In vitro functional characterization of the Na\(^+\)/H\(^+\) antiporters in Corynebacterium glutamicum

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One sentence summary: Corynebacterium glutamicum Mrp1 antiporter showed significant Na\(^+\)/(Li\(^+\))/H\(^+\) antiport activities and an alkaline pH optimum, suggesting its important roles in coping with salt–alkali stress.

ABSTRACT

Corynebacterium glutamicum, typically used as industrial workhorse for amino acid production, is a moderately salt–alkali-tolerant microorganism with optimal growth at pH 7–9. However, little is known about the mechanisms of salt–alkali tolerance in C. glutamicum. Here, the catalytic capacity of three putative Na\(^+\)/H\(^+\) antiporters from C. glutamicum (designated as Cg-Mrp1, Cg-Mrp2 and Cg-NhaP) were characterized in an antiporter-deficient Escherichia coli KNabc strain. Only Cg-Mrp1 was able to effectively complement the Na\(^+\)-sensitive of E. coli KNabc. Cg-Mrp1 exhibited obvious Na\(^+\)/(Li\(^+\))/H\(^+\) antiport activities with low apparent Km values of 1.08 mM and 1.41 mM for Na\(^+\) and Li\(^+\), respectively. The Na\(^+\)/H\(^+\) antiport activity of Cg-Mrp1 was optimal in the alkaline pH range. All three antiporters showed detectable K\(^+\)/H\(^+\) antiport activity. Cg-NhaP also exhibited Na\(^+\)/(Li\(^+\), Rb\(^+\))/H\(^+\) antiport activities but at lower levels of activity. Interestingly, overexpression of Cg-Mrp2 exhibited clear Na\(^+\)/(K\(^+\))/H\(^+\) antiport activities. These results suggest that C. glutamicum Na\(^+\)/(K\(^+\))/H\(^+\) antiporters may have overlapping roles in coping with salt–alkali and perhaps high-osmolarity stress.

Keywords: Corynebacterium glutamicum; Na\(^+\)/H\(^+\) antiporters; function

INTRODUCTION

The monovalent cation/H\(^+\) antiporters play essential physiological roles in the regulation of intracellular pH, adjustment of cell volume and efflux of toxic monovalent cations. They also have roles in establishment of an inwardly-directed sodium electrochemical gradient that drives Na\(^+\)-coupled solute uptake and motility in both prokaryotic and eukaryotic cells (Padan et al. 2005; Swartz et al. 2005; Krulwich and Hicks 2009; Chanroj et al. 2012; Qiu 2012). In bacteria, the monovalent cation/H\(^+\) antiporters generally function as secondary active transporters, which catalyze efflux of cytoplasmic monovalent cations such as Na\(^+\), K\(^+\) or Li\(^+\) in exchange for external H\(^+\), by using the proton-motive force generating from proton-pumping respiratory complexes or ATP hydrolysis (Padan et al. 2005; Slonczewski et al. 2009). Multiple families of bacterial monovalent cation/H\(^+\) antiporters have been characterized by the sequence-based transporter classification database (Saier et al. 2014),
including (i) the cation/proton antiporter-1 family (CPA1), such as Bacillus subtilis NhaK and NhaA (Gouda et al. 2001; Fujisawa et al. 2005), Pseudomonas aeruginosa NhaP (Kuroda et al. 2003; Herz et al. 2004). (ii) The cation/proton antiporter-2 family (CPA2), such as Enteroctococcus hirae NapA (Waser et al. 1992) and Bacillus cereus GerN (Southworth et al. 2001). In addition, the well-characterized Escherichia coli NhaA antiporter also belongs to this family (Padan et al. 2014). (iii) The cation/proton antiporter-3 family (CPA3). Members of this family are usually encoded by six to seven genes, and have diverse designations in various bacterial species, for example Mrp, Mnh, Pha and Sha (Hamamoto et al. 1994; Kosono et al. 2005; Yang et al. 2007; Swartz et al. 2007). (iv) The major facilitator superfamily (MFS), which encompasses several multidrug transporters that catalyze Na⁺/K⁺/H⁺ antiport activity in addition to the efflux of multiple drug substrates, such as E. coli MdhA and B. subtilis TetL (Cheng et al. 1994; Edgar and Bibi 1997). (v) The Ca²⁺/cation antiporter family (CaCA), which contains at least one Ca²⁺/H⁺ antiporter ChaA, and it also has Na⁺/H⁺ antiport activity (Ohyama et al. 1994). (vi) Other Na⁺/H⁺ antiporter families, including NhaB, NhaC, NhaD and NhaH (Pinner et al. 1992; Ito et al. 1997; Herz et al. 2003; Jiang et al. 2013). Most bacterial genomes have multiple genes and operons predicted to encode monovalent cation/H⁺ antiporters, whereas some especially ecologically stressed or versatile bacteria exhibit higher numbers of cation/H⁺ antiporters (Krutwich and Hicks 2009; Mesbah et al. 2009). Although the significant roles of Na⁺/H⁺ antiporters has been widely characterized in many bacteria, the properties of each antiporter and how they advantage particular species of bacteria are not yet known for the diverse cohorts of Na⁺/H⁺ antiporters, let alone additional antiporters with different catalytic profiles.

