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Catalytic Properties of *Staphylococcus aureus* and *Bacillus* Members of the Secondary Cation/Proton Antiporter-3 (Mrp) Family Are Revealed by an Optimized Assay in an *Escherichia coli* Host[▽]

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Monovalent cation proton antiporter-3 (Mrp) family antiporters are widely distributed and physiologically important in prokaryotes. Unlike other antiporters, they require six or seven hydrophobic gene products for full activity. Standard fluorescence-based assays of Mrp antiport in membrane vesicles from *Escherichia coli* transformants have not yielded strong enough signals for characterization of antiport kinetics. Here, an optimized assay protocol for vesicles of antiporter-deficient *E. coli* EP432 transformants produced higher levels of secondary $Na^+(Li^+)/H^+$ antiport than previously reported. Assays were conducted on Mrps from alkaliphilic *Bacillus pseudofirmus* OF4 and *Bacillus subtilis* and the homologous antiporter of *Staphylococcus aureus* (Mnh), all of which exhibited $Na^+(Li^+)/H^+$ antiport. A second paralogue of *S. aureus* (Mnh2) did not. K^+, Ca^{2+}, and Mg^{2+} did not support significant antiport by any of the test antiporters. All three $Na^+(Li^+)/H^+$ Mrp antiporters had alkaline pH optima and apparent K_m values for Na^+ that are among the lowest reported for bacterial Na^+/H^+ antiporters. Using a fluorescent probe of the transmembrane electrical potential ($\Delta\Psi$), Mrp Na^+/H^+ antiport was shown to be $\Delta\Psi$ consuming, from which it is inferred to be electrogenic. These assays also showed that membranes from *E. coli* EP432 expressing Mrp antiporters generated higher $\Delta\Psi$ levels than control membranes, as did membranes from *E. coli* EP432 expressing plasmid-borne NhaA, the well-characterized electrogenic *E. coli* antiporter. Assays of respiratory chain components in membranes from Mrp and control *E. coli* transformants led to a hypothesis explaining how activity of secondary, $\Delta\Psi$-consuming antiporters can elicit increased capacity for $\Delta\Psi$ generation in a bacterial host.

Monovalent cation/H^+ antiporters of bacteria have critical roles in alkali tolerance, efflux of toxic monovalent cations, and establishment of a sodium gradient that can drive solute uptake and motility (44, 46). Members of the family of monovalent cation/H^+ antiporters referred to here as the Mrp family have diverse designations, including Mrp (19), Mnh (17), Pha (48), Sha (31), and Sno (3), and are widely distributed among physiologically diverse prokaryotes, including numerous pathogenic bacteria (56). Important physiological functions have been attributed to the monovalent cation/H^+ activity of Mrp antiporters, since mutational loss or compromise of this activity has been associated with: alkali and Na^+ sensitivity in alkaliphilic *Bacillus* (14) and *Anabaena* (4); Na^+ sensitivity, alkali sensitivity, and a sporulation defect in *Bacillus subtilis* (19, 20, 31, 32); a growth defect in *Staphylococcus aureus* (3, 24); and Na^+ sensitivity and decreased virulence in *Pseudomonas aeruginosa* (33). Several bacterial strains have more than one Mrp system. In *Sinorhizobium* species, where dual systems have been named Pha1 and Pha2, mutational loss of Pha1 results in K^+ sensitivity and a nitrogen fixation defect (48) while loss of Pha2 results in Na^+ sensitivity (67). The recent observation of increased Mrp expression under specific metabolic conditions, e.g., during growth of the archaeon *Methanosarcina acetivorans* on acetate (35), suggests that additional settings in which Mrp has important roles will continue to be identified.

In spite of their distribution and importance, Mrp antiporters are still very incompletely characterized, but they are clearly unique among monovalent cation/H^+ antiporters. Mrp antiporters constitute their own family, the cation proton antiporter-3 (CPA-3) family (TC 2.A.63), in the sequence-based transporter classification (TC) because of particular sequence features and apparent complexity (6, 54). Mrp antiporter systems are encoded by six or seven gene operons that are highly conserved. All the genes are required for full function of Mrp antiporters in Na^+ and alkali resistance (17, 20). Each *mrp* gene product is a hydrophobic membrane protein that is predicted to span the membrane multiple times. Three of them, MrpA, MrpC, and MrpD, exhibit significant sequence similarity to hydrophobic subunits that are part of the H^+-translocating domains of complexes such as respiratory chain complex I and hydrogenases (40, 41, 56). By contrast, all other monovalent cation/proton antiporters are encoded by a single gene with a single gene product that functions as a monomer or homodimer (11, 53). Mrp proteins have been proposed but not yet shown to form a functional complex (17, 56). We have hypothesized that such a complex is a consortium of distinct transporters that includes one or more Na^+/H^+ antiporters (56, 58), which were proposed by Mathiesen and Hägerhäll to be MrpA and MrpD (40). The putative Mrp complex is also

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TABLE 1. Strains and plasmids

Strain or plasmid	Description	Reference or source
E. coli strains		
EP432	*melB*(LiD[a]) *nhaA1*::*kan* Δ*nhaB1*::*cam* Δ*lacZY thr-1*	47
DH5α	F^- ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17* (r_K^- m_K^+) *phoA supE44* λ^- *thi-1 gyrA96 relA1*	Gibco-BRL
KNabc	TG1 (Δ*nhaA* Δ*nhaB* Δ*chaA*)	43
Plasmids		
pMW118	Cloning vector; Amp^r	NipponGene, Toyama, Japan
pMWBSMrp	pMW118(+) derivative; contains the full *mrp* operon from *B. subtilis*	21
pMWOF4Mrp	pMW118(+) derivative; contains the full *mrp* operon from *B. pseudofirmus* OF4	21
pMWSaMnh	pMW118(+) derivative; contains the full *mnh* operon from *S. aureus*	This study
pMWSaMnh2	pMW118(+) derivative; contains the full *mnh2* operon from *S. aureus*	This study
pGEM-3zf	Cloning vector; Amp^r	Promega
pGEM*Bs*-Mrp	pGEM-3Zf(+) derivative; contains the full *mrp* operon from *B. subtilis*	This study
pGEM*Bp*OF4-Mrp	pGEM-3Zf(+) derivative; contains the full *mrp* operon from *B. pseudofirmus* OF4	This study
pGEMMnh	pGEM-3Zf(+) derivative; contains the full *mnh* operon from *S. aureus*	This study
pGEMMnh2	pGEM-3Zf(+) derivative; contains the full *mnh*2 operon from *S. aureus*	This study
pBR322	Cloning vector; Amp^r	New England Biolabs
pGM36	pBR322 derivative; contains the full *nhaA* gene from *E. coli*	12

[a] LiD, Li^+ dependent.

expected to include one or more anion transporters whose particular substrate(s) may be species specific (56, 58). This is suggested by roles of Mrp in bile salt and arsenite resistance in *B. subtilis* and *Agrobacterium tumefaciens*, respectively (19, 20, 27). A multifunctional complex could provide synergies such as the presentation of a large protein surface area on the external membrane surface. This could enhance proton capture in support of the monovalent cation/proton antiport function that supports alkali resistance (56, 58).

