The *Vibrio cholerae* Mrp System: Cation/Proton Antiport Properties and Enhancement of Bile Salt Resistance in a Heterologous Host

Judith Dzioba-Winogradzki^a Olga Winogradzki^a Terry A. Krulwich^b
Markus A. Boin^c Claudia C. Häse^c Pavel Dibrov^a

^aDepartment of Microbiology, University of Manitoba, Winnipeg, Man., Canada; ^bDepartment of Pharmacology and Biological Chemistry, Mount Sinai School of Medicine, New York, N.Y., and ^cDepartment of Biomedical Sciences, College of Veterinary Medicine, Oregon State University, Corvallis, Oreg., USA

Key Words

Vibrio cholerae · Mrp · Na⁺(Li⁺)(K⁺)/H⁺ antiporter ·
Bile resistance · Heterologous expression

heterologous host make Vc-Mrp an attractive experimental model for the further studies of biochemistry and physiology of Mrp systems.

Copyright © 2008 S. Karger AG, Basel

Abstract

The *mrp* operon from *Vibrio cholerae* encoding a putative multisubunit Na⁺/H⁺ antiporter was cloned and functionally expressed in the antiporter-deficient strain of *Escherichia coli* EP432. Cells of EP432 expressing Vc-Mrp exhibited resistance to Na⁺ and Li⁺ as well as to natural bile salts such as sodium cholate and taurocholate. When assayed in everted membrane vesicles of the *E. coli* EP432 host, Vc-Mrp had sufficiently high antiport activity to facilitate the first extensive analysis of Mrp system from a Gram-negative bacterium encoded by a group 2 *mrp* operon. Vc-Mrp was found to exchange protons for Li⁺, Na⁺, and K⁺ ions in pH-dependent manner with maximal activity at pH 9.0–9.5. Exchange was electrogenic (more than one H⁺ translocated per cation moved in opposite direction). The apparent K_m at pH 9.0 was 1.08, 1.30, and 68.5 mM for Li⁺, Na⁺, and K⁺, respectively. Kinetic analyses suggested that Vc-Mrp operates in a binding exchange mode with all cations and protons competing for binding to the antiporter. The robust ion antiport activity of Vc-Mrp in sub-bacterial vesicles and its effect on bile resistance of the

Introduction

Bacterial *mrp* loci encode a phylogenetically distinct group of highly unusual multisubunit cation/proton antiporters (Mrp systems) which exchange cytoplasmic Na⁺, Li⁺ and/or K⁺ ions for extracellular H⁺. Mrp systems are widespread among bacteria and archaea and comprise a separate CPA3 (proton antiporter-3) class of the transporter classification system [Saier et al., 1999]. Other names suggested by independent groups to designate operons homologous to *mrp* are: *pha* (for pH adaptation) in *Sinorhizobium meliloti* [Putnoky et al., 1998], *mnh* (for multisubunit Na⁺/H⁺ antiporter) in *Staphylococcus aureus* [Hiramatsu et al., 1998], *sha* (for sodium-hydrogen antiporter) in *Bacillus* [Kosono et al., 2000]. Here, we will use Mrp (for multiple resistance and pH-related antiporter) to designate all of them. Mrp systems are a relatively recent discovery. Available physiological and genetic data on Mrp systems leave major questions about

this unusual transporter group, as summarized in a recent review [Swartz et al., 2005a].

Mrp systems are proposed to be uniquely complex multi-subunit monovalent cation/proton antiporters [Hiramatsu et al., 1998; Swartz et al., 2005a]. Very recently, complex formation by the *mrpABCDEF*G gene products was demonstrated experimentally in *Bacillus subtilis* [Kajiyama et al., 2007]. There are two major types of *mrp* operons, among which there are species-specific variations [see Swartz et al., 2005a, and references therein]: group 1 with gene order *mrpABCDEF*G (e.g., in bacilli, staphylococci, sinorhizobia) and group 2 with gene order *mrpA*²-*CDEF*G (e.g., in corynebacteria and pseudomonadae). A group 2 operon that encodes the Mrp system of *Vibrio cholerae* (Vc-Mrp) is the subject of this study and is the only Mrp system found among sequenced *Vibrio* strains [Swartz et al., 2005a]. In group 2 operons, the first two genes of the operon are fused yielding *mrpA*², an extended variant of *mrpA* with an additional *mrpB* domain [Swartz et al., 2005a]. In turn, *mrpA* is possibly a result of an earlier fusion of *mrpD* and *mrpB*, so that either type of operon contains two copies of *mrpB* coding region [Swartz et al., 2005a]. Deletion analyses of the *S. aureus* [Hiramatsu et al., 1998] and *B. subtilis* [Ito et al., 2000] operons demonstrated that all the *mrp* gene products are required for the maximal Mrp-associated Na⁺ resistance. These findings indicate that the Mrp antiporters probably function as hetero-oligomeric complexes, in contrast to most other prokaryotic secondary monovalent cation/proton exchangers which are single membrane polypeptides or homo-oligomers [Gerchman et al., 2001; Safferling et al., 2003].

Experiments with whole cells and sub-bacterial vesicles conducted by independent groups showed that the Mrp systems from various sources differ in their specificity toward monovalent cations. In alkaliphilic bacilli [Hamamoto et al., 1994; Ito et al., 2000; Swartz et al., 2007] and in *S. aureus* [Hiramatsu et al., 1998; Swartz et al., 2007] the Mrp system functions as a Na⁺(Li⁺)/H⁺ antiporter. In *B. subtilis* the possibility of Mrp catalyzing both Na⁺/H⁺ and K⁺/H⁺ exchange was raised by physiological studies [Ito et al., 1999], but recent data indicate that *B. subtilis* Mrp only catalyzes Na⁺(Li⁺)/H⁺ antiport [Swartz et al., 2007]. On the other hand, *S. meliloti* Mrp (Pha1) apparently acts as a specific K⁺/H⁺ antiporter [Putnoky et al., 1998]. In all studied cases, however, the Mrp systems operate as typical secondary ion exchangers, being energized by the protonmotive force (Δp). *Bacillus* Mrp antiporters can apparently use the transmembrane electric potential, $\Delta\psi$, as a sole driving force for Na⁺ translocation [Hamamoto et al., 1994; Ito et al., 1999, 2000; Swartz et

al., 2007], which indicates an electrogenic antiport with the stoichiometry H⁺:Na⁺ >1. Sequence similarities between several Mrp proteins and subunits of NADH:quinone oxidoreductases [see Swartz et al., 2005a and cited references] prompted the hypothesis of possible additional primary energization mode of Mrp [Bayer et al., 2006; Ito et al., 2001]. This hypothesis posited that Mrp systems could directly use the energy of a redox reaction for the energization of monovalent cation/proton antiport. However, direct experimental examination did not support this possibility [Ito et al., 2001; Swartz et al., 2005b].

