pH-dependent regulation of the multi-subunit cation/proton antiporter Pha1 system from *Sinorhizobium meliloti*

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The *pha1* gene cluster (*pha1A'-G*) of *Sinorhizobium meliloti* has previously been characterized as a necessary component for proper invasion into plant root tissue. It has been suggested to encode a multi-subunit K⁺/H⁺ antiporter, since mutations in the *pha1* region rendered *S. meliloti* cells sensitive to K⁺ and alkali, and because there is high amino acid sequence similarity to previously characterized multi-subunit cation/H⁺ antiporters (Mrp antiporters). However, the detailed transport properties of the Pha1 system are yet to be determined. Interestingly, most of the Mrp antiporters are highly selective for Na⁺, unlike the Pha1 system. Here, we report the functional expression of the Pha1 system in *Escherichia coli* and the measurement of cation/H⁺ antiport activity. We showed that the Pha1 system is indeed a K⁺/H⁺ antiporter with a pH optimum under mildly alkaline conditions. Moreover, we found that the Pha1 system can transport Na⁺; this was unexpected based on previous phenotypic analyses of *pha1* mutants. Furthermore, we demonstrated that the cation selectivity of the Pha1 system was altered when the pH was lowered from the optimum. The downregulation of Na⁺/H⁺ and K⁺/H⁺ antiport activities upon acidic shift appeared to occur via different processes, which might indicate the presence of distinct mechanisms for the regulation of the K⁺/H⁺ and Na⁺/H⁺ antiport activities of the Pha1 system.

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INTRODUCTION

Monovalent cation/H⁺ antiporters play major roles in pH and cation homeostasis in a wide variety of organisms (Apse & Blumwald, 2007; Brett *et al.*, 2005; Malo & Fliegel, 2006). The involvement of Na⁺/H⁺ antiporters in pH adaptation and Na⁺ extrusion had been extensively studied in *Escherichia coli* (Padan *et al.*, 2001) and several other species, particularly in alkaliphilic bacteria (Krulwich *et al.*, 1999). While most of the antiporters studied to date are encoded by single genes, a wide variety of prokaryotic species possess characteristic multi-gene-encoded cation/H⁺ antiporters called Mrp transporters (Swartz *et al.*, 2005). These are grouped into the Cation Proton Antiporter 3 (CPA-3) family due to their distinctive structural properties (Saier *et al.*, 1999). Mrp antiporters typically consist of six or seven membrane proteins encoded in a single operon. The *mrp* operons are categorized into two major groups (Swartz *et al.*,

2005): group 1, which includes *mrp* from *Bacillus subtilis*, contains seven genes (*mrpABCDEFG*); and group 2, which includes *Vibrio cholerae* Vc-*mrp*, contains six genes (*mrpA'CDEFG*) (Dzioba-Winogradzki *et al.*, 2009). It has been shown that all of the components are required for the Mrp antiporter to be fully functional (Ito *et al.*, 1999, 2000; Putnoky *et al.*, 1998; Yoshinaka *et al.*, 2003). Recently, two groups have experimentally shown that gene products of the *mrp* operon from *Bacillus* species actually form complexes in *B. subtilis* cells (Kajiyama *et al.*, 2007) and in heterologous *Escherichia coli* cells (Morino *et al.*, 2008). Detailed functional analysis of group 1 Mrp antiporters from *Staphylococcus aureus* and several *Bacillus* species (Swartz *et al.*, 2007) determined that these antiporters are electrogenic Na⁺(Li⁺)/H⁺ antiporters with no detectable K⁺ transport activity. In comparison, a group 2 Mrp antiporter, VcMrp, was shown to be a Na⁺(Li⁺)/H⁺ antiporter with low-affinity K⁺/H⁺ antiport activity (Dzioba-Winogradzki *et al.*, 2009).

The Pha1 (pH adaptation 1: formerly named Pha) system from a nitrogen-fixing bacterium, *Sinorhizobium meliloti*, is

Abbreviation: BTP, bis-[tris(hydroxymethyl)methylamino]propane.

