Mrp-dependent Na^+/H^+ antiporters of *Bacillus* exhibit characteristics that are unanticipated for completely secondary active transporters

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Abstract The Na^+/H^+ antiport activity encoded by the seven-gene *mrp* operons of *Bacillus subtilis* and alkaliphilic *Bacillus pseudofirmus* OF4 were cloned into a low copy plasmid, were expressed in several *Escherichia coli* mutant strains and compared side-by-side with similarly cloned *nhaA*, a major secondary antiporter from *E. coli*. All three antiporter systems exhibited electron donor-dependent antiport in a fluorescence-based vesicle assay, with NhaA being the most active. In whole cells of the same antiporter-deficient strain from which the vesicles were made, *E. coli* KNabc, Mrp-mediated Na^+ exclusion was significantly more protonophore-resistant than that conferred by NhaA. The Mrp systems were also more efficacious than NhaA: in supporting anaerobic Na^+ resistance in wild type and a terminal oxidase mutant strain of *E. coli* (SBS2115); and in increasing non-fermentative growth of an NADH dehydrogenase-minus *E. coli* mutant (ANN0222). The results suggest the possibility that the Mrp systems may have both secondary and primary energization capacities. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Na^+/H^+ antiporter; Mrp; *Bacillus subtilis*; *Bacillus pseudofirmus* OF4

1. Introduction

Recent studies of a Na^+/H^+ antiporter by the *mrp* (also referred to in some species as *sha* or *mnh*) operon have raised the question of whether these novel systems might have multiple modes of energization [1,2]. That is, in addition to energization by the electrochemical proton gradient, Δp, exhibited by typical, single gene product, secondary antiporters [3], there may be a primary energization mode. The possibility of a Na^+/H^+ antiporter with a capacity for primary energization could be particularly important under highly alkaline conditions. At high pH, the adequacy of completely Δp-dependent basis for active pH homeostasis and Na^+ resistance is questionable since the total Δp decreases with increasing external pH [4].

The *mrp* operon of alkaliphilic *Bacillus pseudofirmus* OF4 [5] and *Bacillus subtilis* [1,6], which now has homologues in many microorganisms [7], is a seven-gene operon whose 5′-end is homologous to a DNA fragment that was first reported from alkaliphilic *Bacillus halodurans* C125 [8,9]. That original discovery involved restoration, by a cloned fragment that corresponded to three *mrp* genes, of a capacity for alkaliphily, pH homeostasis and Na^+/H^+ antiport activity to a non-alkaliphilic mutant strain of *B. halodurans* C125. The antiport activity was attributed to the first gene, in which the chromosomal mutation of the mutant was found [9]. Subsequent studies of the full operons from *B. subtilis* (*mrp*) [1,2] and *Staphylococcus aureus* (*mnh*) [10] have shown, however, that Na^+ exclusion and antiport depend upon the entire operon. Hiramatsu et al. [10] accordingly suggested that the antiporter might function as a hetero-oligomeric complex. Studies of the *mrp*- and *mnh*-encoded antiports indeed indicate that they can function as secondary, Δp-energized antiporters [1,2,9,10]. Moreover, there are no evident ATP binding motifs. However, significant sequence similarity between many of the *mrp* and *mnh* gene products and the hydrophobic subunits of energy-coupled NADH dehydrogenase complexes (Complex I), as well as other membrane-associated dehydrogenases and hydrogenase, has been noted [9,11–13]. Recently, a novel redox group has been reported in the membrane-embedded segment of Complex I although its specific association with subunits that Mrp resembles has not been specified [14]. The possibility that the putative *mrp* or *mnh* complexes may have a primary energy-coupling mode, in addition to their capacity for secondary antiport, has not been directly explored, let alone excluded. In the current study, the *mrp* operons from *B. subtilis* and *B. pseudofirmus* OF4 were expressed from a low copy plasmid in a Na^+-sensitive and in two different respiratory chain mutants of *Escherichia coli*. The capacities of the *mrp* operon were assessed side-by-side with control vector as well as similarly cloned *E. coli nhaA*, which encodes a major secondary Na^+/H^+ antiporter of *E. coli* [3].