The monovalent cation/proton antiport activity of Mrp systems has important physiological roles in alkali, Na⁺, and K⁺ resistance [Hamamoto et al., 1994; Ito et al., 1999; Kosono et al., 1999; Putnoky et al., 1998] with significant impact on sporulation [Kosono et al., 2000] and symbiotic nitrogen fixation [Putnoky et al., 1998]. Recent analysis of deletion mutant revealed an important role of Mrp in the pathogenesis of *Pseudomonas aeruginosa* [Kosono et al., 2005]. There is another important activity also associated with Mrp, namely its capacity for cholate efflux, documented in *B. subtilis* [Ito et al., 1999, 2000]. It is not yet clear how the Mrp-mediated cholate transport is related to Na⁺ flux through Mrp. Deletion analysis suggests that MrpF, which shows a sequence similarity to Na⁺-coupled bile transporters, may be responsible for cholate transport by itself [Ito et al., 2000]. However, the initial assays of MrpF-dependent cholate and Na⁺ efflux have not shown coupling between these two substrates [Ito et al., 2000].

Detailed biochemical analysis of Mrp antiporters is impeded by the fact that the signals in standard fluorescence-based assays are typically very weak [Hiramatsu et al., 1998; Ito et al., 2001]. In this study, the group 2 Mrp system from *V. cholerae*, Vc-Mrp, has been overexpressed in its functional form in the Na⁺/H⁺ antiport-deficient strain of *E. coli*, EP432 [Pinner et al., 1993]. The robust antiport activity observed in assays of everted membrane vesicles from this transformant made it possible, for the first time, to measure key kinetic parameters of Vc-Mrp and to probe its stoichiometry. Of note, Vc-Mrp showed the ability to transport K⁺ as well as Na⁺ and Li⁺. In addition, it enhanced the resistance of the heterologous host strain to natural bile salts. The latter property had only been shown previously for the *B. subtilis* Mrp system belonging to the group 1 of *mrp* operons, and which conferred resistance to cholate [Ito et al., 1999, 2000]. All these features make Vc-Mrp a very attractive experimental model for the further studies of biochemistry and physiology of Mrp systems.

Fig. 1. Structure of the *mrp* operon in *V. cholerae*. Shown are the genes comprising the operon, their length, names of corresponding loci (according to the TIGR database, <http://www.tigr.org/tigr-scripts/CMR2/GenomeTabs.spl?database=gvc#4>), together with calculated length and molecular mass of putative products, as well as the number of putative transmembrane segments (TMS) for each protein.

Results

Functional Expression of *Vc-mrp* Operon in *E. coli* EP432

The entire *Vc-mrp* operon containing the genes from *mrpA'* to *mrpG* of *V. cholerae* (fig. 1) was cloned under the control of the arabinose-inducible P_{BAD} promoter, and introduced into Na^+/H^+ antiport-deficient *E. coli* EP432. Sequence analyses of the cloned operon revealed several nucleotide substitutions compared to the sequence of the genomic *mrp* published at <http://www.tigr.org> for *V. cholerae* biotype El Tor resulting in a few deviations at the amino acid level, namely, seven following substitutions were found: in *Vc-MrpA'*, I12T (ATC $\rightarrow$ ACC), V657M (GTG $\rightarrow$ ATG), P669A (CCG $\rightarrow$ GCG), and V739I (GTC $\rightarrow$ ATC); in *Vc-MrpD*, P105L (CCC $\rightarrow$ CTC) and Q439P (CAA $\rightarrow$ CCG); in *Vc-MrpE*, I68V (ATC $\rightarrow$ GTC). Independent sequencing of the chromosomal DNA of O395-N1 strain amplified by PCR with the above primers yielded a sequence identical to that of pVcMrp insert. Therefore, all the substitutions reflect the actual difference between genomes of classical strain of *V. cholerae* used in this study and published El Tor biotype. Interestingly, all these substitutions are rather conservative (neutral residue substituted by another neutral residue, or neutral residue substituted by uncharged polar one and vice versa). The *Vc-mrp* sequence from the O395-N1 strain was deposited in GenBank under the accession No. EF546428.

EP432 strain of *E. coli* is hypersensitive to environmental Na^+ and Li^+ due to its inability to maintain low cytoplasmic concentration of these cations caused by the deletion of two Na^+/H^+ antiporters, NhaA and NhaB [Harel-Bronstein et al., 1995; Padan et al., 2001; Pinner et al., 1993]. When grown in the presence of 0.5 mM arabinose, the EP432 transformant in which *Vc-mrp* was expressed showed enhanced resistance toward Na^+ and Li^+ ions (fig. 2), thus indicating that *Vc-Mrp* is functional in

the heterologous host. Protection against added alkali cations conferred by *mrp* expression was pH-dependent with a maximum in alkaline (pH 8.5) medium, which was especially evident with Li^+ (fig. 2). This pH profile indicates that the alkali cation extrusion via *Vc-Mrp* can be energized by the transmembrane electric potential difference, $\Delta\psi$, because the ΔpH component of the total driving force would be zero or even reversed (i.e. more acid inside than outside the cells) at an external pH of 8.5.

Since the *Mrp* system of *B. subtilis* has been implicated in cholate transport [Ito et al., 1999, 2000], resistance of EP432/pVcMrp cells to cholate was examined at different pH values. As shown in figure 3a, *Vc-Mrp* rendered transformants resistant to 7.5–22.5 mM sodium cholate, but only at pH 7.2 in contrast to the protection from alkali cations (fig. 2). Microscopic observation revealed that used concentrations of bile salts did not affect the shape of cells thus excluding possible artifacts of measurements of growth by registering the optical density at 600 nm. The observed difference in pH dependence suggests the possibility that the *Vc-Mrp*-mediated transport of alkali cations and transport of cholate may be in fact two independent activities of the same transport system. Such a suggestion is in accord with previous failure to demonstrate linkage between *Mrp*-dependent transport of Na^+ and cholate in bacilli [Ito et al., 2000].

