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mrp, a Multigene, Multifunctional Locus in *Bacillus subtilis* with Roles in Resistance to Cholate and to Na^+ and in pH Homeostasis

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A 5.9-kb region of the *Bacillus subtilis* chromosome is transcribed as a single transcript that is predicted to encode seven membrane-spanning proteins. Homologues of the first gene of this operon, for which the designation *mrp* (multiple resistance and pH adaptation) is proposed here, have been suggested to encode an Na^+/H^+ antiporter or a K^+/H^+ antiporter. In the present studies of the *B. subtilis mrp* operon, both polar and nonpolar mutations in *mrpA* were generated. Growth of these mutants was completely inhibited by concentrations of added Na^+ as low as 0.3 M at pH 7.0 and 0.03 M at pH 8.3; there was no comparable inhibition by added K^+. A null mutant that was constructed by full replacement of the *mrp* operon was even more Na^+ sensitive. A double mutant with mutations in both *mrpA* and the multifunctional antiporter-encoding *tetA*(L) gene was no more sensitive than the *mrpA* mutants to Na^+, consistent with a major role for *mrpA* in Na^+ resistance. Expression of *mrpA* from an inducible promoter, upon insertion into the *amyE* locus, restored significant Na^+ resistance in both the polar and nonpolar *mrpA* mutants but did not restore resistance in the null mutant. The *mrpA* disruption also resulted in an impairment of cytoplasmic pH regulation upon a sudden shift in external pH from 7.5 to 8.5 in the presence of Na^+ and, to some extent, K^+ in the range from 10 to 25 mM. By contrast, the *mrpA tetA*(L) double mutant, like the *tetA*(L) single mutant, completely lost its capacity for both Na^+- and K^+-dependent cytoplasmic pH regulation upon this kind of shift at cation concentrations ranging from 10 to 100 mM; thus, *tetA*(L) has a more pronounced involvement than *mrpA* in pH regulation. Measurements of Na^+ efflux from the wild-type strain, the nonpolar *mrpA* mutant, and the complemented mutant indicated that inducible expression of *mrpA* increased the rate of protonophore- and cyanide-sensitive Na^+ efflux over that in the wild-type in cells preloaded with 5 mM Na^+. The *mrpA* and null mutants showed no such efflux in that concentration range. This is consistent with MrpA encoding a secondary, proton motive force-energized Na^+/H^+ antiporter. Studies of a polar mutant that leads to loss of *mrpFG* and its complementation in *trans* by *mrpF* or *mrpFG* support a role for MrpF as an efflux system for Na^+ and cholate. Part of the Na^+ efflux capacity of the whole *mrp* operon products is attributable to *mrpF*. Neither *mrpF* nor *mrpFG* expression in *trans* enhanced the cholate or Na^+ resistance of the null mutant. Thus, one or more other *mrp* gene products must be present, but not at stoichiometric levels, for stability, assembly, or function of both MrpF and MrpA expressed in *trans*. Also, phenotypic differences among the *mrp* mutants suggest that functions in addition to Na^+ and cholate resistance and pH homeostasis will be found among the remaining *mrp* genes.

