Identification of a pH regulated Na^+/H^+ antiporter of *Methanococcus jannaschii*

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Abstract The genome of the hyperthermophilic archaeon *Methanococcus jannaschii* contains three Na^+/H^+ antiporter related genes Mj0057, Mj1521 and Mj1275. Comparative sequence alignments revealed that Mj0057 and Mj1521 belong to the NhaP family whereas Mj1275 is a member of the NapA family. The genes were cloned and expressed in the double mutant *Escherichia coli* strain Frag144 (ΔnhaA, ΔnhaB) to analyze their capability of mediating ΔpH driven Na^+ flux in everted vesicles. From the tested clones only Mj0057 displayed Na^+ (Li^+)/H^+ antiporter activity. The transport was pH dependent and occurred at pH 7.0 and below. At pH 6.0 the apparent K_m values for Na^+ and Li^+ were approximately 10 and 2.5 mM, respectively.

Key words: Na^+/H^+ antiporter; pH regulation; *Methanococcus jannaschii*

1. Introduction

Na^+/H^+ antiporters are ubiquitous membrane proteins that exchange sodium for protons. They exist in all three domains of life and are found in the cytoplasmic membrane of almost every cell. In eukaryotes Na^+/H^+ antiporters are also located in the vacuolar membranes as well as in the membranes of the organelles. Since Na^+ and H^+ are most common ions, Na^+/H^+ antiporters play a primary role in osmoregulation, pH control and cell energetics [1,2].

Two Na^+/H^+ antiporters, NhaA and NhaB, have been identified in *Escherichia coli* [3,4]. ChaA, the third protein involved in Na^+ extrusion, is an unspecific Ca^{2+}/H^+ antiporter [5]. *E. coli* mutants deleted in all three genes display a high sensitivity to Na^+ and are affected in ion homeostasis [6].

NhaA, the housekeeping antiporter of *E. coli*, is required for growth in the presence of high salt concentrations (0.9 M Na^+, pH 7.0) and for tolerance of the upper pH limit of pH 8.5 at 0.7 M NaCl. The intracellular pH highly influences the activity of NhaA reflecting a 2000-fold increase of V_{max} between pH 7.0 and 8.5 [3,7]. These properties remain after reconstitution of NhaA into vesicles, suggesting an internal pH sensor. The amino acids involved in pH regulation have been identified either by deleting or replacing the histidine residues of NhaA [7,8].

Although many prokaryotic Na^+/H^+ antiporters have been cloned and studied in antiporter deficient *E. coli* mutants [9–11], very little is known about antiporters from the archaeal kingdom. The only evidence for Na^+/H^+ antiporters in archaea comes from studies using either intact cells or vesicles [12–14], but none of the genes has been cloned and studied in a heterologous system, yet.

The hyperthermophilic archaeon *Methanococcus jannaschii* has three Na^+/H^+ antiporter related genes, Mj1275, Mj1521 and Mj0057. In this study the open reading frames of the putative antiporters were cloned and expressed in the double mutant *E. coli* strain Frag144 (ΔnhaA, ΔnhaB). Subsequently their capability of mediating ΔpH driven sodium transport was assayed.

2. Materials and methods

2.1. Construction of expression plasmids

The genes were amplified from chromosomal DNA of *M. jannaschii* using SAWADY Pwo-DNA-Polymerase (Peqlab). A-tailing of the polymerase chain reaction (PCR) products was performed with Biotherm Taq-Polymerase (Genecraft). The PCR products were ligated into the one step cloning vector pTrcHis2-TOPO (Invitrogen). The following primers were used for amplification: Mj0057 forward: 5′-ATG GAA CTT ATG ATG GCT ATT GGT TAC-3′, reverse: 5′-ATG GTG GGA TTC TTC TTT ATA CTT TG-3′; Mj1275 forward: 5′-ATG GAA AGT TAT TAT TAT GTG TTC TTC-3′, reverse: 5′-ATT TTT AGC TTT TTT ATT ACA TTT TGC-3′; Mj1521 forward: 5′-GTG AAT ATT GTA TTA TTT CTC GGC TAT-3′, reverse: 5′-GGA GGT TGA GGT CTT TCT TTT CAA TAA-3′. The resulting plasmids were named pMj0057, pMj1275 and pMj1521, respectively.