Further, *Vc-Mrp* was found to protect EP432 transformants from conjugated bile salt, sodium taurocholate, a compound naturally occurring in bile (fig. 3b). Of note, in this case *Vc-Mrp* offers no protection until the concentration of added taurocholate exceeds 22.5 mM (fig. 3b). The growth yields of both transformants at 22.5 mM taurocholate is nearly threefold lower than that in the absence of added taurocholate (fig. 3b), but as the taurocholate concentration was raised to the range of 22.5–42.5 mM, *Vc-Mrp*-dependent protection was evident by the better

Fig. 2. Vc-Mrp protects *E. coli* EP432 cells against sodium and lithium ions. Cells were transformed with either pVcMrp (closed symbols) or 'empty' pBAD24 (open symbols) and grown aerobically as described in 'Experimental Procedures' in the presence of 0.5% L-arabinose and indicated concentrations of NaCl or LiCl for 18 h. The growth yield was measured as optical density at 600 nm (OD_{600}). Starting OD_{600} in all cases was approximately 0.05. Plotted are averages of at least four independent experiments. Bars show standard deviation.

Fig. 3. Vc-Mrp confers resistance to cholate in *E. coli* EP432 transformants. Cells were transformed with either pVcMrp (closed symbols) or 'empty' pBAD24 (open symbols) and grown aerobically as described in 'Experimental Procedures' in the presence of 0.5% arabinose and varying concentrations of sodium cholate for 16 h. The growth yield was measured as optical density at 600 nm (OD_{600}). The OD_{600} at the start of the experiments was approximately 0.05 in all cases. Plotted are averages of at least four independent experiments. Bars show standard deviation.

Fig. 4. Activity of Vc-Mrp in everted membrane vesicles. **a** pH profiles of antiport activities mediated by Vc-Mrp. Everted membrane vesicles were isolated from EP432 cells transformed with pVcMrp or 'empty' pBAD24 and assayed with 10 mM of specified salt at various pH values as described in 'Experimental Procedures'. In each case, residual nonspecific activity measured in 'empty' vesicles was subtracted from that registered in Vc-Mrp-containing vesicles and the resulting Vc-Mrp-dependent activity was plotted as a function of pH. Experiments were repeated 3 times for each cation and gave nearly identical results. **b** High concentrations of external KCl do not prevent Na^+/H^+ antiport mediated by Vc-Mrp. Vesicles were assayed with 10 mM NaCl at pH 9.0 in the standard choline chloride experimental medium

supplemented with 140 mM KCl (upper trace) or the same medium containing 280 mM of choline-Cl instead of 140 mM (lower trace) to compensate the difference in osmolarity. Representative data of three independent experiments are shown. **c** K^+ ions compete with Na^+ for the antiporter. Dequenching of AO fluorescence (ΔQ) in response to the addition of varying $[\text{NaCl}]$ was registered at pH 9.0 in the standard choline chloride medium supplemented with indicated concentrations of KCl. Concentrations of choline-Cl were varied accordingly to keep the osmolarity constant. Data were plotted in reciprocal coordinates. Obtained apparent K_m values for Na^+ are: 1.3 mM (at 10 mM KCl), 1.9 mM (at 60 mM KCl), and 2.8 mM (at 140 mM KCl).

growth of Vc-Mrp transformant (fig. 3b). While these data per se do not prove the bile efflux via Vc-Mrp, they are consistent with the notion that Vc-Mrp-dependent protection from bile salts may be achieved by active extrusion via Vc-Mrp, with cholate being a preferable substrate. If so, the additional amidosulfonate group of taurocholate (fig. 3c) could rise the K_m of Vc-Mrp for this bulkier compound. Direct assays of bile salt transport will be needed to examine the role of Vc-Mrp in bile resistance.

Phenotypes of the EP432/pVcMrp transformants described above clearly showed that the arabinose-induced expression of the Vc-Mrp system in *E. coli* resulted in the proper targeting/insertion of Vc-Mrp into the membrane, which preserved its ability to function as a cation/proton antiporter, allowing more detailed characterization of Vc-Mrp antiport in a subcellular experimental model.

Cation-Proton Antiport Catalyzed by Vc-Mrp in Everted Membrane Vesicles

Measurements of Vc-Mrp-mediated cation-proton antiport in inside-out membrane vesicles isolated from

EP432/pVcMrp revealed that both Na^+ and Li^+ ions are good substrates for Vc-Mrp (fig. 4a, closed circles and triangles). In both cases, maximal activity was detected at alkaline pH of the experimental mixture. The pH profile for Li^+/H^+ exchange was shifted toward more alkaline pH by one pH unit relative to Na^+/H^+ antiport. At pH 8.0, Na^+/H^+ antiport reached its half-maximal magnitude while Li^+/H^+ antiport was not detectable at pH <8.5 (fig. 4a). Vc-Mrp also catalyzed measurable K^+/H^+ antiport with the magnitude steadily increasing from pH 7.5 to pH 9.5 (fig. 4a, closed squares). For this set of experiments, the vesicles were isolated and assayed in K^+ -free buffer. Under these conditions, a small background cation-proton antiport activity (i.e. small dequenching) was observed in assays of control 'empty' vesicles isolated from parental EP432 strain (not shown). This background activity never exceeded 18% of AO dequenching and was completely eliminated by introduction of 10 mM K^+ into experimental solutions. The background was measured at every pH examined for each cation in separate control experiments and subtracted from the levels obtained in

Vc-Mrp-containing vesicles to yield data sets plotted in figure 4a. The alkaline pH optimum for Vc-Mrp-mediated antiport with each of its cation substrates is consistent with observations on other Mrp systems, with Mrp roles in pH homeostasis, and with the greater sensitivity of bacteria to the cytotoxic effects of Na^+ at alkaline pH [Swartz et al., 2005a, 2007]. Even if antiport is energized by a $\Delta\psi$ as indicated by experiments below, proton capture from the outside is increasingly difficult as the external pH rises. One proposed rationale for a Mrp complex is the presentation of a large protein surface that might facilitate proton gathering in support of antiport at high pH [Swartz et al., 2005a].