To date, little exploration has been initiated on Mrp activities other than monovalent cation/H^+ antiport, and this activity of Mrp has only been minimally characterized, even for the most intensively studied *B. subtilis* Mrp (19, 20, 21, 31, 34). Like the activities of well-characterized prokaryotic antiporters that are single gene products, Mrp-dependent antiport is secondary antiport that is energized by the respiration-generated electrochemical proton gradient, Δp, as demonstrated in whole-cell and membrane vesicle assays (29, 46, 56). In addition, an imposed transmembrane potential is sufficient to energize Na^+ efflux by *Bacillus* Mrp antiporters from whole cells (14, 19, 20); this is consistent with movement of net charge during Mrp antiport, i.e., with an electrogenic antiport. Electrogenicity is an important property for Na^+/H^+ antiporters that support alkali resistance by generating a pH gradient in which the cytoplasm is more acidic than the external medium (38, 46). However, neither kinetic properties of Mrp antiport nor a definitive in vitro demonstration of electrogenicity has been presented for any Mrp system. The standard fluorescence-based antiporter assays of these properties are usually conducted with everted (inside-out) membrane vesicles from antiporter-deficient *Escherichia coli* strains expressing the test antiporter (12, 52). Such assays of Mrp systems have thus far yielded signals that were too low for kinetic analyses or assays of transmembrane electrical potential (ΔΨ) consumption (17, 21, 33, 34).

Here, an optimized assay for secondary Mrp antiport in *E. coli* EP432 (Δ*nhaA* Δ*nhaB*) (47) facilitated a more detailed characterization of secondary Mrp antiport than has heretofore been possible. The Mrp systems chosen for study were from three gram-positive bacteria, *Bacillus pseudofirmus* OF4 Mrp (Mrp_{BpOF4}), *B. subtilis* Mrp (Mrp_{Bs}), and *S. aureus* Mnh. The study included a second *S. aureus* Mrp system (designated Mnh2), of unknown catalytic capacity or function, that is found in staphylococci. All four Mrp systems are "group 1" Mrp systems that are encoded by canonical seven-gene operons (*mrpA* to *mrpG*) (56). The substrates for Mnh2 were not identified, but results with the other three Mrp systems show that they catalyze secondary, electrogenic $Na^+(Li^+)/H^+$ antiport with a very low apparent K_m value for Na^+. In addition, experiments conducted here demonstrate that *E. coli* EP432 transformants expressing Mrp_{BpOF4} or Mnh from a plasmid exhibit a higher capacity to generate a transmembrane potential, the ΔΨ (inside negative relative to outside), than a control transformant. Plasmid-borne NhaA, the extensively studied electrogenic $Na^+(Li^+)/H^+$ antiporter of *E. coli* (18, 45), produced the same effect on ΔΨ generation. The host response to the presence of an electrogenic antiporter in multicopy is of interest because it mimics situations in which such antiporters are activated in their natural settings. The nature of the host response that results in increased ΔΨ generation in the current experimental system was therefore probed by assays of respiratory chain components in the *E. coli* EP432 transformant expressing Mrp_{BpOF4} versus a control.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The *E. coli* strains used in this study were DH5αMCR (Gibco-BRL), KNabc (Δ*nhaA* Δ*nhaB* Δ*chaA*) (43), and EP432 (Δ*nhaA* Δ*nhaB*) (47) (Table 1). The strains were grown routinely in LBK medium, pH 7.5 (12), at 37°C. The plasmids used in this study were pMW118 (Nippon Gene), pGEM-3Zf(+) (Promega), and the recombinant pGEM-3zf(+) derivatives containing Mrp_{Bs}, Mrp_{BpOF4}, Mnh, or Mnh2 (Table 1). The operons cloned in these four recombinant plasmids of pGEM-3Zf(+) contained the *B. subtilis mrp* operon, the *B. pseudofirmus* OF4 *mrp* operon, the *S. aureus mnh* operon, and the *S. aureus mnh*2 operon, respectively; the operons were cloned behind their own promoters. They were designated, respectively, Mrp_{Bs}, Mrp_{BpOF4}, Mnh, and Mnh2. For the construction of Mrp_{Bs} and Mrp_{BpOF4}, the starting material was a pair of recombinant plasmids constructed earlier, pMWBSMrp and pMWOFMrp, respectively, containing the full operons with

their promoters in the low-copy plasmid pMW118 (21). For isolation of Mrp_{Bs}, pMWBSMrp was digested with EcoRI and XbaI, and then the purified product, containing the *mrp* operon and its promoter region, was ligated into EcoRI- and XbaI-digested pGEM-3Zf(+) using Quick ligase (New England Biolabs). For isolation of Mrp_{BpOF4}, pMWOFMrp was digested with EcoRI and XmaI, and then the purified product, containing the *mrp* operon and its putative promoter region, was ligated into EcoRI- and XmaI-digested pGEM-3Zf(+) using Quick ligase. For construction of a recombinant plasmid bearing *S. aureus* Mnh, PCR was performed on *S. aureus* RF4220 chromosomal DNA with primers SaMnh1-F-NheI and SaMnh1-R-XhoI. SaMnh1-F-NheI (5′-GCT AGC TTG TTA CAT ATT GCG GTG-3′) corresponded to the sequence of the database entry GenBank accession no. DQ659238 and additional nucleotides containing an NheI site at the 5′ end of the sequence. SaMnh1-R-XhoI (5′-CTC GAG TTT TGT GTC TTT TAA GTC TTC CG-3′) corresponded to the complementary sequence of the database entry GenBank accession no. DQ659238 and additional nucleotides containing an XhoI site at the 5′ end of the sequence. The PCR product was ligated into SmaI-digested pMW118. After sequence confirmation, the recombinant plasmid was digested with EcoRI and HindIII and ligated into EcoRI- and HindIII-digested pGEM-3Zf(+). For construction of pGEMSaMnh2, PCR was performed on *S. aureus* RF4220 chromosomal DNA with primers SaMnh2-F-XbaI and SaMnh2-R-XhoI. SaMnh2-F-XbaI (5′-TCT AGA AAC AAA GGA GGC TAA TAA TGA GTT TGG-3′) corresponded to the sequence of the database entry GenBank accession no. DQ659239 and additional nucleotides containing an XbaI site at the 5′ end of the sequence. SaMnh2-R-XhoI (5′-CTC GAG ATC GTT TTG ATA CCA TTT CTT ACG-3′) corresponded to the complementary sequence of the database entry GenBank accession no. DQ659239 and additional nucleotides containing an XhoI site at the 5′ end of the sequence. The PCR product was ligated into SmaI-digested pMW118. After sequence confirmation, the recombinant plasmid was digested with EcoRI and HindIII and ligated in EcoRI- and HindIII-digested pGEM-3Zf(+). 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. The plasmids were transformed into *E. coli* KNabc and EP432 for use in the experiments. Plasmid preparations made from those parallel transformants indicated that the transformant with pGEM*Bs*Mrp consistently had a lower level of plasmid than transformants with the other three *mrp*-bearing plasmids. Therefore, direct quantitative comparisons of the total activity or complementation capacities of Mrp_{Bs} with those of the other systems could not be made.

