Three putative cation/proton antiporters from the soda lake alkaliphile *Alkalimonas amylolytica* N10 complement an alkali-sensitive *Escherichia coli* mutant

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Attempts to identify members of the antiporter complement of the alkali- and saline-adapted soda lake alkaliphile *Alkalimonas amylolytica* N10 have used screens of DNA libraries in antiporter-deficient *Escherichia coli* KNabc. Earlier screens used Na⁺ or Li⁺ for selection but only identified one NhaD-type antiporter whose properties were inconsistent with a robust role in pH homeostasis. Here, new screens using elevated pH for selection identified three other putative antiporter genes that conferred resistance to pH ≥ 8.5 as well as Na⁺ resistance. The three predicted gene products were in the calcium/cation antiporter (CaCA), cation/proton antiporter-2 (CPA2) and cation/proton antiporter-1 (CPA1) families of membrane transporters, and were designated Aa-CaxA, Aa-KefB and Aa-NhaP respectively, reflecting homology within those families. Aa-CaxA conferred the poorest Na⁺ resistance and also conferred modest Ca²⁺ resistance. Aa-KefB and Aa-NhaP inhibited growth of a K⁺ uptake-deficient *E. coli* mutant (TK2420), suggesting that they catalysed K⁺ efflux. For Aa-NhaP, the reversibility of the growth inhibition by high K⁺ concentrations depended upon an organic nitrogen source, e.g. glutamine, rather than ammonium. This suggests that NH₄⁺ as well as K⁺ efflux is catalysed by Aa-NhaP. Vesicles of *E. coli* KNabc expressing Aa-NhaP, which conferred the strongest alkali resistance, exhibited K⁺/H⁺ antiport activity in a pH range from 7.5 to 9.5, and with an apparent *K_m* for K⁺ of 0.5 mM at pH 8.0. The properties of this antiporter are consistent with the possibility that this soda lake alkaliphile uses K⁺(NH₄⁺)/H⁺ antiport as part of its alkaline pH homeostasis mechanism and part of its capacity to reduce potentially toxic accumulation of cytoplasmic K⁺ or NH₄⁺ respectively, under conditions of high osmolarity or active amino acid catabolism.

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INTRODUCTION

Bacteria that grow in soda lakes must be adapted to both high pH and high concentrations of Na⁺, as well as to stresses that are secondary to these major ones, such as low water activity and scarcity of some micronutrients (Grant

& Tindall, 1986; Grant, 2004; Jones *et al.*, 1998). For example, Lake Chahannor, a soda lake in the Inner Mongolia autonomous region of China, has reported pH values in a range from 9.5 to 10.2 and contains more than 5 M Na⁺, high concentrations of Cl[−] and CO₃^{2−}, very low levels of Mg²⁺ and undetectable levels of Ca²⁺ (Ma *et al.*, 2004; Zheng, 1992). *Alkalimonas amylolytica* N10 is a halo-tolerant, alkaliphilic Gram-negative (γ -Proteobacterium) isolate from this lake (Ma *et al.*, 2004). In an earlier study, we sought to identify *A. amylolytica* N10 genes encoding cation/proton antiporters that might participate in alkaline pH homeostasis and Na⁺ resistance (Liu *et al.*, 2005). In other bacteria, a complement of several distinct secondary cation/proton antiporters usually contributes to alkaline pH homeostasis (Padan *et al.*, 2005). Na⁺(Li⁺)/H⁺ antiporters often play dominant roles in both this function and Na⁺(Li⁺) resistance (Padan *et al.*, 2005). K⁺/

Abbreviations: AO, acridine orange; BTP, bis-[tris(hydroxymethyl)methyl-amino]-propane; CaCA, calcium/cation antiporter; CPA, cation/proton antiporter; LBK, Luria broth with potassium instead of sodium; LBC, Luria broth with choline instead of sodium.

The GenBank/EMBL/DDBJ accession numbers for the sequences of the *A. amylolytica* N10 gene products are CaxA (ABG37980), KefB (ABG37986) and NhaP (ABG37987). DNA sequences of the larger cloned fragments containing these genes were deposited as pNAK7 (DG649017), pNAK9/10 (DG649019) and pNAK11 (DG649020) respectively, and a fourth cloned fragment that was not further studied, pNAK8, was deposited as DQ649018.

H⁺ antiporters have also been proposed to participate in alkaline pH homeostasis (Kakinuma & Igarashi, 1995; Nakamura *et al.*, 1984, 1992; Plack & Rosen, 1980) and recently the genes encoding such antiporters have been identified in both non-marine and marine bacteria (Fujisawa *et al.*, 2005; Lewinson *et al.*, 2004; Radchenko *et al.*, 2006a, b). Na⁺/H⁺ and K⁺/H⁺ antiporters can exchange cytoplasmic cations for external protons to achieve a cytoplasmic pH significantly less alkaline than the external pH (Padan *et al.*, 2005). Genomic data from the haloalkaliphilic archaeon *Natronomonas pharaonis* from African soda lakes (Falb *et al.*, 2005) suggest the presence of one Na⁺/H⁺ antiporter of the cation/proton antiporter-1 (CPA1) family and two more from the NhaC family in addition to four K⁺/H⁺ antiporters of the cation/proton antiporter-2 (CPA2) family and one from the cation/proton antiporter-3 (CPA3) family, as classified using the transporter classification system (Ren *et al.*, 2007; Saier *et al.*, 2006). However, the cation specificity, properties and physiological functions of these transporters have yet to be studied experimentally.

