ChinaRxiv 中X

AI translation · View original & related papers at
chinarxiv.org/items/chinaxiv-201809.00120

Functional Identification of a Novel YdjM Superfamily Member as a Na^+/H^+ Antiporter Postprint

Authors: Wang Yanhong, Liu Yanshuang, Shi De-xi, Zhu Baoguo, Lü Baolei, Fu Shiyu, Xu Miao, Wang Wei, Yin Kuide

Date: 2018-09-13T00:00:00+00:00

Abstract

In prokaryotes, Na^+/H^+ antiporters play a crucial role in catalyzing the extrusion of intracellular alkali cations such as Na^+, Li^+, or K^+ in exchange for external protons, thereby reducing the cytoplasmic concentration of toxic alkali metal cations and maintaining intracellular pH homeostasis. To further explore sodium/hydrogen antiporter genes with salt-alkali tolerance in the moderately halophilic bacterium *Halobacillus* Y5 and characterize their function, we first extracted the genomic DNA of this strain, and then obtained a novel sodium/hydrogen antiporter gene *Ha_{ydjM}* through Sau3AI random digestion and functional complementation. Bioinformatic analysis revealed that this gene belongs to the YdjM superfamily and is a membrane protein of unknown function; phylogenetic analysis confirmed that it clusters with YdjM family members from *Halobacillus* sp. Marseille-P3789 (protein accession number WP_{101846656}.1) but forms an independent branch. The study found that this gene could restore the tolerance of *Escherichia coli* mutant strain KNabc to 0.2 M NaCl and 5 mM LiCl, as well as tolerance to alkaline pH 8.0. Functional analysis showed that the protein exhibits pH-dependent Na^+/H^+ antiporter activity, and transport kinetic analysis indicated that the Km values for Na^+, K^+, and Li^+ in KNabc are 0.43 ± 0.05 mM, 0.49 ± 0.06 mM, and 0.64 ± 0.06 mM, respectively, corresponding to affinities of $Na^+ > K^+ > Li^+$. Taken together, we propose that Ha_{ydjM} represents a novel type of Na^+/H^+ antiporter. This study enriches the members of the YdjM superfamily and provides a basis for functional analysis of other unknown membrane proteins.

ChinaRxiv 中X

Full Text

Functional Identification of a Novel Na+/H+ Antiporter from the YdjM Superfamily

WANG Yan-hong, LIU Yan-shuang, SHI De-xi, ZHU Bao-guo, LÜ Bao-lei, FU Shi-yu, XU Miao, WANG Wei, YIN Kui-de

College of Life Science and Technology, Heilongjiang Bayi Agricultural University, Daqing 163319, China

Abstract

In prokaryotes, Na+/H+ antiporters catalyze the efflux of intracellular alkali cations such as Na+, Li+, or K+ in exchange for external protons, playing a vital role in reducing cytoplasmic concentrations of toxic alkali metal cations and maintaining intracellular pH homeostasis. To identify novel Na+/H+ antiporter genes with salt and alkali tolerance from the moderately halophilic bacterium *Halobacillus* Y5, we extracted genomic DNA from this strain and employed random digestion with Sau3AI combined with functional complementation to obtain a novel Na+/H+ antiporter gene designated *Ha_{ydjM}*. Bioinformatic analysis revealed that this gene belongs to the YdjM superfamily and encodes a putative membrane protein of unknown function. Phylogenetic analysis confirmed that it clusters with YdjM family members from *Halobacillus* sp. Marseille-P3789 (protein accession WP_{101846656}.1) while forming an independent branch. Functional studies demonstrated that this gene restored the tolerance of *E. coli* mutant strain KNabc to 0.2 M NaCl and 5 mM LiCl, and conferred tolerance to alkaline pH 8.0.

Activity analysis revealed pH-dependent Na+/H+ antiporter activity, with kinetic analysis showing Km values for Na+, K+, and Li+ in KNabc of 0.43 ± 0.05 mM, 0.49 ± 0.06 mM, and 0.64 ± 0.06 mM, respectively, indicating substrate affinities in the order Na+ > K+ > Li+. In summary, we conclude that Ha_{YdjM} represents a novel Na+/H+ antiporter. This study expands the YdjM superfamily membership and provides a foundation for functional analysis of other unknown membrane proteins.