The sequence of a locus that was reported as part of the *Bacillus subtilis* genome project (21; GenBank accession no. Z93937 and Z93932 [some corrections to the original sequence were noted during the present study and entered into the databank]) is of interest in connection with monovalent cation resistance and cytoplasmic pH regulation in adaption to high pH. This unusual cluster of genes is 5.9 kb long and is predicted to encode seven hydrophobic proteins that are likely to be coordinately expressed as an operon. The special interest in connection with alkali and monovalent cation resistance is based on studies reported by others on diverse homologues of this locus, i.e., a homologue from alkaliphilic *Bacillus* strain C-125 encompassing only the first three genes (10) and full operons designated *pha* (pH adaptation) from *Rhizobium meliloti* (23) and designated *mnh* from *Staphylococcus aureus* that complemented a Na^+/H^+ antiporter-deficient *Escherichia coli* strain (12). In the alkaliphilic *Bacillus* strain C-125 in which this gene family was first identified (10, 16), a crossover event involving the first gene of the partially cloned operon corrected a point mutation in the chromosomal copy of a mutant that had a non-alkaliphilic, pH homeostasis-negative, and Na^+/H^+ antiporter-negative phenotype. By contrast, the recently reported studies of the full homologue from *R. meliloti* (23) arose from characterization of a transposition mutant whose disruption of the first gene in the operon, *phaA*, rendered it unable to invade nodule tissue, exquisitely sensitive to inhibition by K^+, and deficient in diethanolamine-induced K^+ efflux but normal with respect to Na^+-related properties. The data presented by both sets of studies support the respective suggestions that the first gene of the alkaliphile operon encodes an Na^+/H^+ antiporter and that of the *pha* operon encodes a K^+/H^+ antiporter. However, neither study included complementation of mutant phenotypes in *trans* without a recombination event. This is particularly important with this operon because of its unusual complexity compared to other monovalent cation/H^+ antiporter-encoding loci. Indeed, Hiramatsu et al. (12) demonstrated that Na^+-related functions of the *S. aureus mnh* operon expressed in *E. coli* were dependent on the presence of more than just the first gene (12). Also, as noted by the other investigators (10, 23) and observed in our sequence analysis of the *B. subtilis* operon, there is a striking similarity between several of the genes of

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
E. coli		
DH5αMCR	F$^-$ *mcrA*Δ*1* (*mrr-hsd RMS-mcrBC*) ϕ80*dlacZ*ΔM15 Δ(*lacZYA-argF*) *U169 deoR recA1 endA1 supE44* λ *thi-1 gyr-496 relA1*	GIBCO-BRL
B. subtilis		
BD99 (wild type)	*hisA1 thrS trpC2*	A. Garro
JC112	BD99 *tetA*(L)::Cm^r	5
VK123	BD99 *tetA*(L)::Cm^r *mrpA*::Spc^r	This study
VK1	BD99 Δ*mrpA*	This study
VK6	BD99 *mrpA*::Spc^r	This study
VK15	BD99 *mrpF*::Spc^r	This study
VK1/*mrpA*	VK1 *amyE*::p_{spac}-*mrpA*	This study
VK6/*mrpA*	VK6 *amyE*::p_{spac}-*mrpA*	This study
VK6/*mrpF*	VK6 *amyE*::p_{spac}-*mrpF*	This study
VK6/*mrpFG*	VK6 *amyE*::p_{spac}-*mrpFG*	This study
VK15/*mrpF*	VK15 *amyE*::p_{spac}-*mrpF*	This study
VK15/*mrpFG*	VK15 *amyE*::p_{spac}-*mrpFG*	This study
VKN1	BD99Δ*mrp*A–*G*::Spc^r	
VKN1/*mrpA*	VKNI *amyE*::p_{spac}-*mrpA*	
VKN1/*mrpF*	VKNI *amyE*::p_{spac}-*mrpF*	
VKN1/*mrpFG*	VKN1 *amyE*::p_{spac}-*mrpFG*	
pGEM11Zf(+)	Cloning vector (Ap^r)	Promega
pGEM9Zf(−)	Cloning vector (Ap^r)	Promega
pDH88	Cm^r vector for cloning *B. subtilis* chromosomal DNA and integration into the corresponding locus	11
pDR67	*amyE* integration vector with Cm^r gene and p_{spac} promoter upstream of multiple cloning site	14
pDHA1	pDH88 + Δ*mrpA*	This study
pDRA1	pDR67 + *mrpA*	This study
pDRF1	pDR67 + *mrpF*	This study
pDRFG1	pDR67 + *mrpFG*	This study

these operons and those encoding subunits of proton-translocating NADH dehydrogenases (26, 28) and a recently analyzed, putative proton-translocating formate hydrogenlyase system from *E. coli* (2). Hiramatsu et al. (12) proposed that the Na^+/H^+ antiporter is a novel multisubunit secondary transporter that is energized conventionally by the proton motive force. It should not be ruled out, though, that products of these new antiporter-encoding loci may form complexes that under some conditions function as a primary ion extrusion or exchange system that is energized directly, e.g., by electron transport through Mrp components. Alternatively, the complexity of the operon may reflect the presence of genes that encode diverse transporters whose functions all relate to a particular stress. Such an operon might also include specific regulators, sensors, assembly factors, or chaperones that are stable under that stress condition and allow the function of the transporters. This would result in an interdependence among the individual gene products of the type suggested for *mnh* (12).