The first attempts to identify genes encoding antiporters in DNA libraries from *A. amylytica* N10 utilized a screen in the triple antiporter mutant of *Escherichia coli* strain KNabc (Δ nhaA Δ nhaB Δ chaA), in which resistance to Na⁺ or Li⁺ at pH 7.5 was the basis for identifying candidate plasmids that carried antiporter genes of interest (Liu *et al.*, 2005). This is the most frequently used protocol for isolating genes encoding Na⁺(Li⁺)/H⁺ antiporters from bacteria whose genomes have not been sequenced and which lack a genetic system that allows screening in the natural host via transpositional mutants that are alkali-sensitive (Padan *et al.*, 2005). The initial screens in *E. coli* KNabc resulted in identification of only one antiporter, an NhaD-type Na⁺(Li⁺)/H⁺ antiporter with an optimum pH of at least 9.5, which exceeded the limits that the assay could determine definitively (Liu *et al.*, 2005). In addition, the optimum [Na⁺] was unusually high, at about 600 mM. These properties suggested that Aa-NhaD may serve an emergency role, i.e. when acute cytoplasmic alkalinization and adverse Na⁺ elevation have occurred, while other yet to be identified antiporters have important roles in alkaline pH homeostasis and Na⁺ resistance (Liu *et al.*, 2005). Many halotolerant micro-organisms are adapted to high cytoplasmic concentrations of Na⁺ while others maintain cytoplasmic Na⁺ levels that are much lower than the high external concentrations (Ventosa *et al.*, 1998). However, even if *A. amylytica* N10 is adapted to high cytoplasmic Na⁺, it seemed unlikely that it grows robustly with both a high cytoplasmic [Na⁺] and a cytoplasmic pH well above 9 since elevated pH exacerbates Na⁺ toxicity and elevated Na⁺ compromises growth at high pH (Padan *et al.*, 2005).

In the current study, screening of a DNA library of *A. amylytica* N10 was carried out using the same mutant *E. coli* KNabc host but with selection based on alkali resistance, with a view towards broadening the search for antiporters that might contribute to alkali and/or Na⁺

resistance. Since *E. coli* KNabc is deficient in K⁺(Ca²⁺)/H⁺ as well as Na⁺(Li⁺)/H⁺ antiport, this selection might identify antiporters with different cation specificities or Na⁺/H⁺ antiporters with a higher affinity for Na⁺ than Aa-NhaD. Indeed, Aa-NhaD was not identified in this screen but three other genes were identified whose products are predicted to come from three different cation/proton antiporter families of the transporter classification system (Ren *et al.*, 2007; Saier *et al.*, 2006). The capacity of each gene to complement several different growth phenotypes was studied in mutant *E. coli* hosts that have well-defined properties. One of the antiporters, designated Aa-NhaP, was then characterized further in assays of antiport activity in membrane vesicles.

METHODS

Bacterial strains, plasmids and culture conditions. The bacterial strains and plasmids used in this study are shown in Table 1. *Alkalimonas amylytica* N10 was grown at 37 °C, with shaking, in Horikoshi I medium (Horikoshi, 1991). *E. coli* strains DH5 α , KNabc and TK2420 were routinely grown in LB medium with potassium instead of sodium (LBK) at pH 7.5 (Goldberg *et al.*, 1987). In LBC medium, 6 g l⁻¹ of choline chloride (40 mM) replaced the KCl that is present in LBK; the pH of LBC was adjusted by addition of HCl or bis-[tris(hydroxymethyl)methylamino]-propane (BTP). For growth experiments on *E. coli* KNabc in LBK and LBC at different pH values and levels of added NaCl, cells were grown at 37 °C, with shaking, for 9 h, after which the OD₆₀₀ was recorded. When antibiotics were added to the medium for selection or plasmid maintenance, the concentrations used were 100 μ g ampicillin ml⁻¹, 50 μ g chloramphenicol ml⁻¹ and 50 μ g kanamycin ml⁻¹.

Isolation of *A. amylytica* N10 genes that increase alkali tolerance of *E. coli* KNabc. DNA isolation, cloning and restriction analyses were carried out by standard methods (Sambrook *et al.*, 1989). For construction of a DNA library, chromosomal DNA was isolated from *A. amylytica* N10 and partially digested with *Sau*3AI. Fragments in the range of 3–8 kb were isolated and ligated to *Bam*HI-digested pGEM-3Zf(+) cloning vector (Promega). The ligation mixture was used to transform *E. coli* DH5 α under selection on LBK-ampicillin plates (pH 7.5) at 37 °C. After amplification, the mixed plasmid pool from about 5 $\times$ 10⁶ colonies was collected, and used to transform *E. coli* KNabc. The transformant pool was screened for colony-forming cells at pH 8.5–9.0 and 37 °C on LBK-ampicillin-kanamycin plates. Plasmids in transformants from colonies that arose on these plates were retested. After confirmation of their ability to support growth under the same screening conditions, complete sequencing was conducted on the inserts of five complementing plasmids that had distinct restriction patterns. These plasmids were designated pNAK7–11; the sizes of their inserts were 4113 bp, 3566 bp, 5412 bp, 3782 bp and 8473 bp respectively. The plasmid insert sequences were further confirmed to match the sequence of PCR products from chromosomal DNA of *A. amylytica* N10. DNA sequencing was conducted at the Mount Sinai School of Medicine DNA Core Facility. Computer analyses were performed by using the Gene Runner 3.05 program (Hastings Software). Putative ORFs were identified with the ORF Finder program and homologue searches were conducted using BLASTP (Altschul *et al.*, 1990) and the network services of the National Center for Biotechnology Information (NCBI). The secondary structure and the free energy calculations of putative stem-loop structures were analysed using the method of Zuker (2003) (<http://www.bioinfo.rpi.edu/applications/mfold/cgi-bin/rna-form1.cgi>).