Preparation of everted membrane vesicles. Everted membrane vesicles were prepared by breaking cells with a French Pressure cell as described by others (1, 52); the buffer used in the preparations was 10 mM bis-[tris(hydroxymethyl)-methylamino]-propane (BTP) (pH 7.5), 10% glycerol, a protease inhibitor tablet (Roche), and 1 mM phenylmethylsulfonyl fluoride for the assays of ΔΨ generation. For the antiport assays, the buffer used to prepare everted membrane vesicles was 10 mM Tris-HCl (pH 7.5) containing 140 mM choline chloride, 0.5 mM dithiothreitol, 10% glycerol, a protease inhibitor tablet (Roche), and 1 mM phenylmethylsulfonyl fluoride. For storage, glycerol (to 10%, vol/vol) was added to all everted membrane vesicles, and the vesicles were shock frozen in liquid nitrogen and then stored at −80°C. Protein content was measured by the method of Lowry et al., using lysozyme as the standard (37).

Assays of ΔpH-dependent antiport activity. Antiport assays were conducted in 10 mM BTP-sulfate, 140 mM choline-Cl, 5 mM $MgSO_4$, and 1 μM acridine orange at pH 7.0 to 9.0. Measurements were conducted using a Perkin-Elmer LS50B luminescence spectrometer with excitation at 420 nm (10-mm slit) and emission at 500 nm (10-mm slit). Respiration was initiated by the addition of Tris-succinate to a final concentration of 2.5 mM. The high chloride content ensured that the Δp established by the addition of the electron donor was entirely in the form of a ΔpH, acid inside the everted vesicles. Establishment of this ΔpH was monitored by quenching of acridine orange fluorescence. Dequenching of fluorescence, which results from subsequent cation addition, reflected cation-dependent proton movement out of the everted vesicles (12). Addition of 10 mM ammonium chloride was used to dissipate the remaining Δp to bring the fluorescence back to baseline. The concentration of cation yielding the half-maximal dequenching has been validated as a good estimate of the apparent K_m of monovalent cation/H^+ antiporters (51, 61).

Fluorescence-based assays of ΔΨ generation and antiporter-dependent consumption. ΔΨ-dependent fluorescence of oxonol VI was used to measure the generation of a positive inside potential with the addition of 1 mM NADH. Evaluation of electrogenicity was conducted by adding 10 mM NaCl to energized membranes and observing a reversal of the quench. The reversal of quenching represents antiport-dependent consumption of the ΔΨ, which reflects the activity of an electrogenic antiport that can be energized by, and thus partially dissipate, ΔΨ (46). Addition of 10 μM carbonyl cyanide *m*-chlorophenylhydrazone, a protonophore that can abolish the ΔΨ, was used to bring the fluorescence back to baseline. The assay mixture contained 10 mM BTP, 5 mM $MgSO_4$, 200 mM K_2SO_4, 1 μM nigericin (pH 7.5), and 1 μM oxonol VI. Measurements were conducted on a Perkin-Elmer LS50B luminescence spectrometer. The excitation wavelength was set at 580 nm with a 10-mm slit, and emission was at 631 nm with a 10-mm slit (64). The final concentration of vesicle protein was 200 μg/ml.

Assays of respiratory chain activities in everted membrane vesicles. Succinate dehydrogenase assays were carried out according to the method of Hatefi and Stiggall (15) by phenazine methosulfate-mediated bleaching of the electron acceptor 2,6-dichloroinophenol (DCPIP), using a Shimadzu UV-1601 UV-visible spectrophotometer. This approach was also used for assays of malate:quinone oxidoreductase as recently described (57). Assays were conducted at room temperature using 100 μg of vesicle protein in 1 ml of 10 mM BTP-sulfate and 5 mM $MgSO_4$ (pH 7.5). NADH oxidase assays were carried out using the standard method of monitoring A_{340} over time in the presence of 1 mM NADH (66). The NADH-$K_3Fe(CN)_6$ reductase activity was measured at 420 nm with added 10 mM KCN, 1 mM NADH, and 1 mM $K_3Fe(CN)_6$ as described previously (25). The extinction coefficients used for activity calculations were $\varepsilon_{340} = 6.22\ mM^{-1}\ cm^{-1}$ for NADH and deamino-NADH (d-NADH) (reduced nicotinamide hypoxanthine dinucleotide), $\varepsilon_{420} = 1.00\ mM^{-1}\ cm^{-1}$ for $K_3Fe(CN)_6$, and $\varepsilon_{600} = 2\ mM^{-1}\ cm^{-1}$ for DCPIP (2). The NADH analogue d-NADH is a substrate for the proton-pumping Nuo complex but not for the non-proton-pumping Ndh NADH dehydrogenase of *E. coli* (42).

Cytochrome spectra. Absorption spectra were recorded at room temperature with a Perkin-Elmer 550 dual-beam spectrophotometer. Scans were performed at 2 nm/s with a slit width of 2 nm. Samples containing 2 mg/ml of membrane protein in 50 mM Tricine–NaOH (pH 8)–0.1% lauryl maltoside were evaluated. A baseline correction was obtained, and then reduced-minus-air-oxidized difference spectra were obtained by addition of a small amount of sodium dithionite and incubation for 1 min. The following wavelength pairs and millimolar extinction coefficients were used: cytochrome *b*, $\Delta A_{560-575}$ and $\Delta\varepsilon = 17.5$; cytochrome *d*, $\Delta A_{622-650}$ and $\Delta\varepsilon = 18.8$ (16, 28, 49).

RNA preparation and real-time RT-PCR. Cells were grown overnight, and total RNA was isolated after cell lysis with glass beads (0.5 mm) using the RNeasy minikit (QIAGEN). RNA (1 μg) was used for reverse transcription in a reaction mixture of 20 μl. Real-time PCR was performed in a LightCycler (Roche) with a QuantiTect SYBR green reverse transcriptase (RT-PCR) kit (QIAGEN). Primers were designed using the LC probe design software and selected based on an amplicon size of 100 bp and a melting temperature at 55°C. The two genes studied were the succinate dehydrogenase gene (*sdh*) and the 16S rRNA gene (*rrsA*). The primer sequences used were the following: *rrsA* forward, 5′-AGAGATGAGAATGTGC-3′; *rrsA* reverse, 5′-CACTTTATGAGGTCCG-3′; *sdh* forward, 5′-TGCTGAACAAACATGG-3′; and *sdh* reverse, 5′-GTGAC AGGTTGGGATA-3′. Reverse transcription was carried out at 50°C for 30 min, and then amplification conditions were as follows: an initial denaturation step for 15 min at 94°C and then 45 cycles of denaturation at 94°C (15 s), annealing at 55°C (25 s), and elongation at 72°C (10 sec). Melting curve analysis was performed to check for a single amplicon and the absence of primer-dimers. LightCycler analysis software was used for determining crossing points. Data were analyzed by the $2^{-\Delta\Delta C_T}$ method and are presented as fold induction/repression of *sdh*, normalized to *rrsA* levels (36).