Corynebacterium glutamicum is a generally-regarded-as-safe (GRAS) Gram-positive bacterium, and is referred to as the industrial workhorse for amino acid, organic acid and diamine productions (Zahoor et al. 2012; Becker and Wittmann 2015; Eggingel and Bott 2015). In biotechnological application, this organism is often subjected to severe periodic challenges in salinity, pH and osmolarity, resulting in a profound effect on optimal growth and product yields (Varela et al. 2003; Follmann et al. 2009; Franzel et al. 2010). In addition, osmotic stresses imposed by elevated toxic salt concentrations might generate secondary effects, such as reduced water availability and increased oxidative damages, and further exacerbate survival challenges (Xiong and Zhu 2002). Several previous studies have suggested that C. glutamicum has a moderately alkali-tolerant organism with optimal growth at pH 7.0–8.5 (Follmann et al. 2009; Franzel et al. 2010). Our preliminary experiments also confirmed that C. glutamicum had a strong salt–alkali stress tolerance, exhibiting a marked Na⁺ resistance at up to 0.9 M at alkaline pH (Fig. S1, Supporting Information). Considering the potential roles of Na⁺/H⁺ antiporters in the maintenance of cellular pH and cation homeostasis, we speculate that C. glutamicum Na⁺/H⁺ antiporters might have contributed to its resistance to high salt–alkali stresses. The whole genome analyses reveal that C. glutamicum has three putative Na⁺/H⁺ antiporters (Kalinskiw et al. 2003); however, there is no report on the biochemical properties and physiological roles of sodium/proton antiporters from C. glutamicum so far. In this study, we were interested in the potential roles of Na⁺/H⁺ antiporters from C. glutamicum in environmental salt–alkali stresses. By expressing these antiporters in a triple antiporter-deficient E. coli KNabc strain, we provided the first characterization of their biochemical properties in vitro.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids and growth conditions**

The strains and plasmids are listed in Table 1. Corynebacterium glutamicum ATCC13032 was cultured in the standard LB medium (1% yeast extract, 2% peptone, 1% NaCl) at 32 C. E. coli DH5α was used as efficiency competent cells for general cloning, and E. coli KNabc lacking three major Na⁺/H⁺ antiporters (NhaA, NhaB and ChaA) was used as Na⁺/H⁺ antiporter-deficient background strain (Nozaki et al. 1998). The plasmids used for gene cloning were low-copy-number pMW118 and high-copy-number pGEM3zf vector, respectively. The primers used in this study

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<th>Plasmids or strain</th>
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<td>pMW118</td>
<td>Low copy cloning vector, Amp&lt;sup&gt;B&lt;/sup&gt;</td>
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<tr>
<td>pGEM3zf</td>
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<td>Promega</td>
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<tr>
<td>pMW118-Mrp1</td>
<td>pMW118 derivative; contains the full mrp1 operon from C. glutamicum</td>
<td>This study</td>
</tr>
<tr>
<td>pMW118-Mrp2</td>
<td>pMW118 derivative; contains the full mrp2 operon from C. glutamicum</td>
<td>This study</td>
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<tr>
<td>pMW118-NhaP</td>
<td>pMW118 derivative; contains the full nhaP gene from C. glutamicum</td>
<td>This study</td>
</tr>
<tr>
<td>pGEM3zf-Mrp1</td>
<td>pGEM3zf derivative; contains the full mrp1 operon from C. glutamicum</td>
<td>This study</td>
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<tr>
<td>pGEM3zf-Mrp2</td>
<td>pGEM3zf derivative; contains the full mrp2 operon from C. glutamicum</td>
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**Table 1. Strains and plasmids used in this study.**