2.2. Bacterial strains and growth conditions

Everted vesicles were prepared from strain FRAG144 (ΔnhaA::Kan ΔnhaB::Cam). The cells were grown overnight at 30°C in LBK medium (10 g l^{-1} tryptone, 5 g l^{-1} yeast extract, 87 mM KCl) to $A_{600nm} = 1$. After addition of 1 mM isopropyl thiogalactose (IPTG) the cells were grown for 2 h until harvested.

2.3. Complementation

EP432 cells carrying either pMj0057 or pTrcHis2c (Invitrogen) as negative control were grown at 37°C in LBK medium until the A_{600nm} reached 1. The cells were diluted 1000-fold in LBN medium (10 g l^{-1} tryptone, 5 g l^{-1} yeast extract, 70 mM MES/Tris (pH 6.5), NaCl was added as indicated) and grown overnight.

2.4. Preparation of everted vesicles

Everted vesicles were prepared from strain Frag144 essentially as described by Rosen [15]. The vesicles were resuspended in TCDS buffer (10 mM Tris–HCl (pH 7.5), 140 mM choline chloride, 0.5 mM dithiothreitol (DTT), 250 mM sucrose) at a protein concentration of 1.6 mg ml^{-1} and stored in liquid N_2.

2.5. Identification of fusion proteins

Everted vesicles (10 μl) were subjected to sodium dodecyl sulfate–

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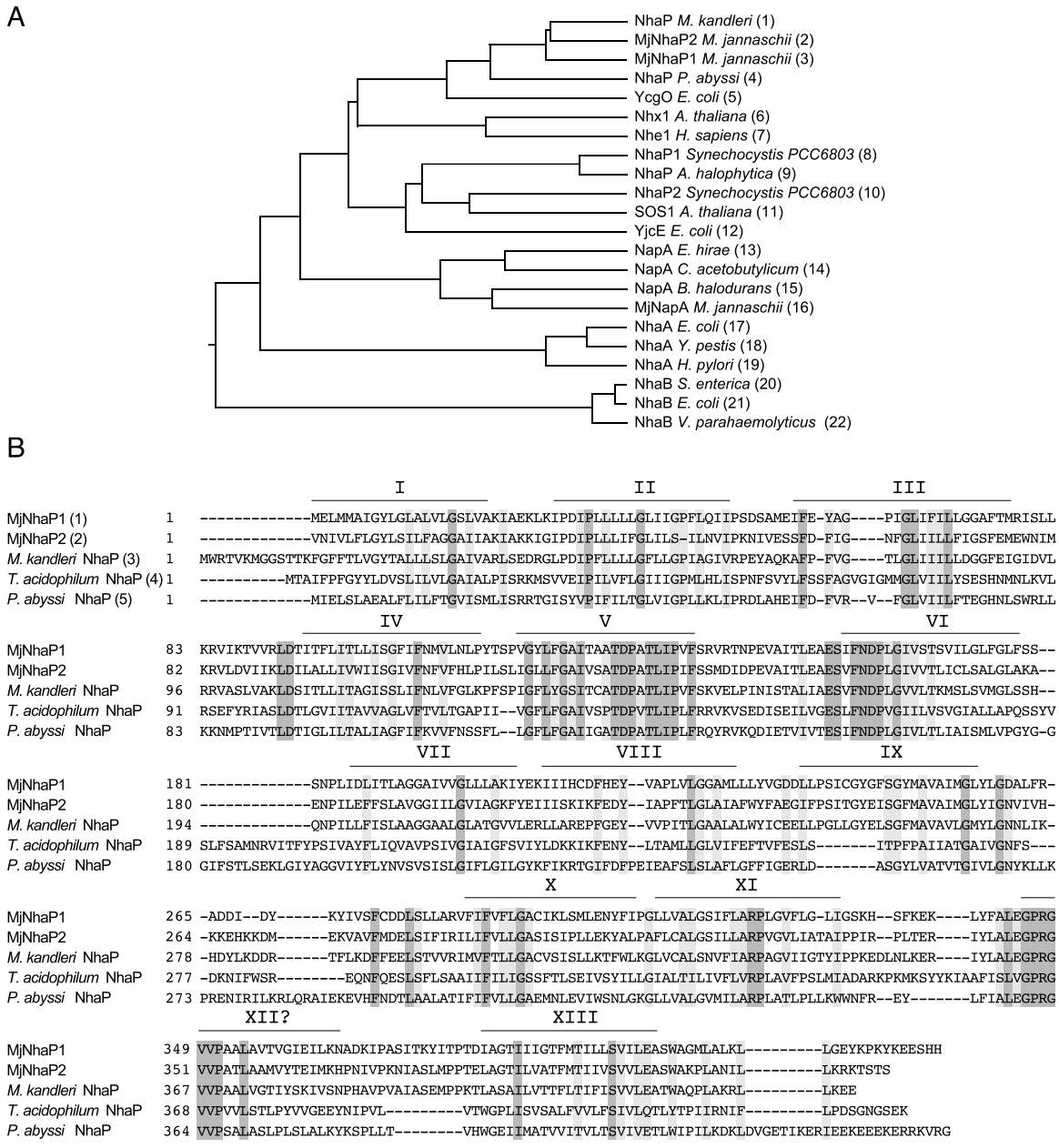