RESULTS AND DISCUSSION

Mrp_{Bs}, Mrp_{BpOF4}, and Mnh complement the Na^+ and alkali sensitivities of *E. coli* EP432. The Na^+-sensitive *E. coli* strain EP432, harboring the plasmid control, is highly sensitive to growth inhibition by NaCl concentrations of 200 mM and above (Fig. 1A). The *E. coli* EP432-Mnh transformant exhibited marked Na^+ resistance at up to 400 mM, with the Mrp_{BpOF4} transformant supporting resistance at up to 600 mM. The Mnh2 transformant did not confer Na^+ resistance (Fig. 1A). The *B. subtilis* Mrp complemented to a lesser extent, which perhaps is related to the lower levels of plasmid in this transformant (see Materials and Methods). All four Mrp systems, including the Mnh2 transformant, supported a high growth yield compared to the control transformant at pHs of up to 9.5 in the absence of added Na^+ (Fig. 1B).

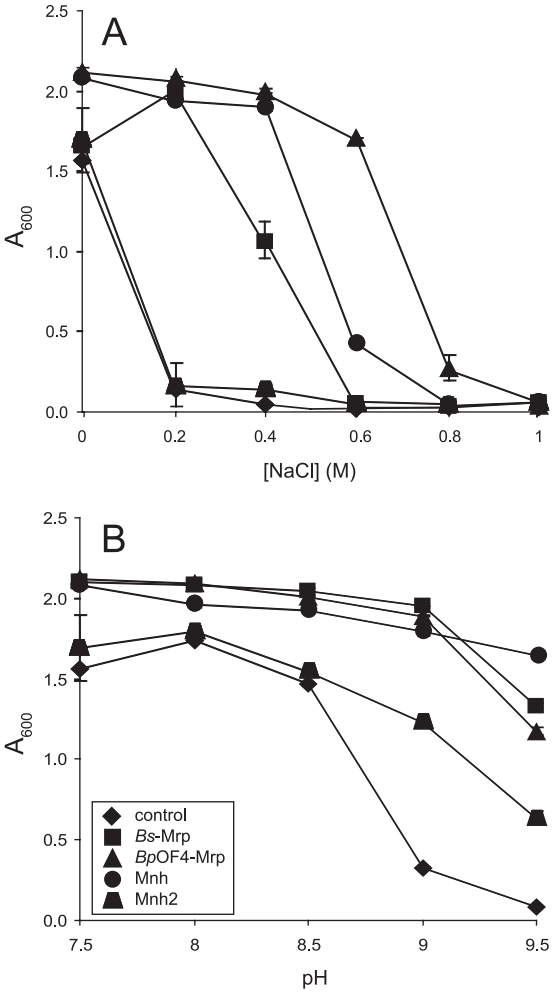

FIG. 1. Effect of NaCl concentration and pH on the growth of *E. coli* EP432 transformants of Mrp-Mnh antiporters. (A) Transformants with empty vector [pGEM-3Zf(+)], or expressing the Mrp operons from *B. subtilis*, *B. pseudofirmus* OF4, or *S. aureus* were grown on LBK medium, pH 7.5, containing added NaCl at the indicated concentrations. (B) LBK with no added NaCl was adjusted to the pH values indicated. The LBK contained approximately 12 mM contaminating Na^+ (62). Cells were grown overnight for 16 h at 37°C, with shaking, after which the A_{600} of the cultures was measured. The error bars indicate standard deviations from duplicate cultures in three independent experiments.

Mrp_{Bs}, Mrp_{BpOF4}, and Mnh catalyze secondary $Na^+(Li^+)/H^+$ antiport with a low apparent K_m for Na^+ and an alkaline pH optimum. Previous in vitro studies of Mrp-dependent antiport activity via the widely used fluorescence assay (12) employed everted vesicles from the triple antiporter mutant *E. coli* KNabc (43) transformed with a low-copy-number plasmid expressing the test operons. In this assay, energization of everted membrane vesicles by addition of an electron donor results in development of a ΔpH, acid in, in the everted vesicles. The ΔpH is monitored by quenching of acridine orange fluorescence until steady state is reached. Antiport is then assessed by the percent dequenching resulting from addition of the monovalent cation substrate for antiport. In the earlier Mrp assays conducted with vesicles of *E. coli* KNabc by our group and others, the percent dequenching that reflected antiport activity was ≤6% dequenching for Mrp_{Bs}/Sha (21, 34), Mrp_{BpOF4} (21), and Mnh (17). Attempts to achieve significantly higher signals for Mrp-dependent antiport in this triple mutant were made using different vectors and induction protocols, but these were without success because high expression of the Mrp antiporters led to poor growth or poor vesicle quality (data not shown). Further attempts to empirically optimize the fluorescence assay for Mrp systems were therefore mounted in the less impaired double mutant *E. coli* EP432. This strain differs from *E. coli* KNabc in retaining a functional *chaA* gene that encodes an $Na^+(Ca^{2+})(K^+)/H^+$ antiporter (23, 50). The background activity in membranes from this strain either can be abolished by the addition of KCl to the assay buffer for antiporters that do not use K^+ as a substrate or is small enough to be subtracted from the dequenching observed in assays with other substrates (Table 2); in the current studies, any small background activity was subtracted. In addition to using *E. coli* EP432, the new antiport assay protocol used the pGEM-3Zf(+) vector instead of the lower-copy-number vector pMW118 and used BTP in the assay buffer instead of Tris, which had been included in our earlier assays (21). The new protocol yielded larger signals for Mrp-dependent activity, i.e., percent dequenching of as high as 40 to 50% (Table 2).

Assays were first performed to characterize the cation specificity of secondary Mrp-dependent antiport. Initial experiments were conducted at pH 8.5 in anticipation of an alkaline optimum for these antiporters. Mrp_{BpOF4} and Mnh exhibited antiport in the presence of test cations Na^+ and Li^+ (2.5 mM) at pH 8.5 (Table 2). Mrp_{Bs} also exhibited significant Na^+- and Li^+-dependent antiport, although the levels were lower than those observed with Mrp_{BpOF4} and Mnh. Significant K^+/H^+ antiport was not observed with any of the three antiporters using a range of K^+ concentrations of from 2 to 400 mM (Table 2 and data not shown). The possibility of modest Mrp-dependent K^+/H^+ antiport activity had been raised by physiological experiments on *B. subtilis* Mrp (19), and the assay data here similarly show a hint but certainly not highly significant levels of such activity, even though some other Mrp systems

TABLE 2. Assays of monovalent cation/proton antiport activity at pH 8.5 in everted membrane vesicles of Mrp-expressing *E. coli* EP432 transformants

Transformant	% Dequenching observed upon addition of[a]:		
	Na^+	Li^+	K^+
Control	2.6 ± 0.7	1.1 ± 0.0	2.6 ± 1.2
Mrp_{Bs}	17.3 ± 2.6	20.3 ± 0.9	4.3 ± 0.5
Mrp_{BpOF4}	37.2 ± 0.8	31.5 ± 1.4	2.6 ± 1.1
Mnh	50.7 ± 0.6	46.7 ± 1.3	1.8 ± 0.5
Mnh2	0	0	0

[a] Vesicles from transformants expressing empty vector [pGEM-3zf(+)] and the Mrp operons from *B. subtilis*, *B. pseudofirmus* OF4, and *S. aureus* (Mnh and Mnh2) were assayed in 2 ml containing 50 mM BTP-sulfate buffer, 140 mM choline chloride, 5 mM $MgCl_2$, 1 μM acridine orange, and 66 μg of vesicle protein. Respiration was initiated by the addition of Tris-succinate to a final concentration of 2.5 mM. After steady-state fluorescence quenching was reached, NaCl, LiCl, or KCl was added to a final concentration of 2.5 mM. The values presented for the subsequent percent dequenching are from duplicate assays from three independent experiments. The percentages represent the average values of the calculated percent dequenching and are shown with the standard deviation of the values.