Table 1. Bacterial strains, plasmids and primers

Strain or plasmid	Relevant phenotype or genotype	Source or reference
<i>A. amylolytica</i> N10	Extreme alkaliphile, Gram-negative	Ma <i>et al.</i> (2004)
<i>E. coli</i> DH5 α	F ⁻ ϕ 80d <i>lacZ</i> Δ <i>M15</i> Δ(<i>lacZYA-argF</i>) <i>U169</i> <i>recA1</i> <i>endA1</i> <i>hsdR17</i> (r ⁻ m ⁺) <i>phoA</i> <i>supE44</i> λ^- <i>thi-1</i> <i>gyrA96</i> <i>relA1</i>	Gibco-BRL
<i>E. coli</i> KNabc	TG1(Δ <i>nhaA</i> Δ <i>nhaB</i> Δ <i>chaA</i>)	Nozaki <i>et al.</i> (1998)
<i>E. coli</i> TK2420	Δ(<i>kdpABC</i>) <i>trkD1</i> Δ <i>trkA</i>	Epstein <i>et al.</i> (1993)
pGEM-3Zf(+))	Cloning vector (Ap ^R)	Promega
pGerN	pGEM-3Zf(+) carrying the <i>gerN</i> gene from <i>B. cereus</i>	Southworth <i>et al.</i> (2001)
pNAK7	pGEM-3Zf(+) carrying a 4113 bp <i>A. amylolytica</i> N10 <i>Sau</i> 3AI fragment at <i>Bam</i> HI site encoding a CaCA transporter homologue	This study
pNAK8	pGEM-3Zf(+) carrying a 3566 bp <i>A. amylolytica</i> N10 <i>Sau</i> 3AI fragment at <i>Bam</i> HI site encoding a SulP transporter homologue	This study
pNAK9	pGEM-3Zf(+) carrying a 5412 bp <i>A. amylolytica</i> N10 <i>Sau</i> 3AI fragment at <i>Bam</i> HI site encoding a KefB homologue	This study
pNAK10	pGEM-3Zf(+) carrying a 3782 bp <i>A. amylolytica</i> N10 <i>Sau</i> 3AI fragment at <i>Bam</i> HI site encoding KefB and LysR proteins	This study
pNAK11	pGEM-3Zf(+) carrying a 8473 bp <i>A. amylolytica</i> N10 <i>Sau</i> 3AI fragment at <i>Bam</i> HI site encoding an NhaP homologue	This study
pYW136	pGEM-3Zf(+) carrying <i>A. amylolytica</i> N10 <i>Aa-caxA</i> with its native Shine–Dalgarno sequence	This study
pYW138	pGEM-3Zf(+) carrying <i>A. amylolytica</i> N10 <i>Aa-kefB</i> with its native Shine–Dalgarno sequence	This study
pYW139	pGEM-3Zf(+) carrying <i>A. amylolytica</i> N10 <i>Aa-nhaP</i> with its native Shine–Dalgarno sequence	This study
Primer	Sequence*	
pNAK7/EF1 (<i>Eco</i> RI)	5'-AAAAGAATTCAATGCGCCAACAGGATCC-3'	This study
pNAK7/XR1 (<i>Xba</i> I)	5'-AAATCTAGAGTATCCATTAAGTCTAGCTGG-3'	This study
pNAK9/SF1 (<i>Sma</i> I)	5'-AAAACCCGGGTTAAAGTTAAATCACAGC-3'	This study
pNAK9/BR1 (<i>Bam</i> HI)	5'-AAAAGGATCCTGCTTAATCCGCAG-3'	This study
pNAK11/EF1 (<i>Eco</i> RI)	5'-AAAAGAATTCACATTGTCTAAATTAGGGGA-3'	This study
pNAK11/XR1 (<i>Xba</i> I)	5'-AAATCTAGACATTGAGTTTCAGCGCTG-3'	This study

*Restriction sites are underlined.

Since part of the pNAK9 insert contained a 2.3 kb fragment that was identical to that in pNAK10, the sequences were merged appropriately. The DNA sequences of the four plasmid inserts were deposited in GenBank under the following accession numbers: DQ649017 for pNAK7; DQ649018 for pNAK8; DQ649019 for pNAK9/10; and DQ649020 for pNAK11. Three of these plasmids, pNAK7, pNAK9/10 and pNAK11, contained genes that were candidates for cation/proton antiporters. These three genes were studied further and their putative products were deposited in GenBank with the following designations and accession numbers: Aa-CaxA, ABG37980; Aa-KefB, ABG37986; and Aa-NhaP, ABG37987.

Cloning the putative antiporter genes. The three putative antiporter gene ORFs, including their native Shine–Dalgarno (SD) sequences, were cloned behind the T7 promoter of pGEM-3Zf(+) (Promega). The PCR primers listed in Table 1 were used to amplify the genes from *A. amylolytica* N10 chromosomal DNA by PCR reactions that used the TaKaRa *Taq* polymerase kit according to the manufacturer's instructions. The *Aa-caxA* gene was cloned as follows. PCR with primer pair pNAK7/EF and pNAK7/XR1 was conducted so that the forward primer contained an *Eco*RI site upstream of the putative SD sequence and the reverse primer contained an *Xba*I site downstream of the stop codon of the *Aa-caxA* coding sequence. The 1.6 kb PCR product was digested with *Eco*RI and *Xba*I, ligated to

pGEM-3Zf(+) vector, linearized with the same enzymes and used to transform *E. coli* DH5 α . The resulting recombinant plasmid was designated pYW136. Similar strategies were used to construct plasmid pYW138 for cloning *Aa-kefB* with primer pair pNAK9/SF1 (*Sac*I) and pNAK9/BR1 (*Bam*HI), and to construct plasmid pYW139 containing *Aa-nhaP* using primers pNAK11/EF1 (*Eco*RI) and pNAK11/XR1 (*Xba*I). The sequences of all the recombinant plasmids were confirmed to be correct.