The GenBank/EMBL/DBJ accession number for the newly determined DNA sequence of *pha1* is AB482223.

distinctive among Mrp antiporters in terms of its relation to K^+ efflux (Putnoky *et al.*, 1998). The *pha1* gene cluster had been identified as a necessary locus for proper invasion of *S. meliloti* into cells of plant nodule tissue (Forrai *et al.*, 1983; Putnoky *et al.*, 1988, 1998). A *fix-2* mutant carrying the Tn5 transposon insertion in the *pha1* cluster showed early abortion of infection threads and formation of empty (bacterium-free) nodules (Putnoky *et al.*, 1988). Physiological analysis revealed that the *fix-2* mutant is K^+ -sensitive at alkaline pH and deficient in K^+ efflux; no perturbation on Na^+ efflux was observed (Putnoky *et al.*, 1998). Based on these observations, the *pha1* cluster is believed to encode a K^+/H^+ antiporter. However, no direct evidence existed to show antiport activity of the Pha1 system. Here, we have conducted kinetic analysis of the Pha1 system using *E. coli* as a host for heterologous expression. Our results clearly demonstrate that the Pha1 system is indeed a K^+/H^+ antiporter that has a pH optimum of approximately 8.5. In addition, we show that the Pha1 system is capable of Na^+/H^+ antiport, although the apparent V_{max} is lower than that for the K^+/H^+ antiport. Furthermore, we demonstrate that the cation selectivity of the Pha1 system is modulated by pH.

METHODS

Bacterial strains, plasmids and growth conditions. *E. coli* strain DH5 α was used for routine cloning, and the cation/ H^+ antiporters-deficient strain TO114 (*nhaA::Km'* *nhaB::Em'* *chaA::Cm'*) (Ohya *et al.*, 1994) was used for the growth assay and membrane vesicle preparation. These strains were routinely grown in Luria-Bertani (LB) medium (1 % tryptone, 0.5 % yeast extract, 1 % NaCl) or LBK (contains 1 % KCl instead of NaCl) medium at 37 °C unless otherwise indicated. LBK100 medium (1 % tryptone, 0.5 % yeast extract, 100 mM KCl, 10 mM bis-[tris(hydroxymethyl)methylamino]propane (BTP), pH 6.5–9.0) and LBNa100 medium (same as LBK100 except 100 mM NaCl was substituted for KCl) containing 0.4 mM IPTG were used for growth assays. Antibiotics were added to the media when required as follows: ampicillin (100 μ g ml⁻¹), kanamycin (25 μ g ml⁻¹), chloramphenicol (25 μ g ml⁻¹) and erythromycin (180 μ g ml⁻¹). Bacterial growth was monitored by measuring OD₆₆₀ with a mini photo 518R (TAITEC Corp.).

For the cloning of the *phaA1–G1* gene cluster, *phaA1–G1* was amplified by PCR from pPP684 (Putnoky *et al.*, 1998) using Vent^R DNA polymerase (New England Biolabs) in two separate fragments. The upstream portion (containing *phaA1* to the *Bam*HI site) was amplified using *pha-1f* (ACACAGATCTGTGACCCGTCGGC-GTCAGTC) and *pha-3r* (ACCAGGATCCGACGCCCGTGGCC) primers, and the downstream portion (containing *Bam*HI site to *phaG1*) was amplified using *pha-4f* (CGTCCGATCCTGTTCTTCGGC) and *pha-2r* (ACACGAATTCCTTCAACCCGGCGCGGGCTTC) primers. These fragments were subcloned into pGEM-T Easy vector (Promega) and sequenced; they were designated pGEM-1f3r (containing the upstream fragment) and pGEM-4f2r (containing the downstream fragment). Both plasmids were digested by *Bam*HI and *Sal*I and the entire *phaA1G1* cluster was reconstructed by joining the upstream and downstream fragments at the *Bam*HI site on pGEM-1f3r, yielding plasmid pGEM-1f2r. The *phaA1G1* cluster was excised from pGEM1f2r by *Bgl*II/*Eco*RI digestion and cloned into the corresponding sites of pTrcHis2C (Invitrogen), resulting in plasmid pTrcHis2C-*pha1*. Plasmid pTrcHis2C-*pha1* and the pTrcHis2C vector control