(e.g., the Pha1 antiporter of *S. meliloti*) are active K^+/H^+ antiporters (48). Neither Mg^{2+}- nor Ca^{2+}-dependent antiport was observed for any of the four antiporters in assays conducted in buffers without added Mg^{2+} (not shown).

Mnh2 from *S. aureus*, for which no earlier studies had been reported, exhibited no activity with any test cation at 2.5 mM at pH 8.5 (Table 2). Mnh2 also did not exhibit antiport activity at pH 8.5 with any of the monovalent cations when added at concentrations of up to 200 mM at a range of pH values of from 7.0 to 9.0. Finally, assays of Mnh2 were conducted in the absence of Mg^{2+} to test the possibility that this system has cation/proton activity that is inhibited by Mg^{2+}. These assays were also negative (not shown). It was interesting that the low background level observed in control vesicles was suppressed in the Mnh2-expressing vesicles. This is consistent with an Mnh2 antiporter whose activity during growth repressed the modest background antiport in the heterologous host. Mnh2 may use an unusual efflux substrate and perhaps may require a cosubstrate on the external side to couple with the H^+ uptake that is inferred for Mnh2 by its capacity to complement alkali sensitivity in *E. coli* EP432 (e.g., a citric-malic or malic-lactate type of exchange). The distinct subset of second Mrp-type systems of which Mnh2 is a member is found only in *Staphylococcus* species (56), so Mnh2 may play a role that is specific to the ecology-metabolism interplay of this pathogen.

The Na^+/H^+ antiport of the three active Mrp antiporters was examined over a range of concentrations of added NaCl. Michaelis-Menten kinetics were observed (Fig. 2). The linear regression of a double-reciprocal plot (Fig. 2, inset) allowed calculation of apparent K_m values at pH 8.5 (Fig. 2). Although the level of Na^+-dependent Mrp_{Bs} antiport was lower than that of the other two Mrp systems, apparent K_m values were of a comparable order of magnitude for all three antiporters at pH 8.5, in the range of 0.06 to 0.12 mM for Na^+ (Fig. 2). These low apparent K_m values for Na^+ (Fig. 2) are within the range of the lowest reported for a prokaryotic monovalent cation/H^+ antiporter (9, 61). This property could facilitate acidification of cytoplasmic pH even at low concentrations of Na^+ (20, 22).

The activity profile of Mrp_{BpOF4} as a function of pH indicated an optimum for Na^+/H^+ antiport between pH 8.0 and 9.0, with greatly reduced activity at pH 7.5 and no activity at pH 7.0 and below (Fig. 3). This profile is consistent with the inability of *B. pseudofirmus* OF4 to grow nonfermentatively at pH 7 (55) and its ability to maintain a cytoplasmic pH of around 8.2 while growing at pH 10.5 (55, 68). By contrast, Mnh exhibited Na^+/H^+ antiport activity over a broader range of pH values, from pH 7.0 through pH 9.0; *S. aureus* exhibits strong Na^+ and alkali resistance that is consistent with this profile (63, 65). Significant Mrp_{Bs}-dependent Na^+/H^+ antiport, albeit less active than the others, was observed at between pH 7.5 and 9.0, with retention of very modest activity relative to the control membranes at pH 7.0 but not 6.5 (Fig. 3). It is notable that a *mrp* null mutant of *B. subtilis* is profoundly sensitive to growth inhibition by Na^+ at pH 7.0, probably accounting for the listing of *mrp* genes among essential genes in experiments conducted in LB medium which contains added Na^+ (20, 30).

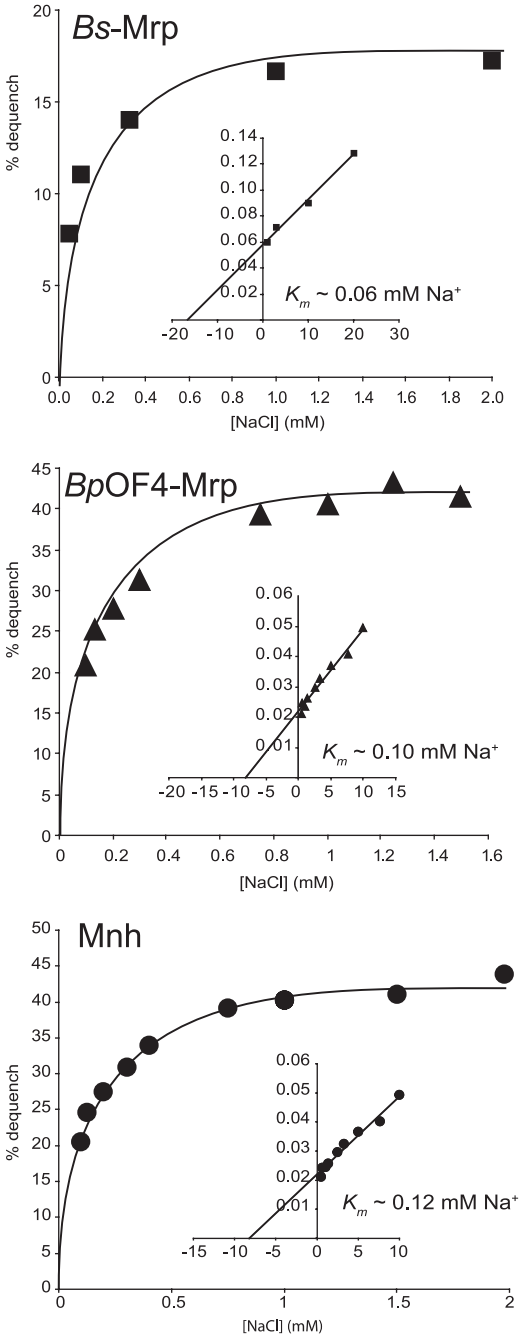

FIG. 2. Na^+/H^+ antiport activity of Mrp antiporters as a function of cation concentration at pH 8.5. Fluorescence-based assays of the Na^+/H^+ antiport activities of the Mrp antiporters in *E. coli* EP432 vesicles were conducted at pH 8.5 over a range of concentrations of added NaCl; the Michaelis-Menten plot is shown, and a reciprocal plot is shown as an inset in each panel. The assay protocol was identical to that described in Table 2, footnote *a*. The standard deviations of the values, which are derived from duplicate assays in at least three independent experiments, were less than 10% of the mean values.