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<th>Strains</th>
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<td>DH5α</td>
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<td>Representative wild-type C. glutamicum strain</td>
<td>Lab stock</td>
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<td>KNabc</td>
<td>E.coli derivative; TGlΔnhaA ΔnhaB ΔchaA</td>
<td>Nozaki et al. 1998</td>
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<td>E.coli KNabc harboring pMW118-Mrp1 vector</td>
<td>This study</td>
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<tr>
<td>KNabc-Mrp2</td>
<td>E.coli KNabc harboring pMW118-Mrp2 vector</td>
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<td>KNabc-NhaP</td>
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**Plasmid or strain**

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<td>Low copy cloning vector, Amp&lt;sup&gt;B&lt;/sup&gt;</td>
<td>This study</td>
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are listed in Table S1 (Supporting Information). The recombinant plasmid of pMW118-Mrp1 was constructed as follows. The whole Mrp1 operon was amplified by PCR from the C. glutamicum ATCC13032 chromosomal DNA with the primers CgMrp1-F-Smal and CgMrp1-R-XbaI, digested with Smal/XbaI, and ligated into Smal/XbaI sites of pMW118 vector, resulting in pMW118-Mrp1 recombinant plasmid. Similar strategies were performed for the construction of other recombinant plasmids derived from pMW118 or pGEM3zf vector. All constructs were confirmed by DNA sequencing, and the recombinant plasmids were transformed into E. coli KNabc for further experiments. Escherichia coli KNabc strain and its derivatives were routinely grown at 37 °C in LBK medium (1% yeast extract, 2% peptone and 0.6% KCl) supplemented with indicated NaCl concentrations. Ampicillin was added to a final concentration of 100 μg ml⁻¹ for the selection of specific transformants.

Preparation of everted membrane vesicles

Everted membrane vesicles were prepared by breaking cells with a French Pressure cell as previous described (Liu et al. 2005; Swartz et al. 2007). Briefly, Mid-exponential phase cultures of E. coli KNabc transformants were harvested by centrifuge, resuspended in 50 ml of TCDG buffer (10 mM Tris-HCl, pH 7.5; 140 mM choline chloride; 0.5 mM dithiothreitol; 10% glycerol), and washed three times. French Press buffer was prepared by adding the following agents to TCDG buffer: 1 mM phenylmethylsulfonyl fluoride (PMSF), a protease inhibitor tablet and a trace amount of DNase. Cells were resuspended in French Press buffer, broken by French Press once at 18 000 p.s.i., suspended in TCDG buffer using homogenizer, and stored at −80 °C before use. Protein content was measured by the method of Lowry et al. (1951) with bovine serum albumin as the standard.

Assay of ΔpH-dependent antiport activity

The antiport activity of everted membrane vesicles was estimated by acridine orange as a fluorescent probe of the transmembrane pH gradient (Goldberg et al. 1987; Jiang et al. 2013). Antiport assays were conducted in 20 mM BTP-sulfate, 140 mM choline chloride, 5 mM MgCl₂, 35 μg everted membrane vesicles and 1 μM acridine orange in a range of pH 6.5–9.0. Fluorescence intensities were constantly monitored with a Hitachi F-7000 fluorescence spectrophotometer at excitation and emission wavelengths of 495 and 530 nm, respectively. Respiration was initiated by the addition of Tris-succinate to a final concentration of 2.5 mM. After the fluorescence quench reached a steady state, appropriate cation substrates were added and the fluorescence dequench percentage was recorded as a representative of relative cation antiport activity. The addition of 10 mM NH₄Cl was used to dissipate the remaining pH gradient to bring the fluorescence back to baseline. As for the measurement of the apparent Kᵣ values for Na⁺ and Li⁺ of Cg-Mrp1 antiporter derived from E. coli KNabc pMW118 derivatives, pH was adjusted to 9.0 based on the highest antiport activity, and the substrate NaCl or LiCl concentrations varied from 0.5 to 10 mM. The fluorescence dequenching observed over a range of 0.5–20 mM NaCl or Na⁺ concentrations was used to calculate apparent Kᵣ values. The double-reciprocal plot was created by plotting the inverse fluorescence dequenching percentage as a function of the inverse cation concentration (Liu et al. 2005).