Fig. 1. A: Phylogenetic relationship between MjNhaP1 and various Na^+/H^+ antiporters from pro- and eukaryotes. The tree was drawn by Megalign (DNA-Star) using the Clustal W algorithm. The accession numbers refer to the arbitrary numeration of the Na^+/H^+ antiporters shown on the tree. (1) NP_613837, (2) NP_248529, (3) NP_247021, (4) NP_126974, (5) NP_415709, (6) NP_198067, (7) P19634, (8) BAA17925, (9) BAB69459, (10) BAA18490, (11) AAF76139, (12) P32703, (13) CAD22163, (14) NP_347084, (15) NP_243710, (16) NP_248271, (17) Np_285709, (18) NP_404112, (19) NP_224165, (20) NP_456313, (21) NP_287425, (22) BAA12073. B: Alignment of the deduced amino acid sequences of Na^+/H^+ antiporters. MjNhaP1 was aligned with four antiporters of the NhaP type. Identical and conserved amino acids were highlighted in dark and light gray, respectively. Predicted membrane spanning regions were marked above the alignment. The multiple sequence alignment was performed with DNA-Star software using the Clustal W algorithm. The predicted membrane spanning regions were obtained from NiceProt (www.expasy.ch). Using the Goldman–Engelman–Steitz algorithm (www.tigr.org), another uncertain transmembrane region was detected that was designated XII? The accession numbers refer to the arbitrary numeration of the antiporters in the alignment: (1) NP_247021, (2) NP_248529, (3) NP_613837, (4) NP_394359, (5) NP_126974.

polyacrylamide gel electrophoresis (SDS–PAGE). The separated proteins were blotted to nitrocellulose (Biometra) and detected with anti-myc antibody coupled to alkaline phosphatase (Invitrogen), according to the manufacturers instructions.

2.6. Measurement of H^+ transport by fluorescence

Fluorescence measurements were performed with 20 μl vesicles in a thermostated (22°C), stirred cuvette containing 2 ml of MTCM buffer (10 mM MES/Tris, 140 mM choline chloride, 5 mM $MgCl_2$, 5 mM KCl, titrated to the indicated pH) and 2 μM acridine orange. The vesicles were loaded with protons by addition of 2 mM Tris–DL-lactate (titrated to the indicated pH). Dequenching was initiated by adding either NaCl or LiCl at the indicated amounts. Full fluorescence was recovered by adding 25 mM NH_4Cl to the cuvette. Measurements were performed with a UDL200 laser (Laser Technik Berlin) using coumarin 102 as laser dye and a MSG801 SD nitrogene laser (Laser