Mrp-dependent Na^+/H^+ antiport is electrogenic. Secondary antiporters that catalyze net H^+ accumulation in support of alkaline pH homeostasis are expected to be electrogenic (46), catalyzing an Na^+/H^+ exchange in which the inward H^+ flux is greater than the outward Na^+ flux during a single turnover. During such an electrogenic exchange, net positive charge is translocated inward. The antiport can be energized by the transmembrane electrical potential, i.e., the $\Delta\Psi$ (negative in-

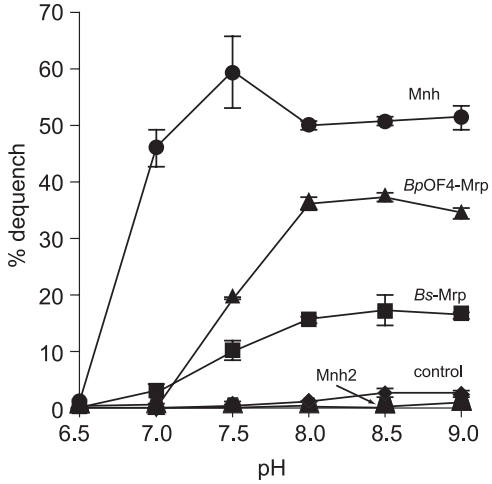

FIG. 3. Na^+/H^+ antiport activity of Mrp antiporters as a function of pH. The assay protocol was as described in the legend to Fig. 2, with the pH of the buffers adjusted to the values indicated. The data shown are for assays with 2.5 mM NaCl. The error bars indicate standard deviations from duplicate assays from three independent experiments.

side in whole cells), thus partially dissipating $\Delta\Psi$ during antiport. Mrp_{Bs} antiport was previously inferred to be electrogenic from whole-cell experiments (19, 20), and its lower level of activity than the Mrp_{Bp} and Mnh in the current study led us to test only Mrp_{BpOF4} and Mnh antiport for electrogenicity in the in vitro assay. The fluorescent $\Delta\Psi$ probe oxonol VI was used; this probe monitors the development of the $\Delta\Psi$, positive inside, that is established in the everted vesicle system upon addition of an electron donor to the respiratory chain. NADH was added to energize the vesicles under conditions in which the presence of nigericin prevents development of a transmembrane pH gradient. The resulting quench in oxonol fluorescence indicates the formation of the $\Delta\Psi$. After the $\Delta\Psi$ reaches steady state, NaCl is added to evaluate whether a reversal of the fluorescence quench occurs in the Mrp-expressing vesicles. Such dequenching monitors Na^+- and Mrp-dependent consumption of the $\Delta\Psi$, i.e., electrogenic Na^+/H^+ antiport, and was observed for membranes containing either Mrp_{BpOF4} or Mnh (Fig. 4).

Mrp_{BpOF4} and Mnh increase $\Delta\Psi$ generation by the *E. coli* EP432 host, as does plasmid-borne NhaA, an electrogenic *E. coli* antiporter. In the fluorescence-based assays of electrogenicity, it was observed that both Mrp_{BpOF4} and Mnh membranes consistently exhibit a larger initial NADH-dependent oxonol quench than the control membranes (see the magnitude of the middle and right traces shown in Fig. 4 compared to that of the control on the left during the interval between NADH addition and addition of NaCl). This indicates that membranes from these two Mrp-expressing transformants of *E. coli* EP432 cells produce a greater respiration-dependent $\Delta\Psi$ than the control transformant. What might account for a greater capacity of the Mrp_{BpOF4} and Mnh vesicles of *E. coli* EP432 to generate a $\Delta\Psi$ than the control vesicles? The cells were all grown under the same conditions. The variable is the presence of electrogenic antiporters. These antiporters would have been very active under the conditions of growth, since the antiporters are expressed from multicopy plasmids, further alkalinization of the pH 7.5 medium occurs during growth (8), and there is sufficient contaminating Na^+ to support Mrp_{BpOF4}- and Mnh-dependent antiport. We hypothesized that the following scenario leads to a higher capacity for $\Delta\Psi$ generation under these conditions: (i) the high activity of $\Delta\Psi$-consuming antiporters depletes the $\Delta\Psi$; (ii) the $\Delta\Psi$ depletion elicits a host response that leads to a compensatory increase in the capacity of the host to generate $\Delta\Psi$, i.e., by increasing levels of key respiratory chain components and/or enzymes that donate electrons to the respiratory chain; and (iii) the cell thus adapts to the high level of antiport activity without compromising other $\Delta\Psi$-dependent functions. Host responses of this type could be mediated by two-component signaling or other regulatory elements that respond directly to reduced $\Delta\Psi$ or reduced total Δp. Alternatively, they could respond to a change in the redox poise of the quinone, NADH-NAD^+, or flavin pools that would be secondary to an increase in the respiratory rate that is expected to occur as the $\Delta\Psi$ is consumed. There are examples of regulatory elements that mediate responses to each of these signals in bacteria (5, 7, 10, 13, 39, 60).

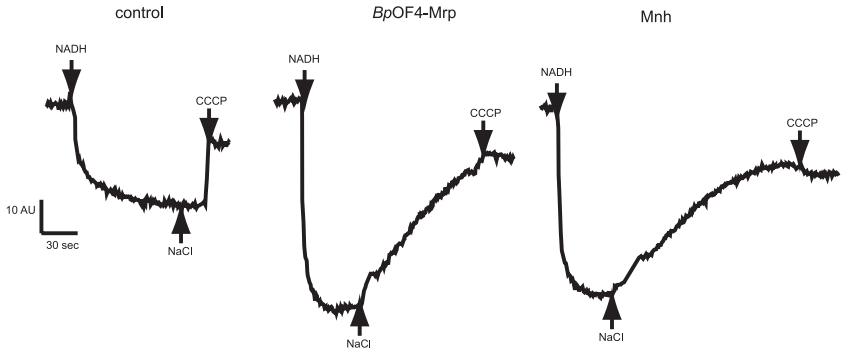

FIG. 4. Electrogenicity of Mrp-dependent Na^+/H^+ antiport. Fluorescence-based assays of everted membrane vesicles from the control, Mrp_{BpOF4}, and Mnh transformants of *E. coli* EP432 were performed in reaction mixtures containing 200 μg vesicle protein/ml in a total of 2 ml. The reaction mixtures contained 50 mM BTP-sulfate, 5 mM $MgSO_4$, 200 mM K_2SO_4, and 1 μM nigericin at pH 7.5, plus 1 μM oxonol VI. To initiate respiration, 1 mM Tris-NADH was added at the first arrow. Once the quenching achieved steady state, 10 mM NaCl was added at the second arrow. The final arrow indicates the addition of 10 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). The traces shown are representative of at least three independent experiments.