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

The *E. coli* strains used in this study were: DH5αMCR (Gibco-BRL), wild type; KNabc, Δ*chaA*Δ*nhaA*Δ*nhaB* [15]; NM81, Δ*nhaA* [16]; SBS2115, Δ*cyo*Δ*cyd*Δ*cbd* (from P.L. Bouquet); and ANN0222, Δ*nuo*Δ*ndh*, a derivative of AN387 ([17] from T. Friedrich). The strains were grown routinely in LBK medium [18] at 37°C. For experiments,

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Abbreviations: Δp, transmembrane electrochemical proton gradient; $\Delta\Psi$, transmembrane electrical potential; BTP, bis tris propane; CCCP, carbonyl cyanide *p*-chlorophenylhydrazone; MIC, minimal inhibitory concentration; Mrp, multiple resistance and pH (*mrp*) locus; TMG, methyl-β-thio-D-galactopyranoside

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E. coli ANN0222 was grown in a mineral salts medium supplemented as described by others [19] and containing 20 mM L-lactate as the major energy and carbon source. For some experiments, growth was in an anaerobic chamber, which was made oxygen-free by adding Gas Pack Plus (BBL Anaerobic System). The plasmids used in this study were: pGM36 [18], pMW118 (Nippon Gene, Toyama, Japan) and three recombinant plasmids of pMW118 containing either *nhaA*, the *B. subtilis mrp* operon, or the *B. pseudofirmus* OF4 operon, cloned behind their own promoters. The recombinant plasmids were designated, respectively, pMWnhaA, pMWBSmrp, and pMWOF4mrp. pMWnhaA was prepared by digesting *nhaA*-containing plasmid pGM36 with *Sph*I and *Eco*RI and cloning the fragment into similarly digested pMW118. For construction of pMWBSmrp, PCR was performed on *B. subtilis* BD99 (wild type) chromosomal DNA with primers BSMRPE1 and BSMRPNB2, described elsewhere [2]. The purified product, containing the *mrp* operon and its putative promoter region, was digested with *Eco*RI and *Bgl*II and then cloned into *Eco*RI–*Bam*HI-digested pMW118. For construction of pMWOF4mrp, PCR was performed on *B. pseudofirmus* OF4 chromosomal DNA with the sets of primers OF4MRPAE1 and OF4MRPK1. OF4MRPAE1 (5′-GGAATTCGTAACTTGACCTAAGCCCTGA-3′) corresponded to the complementary sequence of bp 73–93 of the database entry GenBank accession no. AF097740 and additional nucleotides containing an *Eco*RI site at the 5′-end of the sequence. OF4MRPGK1 (5′-GGGGTACCTAGAGCAAGTGTATCATCTGCTC-3′) corresponded to the complementary sequence of bp 6715–6693 of the database entry GenBank accession no. AF97740 and additional nucleotides containing a *Kpn*I site at the 5′-end of the sequence. The purified PCR product, which contains the whole *mrp* operon and its own promoter region, was digested with *Eco*RI and *Kpn*I and cloned into similarly digested pMW118. For all the plasmid selections, blue–white screening in *E. coli* DH5α was employed and complete DNA sequencing was used to confirm that the plasmids ultimately used were free of errors.

2.2. Assays

Determinations of the minimal inhibitory concentration (MIC) of Na^+ for *E. coli* KNabc were performed in LBK medium as previously described [20]. Everted membrane vesicles were prepared by the method of Ambudkar et al. [21]. Assays of Na^+/H^+ antiport by acridine orange fluorescence were performed as described by others [18]. Determinations of the intracellular concentration of Na^+ were conducted as described by Harel-Bronstein et al. [22]. Cells were grown to the mid-logarithmic phase in LBK–50 mM glucose medium containing 25 mM NaCl. Carrier-free $^{22}Na^+$ (0.5 μCi/ml) was added for 1 h, in the presence or absence of 50 μM carbonyl cyanide *p*-chlorophenylhydrazone (CCCP). Samples were taken, filtered, and then counted by liquid scintillation spectrometry. Non-specific binding of $^{22}Na^+$ in toluenized cells was subtracted. For estimation of the chemiosmotic driving force that persisted in the CCCP-treated cells under the conditions of this experiment, each strain was transformed with the *lacY* bearing plasmid, pTE18, obtained from T.H. Wilson. The accumulation ratio of the lactose analogue methyl-β-thio-D-galactopyranoside (TMG) was determined based on the method of Kashket et al. [23]. [^{14}C]TMG (59 Ci/mol) was added at 2 μM to cells incubated under the exact conditions for the Na^+ exclusion experiments except that only non-radioactive Na^+ was added. The same toluenized cell controls were used. For determinations of oxygen consumption by whole cells, cells of the four types of transformants of *E. coli* KNabc and SBS2115 were grown in LBK–glucose medium to the mid-logarithmic phase of growth, harvested by centrifugation, and resuspended in bis tris propane (BTP) buffer, pH 7.5. Oxygen consumption was measured at 37°C using a Clark type electrode with the addition of glucose, mannitol, or D,L-lactic acid in BTP buffer containing either no additional salts, 10 mM NaCl or 10 mM KCl. Assays of NADH dehydrogenase activity were conducted on everted membrane vesicles by the spectrophotometric assay described earlier [24].