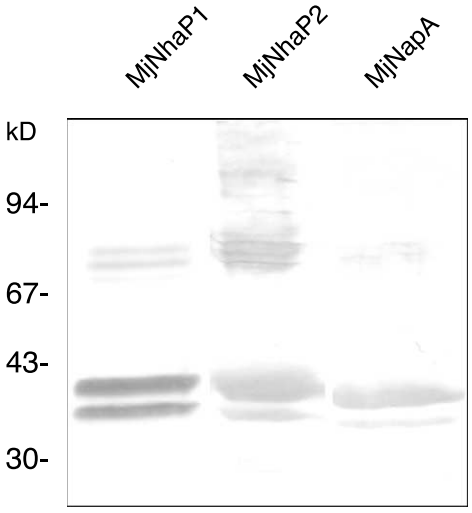

Fig. 2. Immunological detection of MjNhap1, MjNhaP1 and MjNapA. Everted vesicles (10 μl) were subjected to SDS–PAGE. The separated proteins were blotted to nitrocellulose and myc epitopes of recombinant fusion proteins have been detected with anti-myc antibody coupled to alkaline phosphatase.

Technik Berlin) as pump source. Fluorescence of the samples was excited at 493 nm wavelength. The emission spectra were dispersed by a MS125 spectrometer (Lot-Oriel-GmbH) and imaged onto an Instaspec IV CCD camera. To monitor H^+ transport the fluorescence intensity at 530 nm wavelength was used.

3. Results and discussion

Three genes, Mj0057, Mj1521 and Mj1275, whose deduced amino acid sequences are homologous to Na^+/H^+ antiporters, were identified in the genome of *M. jannaschii* [16]. Since the corresponding proteins of Mj0057 and Mj1521 belong to the NhaP family we named them MjNhaP1 and MjNhaP2, respectively. Mj1275 is related to the NapA family and was therefore named MjNapA.

Alignment analysis revealed that MjNhaP1 and MjNhaP2 are 45% identical and have possibly arisen from a gene duplication event. The most closely related genes are putative antiporters of the NhaP type coming from the archaeons *Methanopyrus kandleri* (42–46% identity) and *Pyrococcus abyssi* (27–31% identity). The relationships of Mj0057 and Mj1521 to eukaryotic antiporters like SOS1 (*Arabidopsis thaliana*) and Nhe1 (*Homo sapiens*) are closer (18–21% identity) than to bacterial antiporters like NhaA and NhaB (*E. coli*) (10–16% identity) (Fig. 1A). There is also a quite high degree of identity (20–22%) to *YcgO* (*E. coli*), an antiporter related protein. *YcgO* is not capable in mediating a ΔpH driven sodium flux in everted vesicles but plays a role in osmoregulation [17]. MjNhaP1 and MjNhaP2 are very hydrophobic and comprise of 12 or 13 transmembrane domains TM (I–XIII). Among the NhaP family there are three clusters of high homology, located in TM (V), TM (VI) and the uncertain TM (XII?). TM (V) includes a TDP motif that is widespread among pro- and eukaryotic Na^+/H^+ antiporters and TM (VI) contains the sequence FNDP that is also highly conserved. The GPRGVVP