RESULTS

Corynebacterium glutamicum Mrp1 complemented the defective phenotypes of E. coli KNabc strain

Three putative Na⁺/H⁺ antiporters were identified by searching the genome database of C. glutamicum 13032 based on the highest sequence similarity (Table S2, Supporting Information). These predicted antiporters belong to two different cation/proton antiporter families, including two CPA3 family members (designated as Cg-Mrp1 and Cg-Mrp2, respectively) and one CPA1 family member (Cg-NhaP). Comparative sequence analyses reveal that C. glutamicum CPA3 family members display significant sequence similarity (19%-40% identity) to components of the well-described B. subtilis Mrp, Staphylococcus aureus Mnh and Bacillus pseudofirmus Mrp systems (Swartz et al. 2005, 2007; Morino et al. 2014). Cg-NhaP has 23%-26% sequence identity with other members of NhaP family, such as P. aeruginosa, Vibrio cholera and Methanococcus jannaschii (Kuroda et al. 2004; Resch et al. 2011).

Figure 1. (A) E. coli KNabc cells carrying the blank vector pMW118 or its derivatives were pre-grown at 37 °C for 24 h. The cultures were resuspended in 4 ml liquid LBK medium in the presence of indicated NaCl concentrations with OD₆₀₀ of 0.1, and cultivated at 37 °C. Growth ability was determined by measuring the optical density after 24 h incubation. (B) Overnight pre-cultures were resuspended in 100 mL liquid LBK medium in the presence of 200 mM NaCl with OD₆₀₀ of 0.1, and cultivated at 37 °C. Growth curves were monitored by measuring optical density at the indicated time points. The data are presented as means ± standard deviation (SD) from three independent experiments.
Heterologous complementation experiments were then performed to investigate the catalytic activities of these antiporters. The E. coli KNabc strain lacking three major Na\(^+/\)H\(^+\) antiporters, widely used for the screening of novel Na\(^+/\)H\(^+\) antiporters, is highly sensitive to the presence of Na\(^+\) and fails to grow when the NaCl concentration reaches to 200 mM (Wei et al. 2007). As shown in Fig. 1A, Cg-Mrp1 had the capacity to effectively rescue the growth defects of the Na\(^+\)-sensitive E. coli KNabc strain in the presence of indicated NaCl stress, whereas the other two antiporters were unable to complement the defective phenotype, which was also confirmed by the observation of 24-hr growth curve assays under 200 mM NaCl stress (Fig. 1B). Taken together, these findings suggest that Cg-Mrp1 may play a major role in Na\(^+\) resistance in C. glutamicum, but this remains to be directly tested.

**Corynebacterium glutamicum Mrp1 showed significant Na\(^+\) (Li\(^+)\)/H\(^+\) antiport activities with a low apparent K\(_m\) and an alkaline pH optimum**

Given the important role of Cg-Mrp1 in Na\(^+\) resistance, the cation/proton antiport activities of Cg-Mrp1 were measured in everted membrane vesicles prepared from E. coli KNabc transformants using the described fluorescence-based assay protocol. As shown in Fig. 2A, both Na\(^+/\)H\(^+\) and Li\(^+\)/H\(^+\) antiport activities of Cg-Mrp1 were measured in everted membrane vesicles prepared from E. coli KNabc transformants using the described fluorescence-based assay protocol.
activities were detected in everted membrane vesicles expressing Cg-Mrp1. A smaller level of K⁺/H⁺ antiport activity was also detected for Cg-Mrp1 (Table 2). A previous study has reported that the thermophilic Thermomicrobium roseum Mrp antiporter has the capacity to catalyze Ca²⁺/H⁺ antiport (Morino and Ito 2012), whereas no Ca²⁺/H⁺ antiport activity was observed for Cg-Mrp1 in this assay (data not shown). The everted membrane vesicles containing Cg-Mrp1 exhibited clear pH-dependent Na⁺/H⁺ antiporter activity in the broad range of pH 6.5–9.0, with optimal activity at pH 9.0, a sharply reduced activity at pH 7.0 and no activity at acidic pH (Fig. 2B). Because of the limitations of the heterologous E. coli KNabc system, it is difficult to obtain reliable data for Na⁺/H⁺ antiport at higher or lower pH (Liu et al. 2005; Swartz et al. 2007; Slonczewski et al. 2009). Both Na⁺/H⁺ and Li⁺/H⁺ antiport activities of Cg-Mrp1 were also examined as a function of monovalent cations at pH 9.0. The apparent Kₘ values for Cg-Mrp1 were ~1.08 mM for NaCl (Fig. 2C, inset) and 1.41 mM for LiCl (Fig. 2D, inset), respectively, supporting a high affinity for monovalent cations, which could facilitate the efflux of toxic monovalent cations and the acidification of cytoplasmic pH under complex salt–alkali stress conditions.