3. Results and discussion

3.1. Complementation of Na^+/H^+ antiporter-deficient *E. coli*

The MIC for Na^+ in aerobically grown *E. coli* KNabc, a multiple Na^+/H^+ antiporter mutant lacking functional *nhaA*, *nhaB*, and *chaA*, was 0.18 and 0.08 M, respectively, at pH 7.0 and 8.2 for a transformant with control vector pMW118. Each of the three recombinant plasmids, pMWnhaA, pMWBSmrp and pMWOF4mrp, raised the MIC to 0.9–1.1 M at pH 7.0 and to 0.62–0.68 at pH 8.2. Membrane vesicles were prepared from the four transformants and assayed for secondary Na^+/H^+ antiport activity by a fluorescence assay. In this assay, antiport is assessed by Na^+-dependent dequenching of acridine orange fluorescence, secondary to respiration-induced quenching. Vesicles from transformants of the single antiporter mutant *E. coli* NM81, lacking functional *nhaA* only, were also assayed. As shown in Table 1, the signal was low in *E. coli* KNabc, as had been observed for activity of *mnh* in this strain [10], but there was still a significant antiport mediated by all three recombinant plasmids, with pMWnhaA conferring the greatest activity. The signal was higher for all comparable *E. coli* NM81 transformants, but the pattern was similar to that observed in strain KNabc. These data were consistent with earlier indications from *B. subtilis* Mrp [1,2], as well as homologues from alkaliphilic *B. halodurans* C125 [9] and *S. aureus* [10], that the Mrp-associated Na^+/H^+ antiport activity can function as a secondary antiporter that is energized by respiration-dependent or artificially imposed electrochemical gradients of protons.

Table 1
Assay of Na^+/H^+ antiport activity of everted membrane vesicles by fluorescence of acridine orange

Plasmid	Dequenching upon addition of 10 mM NaCl (%) *E. coli* strain	
	KNabc (Δ*nhaA*Δ*nhaB*Δ*chaA*)	NM81 (Δ*nhaA*)
pMW118	0.17 ± 0.13	8.7 ± 0.6
pMWnhaA	5.21 ± 0.16	45.3 ± 1.2
pMWBSmrp	2.72 ± 0.05	19.7 ± 1.2
pMWOFmrp	2.33 ± 0.25	14.7 ± 2.1

Everted membrane vesicles were prepared from LBK-grown cells of the *E. coli* mutant strains indicated above, transformed by the plasmids listed on the left. The Na^+/H^+ activity was assayed in at least two independent preparations as described under Section 2. Substrate, 2 mM Tris–D-lactate.

As shown in Table 2, Mrp-mediated Na^+ exclusion from whole cells of *E. coli* KNabc was somewhat less effective than the exclusion mediated by NhaA under both aerobic and anaerobic conditions. Most strikingly, the Mrp- but not NhaA-mediated Na^+ exclusion exhibited significant resistance to the presence of 50 μM CCCP. The Δp generated by each transformant was calculated from TMG accumulation ratios. These ratios have been shown to directly reflect the Δp [23]. Under the precise experimental conditions of the Na^+ exclusion, the CCCP treatment reduced the Δp drastically and it concomitantly inhibited NhaA-mediated Na^+ exclusion completely. This complete inhibition by CCCP would be expected for a Mrp system using entirely secondary energization by the Δp unless Mrp is even more kinetically competent than NhaA relative to CCCP. NhaA has been shown to be highly kinetically competent [25]. Thus partial rather than full inhibition of Mrp-mediated Na^+ exclusion was suggestive of a difference in energization. A primary Na^+ pump with no counter-ion coupling is often diagnosed as such by CCCP stimulation. Abolition of the transmembrane electrical potential ($\Delta\Psi$) releases the constraint of that potential on primary electrogenic ion translocation. For a primary, coupled Na^+/H^+ antiport, partial inhibition (rather than stimulation) might occur if H^+