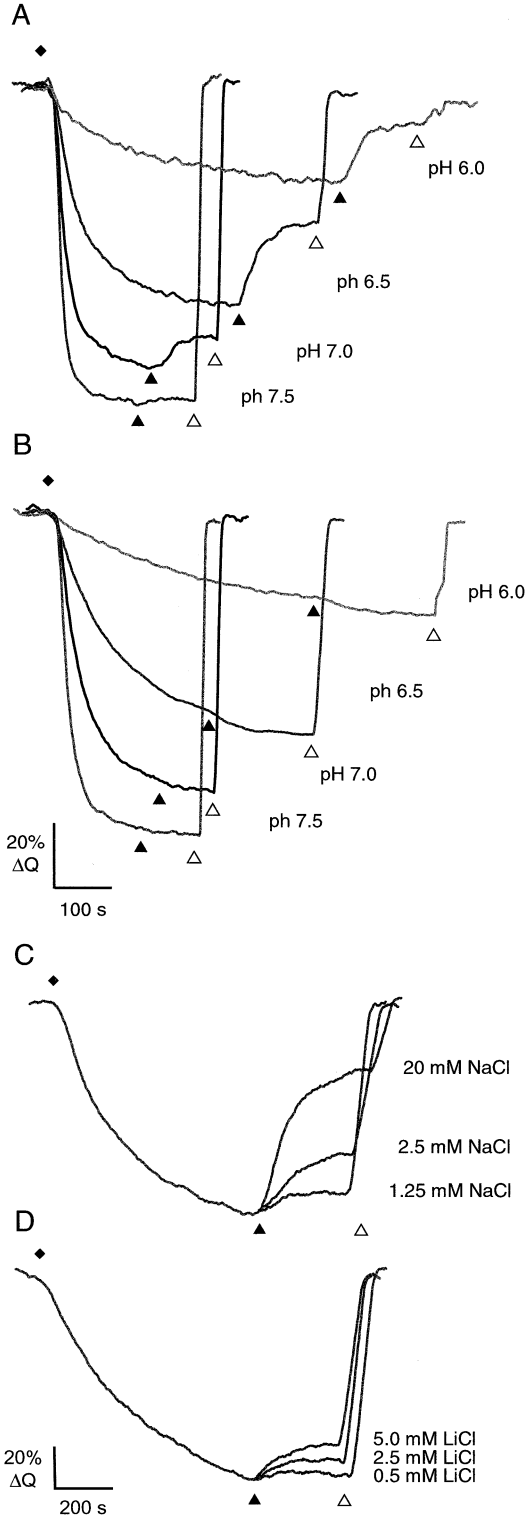

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Fig. 3. Transport properties of MjNhaP1 in everted vesicles. The *E. coli* strain Frag144 was transformed with either pMj0057 (A) or pTrcHis2c (B) as control. The reaction was performed in a 2 ml stirred cuvette thermostated at 22°C. Each assay contained 10 mM MES, 140 mM choline chloride, 5 mM $MgCl_2$, 5 mM KCl (titrated with Tris to the indicated pH), 2 μM acridine orange and membrane vesicles (50 μg protein). Fluorescence quenching was initiated by adding 2 mM Tris–DL-lactate (♦). To measure the Na^+ (Li^+)/H^+ antiporter activity NaCl (LiCl) was added at the times marked by closed triangles (▲) and the H^+ gradient was dissipated by adding 25 mM NH_4Cl (△). For pH dependent transport 20 mM NaCl was added. The measurements for concentration dependent transport (C, D) were performed at pH 6.0.

sequence in TM (XII?) is a unique feature of the NhaP family and is not found in other classes of antiporters (Fig. 1B).

MjNapA is closely related to antiporters from *Enterococcus hirae* and *Clostridium acetobutylicum* (27–31% identity). Compared to MjNhaP1 and MjNhaP1 the homology to SOS1, Na^+/H^+ antiporters and *YcgO* is low (11–14%) and the similarities to NhaA and NhaB are found in the same range. Despite these sequence distances of the NapA family, the antiporter of *E. hirae* mediates ΔpH driven sodium flux in everted vesicles prepared from *E. coli* strain EP432 and also complements the salt sensitive phenotype [10].

To analyze whether the putative transporters display Na^+/H^+ antiport activity we have cloned the open reading frames of Mj0057, Mj1521 and Mj1275 into the bacterial expression vector pTrcHis2Topo, where the genes are under the control of a *trc* promotor. The plasmid provides a C-terminal myc epitope tag for immune detection, followed by a histidine hexamer.