were introduced into TO114 independently (strains TO114/pTrcHis2C-*pha1* and TO114/pTrcHis2C, respectively). We noticed several discrepancies between the newly determined sequence and the previously published sequence of the *phaA1G1* cluster (accession no. X93358) in the vicinity of the *phaA1–B1* junction. Because of these discrepancies, we resequenced that region of the pPP684 plasmid. For resequencing, pPP684 was digested with *Pst*I and *Bam*HI, and 1 kb fragments were subcloned into the corresponding sites of pBluescript SK⁻. DNA sequencings were performed by MacroGen Inc. The newly determined DNA sequence of *pha1* was deposited in the DNA Database of Japan (DDBJ) under accession no. AB482223.

Everted membrane vesicle preparation and Δ pH-dependent antiport activity measurement. Two millilitres of overnight cultures of *E. coli* strains were inoculated into 200 ml fresh LBK100 medium containing appropriate antibiotics and cultured for 2 h, then IPTG was added to 0.4 mM (final concentration) and incubated for a further 3 h. Cells were harvested by centrifugation (3100 g for 10 min). Everted membrane vesicles were prepared as described by Rosen (1986). Protein concentration was measured with the BCA protein assay kit (Pierce) using BSA as a standard. Antiporter activity measurements by acridine orange quenching assay were performed as described by Radchenko *et al.* (2006) with slight modifications. Vesicles (25 μ g protein) were suspended in 2 ml assay buffer (10 mM BTP, 5 mM MgCl₂, 140 mM choline chloride, 1.5 μ M acridine orange at pH 7.0–9.0) and 4 mM Tris/lactate was added to initiate respiration. Time-dependent Δ pH changes were monitored by measuring quenching of acridine orange fluorescence using a Shimadzu RF-5300 PC instrument (Shimadzu) with excitation wavelength at 492 ± 1.5 nm and emission at 525 ± 3.0 nm. Cation-dependent H^+ antiport activities were estimated by monitoring fluorescence dequenching following the subsequent addition of various concentrations of KCl or NaCl. After the dequenching reached saturation, 25 mM ammonium chloride was added to dissipate remaining Δ pH in order to calculate the total quenching value. Antiport activities (dequenching) were expressed as a percentage of the total quenching value, as suggested by Swartz *et al.* (2007). Activities of the Pha1 system were calculated by subtracting the values of TO114/pTrcHis2C vesicles from those of TO114/pTrcHis2C-*pha1* under each condition. Curves were fitted to the mean value measured under each condition by using Kaleidagraph software (Synergy software).

RESULTS

Functional expression of the Pha1 complex in *E. coli*

To assess the transport property of the Pha1 complex, we first attempted to measure the cation/ H^+ antiport activity of everted membrane vesicles prepared from *S. meliloti* cells using an acridine orange fluorescence quenching assay (Rosen, 1986). Unfortunately, although we tested several previously described methods, our attempts to detect K^+/H^+ antiport activity with *S. meliloti* membrane vesicles were not successful (data not shown). We therefore employed *E. coli* TO114, which is deficient in three major cation/ H^+ antiporters (NhaA, NhaB and ChaA) (Ohya *et al.*, 1994) and is therefore frequently used for the analysis of bacterial cation/ H^+ antiporters, as a host for the expression of the Pha1 system.

The coding regions of *phaA1* through *phaG1* from pPP684, which carries the *pha1* gene cluster of *S. meliloti* 41

Fig. 1. Structure of the *pha1* gene cluster on the *S. meliloti* genome. Small arrows (1f, 2r, 3r and 4f) indicate the positions of primers used in this study (pha-1f, pha-2r, pha-3r and pha-4f, respectively; see Methods).

