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Growth Assays of *Vibrio cholerae* Membrane Transporter Mutants in Different pH and Cation Conditions

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Abstract

Vibrio cholerae achieves survival in various saline environments by a network of different Na+ dependent transporters, antiporters, and symporters. The most important transporters for sodium homeostasis are the sodium-translocating NADH-ubiquinone oxidoreductase (VC-NQR) and the secondary sodium pumps Vc-NhaA and Vc-NhaB. It has been difficult to tease out the metabolic role of Vc-NQR as deletion of this dominant contributor to aerobic respiration causes distorting growth defects. By adding L-lactate, we were able to overcome the basic growth defect of a Vc-NQR mutant [1]. Here, we report the growth characteristics of various single and double mutants of *V. cholerae* lacking these transporters in regards to cation resistance in different pH environments, in presence and absence of L-lactate. Overall, Vc-NhaA appears to be instrumental for sodium transport at pH 7.5 and 8.5 and lithium at pH 7.5 in concert with Vc-NQR, and essential at lithium concentrations of 75 mM and higher at pH 8.5 independent of Vc-NQR. In addition, there is some evidence that Vc-NhaA is able to transport potassium at high pH and under high potassium pressure.

Keywords:

Vibrio cholera; Bacterial membrane antiporter; Secondary sodium pumps; Lithium resistance; NQR

Importance

Vibrio cholerae's cation transporters located in the cellular membrane are essential to this pathogens survival in various challenging environments inside and outside of the human host. Exposing mutants, lacking Vc-NhaA, Vc-NhaB, or Vc-NQR, to challenging environments elevates our understanding of which transport proteins confer resilience to specific conditions and can guide the discovery of new drug targets in this or other species.

Introduction

Vibrio cholerae causes the epidemiologically relevant disease cholera, which manifests itself with a severe watery diarrhoea and can lead to the death of the patient.

V. cholerae is endemic to large parts of Southeast Asia, including India, China and Indonesia), and Sub-Saharan Africa [2], but can be found worldwide in estuarine and coastal waters. Environmental resilience contributes to the life cycle of this human pathogen and also guarantees the re-occurrence of *V. cholerae* as a perpetual, lurking threat to human public health. The pathogen is remarkable in its ability to survive in a wide range of saline environments, supported by a plethora of genes that encode for primary and secondary sodium pumps **(Figure 1)**.

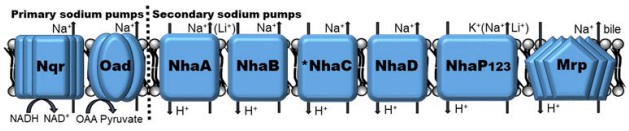

Figure 1: Sodium motive force (SMF) generators located in the inner membrane of *V. cholerae*. Generators of SMF present in *V. cholerae* N16961 and O395 are shown. Primary Na+ pump, Nqr and Na+-translocating oxaloacetate decarboxylase (Oad), generate SMF by direct sodium extrusion. The sodium extrusion activity of NQR is coupled by the NADH oxidation and ubiquinone reduction. The sodium extrusion activity of Oad is coupled by the decarboxylation of oxaloacetate. Na+ (K+)/H+ antiporters, NhaA, NhaB, NhaC and NhaP and Mrp, convert proton motive force into SMF and vice versa. NqrA-F, OadABG and MrpA-F consist of multiple sub-unit proteins. The asterisk represents the Na+/H+ antiporter family NhaC that has not yet been characterized biochemically in *V. cholerae*.

These pumps maintain a sodium and energy gradient across the cellular membrane that enables survival in high sodium (and lithium) environments, energy production, movement, pH

homeostasis - especially under alkaline conditions, and nutrient acquisition [3,4]. While the primary sodium pump, NQR, is not found in *Escherichia coli*, the secondary sodium pumps NhaA and NhaB have been extensively studied in *E. coli* and other bacteria. Although they all share many comparable properties, their functionality differs among the different species. For example in *Pseudomonas aeruginosa*, NQR functions as a proton pump [5].