The lower levels of K^+/H^+ antiport than $\text{Na}^+(\text{Li}^+)/\text{H}^+$ antiport mediated by Vc-Mrp suggest that the latter activity is the major physiological mode of Vc-Mrp. In support of this suggestion, addition of 140 mM KCl into the assay buffer did not prevent the ΔpH response on addition of 10 mM NaCl to Vc-Mrp-containing vesicles at pH 9.0 (fig. 4b, upper trace). The lower magnitude of the respiration-induced ΔpH in K^+ -rich buffer is to be expected since respiration would be occurring concurrently with Vc-Mrp-mediated K^+/H^+ exchange during energization, resulting in a lower steady-state quench (fig. 4b, compare upper and lower traces). Lowered percent of dequenching upon addition of Na^+ in K^+ -rich buffer indicated possible competition between Na^+ and K^+ . This possibility was further analyzed in the next series of experiments, as described in the next section.

Kinetic Behavior of Vc-Mrp Is Consistent with Binding Exchange Mechanism

Since the pH profile of K^+/H^+ exchange mediated by Vc-Mrp differs significantly from that of Na^+/H^+ and Li^+/H^+ exchange, Na^+/H^+ antiport at pH 9.0 was next measured in the presence of three different concentrations of KCl added to the experimental medium. These competition assays clearly demonstrated that K^+ ions compete with Na^+ for the antiporter, affecting its apparent K_m for Na^+ (fig. 4c). Therefore, all three transported cations appear to share the same intramolecular translocation pathway and observed low overall K^+/H^+ exchange through Vc-Mrp (fig. 4a) probably reflects a lower affinity of Vc-Mrp for K^+ ions. The apparent K_m values for Vc-Mrp with each of the three substrate cations were determined as the cation concentration required for a half-maximal AO dequenching at the optimal pH of 9.0. The antiporter exhibited an apparent K_m for Na^+ or Li^+ close to 1.0 mM (fig. 5). Such low K_m values are typical for many bacterial Na^+/H^+ antiporters, including Vc-NhaD [Dzio-

ba et al., 2002; Habibian et al., 2005; Ostroumov et al., 2002] and Ec-NhaA [Dibrov and Taglicht, 1993; Tzuber et al., 2004]. Even in the presence of 140 mM KCl, K_m for Na^+ was 2.8 mM (fig. 4c). Since bacteria usually maintain high levels of cytoplasmic potassium, this K_m value may be closer to the in vivo situation. In contrast, the K_m for Vc-Mrp with K^+ is 68.5 mM, higher by almost two orders of magnitude (fig. 5).

Although the Na^+ -dependent H^+ export from everted vesicles via Vc-Mrp was not prevented by the addition of external K^+ (fig. 4b, c), intravesicular K^+ arrested it completely unless the K^+ ionophore valinomycin was added (fig. 6a). The only difference between this experiment and the one shown in figure 4b is that here 140 mM K_2SO_4 is present *outside and inside* the vesicles. This observation indicates that K^+ and H^+ compete for the binding to the antiporter. One could argue that, alternatively, valinomycin may boost the proton gradient as a driving force for Na^+ transport in K^+ -rich buffer. However, this does not explain the total arrest of the Na^+ -dependent H^+ movement by high internal K^+ in the absence of valinomycin shown in figure 6a. Such arrest does not happen when high $[\text{K}^+]$ is present only in external medium (fig. 4b). Therefore the most plausible explanation is that the flux of H^+ from the intravesicular space is inhibited by the high internal $[\text{K}^+]$. The addition of valinomycin dissipates $\Delta\psi$ on the membrane (fig. 6b, the last addition), thus converting it into ΔpH (more acidic inside the vesicles). This increases internal $[\text{H}^+]$ so that now protons compete favorably with K^+_{in} and the $\text{Na}^+(\text{Li}^+)/\text{H}^+$ antiport can proceed upon the addition of external cation, albeit at a considerably slower rate (compare the lower trace in fig. 6a to that in fig. 4b).

Parallel measurements of $\Delta\psi$ showed that the addition of Li^+ (as shown in fig. 6b) or Na^+ (not shown) did not perturb the membrane potential. These data, together with the results of competition experiments shown in figure 4c, indicate that all transported cations, including protons, compete for the antiporter operating in the binding exchange mode, as suggested for other antiporters [Huang et al., 2003; Hunte et al., 2005] and illustrated by the model diagrammed in figure 6c. Here, C_o and C_i denote two unloaded forms of Vc-Mrp with its cation-binding site accessible to ions from the outer or inner side of the membrane, respectively. At any time, the cation-binding site may be empty or occupied by either 1 K^+ ion, or 1 $\text{Na}^+(\text{Li}^+)$ ion, or by n protons. Only Na^+ -loaded but not Li^+ -loaded forms of antiporter are shown for simplicity. When internal $[\text{K}^+]$ is low, the catalytic cycle includes partial steps $1 \rightarrow 2 \rightarrow 3 \rightarrow 4 \rightarrow 5 \rightarrow 6$ (in the case of

Fig. 5. Determination of kinetic parameters of Vc-Mrp in everted membrane vesicles isolated from EP432 transformant cells. Assays were performed at pH 9.0 with varying concentrations of

NaCl (circles, **a**), LiCl (squares, **b**), or KCl (triangles, **c**) as described in 'Experimental Procedures'. ΔQ = Percent of dequenching of the AO fluorescence.

Fig. 6. Kinetic analysis of Vc-Mrp. **a** A high concentration of intravesicular K^+ prevents cation-proton exchange by Vc-Mrp. Vesicles were isolated from EP432/pVcMrp cells in a high potassium buffer and assayed in the same buffer at pH 9.0. 5 mM Tris-D-lactate was used to energize respiration-dependent proton pumping, then 10 mM NaCl was added to initiate antiport in the absence (upper trace) or presence of 100 nM valinomycin (lower trace).

b Addition of alkali cation (50 mM LiCl) does not perturb the $\Delta\psi$ of the membrane of vesicles. Fluorescence of $\Delta\psi$ -sensitive dye, oxonol V, was monitored instead of AO; all other conditions as in **a**. Representative of five independent experiments is shown. **c** Kinetic model of Vc-Mrp based on the binding exchange mechanism. See the text for discussion.

$Na^+(Li^+)/H^+$ antiport) or $1 \rightarrow 2 \rightarrow 3 \rightarrow 7 \rightarrow 8 \rightarrow 9$ (K^+/H^+ antiport). Due to the competition between K^+ and H^+ , high $[K^+]$ inside prevents protons from binding (thus eliminating steps $1 \rightarrow 2 \rightarrow 3$), but external Na^+ is still able to bind to the antiporter, as it is evident from figure 4b, because of the difference in K_m for Na^+ vs. K^+ (fig. 5). Therefore, under these conditions only homologous K^+/K^+ exchange (route $7 \rightarrow 8 \rightarrow 9$) or heterologous

$K^+/Na^+(Li^+)$ exchange (route $4 \rightarrow 5 \rightarrow 6 \rightarrow 9 \rightarrow 8 \rightarrow 7$) are allowed. Not surprisingly, either of these activities is electroneutral (fig. 6b).