(Putnoky *et al.*, 1988, 1998), were introduced into the expression vector pTrcHis2C under the control of the *trc* promoter (pTrcHis2C-*pha1*). During this cloning process, we noticed that there were several errors in the previously published sequence of the *pha1* cluster (accession no. X93358). These errors include nucleotide substitutions (CG at 1429–30 to GC and G at 3049 to C of X93358) and a single nucleotide insertion (G insertion into downstream of G at 3018 of X93358). The single nucleotide insertion was located near the stop codon of the previously designated *phaA1* ORF (Putnoky *et al.*, 1998), which rendered the previously designated *phaA1* and *phaB1* as a single ORF (*phaA'1*; see Fig. 1). It should be noted that the substitutions and single insertion were identical to the genomic sequence of the *pha1* region from *S. meliloti* 1021 strain (accession no. AL591688) (data not shown). According to these results, the Pha1 system is predicted to be a group 2 Mrp antiporter that is composed of six proteins, rather than a group 1 Mrp containing seven proteins as predicted previously (Putnoky *et al.*, 1998).

The TO114 strains harbouring pTrcHis2C (vector control) and pTrcHis2C-*pha1* were subjected to a growth test at alkaline pH using LBK100 medium to investigate whether the Pha1 complex can function as a cation/H⁺ antiporter in *E. coli* cells. TO114 is sensitive to alkaline pH and high Na⁺ concentration due to the lack of Na⁺/H⁺ antiporters NhaA and NhaB and cation (Ca²⁺, Na⁺, K⁺)/H⁺ antiporter ChaA (Ohyama *et al.*, 1994). TO114 harbouring the pTrcHis2C vector showed growth deficiency at pH 9.0 in LBK100 (Fig. 2a, c), while introduction of the *pha1* cluster into TO114 significantly improved the growth in the same medium (Fig. 2b). This indicates that the Pha1 system can function as a K⁺/H⁺ antiporter as suggested previously (Putnoky *et al.*, 1998). Unfortunately, it was difficult to determine whether the Pha1 system is functional at a lower pH, since no growth retardation was observed up to pH 8.5 with the TO114/pTrcHis2C strain (Fig. 2).

Growth properties of the TO114/pTrcHis2C-*pha1* strain in Na⁺-rich medium (LBNa100) at various pHs were also investigated. Most of the Mrp antiporters studied have been shown to be Na⁺/H⁺ antiporters that play an important role in cytosolic pH homeostasis under alkaline conditions (Dzioba-Winogrodzki *et al.*, 2009; Ito *et al.*,

1999; Kosono *et al.*, 1999; Swartz *et al.*, 2007; Yang *et al.*, 2006). The TO114/pTrcHis2C control strain showed almost no growth at pH 7.0, but the growth of the TO114/pTrcHis2C-*pha1* strain in LBNa100 medium was significantly enhanced at pH values up to 8.5, but not at pH 9.0 (Fig. 2c, d). Growth retardation at pH 9.0 in Na⁺-rich medium might have been due to insufficient Na⁺/H⁺ antiport activity to maintain an adequate cytosolic pH, or it might simply reflect overaccumulation of Na⁺ due to the insufficient Na⁺/H⁺ activity of the Pha1 system in TO114 cells.

Taken together, these results suggest that the *pha1* cluster is functionally expressed in TO114 cells, and that the Pha1 system is able to complement the pH- and Na⁺-sensitivities of the TO114 cells.

pH-dependency of the cation/H⁺ antiport activity of the Pha1 system

To elucidate whether the Pha1 system actually functions as a K⁺/H⁺ or Na⁺/H⁺ antiporter, K⁺/H⁺ and Na⁺/H⁺ antiport activities of Pha1 were evaluated by measuring Na⁺- or K⁺-dependent H⁺ translocation activity of membrane vesicles prepared from the above TO114 strains. Fig. 3 shows a representative result of the measurement using assay buffer adjusted to pH 8.5. While everted membrane vesicles prepared from TO114/pTrcHis2C showed only residual cation/H⁺ antiport activity, vesicles from TO114/pTrcHis2C-*pha1* showed significantly higher K⁺/H⁺ and Na⁺/H⁺ antiport activities (Fig. 3). These results suggest that the Pha1 system has K⁺/H⁺ and Na⁺/H⁺ antiport activities.