Complementation assays in *E. coli* mutant strains. The recombinant plasmids pYW136, pYW138 and pYW139 were transformed into Na⁺(K⁺)(Ca²⁺)/H⁺ antiporter-deficient *E. coli* KNabc and K⁺ uptake-deficient *E. coli* TK2420. The recombinant plasmid pGerN, which contains a Na⁺/H⁺–K⁺ antiporter from *Bacillus cereus* (Southworth *et al.*, 2001) was used as a positive control and the plasmid vector pGEM-3Zf(+) was used as a negative control; GerN confers robust Na⁺ and alkali resistance upon *E. coli* KNabc. For studies of complementation of the Na⁺- and alkali-sensitive phenotypes of *E. coli* KNabc, the test and control transformants were cultured overnight in LBK, pH 7.5, after which the OD₆₀₀ of each culture was adjusted to 2.5 before 10-fold serial dilutions were made with the same medium. One microlitre of each dilution was dotted on LBK plates with different pH values and concentrations of added NaCl. For assays of Ca²⁺ resistance, conducted in *E. coli* KNabc on

plates, Tris-E medium (pH 8.0) containing 100 mM added CaCl_2 was used (Brockman & Heppel, 1968; Waditee *et al.*, 2004). A control transformant of *E. coli* DH5 α that has a wild-type *chaA* gene was used as the positive control. Since *E. coli* ChaA has $\text{Ca}^{2+}/\text{H}^{+}$ antiport activity, it supports calcium resistance (Ivey *et al.*, 1993; Ohyama *et al.*, 1994). Pre-cultures were grown and diluted as described above. The plates for these sets of assays were incubated for the times indicated in the legend to Fig. 3. Tests were also conducted of the complementation or exacerbation of the high K^{+} requirement of K^{+} uptake-deficient *E. coli* TK2420. The inocula for these growth experiments were grown in LBK (pH 7.5) and then diluted 200-fold for growth assays in liquid minimal medium containing a range of added KCl concentrations (Epstein *et al.*, 1993). In some experiments, the $(\text{NH}_4)_2\text{SO}_4$ that is the sole nitrogen source in this minimal medium was replaced by 5 mM glutamine. After 16 h the OD_{600} was recorded. All complementation assays were carried out in duplicate in at least two separate trials.

Preparation and assays of everted membrane vesicles. Everted membrane vesicles were prepared from *E. coli* KNabc transformants by the method described by Rosen (1986). Briefly, cell pellets from one litre of culture were washed twice with 25 ml TCDG buffer containing: 10 mM Tris-chloride (pH 7.5), 140 mM choline chloride, 0.5 mM dithiothreitol and 10 % glycerol. Cell suspensions in 20 ml batches were used for disruption after addition of a protein inhibitor tablet (Roche), 1 mM phenylmethylsulfonyl fluoride and a trace amount of DNase I (Roche). The cell suspensions were passed through a French press cell under 10 000 p.s.i. (69 MPa) pressure. The broken cell suspensions were subjected to two low-speed centrifugations before the vesicles were collected by ultracentrifugation at 250 000 g (Beckman Ti60 rotor), 4 °C for 1 h. The vesicles were suspended in 1 ml TCDG buffer. The protein concentration was measured by the Lowry method using lysozyme as the standard. Monovalent cation/ H^{+} antiport assays of the everted membrane vesicles were carried out in a 2 ml reaction mix containing 10 mM BTP buffer, 140 mM choline chloride, 5 mM MgCl_2 , 1 μM acridine orange and 50 μg vesicle protein ml^{-1} , which were pre-mixed at room temperature. The pH values are indicated for particular experiments; the buffer pH was adjusted with sulfuric acid. Antiport activity was monitored by a fluorescence assay in which acridine orange (AO) was used as a probe of the ΔpH with acid inside the everted vesicles, which is first established by respiration upon addition of an electron donor (2.5 mM Tris-D-lactate) to the reaction mixture. Quenching of the AO fluorescence is observed as the probe is internalized in parallel with the development of this ΔpH , then antiport is assessed as the percentage dequenching of fluorescence that occurs when a cation is added to the reaction mix (Goldberg *et al.*, 1987). Fluorescence was monitored using a Shimadzu RF-5301PC spectrofluorophotometer. Background values from the vector control plasmid vesicle preparations were measured and subtracted. At the end of the assay, 25 mM NH_4Cl was added to establish the baseline as the ΔpH dissipated. At least two assays were conducted on two independent vesicle preparations in each experiment.

RESULTS AND DISCUSSION

Development of a screen based on complementation of alkali sensitivity

The new library of *A. amylolytica* N10 DNA in pGEM-3Zf(+) was first screened at pH 7.5 for clones that complemented the $\text{Na}^{+}(\text{Li}^{+})$ -sensitive phenotype of *E. coli* KNabc, i.e. with the same screen that had yielded Aa-NhaD from an earlier library in pUC18 (Liu *et al.*, 2005). A

large proportion of the complementing clones were found to contain *Aa-nhaD* and no other membrane transporter candidates were identified in this screen. We therefore sought to devise a screen based on alkali resistance. Preliminary growth experiments were conducted on the untransformed host *E. coli* KNabc strain to establish conditions for the screen. Both alkali resistance and Na^{+} resistance of *E. coli* KNabc are expected to be enhanced by the presence of added K^{+} (Harel-Bronstein *et al.*, 1995; Padan & Krulwich, 2000; Padan *et al.*, 2005; Radchenko *et al.*, 2006b). However a side-by-side test of these expectations had not been presented. Assays of *E. coli* KNabc growth were conducted as a function of pH and added NaCl in LB in which added NaCl was replaced with KCl (LBK) versus LB with choline-chloride (LBC) replacing added NaCl or KCl. As expected, growth inhibition by Na^{+} was more pronounced as the growth pH was raised (Fig. 1). Moreover, at every pH tested, the triple antiporter mutant was more sensitive to growth inhibition by Na^{+} in LBC than in the K^{+} -replete LBK medium. In the absence of added Na^{+} , addition of K^{+} led to only a modest increase in growth yield at pH 7.0–7.5, but K^{+} addition strongly increased the growth yield at high pH values. At pH 8.5–9.0 no growth was observed in the absence of