Probing the Electrogenicity of Cation-Proton Antiport via Vc-Mrp

The above kinetic analysis does not determine n , the number of protons exchanged per each alkali cation in a

Fig. 7. Probing the stoichiometry of Vc-Mrp. Vesicles were isolated and assayed for $\Delta\psi$ at pH 9.0 in a sorbitol-based medium devoid of K^+ and Cl^- as described in 'Experimental Procedures'. Diethanolamine at 15 mM was added to the experimental mixture 5 min prior the addition of Oxonol V. Due to the absence of K^+ in the system, protonophore CCCP was added at the end of each measurement instead of valinomycin for the control. All other conditions as in figure 6b. Representative traces of five independent experiments are shown.

single turnover of the catalytic cycle of Vc-Mrp. Growth of EP432 transformants (fig. 2) indicates that Vc-Mrp might use $\Delta\psi$ to drive the export of alkali cations at high external pH, implying that it catalyzes an electrogenic exchange of 1 $Na^+(Li^+)$ per nH^+ ($n > 1.0$). If so, the net transmembrane charge movement should result in (at least transient) dissipation of pre-established $\Delta\psi$ on the membrane upon addition of alkali cation.

In order to probe the electrogenicity of Vc-Mrp antiport, everted EP432 vesicles were isolated and assayed for $\Delta\psi$ in a sorbitol-based medium at pH 9.0. To maximize the respiration-generated $\Delta\psi$, 15 mM diethanolamine was added to the vesicle suspension 5 min prior the addition of the $\Delta\psi$ -sensitive dye oxonol V. For the control experiments, vesicles were isolated from the host EP432 cells transformed with the pBAD24 plasmid without *mrp* insert ('empty' vesicles, left trace in fig. 7). Here, addition of lactate led to rapid generation of $\Delta\psi$ and the subsequent addition of Li^+ resulted in a slow, modest but reproducible depolarization, indicating the presence of electrogenic cation-transporting system(s) operating in the membrane of 'empty' vesicles. A similar modest background effect on ΔpH was noted above. It is not clear at this time what transporters of the EP432 membrane may be responsible for this background activity, but a possible candidate is ChaA, a $K^+(Ca^{2+})(Na^+)/H^+$ antiporter that is active in alkaline media [Ivey et al., 1993; Ohya et al., 1994; Radchenko et al., 2006].

In another control, an artificial electroneutral $Na^+(Li^+)/H^+$ exchanger, monensin, added at 1 μM before lactate, did not affect this response (fig. 7, middle trace). On the other hand, in a separate control experiment the same concentration of monensin caused robust Li^+ -induced proton transfer in vesicles assayed for ΔpH in standard choline-Cl medium (data not shown). Therefore the absence of effect of monensin on $\Delta\psi$ could not be attributed to the lack of its activity, but reflects the electroneutral exchange mediated by this compound. The addition of Li^+ to the Vc-Mrp-containing vesicles resulted in a partial dissipation of respiration-generated $\Delta\psi$. The dissipation was much faster and of significantly higher magnitude than that observed in both 'empty' and monensin controls (fig. 7, compare right trace with others). Li^+ was added to 50 mM in the experiment shown in figure 7 because this concentration saturated the background activity observed in the two controls, but Vc-Mrp-dependent $\Delta\psi$ consumption was also observed when Li^+ was added at 5 mM or when Na^+ or K^+ was used as substrate instead of Li^+ (data not shown). Again, monensin did not affect the background activity in the control vesicles at low concentrations of substrate cation added. These observations support an electrogenic character of the cation-proton antiport catalyzed by Vc-Mrp with the stoichiometry 1 Li^+/nH^+ ($n > 1.0$). The approach exploited in this work is a qualitative one and does not allow for direct determination of n . Reconstitution of purified antiporter into pro-

teoliposomes is required for this task, as it was done earlier for Ec-NhaA [Taglicht et al., 1993] and Ec-NhaB [Pinner et al., 1994]. Although purification of a putative multi subunit transporter such as Vc-Mrp in its functional form poses a significant challenge, this is one of the most important future directions in studies on Mrp systems.

Discussion

This study provides the first detailed characterization of a 'group 2' Mrp system encoded by a six-gene operon with gene order *mrpA'CDEFG* (fig. 1). The results define Vc-Mrp as an electrogenic (exchanging more than one H^+ per each alkali cation) secondary cation-proton antiporter with broad cation specificity (fig. 4, 5). The ability of Vc-Mrp to use K^+ as a substrate (albeit with low affinity), in addition to Na^+ and Li^+ , differentiates it from recently characterized 'group 1' Mrp systems that act as more specific, high-affinity $Na^+(Li^+)/H^+$ antiporters [Swartz et al., 2007]. Vc-Mrp exhibits apparent K_m values for Na^+ and Li^+ that are 1.1–1.3 mM (fig. 5), just a little higher than the submillimolar values displayed by 'group 1' Mrp systems that are among the lowest ever reported for bacterial Na^+/H^+ antiporters [Swartz et al., 2007]. Like the group 1 Mrp systems from *S. aureus* and *Bacillus* species [Swartz et al., 2007], Vc-Mrp exhibits an alkaline pH optimum; it is particularly notable that this optimum of pH 9.0–9.5 is above the pH at which *V. cholerae* exhibits growth [Miller et al., 1984], as will be discussed below. Kinetic analyses in this study provide the first insights into the mechanism of cation exchange catalyzed by Mrp systems. The data suggest that Vc-Mrp operates in a binding exchange mode with all three transported alkali cations competing for the antiporter with protons (fig. 6).

Interestingly, when expressed in a heterologous host, Vc-Mrp not only enhances the resistance of the antiporter-less host cells toward Na^+ and Li^+ (fig. 2), but also protects transformants from growth inhibition by added bile salts (fig. 3). Mrp systems belonging to groups 1 [Ito et al., 1999, 2000] and 2 (this work) may thus share the capacity for bile salt efflux. In order to test this notion in direct experiments we will design assays to measure the bile salt transport in everted EP432 vesicles containing Vc-Mrp. It remains possible that the effects of Mrp on bile salt resistance are mediated by indirect effects of antiport on native bile export systems of the *E. coli* host, but earlier studies of Mrp effects on bile salt efflux in *B. subtilis* support the commonality inferred here.