The antiporter NhaA in *E. coli* (Ec-NhaA) shows highest activity at pH 8.5 and loses activity below pH 6.5 [6], transports two H+ versus one Na+ or Li+ [7], is preferably expressed at higher cytosolic sodium conditions dependent of other sodium pumping systems [8, 9] and is essential against lithium toxicity [10]. Comparable to Ec-NhaA, the NhaA antiporter of *V. cholerae* reveals Na+/Li+ antiport activity that reaches a maximum at a pH of 8.0 [11]. The expression of Vc- NhaA in an *E. coli* mutant lacking nhaA showed restoration of function similar to its wild-type [12]. In comparison to NhaA, Ec-NhaB antiporters display less activity and are therefore often termed "housekeeping" cation exchanger with auxiliary role [13].

Similar to NhaA, NhaB exchanges sodium ions for protons electrogenically, as three protons versus two sodium ions are transported [13-15]. Ec-NhaB does not display pH sensitivity [16]. In contrast, NhaB in *Vibrio alginolyticus* is pH-dependent and reaches its activity maximum at alkaline pH [17]. The role of lithium transport via NhaB-type antiporters is still unclear, but there is evidence that NhaB extrudes lithium at alkaline pH in several *Vibrio* species, including *V. cholerae* [15] and *V. parahaemolyticus* [18], *Pseudomonas aeruginosa* [19], and also *Klebsiella pneumoniae* [20] even if the affinity to Li+ was much lower.

NQR is a primary sodium pump that is formed by a complex of six membrane proteins, encoded by nqrA-F [4,21,22], that accept electrons from NADH and transfer them to the quinone pool while exporting sodium ions across the membrane. This process is efficient at pH<7.5 meanwhile there is proposed leakage of Na+ through the NqrB subunit at pH ≥ 7.5 [23].

Takuda and Unemoto [24] showed that NQR in *Vibrio alginolyticus* was unlikely to export lithium because Na+/H+ antiporter mutants were sensitive to lithium but not sodium. Toulouse et al. [23] proposed that Vc-NQR is able to export lithium, but similar to the sodium leakage, it generated less of a transmembrane voltage possibly due to some cation backflow of the slightly smaller Li+ ions through the B subunit channel of Vc-NQR at a pH between 6.5 and 9 [23].

Nevertheless, the importance of Vc-NQR on lithium toxicity remains unclear and we therefore examined the growth phenotypes of *V. cholerae* mutants lacking the primary sodium pump (Vc- NQR) and/or the secondary Na+ pumps, Vc-NhaA and Vc-NhaB, under different pH and cation concentrations. To compensate for the general growth defect of the Vc-NQR mutant [1], we also performed these growth analyses in the presence of L-lactate.

Materials and Methods

Bacterial strains and culture conditions

Table 1 contains all strains employed in this study.

Table 1: Strains and plasmids used in this study.

Strains	Description	Source or Reference
V. cholerae		
O395N1	O1 classical biotype strain, lacZ−, Smr	Dr. John Mekalanos
ΔnhaA	O395N1, ΔnhaA, Smr,	This study
ΔnhaB	O395N1, ΔnhaB, Smr,	This study
ΔnqrA-F	O395N1, ΔnqrA-F, Smr,	(Barquera, et al. 2002)
ΔnhaA, ΔnhaB	O395N1, ΔnhaA, ΔnhaB, Smr,	This study
ΔnqrA-F, ΔnhaA	O395N1, ΔnqrA-F, ΔnhaA, Smr,	This study
ΔnqrA-F, ΔnhaB	O395N1, ΔnqrA-F, ΔnhaB, Smr,	This study
E. coli		
SM10λpir	Host for suicide cloning vector	(Miller, Mekalanos, 1988)
Plasmid	**Description**	**Source or Reference**
pWM91	Suicide vector, Ampr, oriR6K, mobRP4	(Metcalf, et al. 1996)