Fig. 1. Effects of K^{+} ions on Na^{+} and alkali tolerance in *E. coli* KNabc. *E. coli* KNabc was grown in LBK (upper panel) or LBC (lower panel) with the indicated concentrations of added NaCl at a series of pH values from 7.0 to 9.0. OD_{600} was recorded after 9 h growth at 37 °C with shaking. Results are the means $\pm$ SD of duplicate determinations in two independent experiments.

added K^+ even when Na^+ was also omitted from the medium. At pH 9.0 only a very low level of growth of *E. coli* KNabc was observed under these conditions. The screening conditions chosen to identify antiporters from the *A. amylolytica* N10 were LBK medium at pH 8.5 and 9.0. These conditions might select for antiporters that can use K^+ or Ca^{2+} as an efflux substrate to couple to H^+ uptake that lowers the alkali sensitivity of *E. coli* KNabc. The screen might also select for Na^+/H^+ antiporters that are different from Aa-NhaD in being able to use the low Na^+ content of LBK to achieve H^+ uptake in support of alkali resistance. Five plasmids with distinct inserts were isolated from transformants that grew under these screening conditions and conferred resistance in a rescreening experiment under the same conditions. One of these plasmids, pNAK7, was from the pH 8.5 screen while the other four plasmids, pNAK8–11, were from the pH 9.0 screen.

Sequence analysis of three distinct complementing clones

Complete sequence and homology analyses revealed that two of the plasmid inserts, those of pNAK9 and 10, were overlapping gene sequences. As shown in Fig. 2, there were candidate genes for cation/ H^+ antiporters in pNAK7, pNAK9/10 and pNAK11 but not in pNAK8. pNAK8 is predicted to encode a member of the SulP transporter family whose most closely characterized homologue is a bicarbonate transporter from cyanobacteria (Price *et al.*, 2004). This raises the interesting possibility that bicarbonate transport could be used by prokaryotes for alkaline pH homeostasis as well as for 1-carbon homeostasis. Significant research will be required to test this possibility and it is not pursued here. Further studies focused on the candidate cation/ H^+ antiporter genes in pNAK7, pNAK9/10 and pNAK11. Each of these three genes is listed with a

Fig. 2. Diagrams of the inserts from plasmids bearing *A. amylolytica* N10 genes that complemented the alkali-sensitive phenotype of *E. coli* KNabc. The indicated plasmids are shown with a size scale at the bottom. The predicted ORFs are shown with arrows that indicate the direction of transcription. The closest homologue in a BLAST search, if any, is shown above or below the corresponding arrow.

proposed name in Table 2, which shows the two closest homologues on the basis of sequence similarity followed by the two or three closest homologues for which there are experimental data about structure and/or function. In all instances, the two closest homologues have not yet been characterized.

The candidate gene of interest in the insert from pNAK7 (4.1 kb) was a putative transmembrane protein of 319 amino acids that has strong homology to the K^+ -dependent Ca^{2+}/Na^+ exchangers from the Ca^{2+} /cation (CaCA) transporter superfamily (Cai & Lytton, 2004; Saier *et al.*, 1999). It was designated Aa-CaxA because the related CAX and YRBG branches of the CaCA family contain the closest bacterial homologues among a large group of transporters that are involved in calcium exchange reactions (Cai & Lytton, 2004). Aa-CaxA shows significant sequence similarity (42 % identity) to the putative *E. coli* Na^+/Ca^{2+} exchange protein YrbG, whose membrane topology was determined but whose transport activity has not yet been reported. Aa-CaxA also shows a comparable hydropathy profile and acidic motif but less sequence similarity to two cyanobacterial transporters, Syn-CAX (23 % identity; Table 2) and Ap-CAX (20 % identity), for

which Ca^{2+}/H^+ antiporter activity has been reported (Waditee *et al.*, 2004). It shows slightly less similarity (18 % identity), as do other members of the CaCA family (Cai & Lytton, 2004), to the ChaA antiporter of *E. coli* that has been shown to catalyse $Na^+(Ca^{2+})(K^+)/H^+$ antiport (Ivey *et al.*, 1993; Radchenko *et al.*, 2006a).

The merged sequences from the inserts of pNAK9 and 10 contained a cation/ H^+ antiporter candidate from a different transporter family. The predicted gene product has 661 amino acids and homology to the members of cation/proton antiporter family 2 (CPA2) (Saier *et al.*, 1999). This family includes well-characterized transport proteins, such as the glutathione-gated K^+ efflux KefB and KefC systems from *E. coli*, which have some channel-like properties (Booth *et al.*, 2005). The CPA2 family also contains transporters for which antiport activity has been experimentally supported, e.g. the Na^+/H^+ antiporter NapA from *Enterococcus hirae* (Waser *et al.*, 1992), the Na^+/H^+-K^+ antiporter GerN from *Bacillus cereus* (Southworth *et al.*, 2001), and the Fe^{2+}/H^+ antiporter MagA from *Magnetospirillum* sp. strain AMB-1 (Nakamura *et al.*, 1995). Among the CPA-2 proteins that have been functionally characterized, the protein from *A. amylytica*