The activity of the Pha1 system at various pHs was also assessed by measuring the cation/H⁺ antiport activity with 10 mM KCl or NaCl at pHs 7.0 to 9.0 (Fig. 4). Both K⁺/H⁺ and Na⁺/H⁺ antiport activities were almost undetectable at pH 7.0; however, these activities were enhanced as pH increased. Activity peaked at pH 8.5, and decreased at pH 9.0 (to 43 % and 68 % of the activities at pH 8.5 for the K⁺/H⁺ and Na⁺/H⁺ antiports, respectively). These results agreed with the previous report that the Pha1 system plays an important role in alkaline pH homeostasis (Putnoky *et al.*, 1998). It should be noted that the K⁺ transport activity was higher than Na⁺ transport at pH 8.0–8.5, indicating that the Pha1 system predominantly functions as K⁺/H⁺ antiporter in this range of pH when the same concentration of either cation is present. Interestingly, the degree of pH-dependent change of activity was higher for the K⁺/H⁺ antiport than for the Na⁺/H⁺ antiport. While K⁺/H⁺ antiport activity was increased 3.2-fold at pH 8.5 (18.0 %) compared with that at pH 7.5, where similar K⁺/H⁺ and Na⁺/H⁺ antiport activities were observed, Na⁺/H⁺ antiport activity increased only 2.5-fold under the same condition (13.9 %). These results suggest that the cation selectivity of the Pha1 system to K⁺ and Na⁺ is differentially regulated upon pH shift.

Fig. 2. Growth properties of TO114 cells expressing the Pha1 system in LBK100 and LBNa100 media at various pHs. TO114 cells harbouring an empty vector (pTrcHis2C; a, c) or pTrcHis2C-pha1 (b, d) were grown in LBK100 (a, b) or LBNa100 (c, d) at pH 6.5 (○), 7.0 (□), 7.5 (△), 8.0 (●), 8.5 (■) or 9.0 (▲). The error bars indicate the standard deviations from three independent experiments.

Fig. 3. Cation/ H^+ antiport activity of everted membrane vesicles. Representative results of acridine orange quenching assays with everted membrane vesicles prepared from strains TO114/pTrcHis2C (a) and TO114/pTrcHis2C-pha1 (b) are shown. The assay mixture contained everted membrane vesicles (25 μ g protein), 10 mM BTP, 5 mM $MgCl_2$, 140 mM choline chloride and 1.5 μ M acridine orange. Tris/lactate (final concentration at 4 mM) was added to initiate respiration, and KCl or NaCl (20 mM each, block arrows) was added to monitor K^+ - or Na^+ -dependent H^+ movement. NH_4Cl (final concentration at 25 mM) was added to collapse the pH gradient as indicated.

Fig. 4. pH-dependency of the K^+/H^+ or Na^+/H^+ antiport activity. Cation/ H^+ antiport activities were measured using the acridine orange quenching method at the indicated pHs (7.0–9.0) using 10 mM KCl (white bars) or NaCl (hatched bars). Means $\pm$ SD of more than four experiments are shown. The asterisks indicate significant differences from the K^+/H^+ activity value at the same pH by Student's t test ($P < 0.05$).

Affinity to K^+ of the Pha1 system changes with pH

To explore the nature of the differential regulation of the K^+/Na^+ selectivity by pH shift, kinetic properties of the Pha1 system under different pH conditions were investigated. The antiport activities were measured at pH 7.5 and 8.5 with various concentrations of KCl or NaCl (Fig. 5). Michaelis–Menten-type saturation kinetics were observed in all conditions used here. At pH 8.5, where the highest antiport activities were observed, the apparent V_{max} and K_m were 30 % and 8.23 mM for K^+ , and 19.1 % and 5.47 mM for Na^+ . Interestingly, although V_{max} for Na^+ at pH 7.5, where the Pha1 system showed comparable K^+/H^+ and Na^+/H^+ antiport activities, was decreased to less than half, V_{max} for K^+ was not significantly altered (35.2 %). Furthermore, although the apparent K_m for Na^+ at pH 7.5 was almost unchanged, the apparent K_m for K^+ was significantly increased (63.2 mM). These results confirm that the cation selectivity of the Pha1 system is regulated by pH. Moreover, they indicate that the mechanisms regulating net antiport activities of the Pha1 system under pH shifts are different for K^+ and Na^+ .