The classical biotype Ogawa strain, *V. cholerae* strain O395N1, with partial deletion of ctxA and streptomycin resistance, and its sodium pump mutant derivative strains were cultured in Luria broth (LB Lennox; 10 g/L tryptone (Difco), 5 g/L Yeast extract (Sigma), 10 g/L sodium chloride) or LBB- (non-cationic Luria broth buffered with 60 mM Bis-Tris propane (BTP) hydrochloride) and constant shaking at 200 rpm unless otherwise stated.

It should be noted that without addition of cations, our LBB-media contains residual cations of 13-19 mM Na+ and 16-21 mM K+. 100 µg/ml streptomycin, 100 µg/ml ampicillin, 50 g/l sucrose (BDH), 4 M NaCl, 4 M KCl, 4 M LiCl and 33 mM L(+)− lactic acid (Alfa Aesar) were used where appropriate (Table 2).

Table 2: Primers used in this study.

1VcNhaA	GGGGGGGATCCGTGATTAATGGCAAGAAAGTGAG
2VcNhaA	GACTGACTGACTGACTGACTGACTCATAGGTTTGTCCTTAAATTATG

3VcNhaA	AGTCAGTCAGTCAGTCAGTCTAATCATTTCATAGGCTTTGAAC
4VcNhaA	GGGGGGAGCTCCGATGGGCCACAGAACTGGATCACAC
1VcNhaB	GGGGGGGATCCGATCATGATCCGCTCTGCGCTC
2VcNhaB	AGTCAGTCAGTCAGTCAGTCAGTCCATGATGATTACTCTTTAACTG
3VcNhaB	AGTCAGTCAGTCAGTCAGTCTAAATTTCAATTGAAACTGAAAC
4VcNhaB	GGGGGGAGCTCGCTCTATTTACCTGCCGGTTCCAG

In-frame deletion of nhaA and nhaB in *V. cholerae*

The gene splicing by overlap extension (SOE) method [25,26] was used to generate the single mutants ΔnhaA and ΔnhaB, and double mutant strains, Δnqr/ΔnhaA, Δnqr/ΔnhaB and ΔnhaA/ΔnhaB, essentially as described earlier [27].

Growth analyses

Individual colonies of the wild-type (WT), the single mutants ΔnhaA, ΔnhaB, Δnqr and the double mutants ΔnhaA/ΔnhaB, ΔnhaA/Δnqr, ΔnhaB/Δnqr were grown in 14 × 5 ml LBB- medium that was adjusted to pH 7.5 and supplemented with 5 µl streptomycin. The cultures were grown overnight at 37°C for 24 h with vigorous shaking. The cultures where then adjusted to an optical density at 595 nm (OD595) of 0.5.

For each of the three tested pH, six reagent reservoirs (VWR) with 10 compartments were prepared with LBB- containing lactate or without lactate and adjusted to a cation concentration of 0 mM, 100 mM, 200 mM, 300 mM and 400 mM for NaCl and KCl, or 0 mM, 25 mM, 50 mM, 75 mM and 100 mM for LiCl. Each reservoir provided 2 technical replicates. 180 µl of the appropriate cation broth solution was added into each well of the appropriate column of eighteen 96-well plates, creating two technical replicates per plate. 20 µl of cell solution of each strain was added to the wells of each row.

The plates were lidded and parafilmed before incubation for 24 h at 37°C with shaking. The bacterial growth rates in the plates were measured with a Biorad plate reader at 595 nm and after 0 h, 18 h, and 24 h. The experiment was repeated for a minimum of four times for all pH values.

Statistical analysis

Statistical analysis was performed using GraphPad Prism Version 6.07. A two-way ANOVA and a subsequent Tukey's multiple-comparison test were used to evaluate the results. For all strains, eight to twelve biological replicates, with each having two technical replicates were analyzed. The threshold for significance was $p<0.05$.