The observed differences in pH optima for the protection against bile salts (neutral pH, fig. 3a) and cation/proton exchange measured in everted vesicles (alkaline pH, fig. 4a) are in agreement with earlier indications of a lack of mechanistic coupling between Mrp-mediated Na^+ transport and bile efflux [Ito et al., 2000]. This supports the idea that Mrp systems may in fact be consortia of physically interacting transporters [Swartz et al., 2005a]. There is likely to be some variability in the anion substrates of Mrp as already shown for the cation substrates of Mrp [Swartz et al., 2005a]. Evidence for involvement of *Agrobacterium tumefaciens* Mrp in arsenite resistance may reflect an efflux capacity of this anion instead of (or in addition to) bile salts in some Mrp systems [Kashyap et al., 2006]. Interestingly, bile and arsenite are both substrates of the BART superfamily of transporters [Mansour et al., 2007], so this particular combination may have arisen multiple times. Vc-Mrp has here been shown to be an attractive model for studying the Mrp family. It will be of interest to use this system to explore the molecular mechanism of Mrp-dependent bile extrusion and its association with one or more particular Mrp proteins.

The pH optimum of 9–9.5 found here for the Vc-Mrp-mediated ion exchange deserves a special consideration. We hypothesize that the physiological value of Vc-Mrp may relate to the ecology of the estuarine environment of *V. cholerae*. Estuaries show large fluctuations in physical properties that arise from variable ratios and rates of mixing of brackish and fresh water [Huq et al., 1984]. *V. cholerae* survives and associates with copepods and plankton but does not grow under many of the conditions found in the estuaries in which it is endemic [Binsztein et al., 2004; Huq et al., 1984; Miller et al., 1984]. The pH optimum of 9–9.5 is within the pH range reached in estuaries but above the range for *V. cholerae* growth [Miller et al., 1984]. This high pH optimum is consistent with that of other Mrp systems that are associated with alkali resistance [Hamamoto et al., 1994; Swartz et al., 2005a, 2007]. It is therefore plausible that Vc-Mrp is critical for survival of this pathogen during periods of alkaline swings that occur in the natural setting for a key part of its life cycle. To examine this possibility, it would be interesting to construct a Vc-Mrp knockout mutant of *V. cholerae* and compare its survival to that of the wild-type strain under conditions that mimic the combined estuarine challenges of high pH, low nutrient levels and temperature, sometimes combined with low oxygen content. In view of the K^+/H^+ antiport capacity of Vc-Mrp shown in this study, H^+ uptake to support alkali resistance could

be achieved using the outwardly directed K^+ gradient when oxygen depletion is encountered in a deep current at alkaline pH.

Experimental Procedures

Materials

All chemicals were from Sigma Chemical Co. or Fisher Scientific. Restriction nucleases were from Gibco-BRL, MBI Fermentas or New England Biolabs. Protein content in membrane vesicles was determined by the Bio-Rad Detergent Compatible Protein Assay Kit.

Bacterial Strains and Culture Conditions

The Na^+/H^+ antiporter-deficient (*melBLid*, Δ NhaB1, *cam*^R, Δ NhaA1, *kan*^R, Δ *lacZY*, *thr1*) strain of *E. coli* EP432 was kindly provided by Dr. E. Padan (Hebrew University of Jerusalem, Israel). For routine cloning and plasmid construction, *E. coli* DH5 α (US Biochemical Corp.) was used as host. *V. cholerae* strain used was O395-N1 [Häse and Mekalanos, 1998], which is the classical O395 Ogawa strain with a partial deletion of the *ctxAB* operon and translational *toxT::lacZ* fusion (O395N1-TZ) [Häse and Mekalanos, 1998]. If not otherwise indicated, EP432 cells were grown aerobically at 37°C in LBK (modified L broth in which NaCl was replaced by KCl [Padan et al., 1989]) supplemented with 100 μ g/ml ampicillin, 30 μ g/ml kanamycin, 17.5 μ g/ml chloramphenicol and 0.5% (w/v) arabinose. For the phenotype analysis, various concentrations of NaCl, LiCl or sodium cholate were added to the LBK medium described above and buffered at desired pH with 60 mM of Bis-Tris propane/HCl. For the induction of the expression of Vc-Mrp, EP432 transformants were grown in LBK at 35°C with moderate aeration. At OD₆₀₀ ~0.200, L-arabinose was added to a final concentration of 0.5% (w/v) and then after 30 min more, NaCl was added to a final concentration of 0.3 M. Cells were harvested at OD₆₀₀ of 2.2–2.5 and immediately used for the isolation of vesicles.

Cloning of *Vc-mrp*

Sequence data for *V. cholerae* were from the Institute for Genomic Research website at <http://www.tigr.org>. The entire *mrpA'*-G region was amplified by PCR from chromosomal DNA of *V. cholerae* O395-N1 as the template and directly cloned into the pBAD-TOPO vector (Invitrogen), yielding pVcMrp construct which was used for the expression studies. Primers used for cloning were: 5'-VCA0157 (TA ATG AAG ACA GGA CTC GAT AAG C) and 3'-VCA0152 (ATA GGC AGC AAA CTA CTT CTG CG).

Isolation of Membrane Vesicles and Assays of Antiport Activity

Inside-out membrane vesicles were prepared by passing a bacterial suspension through a French press (Aminco) and assayed for cation-proton antiport activities essentially as described previously [Dzioba et al., 2002; Habibian et al., 2005; Ostroumov et al., 2002] with minor modifications. The antiport activities were monitored by the acridine orange (AO) fluorescence dequenching. Vesicles were obtained and stored in a buffer containing 140 mM choline-Cl, 10% (w/v) glycerol and 20 mM Tris-HCl, pH 7.5. For $\Delta\psi$ measurements, vesicles were isolated in K^+ -free, Cl^- -free

buffer where choline-Cl was substituted by sorbitol. For some experiments, vesicles were isolated in the potassium-rich buffer containing 140 mM K_2SO_4 instead of sorbitol.