The tagged proteins have predicted molecular masses of 50 kDa (MjNhaP1), 50 kDa (MjNhaP2) and 46 kDa (MjNapA). However, in SDS–PAGE these extremely hydrophobic proteins migrate faster than expected, a property that has been reported for NhaA, NhaB and various other membrane proteins [3,18]. The apparent molecular weights of the monomeric forms are 40 kDa (MjNhaP1), 40 kDa (MjNhaP2) and 38 kDa (MjNapA) (Fig. 2). Interestingly the monomers of all three proteins appear as a double band. We were not able to prevent this, neither by using protease inhibitors nor by utilizing protease deficient strains. Maybe the turnover of the proteins occurs already during induction of the bacteria. A second translation product may also appear due to the presence of internal start codons, which was demonstrated for an archaeal potassium channel recently [19].

To study the functional properties of the putative Na^+/H^+ transporters the plasmids pMj0057, pMj1521, pMj1275 and the control vector pTrcHis2c were introduced into the *E. coli* mutant strain Frag144. This strain displays no antiporter activity in the pH range from 6.0 to 8.0, when the unspecific K^+/H^+ antiporter is saturated by 5 mM KCl. From each transformant everted vesicles were prepared and assayed for Na^+/H^+ antiport activity.

Only cells transformed with pMj0057 differed significantly from the control (Fig. 3A, B) and showed a fluorescence recovery after addition of NaCl. We found that MjNhaP1 is active at pH 7.0 and below, whereas above pH 7.0 MjNhaP1 is shut off.

The rate of dequenching after NaCl addition was strictly dependent on the concentration (Fig. 3C) and allowed the estimation of the K_m from the initial rate of dequenching, which was 10 mM at pH 6.0. Lithium was also transported but the rates of dequenching were three times lower at 2.5 mM than for NaCl (Fig. 3D). However, the affinity for lithium was higher with a K_m of approximately 2.5 mM. Lithium concentrations above 5 mM did not lead to a further increase of dequenching but resulted in a decrease, indicating that the antiporter is inhibited above 5 mM LiCl.

Since MjNhaP1 is active in everted vesicles, we tried to complement the salt sensitive phenotype of the *E. coli* mutant strain EP432. When transformed with control plasmid pTrcHis2c the cells grew in presence of up to 150 mM NaCl at pH 6.5. However, EP432 transformed with pMj0057 did not exhibit an increased salt tolerance compared to the control. It has been described previously that the cytoplasmic pH of *E. coli* is maintained between pH 7.6 and 7.8 over a wide range of external pH [20], which would lead to inactivation of MjNhaP1. We conclude that functional complementation can therefore not be used to clone antiporters, which are active below the cytoplasmic pH of *E. coli*.

The only described prokaryotic Na^+/H^+ antiporter, which is activated at acid pH, comes from *Halobacterium halobium* [21]. It is sensitive to both, external and internal pH and only a combination of ΔpH and $\Delta\phi$ activates this unidirectional antiporter. However, *H. halobium* grows in extreme salt-rich environments with very special requirements to the organism. Thus a comparison to *M. jannaschii*, living in sea water, is difficult.

With a K_m for Na^+ of 10 mM MjNhaP1 is active before an intracellular increase of the Na^+ concentration leads to toxic effects, suggesting its function in sodium extrusion. Recently a P-type ATPase of *M. jannaschii* with a pH optimum of 4.0 has been described, which could drive this process [22]. On the other hand the pH sensor could enable MjNhaP1 to prevent acidification of the cytoplasm, a feature described for Na^+/H^+ antiporters of the Nhe family (*H. sapiens*) and sod2 (*Schizosaccharomyces pombe*) [2,23].

At present we can say that MjNhaP1 has at least an intracellular pH sensor and that $\Delta\mu_H^+$ can drive Na^+ flux. Whether its physiological function is the extrusion of Na^+ or the regulation of the cytoplasmic pH remains to be cleared. In any case MjNhaP1 resembles more a eukaryotic than a prokaryotic antiporter with respect to regulation and sequence. Its response to pH makes it an interesting candidate for future studies about pH regulation of Na^+/H^+ antiporters.

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