Keywords: Na+/H+ antiporter, membrane protein, functional identification, YdjM superfamily, *Halobacillus*

Introduction

Na+/H+ antiporters are carrier membrane proteins that couple proton (H+) influx with antiport of Na+/H+ or symport of Na+/OH- [1-4]. Most function as Na+-dependent H+ transporters or H+-dependent Na+ transporters and are widely distributed in bacterial membrane vesicles, eukaryotic mitochondria, and animal and plant cells or tissues [5]. In high-salt environments, they maintain

normal physiological pH in the cytoplasm, making them biologically essential [6].

To date, reported Na+/H+ antiporter families in prokaryotes mainly include the NhaA family, MFS family, Cpa1 family, Cpa2 family, and Cpa3 family [7]. The NhaA family represents the most important Na+/H+ antiporter in many enteric bacteria and *E. coli*. In the microbial world, NhaA from *E. coli* is the most thoroughly studied Na+/H+ antiporter and was the first bacterial Na+/H+ antiporter discovered [8]. Additionally, other Na+/H+ antiporters from the NhaA family have been identified in many pathogenic microorganisms, including *Vibrio cholerae*, *Clostridium tetani*, *Helicobacter pylori*, and *Salmonella typhi* [9]. Beyond the primary NhaA family in *E. coli*, other antiporters such as NapA, NhaP, NhaC, NhaD [10], NhaB, and NhaE [11] have been discovered in prokaryotes. Etana Padan et al. [12] noted these proteins share little or no homology with the NhaA family. In *E. coli*, three major Na+/H+ antiporters are particularly important: NhaA [12], NhaB [13], and ChaA [14]. NhaA enables *E. coli* to maintain growth under high salt (Na+/Li+) stress and is essential for growth in alkaline environments in the presence of Na+ [15]. NhaB, a 504-amino acid transmembrane protein with twelve TMS, functions only at low Na+ concentrations or low pH. ChaA exhibits Na+ (Ca2+)/H+ antiporter activity under alkaline conditions [14] and its expression affects intracellular K+ concentration; deletion of *chaA* inhibits K+ efflux, indicating ChaA also possesses K+/H+ antiporter activity. Consequently, loss of any of these Na+/H+ antiporters impairs the salt resistance of *E. coli*, demonstrating their indispensable roles. Therefore, researchers commonly use salt-sensitive mutant strains such as EP432 (with deleted *nhaA* and *nhaB* genes) or KNabc (with deleted *nhaA*, *nhaB*, and *chaA* genes) for functional complementation screening and identification of Na+ (Li+, K+)/H+ antiporters. This study employed functional complementation to screen for novel Na+/H+ antiporter genes from the moderately halophilic bacterium *Halobacillus* Y5, functionally characterized the identified gene, and thereby expanded the YdjM family membership while providing a theoretical foundation for similar protein studies.

Materials and Methods

Strains and Plasmids

The moderately halophilic bacterium *Halobacillus* Y5 was isolated and preserved in our laboratory. The salt-sensitive strain *E. coli* KNabc (Δ*nhaA*, Δ*nhaB*, Δ*chaA*) was provided by the Microbiology Laboratory of Northeast Agricultural University. KNabc/pUC18 was obtained by chemical transformation of plasmid pUC18 into competent KNabc cells. KNabc/Ha_{YdjM} was the target strain constructed in this study.

Reagents and Kits

Restriction endonucleases (Sau3AI), T4 DNA ligase, and Calf Intestinal Alkaline Phosphatase (CIAP) were purchased from TaKaRa (Japan). DNA gel extraction kits were purchased from Omega (USA). All other reagents were analytical grade.

Culture Media

LBK medium contained 1% peptone, 0.65% KCl, and 0.5% yeast extract (natural pH), sterilized at 121°C for 20 min, used for *E. coli* KNabc cultivation. SG medium contained 1% peptone, 0.5% yeast extract, 0.5% casein, 0.2% KCl, 0.3% sodium citrate, 2% $MgSO_4 \cdot 7H_2O$, and 3% NaCl (pH 7.2-7.4), sterilized at 121°C for 20 min, used for *Halobacillus* Y5 cultivation.