In order to achieve a greater understanding of the activities of C. glutamicum Na⁺/H⁺ antiporters, the activity and substrate specificity of other two antiporters were also examined in the everted membrane vesicles derived from E. coli KNabc transformants carrying pMW118 derivatives (Table 2). The assays revealed that Cg-Mrp2 exhibited small Na⁺/(K⁺)/H⁺ antiport activity, but no obvious Li⁺/H⁺ and Rb⁺/H⁺ antiport activities. Cg-NhaP had the modest ability to transport all four monovalent cations, with a higher K⁺/H⁺ than Na⁺/H⁺ exchange activity. Notably, some K⁺/H⁺ antiport activity was also detected for Cg-Mrp1 antiporter, supporting its possible role in the regulation of cellular potassium ion concentrations.

**Overexpression of C. glutamicum Mrp2 exhibited clear Na⁺/(K⁺)/H⁺ antiport activities**

In a preliminary study, we noted instances in which the expression level had a strong effect on Mrp-dependent antiporter effects on the growth phenotypes of E. coli Na⁺-sensitive KNabc derivatives. We therefore compared the profiles of antiport when the high-copy-number supported by pGEM3zf was used instead of the low-copy-number pMW118 for some of the strains. Spot assay experiments for E. coli KNabc carrying pMW118 derivatives showed similar results as before (compare Fig. 1 with Fig. 3A, up panel). Interestingly, the E. coli KNabc-pGEM3zf-Mrp2 strain could partially complement growth defects of the Na⁺-sensitive KNabc strain, especially under low salt stress such as 50 mM NaCl, while overexpression of Cg-Mrp1 led to poor growth under lower NaCl conditions (Fig. 3A, bottom panel). The antiport activities of Mrp-dependent antiporters derived from E. coli KNabc pGEM3zf derivatives were further assessed (Fig. 3B). An increase in the Na⁺/H⁺ and K⁺/H⁺ antiport activities were observed for Cg-Mrp2, reaching up to 7.3 ± 0.7% and 6.5 ± 0.4%, respectively. In addition, Cg-Mrp2 exhibited a low level of Li⁺/H⁺ antiport activity which is usually found together with Na⁺/(K⁺)/H⁺ antiporters. The above results suggest that Cg-Mrp2 catalyzes both Na⁺/H⁺ and K⁺/H⁺ antiport. However, the Na⁺/H⁺ and Li⁺/H⁺ antiport activities of Cg-Mrp1 derived from E. coli KNabc pGEM3zf derivatives were evidently lower than those of E. coli KNabc pMW118 derivatives, probably caused by overly high expression levels of Cg-Mrp1 in the pGEM3zf vector.

**DISCUSSION**

In this study, we investigated the properties of three putative Na⁺/H⁺ antiporters from C. glutamicum in vitro, and only Cg-Mrp1 was observed to restore the growth defects of Na⁺-sensitive E. coli KNabc strain. Furthermore, the everted membrane vesicles of E. coli KNabc containing Cg-Mrp1 showed significant Na⁺/H⁺ antiport activity with an optimal activity at alkaline pH, partly consistent with the optimal growth pH of C. glutamicum. Taken together, Cg-Mrp1 might play a major role in Na⁺ resistance and the optimum activity of Cg-Mrp1 at alkaline pH suggests its possible role in pH homeostasis of C. glutamicum under alkaline pH stress.