For Δ pH measurements, aliquots of vesicles (200 μ g of protein) were added to 2 ml of an assay buffer containing 50 mM buffer Bis-Tris propane, adjusted to the indicated pH, 140 mM choline-Cl, 10% glycerol (w/v), 10 mM KCl, 5 mM $MgSO_4$, and 0.5 μ M AO. Respiration-dependent formation of the Δ pH was initiated by the addition of 10 mM Tris-D-lactate, and the resulting quenching of AO fluorescence was monitored with the Shimadzu RF-1501 spectrofluorophotometer (excitation at 492 nm and emission at 528 nm). Antiport activity was assessed as the percent of dequenching, i.e., dissipation of the Δ pH when test cations were added. NaCl, LiCl or KCl were added: at 10 mM in the pH-profile determinations; at 0.5–25 mM for the determinations of half-maximal effective concentrations of Na^+ or Li^+ ; and at 25–180 mM for the determinations of half-maximal effective concentration of K^+ . For K^+/H^+ antiport assays, KCl was excluded from the experimental medium. The antiport activities are expressed as percent reversal of the lactate-induced fluorescence quenching, as percent of dequenching.

Measurements of Transmembrane Electric Potential Difference

The $\Delta\psi$ -sensitive dye oxonol V was used to monitor effects of Vc-Mrp-mediated cation-proton antiport on the respiration-generated formation of $\Delta\psi$. In this case, vesicles were prepared in the sorbitol-based experimental medium without K^+ and Cl^- , resuspended in 2 ml of the same medium supplemented with 5 mM $MgSO_4$ and 15 mM diethanolamine, pH 9.0, and pre-incubated for 5 min at room temperature before addition of 1.0 μ M oxonol V. Excitation was at 595 nm and emission was monitored at 630 nm. In some experiments, vesicles were isolated and assayed for $\Delta\psi$ in the potassium-rich buffer (described above).

Acknowledgments

This research was supported by grants from NSERC, Natural Sciences and Engineering Research Council of Canada (to P.D. and J.D.-W.), Ellison Medical Foundation, NIH grant AI-063120-01A2 (to C.C.H.), and NIH grant GM28454 (to T.A.K.).

References

- Bayer AS, McNamar P, Yeaman MR, Lucindo N, Jones T, Cheung AL, Sahl HG, Proctor RA: Transposon disruption of the complex I NADH oxidoreductase gene (*snoD*) in *Staphylococcus aureus* is associated with reduced susceptibility to microbicidal activity of thrombin-induced platelet microbicidal protein 1. *J Bacteriol* 2006;188:211–222.
- Binsztein M, Costagliola MC, Pichel M, Jurquiza V, Ramirez FC, Akselman R, Vacchino M, Huq A, Colwell R: Viable but nonculturable *Vibrio cholerae* O1 in the aquatic environment of Argentina. *Appl Environ Microbiol* 2004;70:7481–7486.