Genomic DNA Extraction from *Halobacillus* Y5

Halobacillus Y5 was cultured in SG medium at 37°C with shaking at 140 rpm for approximately 36 h. Cells were harvested by centrifugation at 4°C, 2600×g for 10 min. Genomic DNA was extracted following the procedure described in reference [16], examined by 1% agarose gel electrophoresis, and stored at -20°C for subsequent use.

Screening for Novel Na+/H+ Antiporter Genes

A genomic library of *Halobacillus* Y5 was constructed following the method of Wang et al. [17] to screen for positive clones harboring Na+/H+ antiporter genes.

Sequence Analysis of Novel Na+/H+ Antiporter Genes

The plasmid from positive clones was extracted and sequenced. Sequence alignment and analysis were performed using the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Open reading frames (ORFs) and multiple amino acid sequence alignments were analyzed using DNAman 6.0. Protein molecular weight and theoretical isoelectric point were predicted using http://web.expasy.org/protparam/. Promoter sequences were predicted using http://www.fruitfly.org/seq_{tools}/promoter.html. Transmembrane regions were predicted using http://mobyle.pasteur.fr/cgi-bin/portal.py#forms::wordcount. A phylogenetic tree was constructed using the Neighbor-Joining method in MEGA 5.1 software [18].

Salt and Alkali Tolerance Assays

The salt and alkali tolerance capabilities of the novel Na+/H+ antiporter gene were tested using KNabc/pUC18 as a negative control. For salt tolerance assays, LBK medium supplemented with various concentrations of NaCl or LiCl was used. For alkali tolerance assays, LBK medium was adjusted to different pH

values with Tris-HCl. Cultures were inoculated and incubated at 37°C, 140 rpm for 16-18 h, after which OD_{600} values were measured [19].

Measurement of Na+/H+ Antiporter Activity

Inverted membrane vesicles were prepared from strains harboring the novel gene and controls [20]. Na+/H+ antiporter activity was measured using the acridine orange (AO) fluorescence quenching method [21]. Briefly, fluorescence parameters were set with excitation (EX) at 492 nm and emission (EM) at 526 nm. To a quartz cuvette containing 2.5 mL buffer B, 2 µM AO and 40 µg inverted membrane vesicles were added and mixed. The respiratory substrate Tris-D-lactate (5 mM final concentration) was added to generate a transmembrane pH gradient, and ΔpH changes were monitored using a fluorescence spectrophotometer. When fluorescence intensity reached equilibrium, 5 mM Na+, K+, or Li+ was added to disrupt ΔpH. The degree of fluorescence recovery indicated whether the protein possessed Na+ (Li+, K+)/H+ antiporter activity and its magnitude.

Determination of Km Values

Km values indicate the affinity between protein and substrate; larger values correspond to weaker affinity. Under optimal pH conditions, transport activity was measured at various concentrations of Na+, K+, and Li+. Ion concentration was plotted on the x-axis and antiporter activity on the y-axis for non-linear regression analysis to calculate Km values.

Results

Screening for Novel Na+/H+ Antiporter Genes

Using LB solid plates containing 0.2 M NaCl, approximately 150 positive clones that restored growth of *E. coli* KNabc were obtained. Sequence analysis identified one clone as a potential novel Na+/H+ antiporter gene, designated pUC-HaY. The insert was sequenced by Beijing Huada Gene Biotechnology Co., Ltd. The foreign DNA fragment in pUC-HaY was 3441 bp and contained three putative ORFs. ORF1 encoded a glycine betaine transporter gene *opuD* (previously published [17]), ORF2 encoded an NmrA family transcription factor (not studied further as it generally lacks Na+/H+ antiporter activity), and ORF3 encoded a hypothetical protein from the YdjM superfamily (accession MH536807). Following the strategy shown in [Figure 1: see original paper], primers with restriction sites were designed for ORF3 (ORF3-F and ORF3-R, shown as horizontal lines; italicized sequences indicate XbaI (TCTAGA) and KpnI (GGTACC) sites; the box indicates the SD sequence). ORF3 was subcloned and designated Ha_{YdjM} for further study.

Sequence Analysis of Ha_{YdjM}

Multiple alignment of Ha_{YdjM} with proteins from other *Halobacillus* species using DNAMAN revealed homologies of 86%, 65%, 57%, 58%, and 57% with proteins WP_{101846656}.1, WP_{082235595}.1, WP_{035544181}.1, WP_{085031358}.1, and WP_{014641626}.1, respectively [Figure 2: see original paper].

