunit will not be clarified without further data.

The Na\(^+\)-NQR enzyme has now been isolated from the *V. alginolyticus*, *V. harveyi* and *V. cholerae* ([12,17] and Barquera et al., unpublished results). In all cases, the six predicted subunits are present in the isolated enzyme. The purified enzyme from *V. cholerae* has similar catalytic properties as reported for the *V. harveyi* enzyme. Both Na\(^+\)-NQRs share the same cofactors, FAD, and FeS center and possibly covalently bound flavin (FMN) [12]. NqrCs from *V. harveyi* and *V. cholerae* both contain FMN ([12] and Barquera et al. unpublished results). Preliminary evidence indicates that this flavin participates in electron transfer. A recent paper by Nakayama et al. [18] also indicates the presence of covalently bound flavins (possibly FMN) in both the B and C subunits of Na\(^+\)-NQR from *V. alginolyticus*. However, almost nothing is known about the function of the other subunits, NqrA, D and E.

The Na\(^+\)-NQRs from *V. alginolyticus* and *V. harveyi* have been reconstituted into phospholipid vesicles. Under these conditions the enzyme could be clearly seen to function as a primary sodium pump with a Na\(^+\)/e\(^-\) stoichiometry of 1 [12,19–21].

The formation of ΔpH by the reconstituted Na\(^+\)-NQRs was increased by CCCP (carbonyl cyanide m-chlorophenylhydrazone) [12,19,20] and decreased by the sodium ionophore ETH-157 [12]. These findings indicate that the interior alkalinization in the vesicles is due to proton efflux – a secondary process – that discharges the ΔΨ created by sodium translocation. The enzyme does not generate ΔpH directly at all; in fact, the protons required for the formation of ubiquinol are taken up from the cytosolic side of the membrane, in contrast to what would be expected for a proton motive enzyme.

Na\(^+\)-NQR is not an essential enzyme for survival of *Vibrio* species; however, it seems to have an important role in the regulation of sodium homeostasis. Tokuda [22] has isolated a *V. alginolyticus* mutant with a disrupted Na\(^+\)-NQR subunit F. The mutant cells were able to grow at high sodium concentration and alkaline pH, to expel sodium, and to perform oxidative phosphorylation. However, these cells were no longer resistant to uncouplers. Recently Häse and Mekalanos [23,24] have obtained *V. cholerae* mutants in which Na\(^+\)-NQR has been interrupted by transposon mutagenesis. The mutant cells were able to grow although the growth was inhibited at low and high NaCl concentrations, suggesting a possible role of Na\(^+\)-NQR in the cellular sodium regulation.

The central question about NQR function is how the translocation of sodium across the membrane is coupled to the redox reaction. Another important question is how sodium is conducted to and from the actual sites of coupling. A number of cation

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channels have been studied, but most of these are passive conductances and the mechanisms which convey sodium through the membrane in NQR could be entirely different. Like primary proton pumps, \( \text{Na}^+\)–NQR couples a redox reaction to the translocation of ions against a membrane gradient. \( \text{Na}^+\)–NQR is an attractive system for the study of such chemiosmotic enzymes. NQR has a number of advantages as an experimental system. The enzyme’s protein structure is relatively simple and it can be purified in an active form. Also, the DNA sequences of \( \text{Na}^+\)–NQR from several organisms have been published, which opens the possibility of using genetic manipulation to answer specific structure–function questions.

2.3. Antiporters

Sodium–proton antiporters are ubiquitous membrane proteins that play an important role in the ion homeostasis of all living cells. Most bacterial antiporters exchange sodium for protons across the membrane in an electrogenic process that results in the expulsion of sodium driven by the electrochemical potential [25].

In *Escherichia coli* a system of two antiporters (NhaA and NhaB) is well established. At high external pH and high sodium concentrations, the *nhaA* gene is induced. NhaA seems to regulate the internal pH, acidifying the cytosol through electrogenic exchange of sodium for protons. The expression of NhaA is regulated by *nhaR*, which is a member of the LysR family of transcription regulators. A strain in which *nhaA* was deleted was unable to grow at high pH in the presence of 100 mM NaCl. Using this strain, Padan and Schuldiner [25] identified a second antiporter locus: *nhaB*. NhaB exchanges sodium for protons in an electroneutral manner and is expressed constitutively. A double mutant which lacked both NhaA and NhaB was constructed. Although this strain was sensitive to high pH, sodium transport was restored in the presence of a high potassium concentration or in a medium of high osmolarity [26]. This suggested that the system for sodium regulation in *E. coli* is more complex than previously reported. On this basis, it was proposed that NhaA and NhaB also have a role in maintaining the intracellular osmolarity and that a second unrelated enzyme maintains sodium balance under conditions of high osmotic pressure [26].