- Dibrov P, Taglicht D: Mechanism of Na⁺/H⁺ exchange by *Escherichia coli* NhaA in reconstituted proteoliposomes. *FEBS Lett* 1993;336: 525–529.
- Dzioba J, Ostroumov E, Winogrodzki A, Dibrov P: Cloning, functional expression in *Escherichia coli* and primary characterization of a new Na⁺/H⁺ antiporter, NhaD, of *Vibrio cholerae*. *Mol Cell Biochem* 2002;229:119–124.
- Gerchman Y, Rimon A, Venturi M, Padan E: Oligomerization of NhaA, the Na⁺/H⁺ antiporter of *Escherichia coli* in the membrane and its functional and structural consequences. *Biochemistry* 2001;40:3403–3412.
- Habibian R, Dzioba J, Barrett J, Galperin MY, Loewen PC, Dibrov P: Functional analysis of conserved polar residues in Vc-NhaD, Na⁺/H⁺ antiporter of *Vibrio cholerae*. *J Biol Chem* 2005;280:39637–39643.
- Hamamoto T, Hashimoto M, Hino M, Kitada M, Seto Y, Kudo T, Horikoshi K: Characterization of a gene responsible for the Na⁺/H⁺ antiporter system of alkalophilic *Bacillus* species strain C-125. *Mol Microbiol* 1994;14: 939–946.
- Harel-Bronstein M, Dibrov P, Olami Y, Pinner E, Schuldiner S, Padan E: MH1, a second-site revertant of an *Escherichia coli* mutant lacking Na⁺/H⁺ antiporters (Δ nhaA Δ nhaB), regains Na⁺ resistance and a capacity to excrete Na⁺ in a $\Delta\mu_{\text{H}^+}$ -independent fashion. *J Biol Chem* 1995;270:3816–3822.
- Häse CC, Mekalanos JJ: TcpP protein is a positive regulator of virulence gene expression in *Vibrio cholerae*. *Proc Natl Acad Sci USA* 1998;95:730–734.
- Hiramatsu T, Kodama K, Kuroda T, Mizushima T, Tsuchiya T: A putative multisubunit Na⁺/H⁺ antiporter from *Staphylococcus aureus*. *J Bacteriol* 1998;180:6642–6648.
- Huang Y, Lemieux MJ, Song J, Auer M, Wang DM: Structure and mechanism of the glycerol-3-phosphate transporter from *Escherichia coli*. *Science* 2003;301:616–620.
- Hunte C, Screpanti E, Venturi M, Rimon A, Padan E, Michel H: Structure of a Na⁺/H⁺ antiporter and insights into mechanism of action and regulation by pH. *Nature* 2005;435: 1197–1202.
- Huq A, West PA, Small EB, Huq MI, Colwell RR: Influence of water temperature, salinity, and pH on survival and growth of toxigenic *Vibrio cholerae* serovar O1 associated with live copepods in laboratory microcosms. *Appl Environ Microbiol* 1984;48:420–424.
- Ito M, Guffanti AA, Krulwich TA: Mrp-dependent Na⁺/H⁺ antiporters of *Bacillus* exhibit characteristics that are unanticipated for completely secondary active transporters. *FEBS Lett* 2001;496:117–120.
- Ito M, Guffanti AA, Oudega B, Krulwich TA: *mrp*, a multigene, multifunctional locus in *Bacillus subtilis* with roles in resistance to cholate and to Na⁺ and pH homeostasis. *J Bacteriol* 1999;181:2394–2402.
- Ito M, Guffanti AA, Wang W, Krulwich TA: Effects of nonpolar mutations in each of the seven *Bacillus subtilis* *mrp* genes suggest complex interactions among the gene products in support of Na⁺ and alkali but not cholate resistance. *J Bacteriol* 2000;182:5663–5670.
- Ivey DM, Guffanti AA, Zemsky J, Pinner E, Karpel R, Padan E, Krulwich TA: Cloning and characterization of a putative Ca²⁺/H⁺ antiporter gene from *Escherichia coli* upon functional complementation of Na⁺/H⁺ antiporter-deficient strains by the overexpressed gene. *J Biol Chem* 1993;268:11296–11303.
- Kajiyama Y, Otagiri M, Sekiguchi J, Kosono S, Kudo T: Complex formation by the *mrp*ABCDEF gene products, which constitute a principal Na⁺/H⁺ antiporter in *Bacillus subtilis*. *J Bacteriol* 2007;189:7511–7514.
- Kashyap DR, Botero LM, Lehr C, Hassett DJ, McDermott TR: A Na⁺:H⁺ antiporter and a molybdate transporter are essential for arsenite oxidation in *Agrobacterium tumefaciens*. *J Bacteriol* 2006;188:1577–1584.
- Kitada M, Kosono S, Kudo T: The Na⁺/H⁺ antiporter of alkalophilic *Bacillus* sp. *Extremophiles* 2000;4:253–258.
- Kosono S, Haga K, Tomizawa R, Kajiyama Y, Hatano K, Takeda S, Wakai Y, Hino M, Kudo T: Characterization of a multigene-encoded sodium/hydrogen antiporter (*sha*) from *Pseudomonas aeruginosa*: its involvement in pathogenesis. *J Bacteriol* 2005;187:5242–5248.
- Kosono S, Morotomi S, Kitada M, Kudo T: Analyses of a *Bacillus subtilis* homologue of the Na⁺/H⁺ antiporter gene which is important for pH homeostasis of alkalophilic *Bacillus* sp. C-125. *Biochim Biophys Acta* 1999;1409: 171–175.
- Kosono S, Ohashi Y, Kawamura F, Kitada M, Kudo T: Function of a principal Na⁺/H⁺ antiporter, *ShA*, is required for initiation of sporulation in *Bacillus subtilis*. *J Bacteriol* 2000;182:898–904.
- Mansour NM, Sawhney M, Tamang DG, Vogl C, Saier MH Jr: The bile/arsenite/riboflavin transporter (BART) superfamily. *FEBS J* 2007;274:612–629.
- Miller CJ, Drasar BS, Feachem RG: Response of toxigenic *Vibrio cholerae* O1 to physicochemical stresses in aquatic environments. *J Hyg (Lond)* 1984;93:475–495.
- Ohyama T, Igarashi K, Kobayashi H: Physiological role of the *chaA* gene in sodium and calcium circulations at a high pH in *Escherichia coli*. *J Bacteriol* 1994;176:4311–4315.
- Ostroumov E, Dzioba J, Loewen PC, Dibrov P: D344 and T345 are critical for cation exchange mediated by NhaD, Na⁺/H⁺ antiporter of *Vibrio cholerae*. *Biochim Biophys Acta* 2002;1564:99–106.
- Padan E, Maisler N, Taglicht D, Karpel R, Schuldiner S: Deletion of *ant* in *Escherichia coli* reveals its function in adaptation to high salinity and an alternative Na⁺/H⁺ antiporter system(s). *J Biol Chem* 1989;264:20297–20302.
- Padan E, Venturi M, Gerchman Y, Dover N: Na⁺/H⁺ antiporters. *Biochim Biophys Acta* 2001; 1505:144–157.
- Pinner E, Kotler Y, Padan E, Schuldiner S: Physiological role of *nhaB*, a specific Na⁺/H⁺ antiporter in *Escherichia coli*. *J Biol Chem* 1993; 268:1729–1734.
- Pinner E, Padan E, Schuldiner S: Kinetic properties of NhaB, a Na⁺/H⁺ antiporter from *Escherichia coli*. *J Biol Chem* 1994;269:26274–26279.
- Putnoky P, Kereszt A, Nakamura T, Endre G, Grosskopf E, Kiss P, Kondorosi A: The *pha* gene cluster of *Rhizobium meliloti* involved in pH adaptation and symbiosis encodes a novel type of K⁺ efflux system. *Mol Microbiol* 1998;28:1091–1101.
- Radchenko MV, Tanaka K, Waditee R, Oshimi S, Matsuzaki Y, Fukuhara M, Kobayashi H, Takabe T, Nakamura T: Potassium/proton antiport system of *Escherichia coli*. *J Biol Chem* 2006;281:19822–19829.
- Safferling M, Griffith H, Jin J, Sharp J, De Jesus M, Ng C, Krulwich TA, Wang DN: TetL tetracycline efflux protein from *Bacillus subtilis* is a dimer in the membrane and in detergent solution. *Biochemistry* 2003;42:13969–13976.
- Saier MH, Eng BH, Fard S, Garg J, Haggerty DA, Hutchinson WJ, Jack DL, Lai EC, Liu HJ, Nusinew DP, Omar AM, Pao SS, Paulsen IT, Quan JA, Sliwinski M, Tseng TT, Wachi S, Young GB: Phylogenetic characterization of novel transport protein families revealed by genome analyses. *Biochim Biophys Acta* 1999;1422:1–56.
- Swartz TH, Ikewada S, Ishikawa O, Ito M, Krulwich TA: The Mrp system: a giant among monovalent cation/proton antiporters? *Extremophiles* 2005a;9:345–354.
- Swartz TH, Ito M, Hicks DB, Nuqui M, Guffanti AA, Krulwich TA: The Mrp Na⁺/H⁺ antiporter increases the activity of the malate: quinone oxidoreductase of an *Escherichia coli* respiratory mutant. *J Bacteriol* 2005b; 187:388–391.
- Swartz TH, Ito M, Ohira T, Natsui S, Hicks DB, Krulwich TA: Catalytic properties of *Staphylococcus aureus* and *Bacillus* members of the secondary cation-proton antiporter-3 (Mrp) family are revealed by an optimized assay in an *Escherichia coli* host. *J Bacteriol* 2007;189: 3081–3090.
- Taglicht D, Padan E, Schuldiner S: Proton-sodium stoichiometry of NhaA, an electrogenic antiporter from *Escherichia coli*. *J Biol Chem* 1993;268:5382–5387.
- Tzuberly T, Rimon A, Padan E: Mutation E252C increases drastically the K_m value for Na⁺ and causes an alkaline shift of the pH dependence of NhaA Na⁺/H⁺ antiporter of *Escherichia coli*. *J Biol Chem* 2004;279:3265–3272.