The neighbor-joining phylogenetic tree [Figure 3: see original paper] showed that Ha_{YdjM} clusters with YdjM from *Halobacillus* sp. Marseille-P3789 (accession WP_{101846656}.1) but forms an independent branch. Therefore, Ha_{YdjM} likely represents a novel member of the YdjM superfamily.

Most Na+/H+ antiporters are transmembrane proteins composed of hydrophobic amino acids with multiple transmembrane segments. To verify this for Ha_{YdjM}, transmembrane analysis was performed, revealing five transmembrane regions [Figure 4: see original paper]. Hydrophobicity analysis confirmed the encoded protein is hydrophobic [Figure 5: see original paper].

Ha_{YdjM} encodes a 203-amino acid protein with a molecular weight of 23,637.42 Da and an isoelectric point (pI) of 9.33. The transmembrane region spans amino acids 31-150, including TMS I (31-48), TMS II (61-80), TMS III (90-104), TMS IV (111-128), and TMS V (133-150) .

Salt and Alkali Tolerance Testing

Although Ha_{YdjM} was predicted to be a transmembrane protein with salt-alkali tolerance, ORF2 was also tested for experimental rigor. Only results on LBK solid plates containing 0.2 M NaCl and 5 mM LiCl are shown [Figure 6: see original paper]. Ha_{YdjM} conferred salt tolerance to the salt-sensitive strain, whereas ORF2 failed to grow under these conditions, consistent with predictions. Alkali tolerance assays [Figure 7: see original paper] demonstrated growth up to pH 8.0, confirming that inverted membranes from Ha_{YdjM} could be prepared for activity measurements.

Na+/H+ Antiporter Activity Assay

Inverted membrane vesicles (ISO) were prepared from KNabc/Ha_{YdjM} and control KNabc/pUC18. Using acridine orange (AO) as a fluorescent probe, monovalent ion Na+ (Li+, K+)/H+ antiport activity was measured. When fluorescence quenching reached its minimum, addition of Na+ (Li+, K+) solutions caused varying degrees of fluorescence recovery in KNabc/Ha_{YdjM} inverted membranes, while no change was observed in the negative control KNabc/pUC18 [Figure 8: see original paper]. These results demonstrate that KNabc/Ha_{YdjM} can transport monovalent Na+, Li+, and K+, consistent with transmembrane predictions, confirming Na+/H+ antiporter activity.

Since pH typically affects Na+ (Li+, K+)/H+ antiporter activity, we measured transport activity at different pH values (7.0-8.5). Activity for all mono-

valent cations (Na+, Li+, K+) varied with pH, reaching maximum activity at pH 8.0 [Figure 9: see original paper]. Thus, KNabc/Ha_{YdjM} exhibits pH-dependent transport activity for Na+, Li+, and K+ that changes with pH.

Determination of Km Values for Ha_{YdjM}

Having established that Ha_{YdjM} transports Na+, Li+, and K+ with different efficiencies, we investigated substrate preferences. Km studies revealed that lower values indicate stronger substrate affinity. Using GraphPad Prism 6.01 software and the Michaelis-Menten equation, Km values were calculated as 0.43 ± 0.05 mM, 0.49 ± 0.06 mM, and 0.64 ± 0.06 mM for Na+, K+, and Li+, respectively [Figure 10: see original paper], indicating substrate affinity in the order Na+ > K+ > Li+.

Discussion

In this study, we constructed a genomic library from the moderately halophilic bacterium *Halobacillus* Y5 and identified a novel Na+/H+ antiporter gene, *Ha_{ydjM}*, through functional complementation. Analysis revealed that Ha_{YdjM} from *Halobacillus* Y5 belongs to the YdjM superfamily and represents a novel member, showing highest amino acid sequence homology with YdjM from *Halobacillus* sp. Marseille-P3789 (accession WP_{101846656}.1) while forming an independent phylogenetic branch. When expressed in *E. coli* mutant strain KNabc, Ha_{YdjM} restored growth in 0.2 M NaCl and 5 mM LiCl and conferred alkaline tolerance. Functional studies demonstrated pH-dependent Na+/H+ antiporter activity, with Km analysis revealing substrate affinity in the order Na+ > K+ > Li+. Therefore, we conclude that *Ha_{ydjM}* encodes a novel YdjM superfamily member with Na+/H+ antiporter function.