Results and Discussion

Overall growth phenotypes of the V. cholerae strains as a function of cation concentration, pH and lactate

Overall, all tested *V. cholerae* strains grew best at slightly acidic conditions **(Figure 2)**, which is not surprising as *V. cholerae* contains a complex acid tolerance response that involves multiple factors, including OmpU, RecO, the cad system, HepA, GshB, and NhaP-antiporters [28–31] and thus is well equipped to withstand acidic conditions. At pH 6.5 and 7.5, the wild-type generally profited from moderate to high cation concentrations up to 400 mM (NaCl and KCl) and 100 mM LiCl, regardless whether lactate was present **(Figures 2 and 3)**

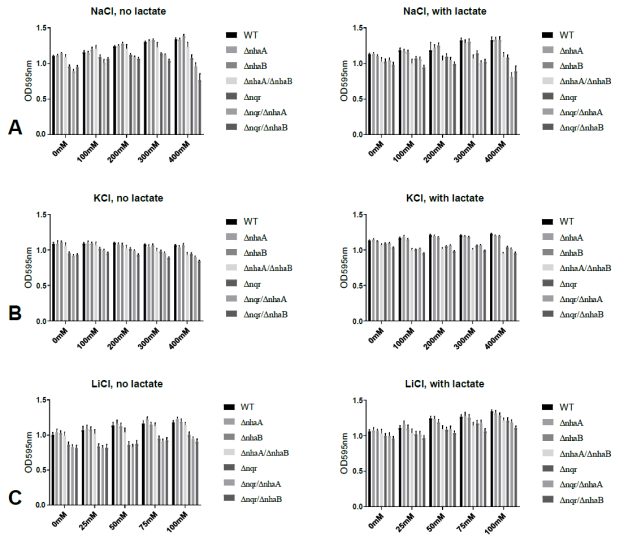

Figure 2: *Vibrio cholerae* wild-type and mutant strains grown with constant aeration at 37°C for 24 hours at pH 6.5 with 0-400 mM sodium chloride (panel A), 0-400 mM potassium chloride (panel B), or 0-100 mM lithium chloride (panel C). In addition, panels on the right side show strains grown with 33 mM lactate, while panels on the left side are grown without any lactate. Bars represent means plus standard errors of the means (SEM).

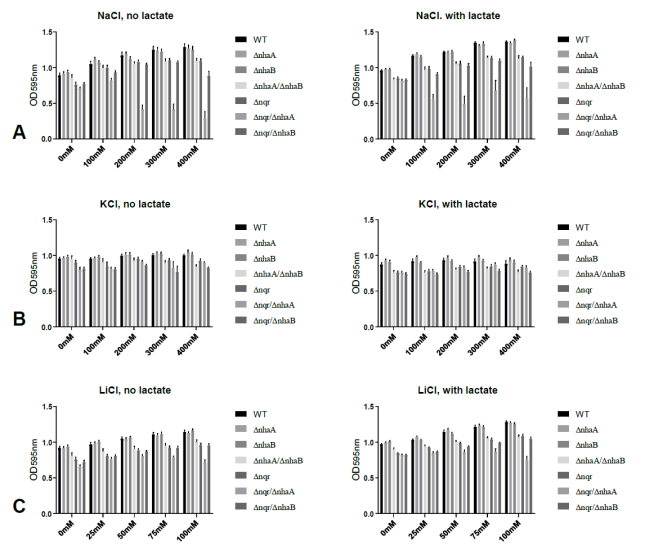

Figure 3: *Vibrio cholerae* wild-type and mutant strains grown with constant aeration at 37°C for 24 hours at pH 7.5 with 0-400 mM sodium chloride (panel A), 0-400 mM potassium chloride (panel B), or 0-100 mM lithium chloride (panel C). In addition, panels on the right side show strains grown with 33 mM lactate, while panels on the left side are grown without any lactate. Bars represent means plus standard errors of the means (SEM).