Sodium–proton antiporters have also been reported in a number of moderate and extreme alkalophiles where they also appear to have a role in maintaining pH homeostasis. For example, *Vibrio* species have developed similar systems in order to survive in high salt environments. Nakamura et al. [27] reported the cloning and sequencing of the *nhaB* gene from *V. alginolyticus*. This gene was able to functionally complement a mutant strain of *E. coli*, which lacked both *nhaA* and *nhaB*. *V. parahaemolyticus* has been reported to be able to restore growth in an *E. coli* mutant which lacked both *nhaA* and *nhaB* [28]. The activity of NhaD was pH-dependent with its highest activity at pH 8.5–9, indicating that this protein is optimized for the more basic conditions which the *Vibrio* face in their natural environment [28].

The now completed genome sequence of *V. cholerae* revealed gene homologs of *nhaA*, *nhaB*, and *nhaB*. Recently, NhaA from *V. cholerae* was found to function in a similar way to the homolog from *E. coli* [29]. Although wild-type *V. cholerae* tolerates high lithium concentrations, a mutant from which *nhaA* was deleted was not able to grow under these conditions, even though it was not sensitive to changes in sodium concentration and pH. They concluded that NhaA is important for survival of the organism in its natural habitat, but that this is not the only enzyme involved in sodium regulation [29]. Similarly, *V. cholerae* mutant strains carrying insertions in the *nhaR* gene showed sensitivity to lithium chloride at alkaline conditions. Furthermore, an *nhaR* mutation had no detectable effect on the virulence of the *V. cholerae* strain in an animal model [30].

3. Sodium motive force-dependent systems

3.1. Motility and virulence

Expression of the two main virulence factors of *V. cholerae*, cholera toxin (CT) and toxin-coregulated pili (TCP), is strongly influenced by environ-
mental conditions and is coordinately regulated via a cascade of regulatory proteins. The ToxR/S and TcpP/H proteins activate expression of the toxT gene, encoding ToxT, a key regulatory protein in the regulon. 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 proteins, ToxS and TcpH, respectively, that stimulate their activities. The ToxT protein is a cytoplasmic AraC-like transcriptional activator that directly activates the ctx and tcpA promoters, leading to expression of CT and TCP, respectively [31,32,24]. The mechanisms of the signal transduction events leading to the repression of these virulence factors by environmental clues are not yet understood. As ToxR/S and TcpP/H are integral membrane proteins, they might be able to directly respond to changes in the surrounding media perhaps by changing their conformation and/or dimerization. Some of the environmental conditions that negatively affect the ToxR regulon, such as bile and pH, are known to positively affect motility [33,34].

Motility is an important virulence factor in many pathogenic species and in some cases is inversely regulated with the expression of virulence traits [35]. Although the role of motility of V. cholerae in its ability to cause cholera has not been clearly estab-
lished, the production of CT and TCP is known to be affected by the motility phenotype of the bacteria. At least two ToxR-regulated genes on the TCP-ACF island, tcpI and acfB, encode proteins with homology to chemoreceptors, and mutations in these two genes positively affect motility of V. cholerae as assayed by swarm plate assays [36,37]. Furthermore, toxR mutant strains display a hypermotile phenotype, whereas some spontaneous hypermotile mutants lacked expression of CT and TCP under normally inducing conditions [38]. Conversely, some non-motile mutants showed constitutive expression of CT and TCP at alkaline conditions and increased toxT transcription [23,38].

We recently demonstrated that mutations in the nqr gene cluster, encoding a primary sodium extrusion pump (as well as a specific inhibitor of this pump) of V. cholerae, resulted in increased virulence factor expression [23,24]. It may be that the observed effects of the nqr mutation on toxT transcription are mediated indirectly via motility. Unlike E. coli, the single polar flagellum of V. cholerae is energized by SMF via translocation of sodium ions [23,39]. Because the activity of the NQR enzyme is believed to generate a SMF that can energize flagellar rotation, perhaps a lack of the NQR activity reduces SMF, which in turn slows flagella (Fig. 3). Consistent with this idea, inhibition of flagellar rotation by the addition of phenamil (aamiloride homolog and 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 non-motile phenotypes led to increases in toxT expression and CT production under in vitro expression conditions [23,38]. These observations led to the hypothesis that perhaps Na⁺ flux through the flagellum might be a signal for gene regulation and that the V. cholerae flagellum might act as a voltmeter, sensing even very small changes in membrane potential, and that a resulting change in flagella activity affects virulence gene expression [23,40]. However, we recently showed that an a-flagellate mutant derivative strain exhibited very similar increases in toxT expression levels after the addition of membrane-active drugs as the parental strain (Häse, unpublished results), indicating that although the membrane potential does appear to be sensed, the flagellum is not required for this effect. Furthermore, the a-flagellate mutant derivative strain responded to the addition of the ionophores as well as phenamil similarly to the motile parent strain (Häse, unpublished results). It is known that amiloride and its analogs inhibit bacterial antiporters [41,42], thus potentially affecting the overall membrane bioenergetics. Moreover, elevated toxT expression was observed in strains carrying a nqr mutation in combination with motility defects (Häse, unpublished results). These results indicate that the flagellum is not required for the effects of changes in the membrane energy levels on toxT transcription. However, the monitoring of ΔΨ by an as yet uncharacterized mechanism might provide V. cholerae with the necessary clues that convert this organism from its environmental to its pathogenic phase.