Many protein families remain functionally uncharacterized, and discovering their functions represents a fundamental task in post-genomic biology, particularly for widely distributed families [22]. Protein Ha_{YdjM} belongs to the YdjM superfamily but functions differently from known members, representing a new type of protein. YdjM superfamily proteins are typically metal-dependent enzymes with diverse types and mechanisms, such as acetylases [23] and deacetylases surrounded by multi-chain α-helices and β-sheets, with divalent metal ions as cofactors involved in cancer and epigenetic regulation. Huang et al. [22] reported that DUF89 proteins exhibit metal-dependent phosphatase activity in metabolic damage control, existing as standalone or fused proteins and classified into subfamilies based on different metal preferences. Other metal-dependent phosphatases are involved in extracellular phosphokinase regulation [24]. A metal-dependent hydrolase from the thermophilic fungus *Malbranchea cinnamomea* showed glycosidase activity in the presence of 1 mM Mn^{2+} rather than hydrolytic activity [25], demonstrating that sequence homology does not guarantee functional similarity. The CbaA hydrolase from *Pigmentiphaga* sp. strain DL-8, encoding 339 amino acid residues, is activated by Mg^{2+}, Ni^{2+},

Ca^{2+}, and Zn^{2+}, with conserved histidine 288 and glutamic acid 301 serving as proton donors and acceptors. Deletion of *cbaA* prevents degradation of 6-chloro-2-benzoxazolinone (CDHB), establishing CbaA as essential for CDHB degradation [26]. In eukaryotes, methionine aminopeptidase from *Trypanosoma brucei* is also metal-dependent and important for post-translational protein processing and drug discovery [27]. Our study of Ha_{YdjM} from *Halobacillus* Y5 differs from these reported proteins. Although sequence homology suggests metal-dependent enzyme activity, our functional complementation screening using a salt-sensitive mutant specifically selected for Na+/H+ antiporter activity. The positive clone complemented the mutant phenotype and exhibited Na+/H+ antiporter activity, making Ha_{YdjM} the first reported YdjM family protein in China with Na+/H+ antiporter activity. This study supplements and enriches YdjM family members, establishing a solid theoretical foundation for future research.

[1] Uzdavinys P, Coincon M, Nji E, et al. Dissecting the proton transport pathway in electrogenic Na+/H+ antiporters. Proceedings of the National Academy of Sciences of the United States of America, 2017, 114(7): 1101-1110.
[2] Padan, E. & Schuldiner, S. Molecular physiology of Na+/H+ antiporters, key transporters in circulation of Na+ and H+ in cells. Biochim. Biophys. Acta, 1994, 1185(2): 129-151.
[3] Ito, M., Guffanti, A. A., Oudega, B. & Krulwich, T. A. mrp, a multigene, multifunctional locus in Bacillus subtilis with roles in resistance to cholate and to Na+ and in pH homeostasis. J. Bacteriol. 1999, 181(8): 2394-2402.
[4] Padan, E., Bibi, E., Ito, M. & Krulwich, T. A. Alkaline pH homeostasis in bacteria: new insights. Biochim. Biophys. Acta, 2005, 1717(2): 67-88.
[5] Jr S M, Reddy V S, Tamang D G, et al. The transporter classification database. Nucleic Acids Research, 2014, 42(Database issue): 251-258.
[6] Quinn M J, Resch C T, Sun J, et al. NhaP1 is a K+(Na+)/H+ antiporter required for growth and internal pH homeostasis of Vibrio cholerae at low extracellular pH[J]. Microbiology, 2012, 158(4): 1094-1105.
[7] 杨礼富，赵百锁，杨苏声．细菌钠离子输出系统的类型及其可能机制．微生物学报，2007, 47(6): 1110-1114.
[8] Padan E. Functional and structural dynamics of NhaA, a prototype for Na+ and H+ antiporters, which are responsible for Na+ and H+ homeostasis in cells. Biochimica Et Biophysica Acta, 2014, 1837(7): 1047-1062.
[9] Dwivedi M, Sukenik S, Friedler A, et al. The Ec-NhaA antiporter switches from antagonistic to synergistic antiport upon a single point mutation. Scientific Reports, 2016, 6:23339.
[10] Zhang H, Wang Z, Wang L, et al. Cloning and identification of a novel NhaD-type Na+/H+ antiporter from metagenomic DNA of the halophilic bacteria in soil samples around Daban Salt Lake. Extremophiles, 2014, 18(1): 89-98.
[11] Sousa P M, Videira M A, Vorburger T, et al. The novel NhaE-type Na+/H+ antiporter of the pathogenic bacterium Neisseria meningitidis. Archives of Microbiology, 2013, 195(3):
[12] Pinner E, Carmel O, Bercovier H, et al. Cloning, sequencing and expression