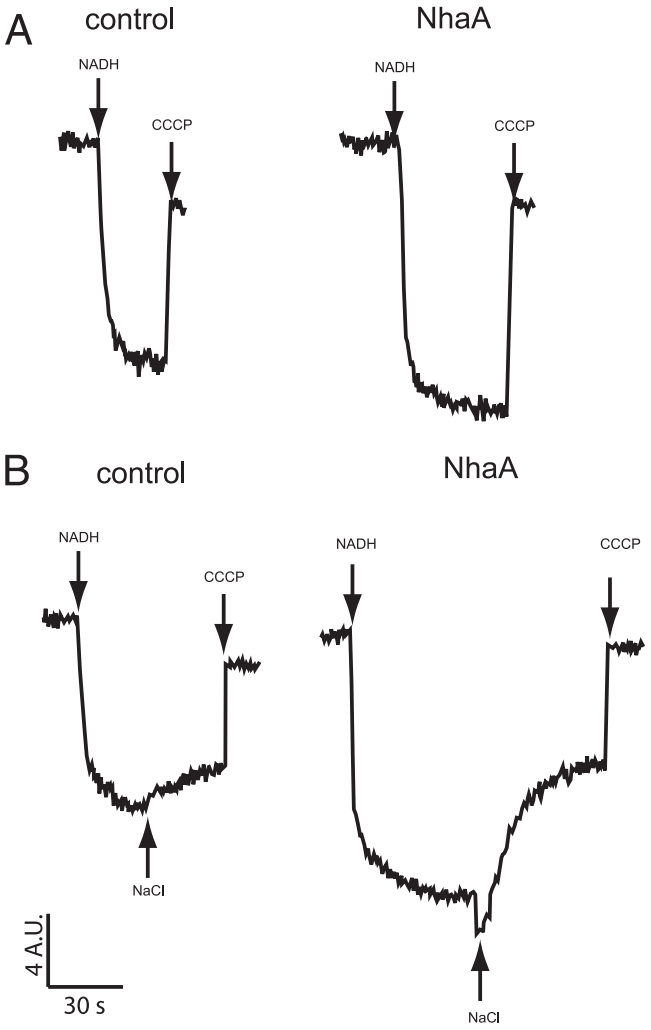

FIG. 5. NhaA-dependent effects on $\Delta\Psi$ generation and Na^+-dependent $\Delta\Psi$ consumption. Fluorescence-based assays of everted membrane vesicles from control and NhaA transformants of *E. coli* EP432 were performed exactly as described in the legend to Fig. 4 except that the vector was pBR322 instead of pGEM3Z(f+) and only $\Delta\Psi$ generation was assessed in the experiments shown in panel A, whereas both $\Delta\Psi$ generation and Na^+-dependent $\Delta\Psi$ consumption were assessed in those shown in panel B. The traces shown are representative of at least three independent experiments. CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

A major prediction of this hypothesis is that an effect similar to that of Mrp antiporters on the capacity of *E. coli* EP432 to generate $\Delta\Psi$ should be observed if a native electrogenic $Na^+(Li^+)/H^+$ antiporter is expressed from a plasmid in *E. coli* EP432 under the same growth conditions. NhaA was chosen because it is well established to be a secondary electrogenic antiporter that is a single gene product with a role in Na^+ resistance at high pH (12, 59) and is structurally unrelated to Mrp-Mnh (6). The recombinant and control plasmids chosen for the NhaA experiments were those that have been used extensively in the characterization of wild-type and mutant NhaA activities in *E. coli* EP432 (26). The vector, pBR322, was a lower-copy-number plasmid than the pGEM-3Zf(+) used for the Mrp assays. Nonetheless, as shown in Fig. 5A, membranes containing NhaA generate a $\Delta\Psi$ that is distinctly and consistently greater than that pf the control; the $\Delta\Psi$ was an average of 20% greater in the NhaA-containing membranes than in control membranes in assays of three separate membrane preparations. Upon addition of Na^+, the $\Delta\Psi$-consuming nature of NhaA-mediated electrogenic antiport was also evident (Fig. 5B).

Respiratory chain components are elevated in the Mrp_{BpOF4} and Mnh transformants of *E. coli* EP432. We next explored the hypothesized increase in activity and expression of respiratory chain components in Mrp_{BpOF4} and Mnh vesicles versus control vesicles via enzymatic and spectral assays. NADH oxidation was first assayed by measurements of the decrease in A_{340} upon addition of NADH to oxygenated vesicles. This measured total respiratory chain activity from NADH oxidation through the terminal oxidases. NADH oxidation rates were significantly higher in both Mrp_{BpOF4} and Mnh vesicles than in control vesicles (Table 3). NADH:ferricyanide reductase activity was then measured by addition of NADH or d-NADH, with ferricyanide reduction monitored via changes in A_{420}; cyanide was present to inhibit the terminal oxidases. NADH-mediated activity assesses the total NADH dehydrogenase activity, i.e., activity of the proton-translocating Nuo complex plus the additional Ndh that does not pump protons (42), whereas d-NADH-mediated activity assesses only the proton-translocating Nuo complex I. No statistically significant difference in these two assay results was observed between the Mrp-containing membranes and control membranes in the average of four independent preparations (Table 3); however, it was noted that in each separate membrane preparation there was a 1.5-fold-higher activity of both NADH:ferricyanide and d-NADH:ferricyanide activity in the Mrp_{BpOF4} and Mnh membranes relative to the control. A marked increase in succinate dehy-

TABLE 3. Effect of *mrp-mnh*-bearing plasmid on respiratory enzyme activities and cytochrome contents of the heterologous *E. coli* EP432 host cell

Prepn	Activity[a]					Fold increase[a]	
	NADH oxidase (nmol NADH/mg protein/min)	NADH:ferricyanide (nmol NADH/mg protein/min)	d-NADH:ferricyanide (nmol d-NADH/mg protein/min)	Succinate dehydrogenase (nmol succinate/mg protein/min)	Malate:quinone oxidoreductase (nmol L-malate/mg protein/min)	Cytochrome *b* content	Cytochrome *d* content
Control	226 ± 81	1,097 ± 323	629 ± 65	113 ± 113	0.2 ± 0.1	1.0	1.0
Mrp_{BpOF4}	500 ± 81***	1,258 ± 81	677 ± 81	1,387 ± 806***	0.2 ± 0.3	3.1 ± 1.5	3.0 ± 1.7
Mnh	484 ± 97***	1,177 ± 129	661 ± 210	1,210 ± 968**	0.2 ± 0.1	3.5 ± 1.2**	3.3 ± 1.0**

[a] **, $P > 0.05$ using a two-tailed Student *t* test; ***, $P > 0.005$ using a two-tailed Student *t* test. Values are means and standard deviations.