Table 2. Homologues of the putative cation/ H^+ antiporters encoded by the complementing *A. amylytica* N10 genes

Name and size (aa)	Description	Identity % (amino acids overlap)	Similarity % (amino acids overlap)	Species	Accession no. of homologue
Aa-CaxA (319)	Putative Ca^{2+}/Na^+ antiporter	58 (187/318)	75 (240/318)	Alpha proteobacterium HTCC2255	ZP_01449988
	Putative Na/Ca transporter (CaCA family)	55 (176/319)	74 (239/319)	Alteromonadales bacterium TW-7	ZP_01612155.1
	*YrbG, hypothetical Ca^{2+}/Na^+ antiporter	42 (136/319)	65 (208/319)	<i>Escherichia coli</i> F11	ZP_00720333.1
	* Ca^{2+}/Na^+ antiporter SynCAX	23 (81/344)	42 (146/344)	<i>Synechocystis</i> sp. PCC 6803	BAA18148
Aa-KefB (661)	Sodium/hydrogen exchanger family/TrkA domain protein	55 (360/654)	70 (457/654)	<i>Methylococcus capsulatus</i> str. Bath	YP_114199.1
	Putative glutathione-regulated potassium efflux system K^+/H^+ antiporter	45 (299/655)	62 (409/655)	<i>Nitrococcus mobilis</i> Nb-231	ZP_01126363.1
	*Glutathione-gated K^+ efflux system KefB	29 (179/603)	49 (300/603)	<i>Escherichia coli</i> K-12	P45522
	*Glutathione-gated K^+ efflux system KefC	30 (163/529)	48 (259/529)	<i>Escherichia coli</i> K-12	NP_414589
Aa-NhaP (574)	NhaP-type Na^+/H^+ antiporter	56 (326/573)	76 (436/573)	<i>Idiomarina loihiensis</i> L2TR	AAV82810.1
	NhaP-type Na^+/H^+ and K^+/H^+ antiporter	45 (254/560)	64 (360/560)	<i>Hahella chejuensis</i> KCTC 2396	ABC30246.1
	*Putative transporter YcgO (CvrA)	41 (234/573)	61 (352/573)	<i>Escherichia coli</i> CFT073	NP_753545
	* K^+/H^+ antiporter VpNhaP2	40 (229/572)	61 (349/572)	<i>Vibrio parahaemolyticus</i> RIMD2210633	BAC61130
	* $Na^+(Li^+)/H^+$ antiporter MjNhaP1	24 (100/416)	43 (180/416)	<i>Methanococcus jannaschii</i>	Q60362

*Antiporters cited in published studies: YrbG (Ruknudin & Schulze, 2002; Saaf *et al.*, 2001), SynCAX (Waditee *et al.*, 2004), KefB (MacLean *et al.*, 1998), KefC (Miller *et al.*, 1997), YegO (Verkhovskaya *et al.*, 2001), VpNhaP2 (Radchenko *et al.*, 2006b), MjNhaP1 (Hellmer *et al.*, 2002).

N10 most closely resembles *E. coli* Kef B (29 % identity, 49 % similarity) (Table 2) and hence was designated Aa-KefB.

The insert in pNAK11 was the largest of the inserts (Fig. 2) and contained a gene encoding a protein that was designated Aa-NhaP. This predicted product has 574 amino acids and homology to members of the monovalent cation/proton antiporter-1 (CPA1) family (Saier *et al.*, 1999). The closest homologues among characterized CPA1 antiporters are YcgO (41 %) from *E. coli* and NhaP2 (40 %) from *Vibrio parahaemolyticus*. YcgO plays a role in cell volume regulation at low osmolarity and was suggested to translocate Na^+ and K^+ (Verkhovskaya *et al.*, 2001). Vp-NhaP2 is exclusively a K^+/H^+ antiporter whose proposed function is protection against adversely high concentrations of K^+ at alkaline pH (Radchenko *et al.*, 2006b). Aa-NhaP has much less similarity (24 % identity) to MjNhaP, an $\text{Na}^+(\text{Li}^+)/\text{H}^+$ antiporter that is only active at pH 7.0 or below (Hellmer *et al.*, 2002).

Complementation studies with the cloned transporter genes

The individual *Aa-caxA*, *Aa-kefB* and *Aa-nhaP* genes, cloned in pGEM-3Zf(+) with their own ribosome-binding sites behind the T7 promoter (in plasmids pYW136, pYW138 and pYW139 respectively), were tested for their ability to complement several phenotypes of *E. coli* mutant strains. The empty vector was the negative control and the *B. cereus* $\text{Na}^+/\text{H}^+-\text{K}^+$ antiporter GerN (Southworth *et al.*, 2001) was a positive control for all but one of the plate assays. *E. coli* DH5 α , which has a wild-type *chaA* gene, was the control for the Ca^{2+} sensitivity assay.

Aa-caxA complementation profile. The complementation profile for pYW136, expressing *Aa-caxA*, in assays conducted in *E. coli* KNabc, was modest complementation of Na^+ sensitivity at pH 7.5 and no complementation at pH 8.0 (Fig. 3 A, B); significant complementation of alkali-sensitivity in the absence of added Na^+ (Fig. 3C); and

Fig. 3. Complementation capacities of *A. amylytica* N10 genes in alkali-, Na^+ - and Ca^{2+} -sensitive *E. coli* KNabc. Plate assays were conducted as described in Methods. The test plasmids were pYW136 (*Aa-CaxA*), pYW138 (*Aa-KefB*) and pYW139 (*Aa-NhaP*), pGerN (positive control except for panel D) and pGEM-3Zf(+) (negative control). *E. coli* DH5 α transformed with an empty vector (pMW118 carrying a kanamycin resistance cassette) was the control in D. The conditions were as indicated above the four panels. Panels A–C show the results of experiments terminated after 40 h of incubation at 37 °C. Panel D shows the results of an experiment, conducted only with pYW136 and controls, terminated after 7 days.