An exception to that was noticeable at pH 6.5, where no strain reacted to increasing potassium concentrations (**Figure 2B**, left panel), except when lactate was present (**Figure 2B**, right panel). Lactate addition aims to replenish the quinone pool via L-lactate dehydrogenase when nqr is deleted. Overall, it slightly increased the growth of strains at all pH and cation conditions, likely because it supported respiration efforts while also being utilized as carbon source.

Growth phenotypes of single and double deletions of nhaA, nhaB and nqr of *V. cholerae* strain O395N1 as a function of sodium concentration, pH and lactate

Similarly to the wild-type, increasing concentrations of sodium slightly improved growth of all mutants, with minor exceptions at high sodium concentrations. At pH 6.5 and the addition of 400 mM NaCl, both the Δnqr/ΔnhaA and Δnqr/ΔnhaB double mutants displayed slight sodium sensitivity and the addition of lactate partially corrected the growth of Δnqr/ΔnhaB, although not being statistically different to Δnqr/ΔnhaA ($p=0.9$) **(Figure 2A)**.

There was a statistical difference to the Δnqr single mutant and the ΔnhaA/ΔnhaB double mutant that was not present with 300 mM sodium, rendering this small change noteworthy **(Figure 2A)**. It seems that Vc-NQR plays some role at high sodium and pH 6.5, with support from NhaA and auxillary help by NhaB **(Figure 2A)**.

However overall, sodium environments did not seem to have much effect on any of these strains, highlighting the well-coordinated acid and sodium tolerance response in this organism that is likely relying on an abundance of membrane proteins including the ones highlighted in this study **(Figure 1)**.

At pH 7.5, sodium transport activity seems to rely on the simultaneous presence of Vc- NhaA and Vc-NQR, as addition of lactate only slightly restored growth of the Δnqr/ΔnhaA double mutant when ≥ 100 mM sodium is present **(Figure 3A)**. Recent findings have shown that at pH 7.5 and higher, NQR could experience some Na+ backflow, making its pumping less effective [23], however our results suggest that Vc-NQR activity is still required when nhaA is deleted. This confirms our earlier findings that were based on transcriptome analysis and suggested that Vc-NhaA complements the sodium pumping activity of Vc-NQR [32].

The only moderate growth reduction of the Δnqr/ΔnhaA double mutant **(Figure 3A)** could be explained by the likely pH-independent Vc-NhaB sodium pump and/or additional antiporters, such as Vc-NhaD, that could be collaborating under these conditions. Earlier work by Dzioba et al. [33] using everted membrane vesicles suggested that the Vc-NhaD antiporter can export sodium at pH 7.5 (and 8.5), but abolishes all activity at pH 6.5. More in depth investigations into the roles of Vc-NhaD with Vc-NhaA, Vc-NhaB and Vc-NQR for *V. cholerae* growth would be very valuable, as most previous studies were either done with membrane vesicles, in *E. coli*, or using cell counts on agar plates.

The most dramatic effects on bacterial growth were found at pH 8.5 **(Figure 4A)**. Without lactate, all strains that lack Vc-NQR were moderately growth inhibited at pH 8.5 in comparison to pH 7.5 and 6.5, regardless of sodium concentration **(Figure 4A, left panel)**. The addition of lactate mostly recovered that deficit, indicating that much of that pH sensitivity is related to loss of respiration in the absence of Vc-NQR **(Figure 4A, right panel)**.

With the addition of lactate the growth performances of all strains resembled those observed at the pH 7.5 condition **(Figures 3A and 4A, left panel)**, with only the Δnqr/ΔnhaA double mutant being moderately growth deficient regardless of sodium addition. This again could be explained by the necessity of Vc-NhaA in combination with Vc-NQR, and a relatively inferior role of Vc-NhaB. Lastly, the generation of a proton- gradient across the cell membrane is difficult at high pH, as the cell environment is more alkaline than the cytoplasm, and this is likely reflected in the diminished overall bacterial growth at pH 8.5 **(Figure 4A)**.