of the nhaA and nhaR genes from Salmonella entiritidis. Archives of Microbiology, 1992, 157(4):
[13] Cui Y, Cheng B, Meng Y, et al. Expression and functional analysis of two NhaD type antiporters from the halotolerant and alkaliphilic Halomonas sp. Y2. Extremophiles, 2016, 20(5): 1-9.
[14] Ohyama T, Igarashi K, Kobayashi H. Physiological role of the chaA gene in sodium and calcium circulations at a high pH in Escherichia coli. Journal of Bacteriology, 1994, 176(14): 4311-4315.
[15] 徐宁, 程海娇, 刘清岱, 等. 细菌 Na+/H+ 逆向转运蛋白的研究进展. 微生物学通报, 2015, 42(10): 2002-2011.
[16] Fakharany E E, Hassan M. A universal method for extraction of genomic DNA from various microorganisms using lysozyme. New Biotechnology, 2016, 33:S210-S210.
[17] 王艳红, 曹宁, 贾桂燕, 等. 中度嗜盐菌 Halobacillus Y5 甘氨酸甜菜碱转运蛋白 opuD 基因的克隆及功能分析. 中国生物制品学杂志, 2017, 30(3): 258-263.
[18] Som A, Fuellen G. The effect of heterotachy in multigene analysis using the neighbor joining method. Molecular Phylogenetics & Evolution, 2009, 52(3):846-851.
[19] Meng L, Meng F, Zhang R, et al. Characterization of a novel two-component Na+(Li+, K+)/H+ antiporter from Halomonas zhaodongensis. Scientific Reports, 2017, 7(1):
[20] Rosen B P. Ion extrusion system in Escherichia coli. Methods in Enzymology, 1986, 125(1): 328-336.
[21] Wang Y, Song N, Yang L, et al. A novel NhaD-type Na+/H+ antiporter from the moderate halophile and alkaliphile Halomonas alkaliphila. Canadian Journal of Microbiology, 2017, 63(7):596-607.
[22] Huang L, Khusnutdinova A, Nocek B, et al. A family of metal-dependent phosphatases implicated in metabolite damage-control. Nature Chemical Biology, 2016, 12(8): 1-8.
[23] López J E, Sullivan E D, Fierke C A. Metal-dependent Deacetylases: Cancer and Epigenetic Regulators. Acs Chemical Biology, 2016, 11(3): 706-729.
[24] Li R, Gong Z, Pan C, et al. Metal-dependent protein phosphatase 1A functions as an extracellular signal-regulated kinase phosphatase. Febs Journal, 2013, 280(11):
[25] Mahajan C, Basotra N, Singh S, et al. Malbranchea cinnamomea : A thermophilic fungal source of catalytically efficient lignocellulolytic glycosyl hydrolases and metal dependent enzymes. Bioresource Technology, 2015, 200: 55-63.
[26] Dong W, Wang F, Huang F, et al. Erratum for Dong et al. Metabolic Pathway Involved in 6-Chloro-2-Benzoxazolinone Degradation by Pigmentiphaga sp. Strain DL-8 and Identification of the Novel Metal-Dependent Hydrolase CbaA. Appl Environ Microbiol, 2016, 82(14): 4169-4179.
[27] Marschner A, Klein C D. Metal promiscuity and metal-dependent substrate preferences of Trypanosoma brucei, methionine aminopeptidase 1. Biochimie, 2015, 115: 35-43.

Note: Figure translations are in progress. See original paper for figures.

ChinaRxiv 中X

Source: ChinaXiv —Machine translation. Verify with original.