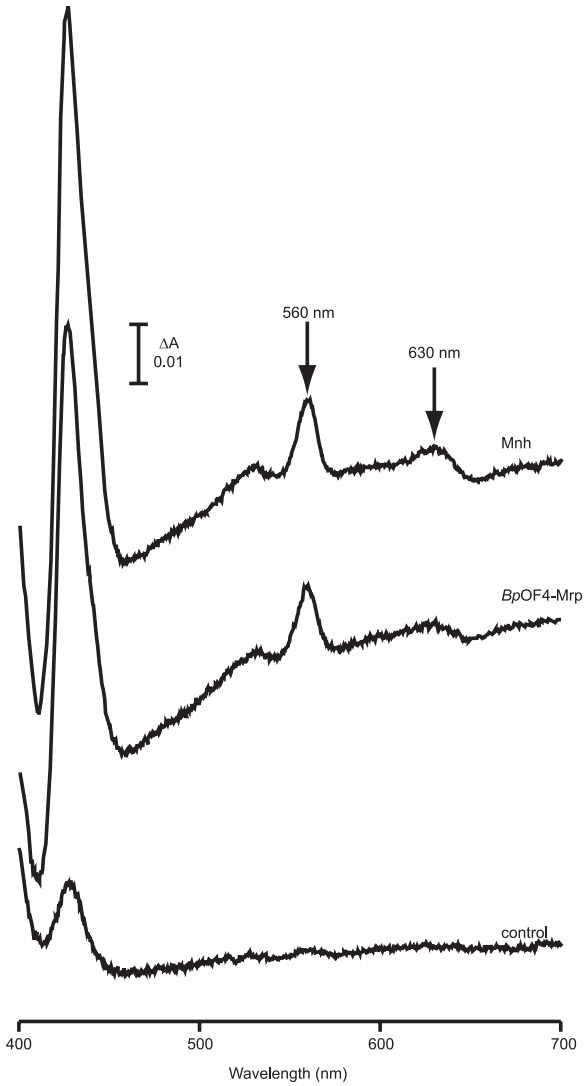

FIG. 6. Sodium dithionite reduced-minus-air-oxidized spectra of everted membrane vesicles of control or Mrp-Mnh-expressing *E. coli* EP432. Everted membrane vesicles (2 mg/ml) were suspended in 50 mM Tricine (pH 8.0) containing 0.1% dodecyl maltoside. Dithionite was added, and spectra were recorded after 1 min of incubation at room temperature. The traces shown are representative of at least three independent experiments. The peak at 560 nm corresponds to cytochrome *b*, and the peak at 630 nm corresponds to cytochrome *d*.

drogenase activity was found in the Mrp_{BpOF4} and Mnh membranes relative to the control using the DCPIP reduction assay. By contrast, no activity was detected for malate:quinone oxidoreductase (Table 3), whose activity had earlier been shown to be increased in an Mrp_{BpOF4}-dependent manner in the respiratory-deficient mutant *E. coli* ANN0222 (57). The cytochrome content of the membrane vesicle preparations was evaluated from dithionite reduced-minus-oxidized absorption spectra. Both Mrp_{BpOF4} and Mnh membranes showed a threefold increase in the cytochrome *b* peak at 560 nm and a threefold increase in the cytochrome *d* peak at 630 nm relative to control membranes (Fig. 6 and Table 3). Since one of the elevated cytochromes, cytochrome *d*, is part of the terminal oxidase complement, it is likely that the overall increase in respiratory chain activity from NADH to oxygen (Table 3) involves increases in the levels of the terminal oxidases; the increased cytochrome *b* could correspond to cytochromes associated with both succinate dehydrogenase and the terminal oxidases, cytochromes *bd* and *bo*.

To specifically assess whether an increase in transcription of respiratory chain components is part of the host cell response to the presence of an active Mrp system, the succinate dehydrogenase locus (*sdh*) was chosen because of the large overall increase in its enzymatic activity (Table 3). RT-PCR measurements were conducted on *sdh* in control cells and in cells expressing either Mrp_{BpOF4} or Mnh, as described in Materials and Methods. The results of three independent experiments showed that the average level of *sdh* mRNA is threefold higher in cells expressing Mrp_{BpOF4} and Mnh than in control cells, when normalized to levels of 16S rRNA *rrsA* (data not shown). The assays demonstrate that transformants of *E. coli* EP432 expressing Mrp_{BpOF4} or Mnh from a multicopy plasmid express and incorporate higher levels of respiratory chain components into their membranes than a control transformant during growth on media that support high Mrp activity. The electrogenic properties shown for Mrp antiporters in this study lead to the expectation that these antiporters will have a significant $\Delta\Psi$-consuming effect when active and present in multicopy. It is a plausible hypothesis, therefore, that it is this antiporter-mediated $\Delta\Psi$ depletion that elicits a host response that counters the $\Delta\Psi$ depletion by enhancing the host capacity for $\Delta\Psi$ generation.

Is it also plausible that analogous $\Delta\Psi$ depletion and compensation are part of the normal physiologic adaptation to an increase in pH and/or Na^+ in which a major chromosomally encoded antiporter is activated? Several observations are consistent with this scenario in *E. coli*. First, *E. coli* with a wild-type complement of antiporters exhibits an alkali-sensitive phenotype in Na^+-containing media upon deletion of the ArcAB two-component system (69). ArcAB responds to the redox poise of the quinone pool and derepresses respiratory components, including *sdh* and *cyd*, when the pool is relatively oxidized (10, 39); this could occur as $\Delta\Psi$ depletion by activated NhaA accelerates respiration, leading to a more oxidized quinone pool. Second, in *E. coli* that is wild type for two-component systems, alkaline pH is lethal for *E. coli* expressing a mutant *nhaA* that has very high Na^+/H^+ antiport activity across a broad pH spectrum, i.e., has lost a characteristic feature whereby its own activity is down-modulated as a function of pH (51). Padan and colleagues have suggested that $\Delta\Psi$ depletion that exceeds the cell's capacity for compensation is the basis for the alkali-lethal phenotype. The reduced ΔpH at high pH lowers the total Δp and thus unregulated $\Delta\Psi$ depletion would be especially detrimental (46, 51). In *S. aureus* cells, too, there is an observation that best fits a scenario in which Mnh activity leads to a host response to the threat of $\Delta\Psi$ depletion, i.e., a host response that increases the $\Delta\Psi$. Bayer et al. (3) observed that the $\Delta\Psi$ generated by wild-type *S. aureus* in an Na^+-replete medium is larger than that of a mutant with a disrupted *mnh* locus. They suggested that Mnh itself functions as a $\Delta\Psi$-generating NADH dehydrogenase rather than as a secondary, $\Delta\Psi$-consuming antiporter, thus directly accounting for the greater $\Delta\Psi$. However, Mnh and Mrp have been found to be devoid of NADH dehydrogenase activity them-

selves (14, 17, 21, 57). This is expected, since they lack an apparent NADH binding site or flavin motifs that are usually found in all types of NADH dehydrogenases. We cannot rule out the possibility that in the natural host, Mnh recruits a cytoplasmic module containing the requisite redox centers and NADH binding site to transform into a primary $\Delta\Psi$-generating element as modeled by Bayer et al. (3). However, our data here show that a bona fide secondary, electrogenic antiporter, NhaA, increases the $\Delta\Psi$-generating capacity in a manner similar to that of Mnh and Mrp. Moreover, succinate dehydrogenase activity increases most substantially, not NADH dehydrogenase activity. In an earlier study, $Mrp_{Bp\mathrm{OF4}}$ was shown to increase $\Delta\Psi$ generation in an *E. coli* strain lacking both NADH dehydrogenases; Mrp did not restore NADH dehydrogenase activity but led to a large increase in malate:quinone oxidoreductase (57). We thus consider it likely that in both natural and heterologous settings, activated (and/or overexpressed) electrogenic antiporters elicit responses to $\Delta\Psi$ depletion that lead to gene expression changes. The net result is maintenance of, or even an increase in, $\Delta\Psi$ under conditions in which the antiporters are most active. It should be possible to test this hypothesis of a "systems" response to $\Delta\Psi$ depletion in *S. aureus* and alkaliphilic *Bacillus pseudofirmus* OF4 and, if it is supported, to identify the signaling path(s) involved.

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