modest complementation of Ca^{2+} sensitivity (Fig. 3D). Given the undetectable levels of Ca^{2+} and very low levels of Mg^{2+} in the natural environment of *A. amylolytica* N10, it is unlikely that a robust efflux protein for these important micronutrients has an important physiological role. Possibly the low complementation activity reflects this. Alternatively, it is possible that under physiological conditions in the natural host, Aa-CaxA is used to accumulate Ca^{2+} in exchange for cytoplasmic Na^+ ; the modest beneficial effect on alkali sensitivity could be an indirect effect of lowering cytoplasmic $[\text{Na}^+]$. A test of the Aa-caxA effect in the K^+ uptake-deficient strain *E. coli* TK2420 showed that Aa-caxA modestly enhanced growth of the mutant in the presence of limiting $[\text{K}^+]$ (Fig. 4A). This is consistent with the possibility that the exchange catalysed by Aa-CaxA can use K^+ as part of the coupling ion complement, as has been demonstrated for some other

antiporters such as *B. cereus* GerN (Southworth *et al.*, 2001), *B. subtilis* CzcD (Guffanti *et al.*, 2002) and *Aphanotece halophytica* NapA1-1 (Wutipraditkul *et al.*, 2005). In this scenario Aa-CaxA might be a $\text{Na}^+/\text{Ca}^{2+}-\text{K}^+$ exchanger, but it is also possible that the beneficial effect of Aa-CaxA at limiting K^+ is an indirect effect of reduced cytoplasmic Na^+ rather than actual K^+ uptake. Attempts to demonstrate Ca^{2+} or $\text{Mg}^{2+}/\text{H}^+$ antiport by assays described for cyanobacterial Syn-Cax (Waditee *et al.*, 2004) were negative, with or without added K^+ at a range of alkaline pH values (data not shown).

Aa-kefB complementation profile. The pYW138 plasmid in which Aa-KefB is encoded conferred significant Na^+ resistance in *E. coli* KNabc at both pH 7.5 and 8.0 and also conferred significant alkali resistance in LBK medium without added Na^+ (Fig. 3 A–C). Growth studies of the Aa-kefB transformant of *E. coli* TK2420 showed that this gene exacerbated the requirement for high $[\text{K}^+]$ in the medium since growth was lower than that of the empty vector control at <40 mM added K^+ (Fig. 4A). This is consistent with a K^+ efflux capacity as found for other Kef proteins (Bakker *et al.*, 1987). Perhaps Aa-KefB is a $\text{K}^+(\text{Na}^+)/\text{H}^+$ antiporter, which would account for all the complementation data, but we have not yet found assay conditions that demonstrate such activity. We note that there is no obvious accessory protein encoded in an operon with Aa-KefB of the kind that have been identified for the subset of CPA-2 proteins that include *E. coli* KefB and KefC (Miller *et al.*, 2000). Neither of the small ORFs upstream of Aa-kefB (Fig. 2) resembles the ancillary proteins of the *E. coli* KefB and KefC homologues. However, many members of the CPA-2 antiporter family for which antiport activity has been demonstrated (e.g. GerN and NapA) lack apparent auxiliary proteins (Southworth *et al.*, 2001; Waser *et al.*, 1992).

Aa-nhaP complementation profile. The plasmid pYW139, whose insert encodes Aa-NhaP, complemented both the Na^+ - and alkali-sensitive phenotypes of *E. coli* KNabc better than any of the other *A. amylolytica* N10 genes albeit not as strongly as the positive control (Fig. 3 A–C). Most strikingly, Aa-NhaP strongly impaired growth of K^+ uptake-deficient *E. coli* TK2420, inhibiting growth almost completely even in the presence of 40 mM added K^+ (Fig. 4A). The failure to reverse the growth inhibition by Aa-NhaP with high concentrations of K^+ indicated that the inhibition was not solely a result of robust Aa-NhaP-dependent K^+ extrusion via a presumed antiport mechanism. We noted that the Aa-NhaP transformant of *E. coli* TK2420 grew identically to the control transformant in the undefined LBK maintenance medium. LBK contains an organic nitrogen source in the form of a mixture of amino acids and peptides, whereas the minimal medium used for complementation assays contains $(\text{NH}_4)_2\text{SO}_4$ as the sole nitrogen source. Since numerous K^+ transporters can also use NH_4^+ as a substrate (Buurman *et al.*, 1991; Epstein, 2003; Wei *et al.*, 2003), a plausible hypothesis for the

Fig. 4. Complementation assays of the high $[\text{K}^+]$ requirement of the K^+ uptake-deficient *E. coli* TK2420 mutant strain with the cloned antiporter candidate genes from *A. amylolytica* N10. (A) *E. coli* TK2420 transformants with pYW136, pYW138, pYW139 or pGEM-3Zf(+) were grown in standard minimal medium (Epstein *et al.*, 1993), containing the indicated concentrations of added KCl, at 37 °C. The OD_{600} values were recorded after 16 h. Results are the mean $\pm$ SD of duplicate determinations from at least three independent growth experiments. (B) *E. coli* TK2420 transformants with either pGEM-3Zf(+) (control) or pYW139 (Aa-NhaP) were grown in minimal medium in which glutamine replaced $(\text{NH}_4)_2\text{SO}_4$ and KCl was added at the indicated concentrations. Two independent experiments were conducted as described for A.