Interestingly, when the TcpP/H molecules alone were used to activate the toxT gene, increased gene expression was observed under conditions that are believed to change the membrane sodium bioenergetics of the bacteria, such as a nqr mutation, monensin, and phenamil [23]. These results suggest that these effects might be mediated through TcpP/H. Furthermore, the ability of the TcpP/H proteins to activate toxT was greatly diminished in the presence of high NaCl concentrations in the growth medium [23], suggesting that TcpP/H may directly sense elevated Na⁺ ion concentrations. However, TcpP/H-mediated activation of the toxT reporter construct did not respond to elevated NaCl concentrations in an 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 high 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 levels of intracellular Na⁺ or the level of sodium gradient across the membrane.

3.2. Other

In E. coli, proton motive force (PMF) is used for a variety of energy-dependent processes, including nu-
tient uptake, protein secretion via the Sec machinery, phage transduction, and efflux pumps. It is not yet clear whether similar processes in \textit{V. cholerae} are, like the flagellar system, solely dependent on SMF or are using PMF and/or SMF. \textit{V. cholerae} exports a variety of proteins into the extracellular milieu, including CT, protease, chitinase, and DNases, some of which play key roles in the pathogenesis of this organism. A Sec-like system is believed to be important in translocation across the cytoplasmic membrane, whereas a typical type II secretion apparatus is necessary for the crossing of the outer membrane. We have preliminary results indicating that the secretion of a major extracellular protease, HA/protease, of \textit{V. cholerae} is strongly reduced under conditions that do not favor the generation of a strong SMF, such as different growth conditions and the addition of ionophores (Häse, unpublished results). This indicates a potential role of sodium bioenergetics in the secretion of various proteins, including the main virulence factors, in \textit{V. cholerae}. Consistent with this, it was suggested that the electrochemical potential of Na$^+$, but not that of H$^+$, is important for protein translocation in \textit{V. alginolyticus} [43].

The cholera toxin genes are encoded on the genome of a filamentous phage, CTX$\Phi$, that uses TCP as its receptor [44]. By using a derivative phage that carries a kanamycin resistance marker in place of the ctx genes, transduction events can easily be scored quantitatively [24]. Incubations of a \textit{V. cholerae} strain strongly expressing the tox$\!T$ gene, and thus TCP, with monensin and/or CCCP resulted in reduced phage transduction events using the CTX$\Phi$-Kan derivative phage (Limburg et al., unpublished results). These very preliminary results indicate that perhaps phage entry into the host cells requires sodium membrane bioenergetics. This could have very important implications in the epidemiology of cholera as it has been suggested that environmental reservoirs of non-toxinogenic strains might be converted to potentially pathogenic organisms by uptake of the CTX$\Phi$ phage. Thus, environmental conditions that stimulate the induction of a strong sodium cycle of energy prior to epidemics could favor this event.

\textit{V. cholerae} can efflux of a variety of compounds and the genes for a putative multidrug pump have been cloned and characterized [45]. Indeed, the genome of \textit{V. cholerae} shows gene homologs of several putative drug pumps. We recently demonstrated that resistance of \textit{V. cholerae} to certain drugs, such as nalidixic acid, tetracycline, and ethidium bromide, is influenced by conditions that affect the formation of a SMF (Brown and Häse, unpublished results). These preliminary observations suggest a potential role of sodium bioenergetics in the activities of at least some MDR pumps in \textit{V. cholerae}. Understanding the energy requirements of these types of efflux systems clearly has broad implications not only for the treatment of infectious diseases.

4. Conclusions

Important roles of sodium bioenergetics in the environmental as well as pathogenic phases of \textit{V. cholerae} have been demonstrated. Indeed, this organism has a strict growth requirement for sodium and appears to use a direct sensing mechanism of Na$^+$ levels, perhaps by monitoring SMF across the cytoplasmic membrane, to either repress or strongly express its main virulence factors. 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 extra-host environment or the human gut. It has been argued that one of CT’s functions is to generate a high Na$^+$ environment for \textit{V. cholerae} in the lumen of the intestine. It is clear that cholera toxin causes electrolyte levels in the intestinal lumen to increase, and perhaps this milieu is a more favorable environment for the intra-intestinal growth of \textit{V. cholerae}.

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