DISCUSSION

The Pha1 system had long been considered to be a K^+/H^+ antiporter because *pha1* mutants were unable to extrude K^+ under alkaline conditions (Kosono *et al.*, 2000), and the *pha1* cluster shows high sequence similarity to other Mrp antiporters (Hiramatsu *et al.*, 1998; Kosono *et al.*, 2005; Swartz *et al.*, 2005). Nonetheless, no direct evidence for this has previously been presented. Here, taking advantage of the *E. coli* strain TO114, which lacks three major cation/ H^+ antiporters (Ohya *et al.*, 1994), we have demonstrated that the Pha1 system indeed has $K^+/$

Fig. 5. Comparison of the kinetic properties of Pha1 system at pH 7.5 and 8.5. Cation/ H^+ antiport activities were measured with 2.5–40 mM KCl ($\square$) or NaCl ($\circ$) at pH 7.5 (a) and 8.5 (b). Means $\pm$ SD of more than four experiments are shown. The apparent V_{max} and K_m for K^+ were 35.2 % and 63.2 mM at pH 7.5, and 30 % and 8.23 mM at pH 8.5. The apparent V_{max} and K_m for Na^+ were 7.85 % and 4.06 mM at pH 7.5, and 19.1 % and 5.47 mM at pH 8.5. Note that up to 80 mM KCl was used for the assay to estimate the V_{max} and K_m for K^+ .

H^+ antiport activity. Moreover, we have shown that Pha1 can also transport Na^+ , which was rather surprising since Na^+ -related deficiencies have not yet been reported for *pha1* mutants (Putnoky *et al.*, 1998).

Fig. 4 shows that the cation/ H^+ antiport activity of the Pha1 system is pH-dependent, i.e. lower at neutral pH and significantly increased at alkaline pH. This type of pH-dependency is frequently observed for bacterial cation/ H^+ antiporters that have important roles in alkaline pH homeostasis (Padan *et al.*, 2001; Radchenko *et al.*, 2006). These observations support the previous suggestion that the Pha1 system is required to cope with an alkaline environment (Putnoky *et al.*, 1998). Interestingly, the K^+/H^+ antiport activity was significantly decreased at pH 9.0 compared with that at pH 8.5, where maximum antiport activity was observed (43 % of that at pH 8.5) (Fig. 4). In contrast, antiport activities of Mrp antiporters from *Bacillus* species (Bs-Mrp and BpOF4-Mrp), *S. aureus* (Mnh) and *V. cholerae* (VcMrp) were rather constant or increased as pH was shifted to above pH 9.0 (Dzioba-Winogradzki *et al.*, 2009; Swartz *et al.*, 2007). These Mrp antiporters are implicated in the tolerance of constant or transient extreme alkaline conditions in these organisms (Dzioba-Winogradzki *et al.*, 2009; Hiramatsu *et al.*, 1998; Ito *et al.*, 1999, 2001; Kosono *et al.*, 1999). Taken together, our results indicate that the Pha1 system has a role in the tolerance of mild alkaline conditions, rather than extreme alkaline tolerance as observed in the above-mentioned Mrp antiporters.

It has been shown that the *S. meliloti* genome contains another *mrp*-like gene cluster, named *pha2* (Kosono *et al.*, 2005; Yang *et al.*, 2006). The *pha2* cluster encodes proteins that demonstrate high similarity to the group 1 Mrp antiporters. Recently, Yang *et al.* (2006) suggested that the Pha2 system is a $Na^+(Li^+, K^+)/H^+$ antiporter because a *pha2* mutant of *Sinorhizobium fredii* RT19 was sensitive to Na^+ and K^+ , and everted membrane vesicles of the mutant exhibited almost no Na^+ and Li^+/H^+ antiport activity and reduced K^+/H^+ antiport activity. Given that the *pha2* loci of *S. meliloti* and *S. fredii* share significantly high similarity (average: 81.1 % identity), it is highly likely that the *S. meliloti* Pha2 system also has a similar activity. It is noteworthy that Putnoky *et al.* (1998) reported that alkaline pH sensitivity in K^+ -rich media of the *pha1* mutant was ameliorated by the addition of Na^+ to the medium. This could have been due to the compensation for the loss of K^+/H^+ antiport activity of the Pha1 system by the Na^+/H^+ antiport activity of the Pha2 system. Although the roles of the Pha2 system in *S. meliloti* cells remain elusive, it is likely that the Pha1 system plays a major role in alkaline pH homeostasis under K^+ -rich conditions, such as inside plant cells, and the Pha2 system would take over that role when Na^+ concentration increased. More detailed kinetic studies and elucidation of the physiological role of the Pha2 system will be needed to clarify this point.