Table 2
Determination of intracellular Na^+ concentration in various transformants of Na^+/H^+ antiporter-deficient *E. coli* KNabc under aerobic and anaerobic growth conditions

Strain	Cytoplasmic Na^+ (mM)				Δp (mV) calculated from TMG accumulation			
			+CCCP (50 μM)				+CCCP (50 μM)	
	aerobic	anaerobic	aerobic	anaerobic	aerobic	anaerobic	aerobic	anaerobic
KNabc/pMW118	24.3 ± 1.7	26.2 ± 2.3	25.0 ± 1.4	28.6 ± 6.4	−151 ± 5	−119 ± 4	−16 ± 2	−19 ± 5
KNabc/pMWnhaA	4.5 ± 0.8	6.5 ± 1.2	24.9 ± 1.1	22.1 ± 3.4	−149 ± 7	−117 ± 3	−15 ± 4	−16 ± 2
KNabc/pMWBSmrp	5.9 ± 1.4	10.6 ± 3.4	13.4 ± 2.3	14.7 ± 1.1	−150 ± 6	−114 ± 3	−16 ± 5	−18 ± 4
KNabc/pMWOF4mrp	7.1 ± 1.6	7.3 ± 2.1	16.9 ± 2.4	15.5 ± 3.5	−151 ± 9	−116 ± 5	−16 ± 4	−17 ± 3

Cells were grown aerobically or anaerobically in LBK–50 mM glucose medium containing 25 mM NaCl as described in Section 2. Values are presented ± standard deviations.

capture and translocation through the antiporter are obligatory for completion of the catalytic cycle. The reaction sequence might involve initial electrogenic Na^+ extrusion followed by rapid inward H^+ translocation through a proton pathway within the putative Mrp complex. Disruption of the cycle and decreased Na^+ extrusion might result from treatment with CCCP or valinomycin+K^+ [26] if they reduce the $\Delta\Psi$ available to energize the H^+ translocation step.

3.2. *Mrp-dependent enhancement of Na^+ resistance and aerobic growth of respiratory chain mutants of E. coli*

The absence of evident nucleotide binding motifs makes it unlikely that Mrp complexes are A(G)TPases, but if they conserve redox energy, they might enhance the aerobic growth of respiratory chain mutants of *E. coli*. If they are primary Na^+/H^+ antiporters of any type they should support Na^+ resistance in such mutants under conditions in which the Δp is too low for efficacy of NhaA. These possibilities were examined in transformants of *E. coli* SBS2115, lacking the three terminal oxidases *cyd*, *cyo*, and *cbd*, and of *E. coli* ANN0222, lacking *nuo* and *ndh*, respectively, encoding the energy-coupled and non-energy-coupled NADH dehydrogenases. *E. coli* SBS2115 was not complemented for aerobic growth on succinate by any of the plasmids.

None of these transformants exhibited any oxygen consumption, nor was oxygen consumption increased in any of the transformants of *E. coli* strains in the study possessing a wild type respiratory chain complement (e.g. *E. coli* KNabc). However, when transformants of wild type *E. coli*, DH5α, and mutant strain SBS2115 were grown anaerobically on increasing concentrations of Na^+, the transformants expressing *mrp* operons grew better than control and pMWnhaA transformants at the higher concentrations of Na^+ (Fig. 1). This was particularly pronounced in *E. coli* SBS2115 which exhibited less growth in general than the wild type strain (hence the use of lower pH and [Na^+]), but showed insignificant growth at 0.6 M Na^+ unless expressing a *mrp* operon. In *E. coli* mutant strain ANN0222, which lacks NADH dehydrogenases, the results were even more striking inasmuch as there was partial complementation of the growth deficit on several non-fermentative substrates in the presence of low, non-cytotoxic Na^+ concentrations. This is shown for transformants of the NADH dehydrogenase-minus strain growing aerobically on L-lactate in the presence of 100 mM Na^+ in Fig. 2. No NADH dehydrogenase activity was detected in membranes of any of the transformants. Nor was there any Mrp-dependent increase in the NADH dehydrogenase activity of the *E. coli* KNabc transformants, regardless of the cations added. This is consistent with the absence of a detectable loss of such activity in a *mrp* mutant of alkaliphilic *Bacillus* C125, in which the first part of the operon was first identified [9]. It is in contrast to reported primary Na^+ extrusion capacities that are proposed to be intrinsic to NADH dehydrogenase complexes [27–29].