Fig. 5. Assay of K^+/H^+ , Na^+/H^+ and Li^+/H^+ antiport activity of Aa-NhaP. Assays were conducted at pH 7.5 on everted membrane vesicles from *E. coli* KNabc transformed with either pYW139 (top row) or pGEM-3Zf(+) (bottom row). At the time points indicated by the downward arrows at the top, Tris-D-lactate was added to a final concentration of 2.5 mM to initiate respiration. The downward arrows, after the consequent AO quenching had levelled off, indicate the addition of 10 mM NaCl, LiCl or KCl. NH_4Cl was added 60 s later (indicated by the upward arrow) to dissipate the pH gradient. AU, arbitrary units of fluorescence quenching.

growth inhibition is that NH_4^+ is also an efflux substrate for Aa-NhaP, perhaps even a preferred one relative to K^+ . Even in the presence of added K^+ such an antiporter might inhibit growth of *E. coli* TK2420 in the complementation medium by using its NH_4^+/H^+ antiport activity to exclude the NH_4^+ in the medium, thereby depriving the bacterium of a nitrogen source. If this is the case, growth of the *E. coli* TK2420 transformant with Aa-NhaP on high $[K^+]$ should be restored if the $(NH_4)_2SO_4$ in the medium is replaced with glutamine. Indeed, when 5 mM glutamine was the nitrogen source, the adverse effect of Aa-NhaP on growth of *E. coli* TK2420 was still observed at low added $[K^+]$ but now higher concentrations of K^+ reduced the growth inhibition at 16 h; at 24 h, growth of the Aa-NhaP transformant was comparable to the control in the presence of 40 mM added K^+ (Fig. 4B). The overall

complementation pattern suggested that Aa-NhaP is a highly active $K^+(NH_4^+)/H^+$ antiporter that may be able to use Na^+ as a non-optimal efflux substrate.

Aa-NhaP-dependent antiport activity in membrane vesicles

Fluorescence-based assays of Aa-NhaP-dependent antiport activity used AO quenching to assess development of a pH gradient, acid in, across the everted vesicles when respiration was initiated (first downward arrow in the traces in Fig. 5). The % dequenching of the fluorescence in response to added cation was used to assess antiport activity. An antiport mechanism was testable for K^+/H^+ and Na^+/H^+ antiport in these fluorescence-based assays of everted membrane vesicles expressing either a control

Fig. 6. The pH and kinetic profile of the K^+/H^+ antiporter activity of Aa-NhaP. (A) K^+/H^+ antiport activity was assayed as described in the legend to Fig. 5, except that the assays were conducted at the different pH values shown. The pH of the BTP buffer was adjusted by addition of sulfuric acid. The values are the mean $\pm$ SD of duplicate determinations from at least two independent experiments. (B) K^+/H^+ antiporter assays were conducted in BTP buffer at pH 8.0, where the background activity of the vector control was zero. The Michaelis–Menten plot is shown with a reciprocal plot as an insert. The standard deviations derived from duplicate assays in two experiments were less than 10% of the mean values and are not shown.

empty vector or pYW139; NH_4^+ cannot be tested as a substrate in these assays because its addition abolishes the pH gradient that is used to monitor activity. Initial experiments were conducted at pH 7.5 to assess the cation specificity of the antiport. As shown in Fig. 5, vesicles expressing Aa-NhaP exhibited clear fluorescence dequenching in response to K^+ addition whereas the control exhibited none. Although addition of Na^+ to the Aa-NhaP vesicles did not elicit a sharp upward dequench as is usually observed, there was a slow dequench that consistently exceeded that observed in the control vesicles; this may reflect a modest capacity to use Na^+ as a substrate. No activity with Li^+ was observed in comparison to the control. The pH profile for K^+/H^+ activity in the heterologous system exhibited an optimum at pH 7.5 (Fig. 6A), which would probably be non-physiological for the natural extremophile host. The cytoplasmic pH of *A. amylolytica* N10 has not yet been determined when it is growing at its optimal growth pH of 10. However, other extreme alkaliphiles exhibit cytoplasmic pH values near 8 in that range of outside pH (Krulwich, 1995; Krulwich *et al.*, 2007; Yumoto, 2002). The broad pH optimum for Aa-NhaP and retention of significant activity at pH 9.5 (the limit for the assay) are consistent with a possible role in pH homeostasis in the natural host (Fig. 6A). The activity of Aa-NhaP was studied as a function of $[\text{K}^+]$ and exhibited Michaelis–Menten kinetics with an apparent K_m of about 0.5 mM (Fig. 6B). This apparent K_m is in the same range of low values found for well-studied Na^+/H^+ antiporters from *E. coli* that have important roles at elevated pH (Padan *et al.*, 2001). Until a genetic system is available for *A. amylolytica* N10, proposed roles remain untested. Aa-NhaP could play a role in osmo-adaptation and could also function in alkali resistance, playing an adjunct role to the Na^+/H^+ antiporters that are expected to play a major role in both alkali and Na^+ resistance. For example, once Na^+/H^+ antiporter activity has generated a substantial, inwardly directed Na^+ gradient and a lower cytoplasmic pH than external pH, Aa-NhaP could reduce the cytoplasmic pH further using the outwardly directed K^+ gradient to power or partially power H^+ uptake. Finally, we hypothesize that Aa-NhaP also catalyses NH_4^+/H^+ antiport, a possibility that merits further examination. When cells are actively catabolizing amino acids at high pH, accumulation of cytoplasmic NH_4^+ poses a problem as it would be expected to inhibit central metabolic pathways as well as pH homeostasis itself. Exchange of cytoplasmic NH_4^+ for external H^+ would mitigate these effects. It will be of interest and importance to use biochemistry and genetics to determine the extent to which transporters currently characterized as using K^+ as a substrate also use NH_4^+ and to evaluate the physiological impact of the use of NH_4^+ .

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