The apparent K_m values for K^+ and Na^+ were determined to be 8.23 mM and 5.47 mM at pH 8.5, respectively (Fig. 5). These results indicate that the Pha1 system is a relatively low-affinity $K^+(Na^+)/H^+$ antiporter compared with other functionally characterized Mrp antiporters (Dzioba-Winogrodzki *et al.*, 2009; Swartz *et al.*, 2007). Interestingly, Mrp antiporters exhibiting high affinity for Na^+ (K_m 0.06 mM for Bs-Mrp from *B. subtilis*, 0.10 mM for BpOF4-Mrp from *Bacillus pseudofirmus* and 0.12 mM for Mnh from *Staphylococcus aureus* at pH 8.5) are highly selective for Na^+ and Li^+ and have been shown to have almost no K^+ transport activity (Swartz *et al.*, 2007). In contrast, Vc-Mrp from *V. cholerae*, which has low-affinity K^+ transport activity (apparent K_m 68.5 mM), was shown to have significantly lower affinity for Na^+ than other Mrp antiporters (apparent K_m 1.30 mM at pH 9.0, where the antiporter exhibits the highest activity) (Dzioba-Winogrodzki *et al.*, 2009), albeit higher than that of the Pha1 system. It is possible that the lower Na^+ affinity is a common characteristic of Mrp antiporters that have K^+/H^+ antiport activity. These observations might suggest that the Pha1 system has acquired K^+ affinity by sacrificing Na^+ affinity, or other Mrp antiporters have lost K^+ affinity in order to be highly selective for Na^+ , during molecular evolution.

The molecular mechanisms for cation discrimination of Mrp antiporters are largely unknown. Recently, Dzioba-Winogrodzki *et al.* (2009) demonstrated competition between Na^+ and K^+ in the cation/ H^+ antiport activity of VcMrp, which suggests that Na^+ and K^+ share a common binding site. Interestingly, our result showed that only K^+ affinity was altered when pH was lowered from the optimum pH, while the K_m for Na^+ was unaffected. Based on these observations, together with the fact that the K^+ ion is larger than the Na^+ ion, one possible explanation for this observation is that the cation selectivity of the Pha1 system is regulated by modulating the size of the 'cation-binding pocket' upon pH shift. For example, when acidified, the size of the pocket may become smaller so that K^+ is less likely to bind due to the size restriction but not small enough to impede the binding of Na^+ to the pocket. Further analysis regarding the structure of the cation-binding site would be needed to clarify this point. Also, we have shown that K^+/H^+ and Na^+/H^+ antiport activities were downregulated as pH was lowered, but these activities appeared to be regulated in different manners. In the case of the K^+/H^+ antiport, affinity for K^+ was decreased and V_{max} was maintained at the same level, while in the case of the Na^+/H^+ antiport, V_{max} was decreased but affinity for Na^+ was maintained at a similar level (Fig. 5). These results suggest that the 'shutting off' mechanisms of the Pha1 system upon acidic shift are different between Na^+/H^+ and K^+/H^+ antiport activities. It is difficult to explain this observation by simply assuming the pH-dependent contraction of the cation-binding pocket or pore size, since that would not only reduce K_m but would also be likely to reduce V_{max} . Although it is highly

speculative at this point, it is possible that these cations are transported via distinct mechanisms. We are currently investigating how the molecular assembly of the Pha1 system is affected by pH, and are also attempting to identify the key subunit for cation discrimination to elucidate the molecular mechanisms under the regulation of antiport activity.

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