Interestingly, the two other antiporters had no significant effect on the growth phenotypes of E. coli KNabc in response to salt stress, and the fluorescence-based activity assays revealed that both Cg-Mrp2 and Cg-NhaP had a weak capacity to transport Na⁺ out of the cytoplasm. The failure to effectively rescue growth defects of E. coli KNabc under different NaCl conditions might be attributed to insufficiency of the Na⁺/H⁺ antiport activity levels to cope with such salt challenge. Expression of Cg-Mrp2 at higher levels improved its Na⁺/H⁺ antiport activity somewhat and partially complemented the growth defects of E. coli KNabc under low NaCl concentration. Cg-Mrp2 might play a specific role in monovalent cation transport when the environmental NaCl stress is not apparent. Further, three of the Na⁺/H⁺ antiporters exhibited detectable K⁺/H⁺ antiport activities, raising the possibility that these antiporters contribute to high KCl resistance. In these assays, we found that Cg-Mrp1, Cg-Mrp2 and Cg-NhaP had a broad substrate profile for monovalent cations, although the levels were different. These above results indicate that C. glutamicum may use a variety of regulatory strategies, such as functional redundancy or compensatory upregulation, to coordinate the activities of these three Na⁺/H⁺ antiporters in response to specific stresses (Kruilwich and Hicks 2009). The elucidation of the mechanism and how they affect Na⁺/H⁺ antiporters and, in turn, C. glutamicum cellular physiology and homeostasis in vivo, will help us provide new insights.

**Table 2. Substrate specificity of C. glutamicum putative Na⁺/H⁺ antiporters.**

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<tr>
<th>Strains</th>
<th>Li⁺</th>
<th>Na⁺</th>
<th>K⁺</th>
<th>Rb⁺</th>
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<tr>
<td>KNabc-pMW118</td>
<td>0.22 ± 0.04</td>
<td>1.38 ± 0.22</td>
<td>2.01 ± 0.05</td>
<td>0.20 ± 0.03</td>
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<tr>
<td>KNabc-Mrp1</td>
<td>10.18 ± 0.43</td>
<td>13.49 ± 0.66</td>
<td>4.19 ± 0.18</td>
<td>0.78 ± 0.15</td>
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<tr>
<td>KNabc-Mrp2</td>
<td>0.43 ± 0.10</td>
<td>2.09 ± 0.32</td>
<td>3.99 ± 0.29</td>
<td>0.73 ± 0.04</td>
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<tr>
<td>KNabc-NhaP</td>
<td>2.63 ± 0.16</td>
<td>2.39 ± 0.19</td>
<td>4.61 ± 0.49</td>
<td>2.16 ± 0.37</td>
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*The antiport assays were performed with 5 mM indicated cation substrates at pH 9.0. Data shown are mean ± SD of duplicate determinations from three independent experiments.*
Figure 3. (A) Growth assay of E. coli KNabc cells carrying low copy vector pMW118 derivatives or high copy vector pGEM3zf derivatives. Ten-fold serial dilutions of mid-exponential phase cells were respectively spotted on the LBK agar plates containing the indicated concentrations of NaCl, and the plates were incubated at 37°C for 24 h before being photographed. (B) Fluorescence-based assays of Na⁺ (K⁺, Li⁺)/H⁺ antiport activity in everted membrane vesicles derived from E. coli KNabc-pGEM3zf derivatives. The data are representative of three independent experiments.

into their physiological significance. Further studies about these efforts are ongoing in our lab.

SUPPLEMENTARY DATA
Supplementary data are available at FEMSLE online.

ACKNOWLEDGEMENTS
We are thankful to Professor Ning Chen (Tianjin University of Science and Technology, Tianjin) for the kind gift of C. glutamicum ATCC13032 strain. We are grateful to Professor Terry Krulwich (Icahn School of Medicine at Mount Sinai, New York) for the kind gifts of E.coli KNabc strain, pMW118 and pGEM3zf, and critical reading of this manuscript. This study was supported by ‘Hundred Talents Program’ of the Chinese Academy of Sciences and the National Natural Science Foundation of China (no. 31500044).

Conflict of interest. None declared.

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