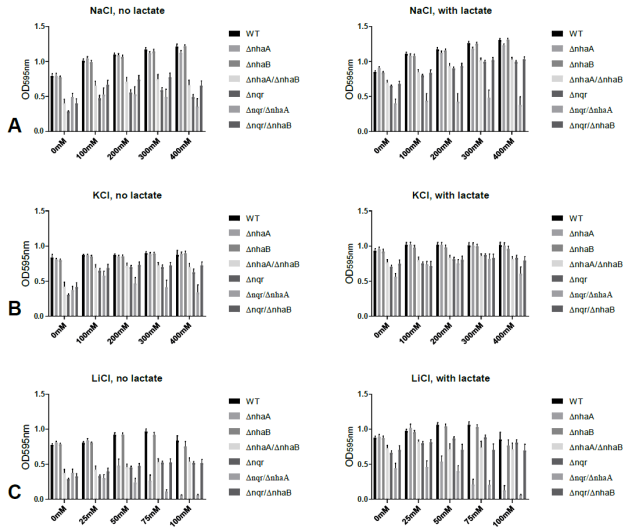

Figure 4: *Vibrio cholerae* wild-type and mutant strains grown with constant aeration at 37°C for 24 hours at pH 8.5 with 0-400 mM sodium chloride (panel A), 0-400 mM potassium chloride (panel B), or 0-100 mM lithium chloride (panel C). In addition, panels on the right side show strains grown with 33 mM lactate, while panels on the left side are grown without any lactate. Bars represent means plus standard errors of the means (SEM).

Growth phenotypes of single and double deletions of nhaA, nhaB and nqr of *V. cholerae* strain O395N1 as a function of potassium concentration, pH and lactate

As expected, across pH 6.5 and 7.5, all strains tolerated increasing concentrations of potassium well **(Figures 2B and 3B)**, suggesting that neither of the investigated proteins has a principal role in potassium transport at those pH conditions.

At pH 8.5, all strains, including the Δnqr mutant, grew better with the addition of potassium **(Figure 4B)**, which could underline the importance of the potassium transporting.

NhaP homologues that help maintain the transmembrane voltage in absence of sodium (proton motive force) [27, 31,34]. Without lactate, lack of Vc-NQR does not interfere with growth when potassium concentrations increased, but if both Vc-NQR and Vc- NhaA are missing growth was reduced compared to the experiments with sodium **(Figure 4B vs. Figure 4A**, left panel). In addition, when lactate was added a slight sensitivity at the highest potassium concentration remained with that double mutant **(Figure 4B**, right panel). This could hint at some potassium-transporting capacity of Vc-NhaA at high pH under high potassium pressure, which might be masked by strong NhaP2 activity under those conditions [34]. This would be a remarkable new finding and these intriguing observations should be further investigated.

Growth phenotypes of single and double deletions of nhaA, nhaB and nqr of *V. cholerae* strain O395N1 as a function of lithium concentration, pH and lactate

Addition of lithium chloride was evaluated because Li+ is an analogue of Na+ and toxic to bacterial cells even at low concentrations. Lithium concentrations of 25 to 100 mM were evaluated in these experiments as higher concentrations tended to be lethal for these strains. At pH 6.5, strains did not show any sensitivities to increasing concentrations of lithium, regardless of the presence of lactate **(Figure 2C)**. This indicates that none of the proteins evaluated in this study are essential against lithium toxicity at acidic pH.