Taken together, the results presented here suggest that, whereas Mrp-mediated Na^+/H^+ antiport can be energized by respiration as a typical secondary antiport, there may be an additional mode that is partially resistant to the effects of CCCP. Without restoring the missing activity of particular

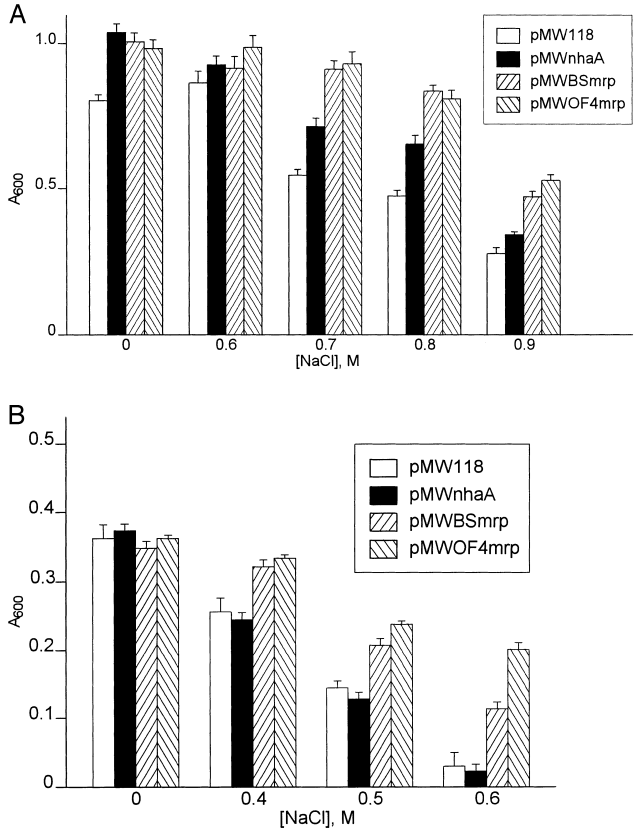

Fig. 1. Anaerobic growth of *E. coli* wild type and SBS2115 (Δ*cyd*Δ *cyo*Δ*cbd*), transformed with control plasmid or pMWnhaA, pMWBSmrp, or pMWOF4mrp, on LBK–glucose medium in the presence of various concentrations of added Na^+. A: *E. coli* DH5α (wild type) was grown on LBK+50 mM glucose at pH 8.2 for 15 h in the presence of the indicated concentrations of added Na^+ before the A_{600} was recorded. Values for six independent experiments are shown with the error bars indicating the standard deviation. B: The same experiment was conducted with transformants of mutants *E. coli* strain SBS2115 except that the medium was at pH 7.0.

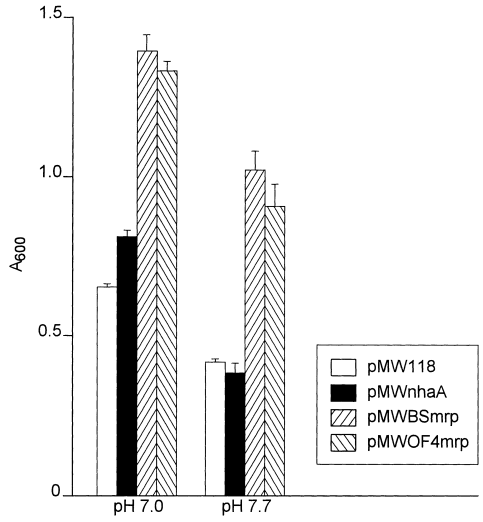

Fig. 2. Growth of transformants of *E. coli* mutant ANN0222 (Δ*ndh*Δ*nuo*) on semi-defined medium containing L-lactate as the major carbon source. Cells were grown aerobically on the L-lactate-containing medium described under Section 2, at either pH 7.0 or 7.7 for 18 h. The A_{600} was then recorded. The average of six experiments is shown with error bars indicating standard deviations.

respiratory chain mutants, this putative primary energization mode can increase non-fermentative growth and/or at least better support Na^+ exclusion in such mutants to extents not observed with the completely secondary, single gene product, Na^+/H^+ antiporter NhaA.

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