At pH 7.5, growth of the Δnqr/ΔnhaA double mutant did not improve with increasing lithium concentrations in contrast to all other strains **(Figure 3C)**. This difference is particularly evident with the addition of lactate **(Figure 3C**, right panel). The Δnqr/ΔnhaA double mutant displayed slightly inferior growth performance in the presence of 100 mM lithium in comparison to the no lithium control, while all single mutants and the ΔnhaA/ΔnhaB double mutant grew well **(Figure 3C**, right panel). This suggests that both NhaA and NQR are somewhat involved with lithium export at this pH.

More remarkable were the changes at pH 8.5 **(Figure 4C)**. All strains tolerated a lithium concentration of 25 mM well. However, only the wild-type strain and the ΔnhaB mutant grew favorably at lithium concentrations up to 75 mM, while 100 mM generally stunted growth minimally. The addition of lactate recovered the growth of strains that lack NQR with the exception of the Δnqr/ΔnhaA double mutant and the ΔnhaA single mutant at lithium concentrations of 50 mM and higher **(Figure 4C**, right panel).

This suggests that NhaA is crucial for lithium expulsion at high pH, supporting Herz et al. [11], who suggested that NhaA is a pH-dependent Li+ transporter, while neither NQR, nor NhaB are very effective at lithium transport. These findings are in accordance with Toulouse et al. [23] who proposed that the NqrB subunit leaks lithium due to its small size at pH 6.5 to 9.0. Curiously, the ΔnhaA/ΔnhaB double mutant did experience much less dramatic lithium sensitivity than expected **(Figure 4C)**, encouraging further investigations into lithium transport activities of these antiporters in the future.

Conclusion

As *V. cholerae* lacks the gene for a Complex I type of enzyme, Vc-NQR is likely the major respiratory enzyme that transfers electrons from NADH to quinone while simultaneously translocating sodium ions across the cellular membrane. Loss of Vc- NQR has major implications in cellular respiration and Na+ homeostasis, which can be visually observed by much slower growth and thus smaller colony-size on “regular” LB agar plates (data not shown). With the addition of lactate, we aimed at replenishing the quinone pool via L-lactate dehydrogenase (1).

In our earlier work, we concluded that loss of Vc-NQR did not affect osmotic stress resistance and suggested, based on transcriptome analysis, that NhaA possibly complements the sodium transport activity of NQR (32). Indeed the present study

indicates that NQR is relevant for sodium transport at pH 7.5 and 8.5, as it supported NhaA, which was essential at high pH. It had been earlier suggested that, when expressed in *E. coli*, the *V. cholerae* NQR and NhaA proteins collaborate to confer sodium and lithium resistance [11]. However, in V. cholerae, we had shown that NQR at pH 7.8 is specific for sodium but less important for lithium [35] and in the present study we found that to be also true at pH 8.5.

Generally, none of the transport proteins Vc-NhaA, Vc-NhaB, or Vc-NQR seem to have essential cation-transporting activities at pH 6.5, or the effects of their deletions are appropriately covered by other antiporters, e.g. NhaD, the NhaC family, Mrp, NhaP, etc. We present evidence that NhaA might be able to transport potassium at high pH and potassium levels. Our earlier results strongly implied that potassium resistance of *V. cholerae* at a wide range of pH is mainly conferred by NhaP1 [27], NhaP2 [34,36] and NhaP3 [31]. It is however possible that these NhaP-type antiporters, annotated Kef-type potassium transporters [37] and possibly unidentified *V. cholerae* homologues to *E.coli's* potassium export systems [38,39] could mask possible potassium pumping activity of Vc-NhaA and this finding should be investigated in the future.

The biggest cation sensitivities of our mutant strains were observed at pH 8.5. NhaA was not only essential for sodium transport but also lithium, when lithium concentrations were 50 mM or higher. Curiously, the ΔnhaA/ΔnhaB double mutant was not as sensitive to increasing lithium concentrations as expected, and the role of NhaB in lithium transport is still rather obscure. More investigations to better understand these observations and respective cation homeostasis are warranted to further elucidate how *V. cholerae* navigates in a wide range of challenging environmental conditions.

Acknowledgments

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