In the present studies on the *B. subtilis* operon, we have examined the properties of a mutant with a null mutation in the operon. We have also focused on the first gene and the final two genes of the operon, generating mutants that could be complemented in *trans*. Since roles in pH homeostasis and Na^+ and cholate resistance were found, the name *mrp* is proposed for the operon, for "multiple resistance and pH adaptation locus."

MATERIALS AND METHODS

Bacterial strains and growth conditions. The wild-type and mutant strains of *B. subtilis* used in this study are listed in Table 1. The *mrp* mutants included a null strain (VKN1), mutants with polar and nonpolar mutations in *mrpA* (VK6 and VK1), a mutant with a polar mutation in *mrpF* (VK15), and a double mutant with mutations in *mrpA* and *tetA*(L) (VK123); these mutants were all made as described below. *B. subtilis* JC112 is a wild-type strain in which the chromosomal *tetA*(L) locus was replaced with a chloramphenicol resistance cassette (5); this strain was included in some of the growth studies and in the pH homeostasis studies. Routine growth of all these strains was carried out at 30°C, with shaking, in TKM medium (Tris-potassium malate, no added Na^+). For growth experiments, pH shift experiments, and solute efflux experiments, either TTM medium (Tris-Tris malate, 1 mM potassium phosphate, no added Na^+) or TKM medium, described previously (5), served as the base to which the indicated additions were made for specific protocols; yeast extract was added to these media at 0.1% (wt/vol). In completely synthetic media (including Spizizen salts-based media [24]) that supported rapid growth of the wild type and substantial growth of JC112, VK1, and VK15, VK6 did not exhibit significant growth. The inability of VK6 to grow in such media was not overcome by the use of glucose instead of malate as the carbon source or by elevation of the phosphate concentration (data not shown). For experiments including constructs into which *mrp* genes were introduced into the *amyE* locus under control of the p_{spac} promoter, 200 μM isopropyl-β-D-thiogalactoside (IPTG) was included in the growth media as well as in the dilution buffers for efflux experiments. If the IPTG was omitted, there was only marginally significant complementation by the constructs that complemented substantially or completely when IPTG was added.

Complementation and resistance studies. For the determinations of NaCl sensitivity, TKM medium at pH 7.0 or pH 8.3 was supplemented with different concentrations of NaCl. Cultures (2 ml) were grown in 15-ml conical tubes with shaking at 30°C. They were inoculated with 10 μl of an 8-h culture grown in TKM medium (pH 7.0), and the absorbance at 600 nm was read after 15 h. The MIC was defined as the minimal NaCl concentration that completely prevented growth after 15 h of incubation. For the determination of growth sensitivity to cholate, 2 ml of TKM (pH 7.0), with or without the addition of 0.08% (wt/vol) cholate, was inoculated with 50 μl of an 8-h culture grown on TKM (pH 7.0). The incubation, in 15-ml conical tubes, was conducted with shaking at 30°C. The absorbance at 600 nm was recorded after 6 h of incubation.

Construction of mutant strains. For each type of mutant, the phenotype of the strain used in the studies was the same as several others from the construction protocol. Each mutant that was used in the subsequent studies was shown to contain the expected sequence. The sequencing was conducted at the Utah State Biotechnology Center (Logan, Utah) with an ABI-100 model 377 Sequencer.

(i) The *mrp* operon null strain, VKN1. Strain VKN1 was constructed by gene splicing via overlap extension (gene SOEing), as described previously (13). Two independent PCRs were performed on wild-type DNA with the sets of primers shown in Fig. 1, BSMRPNE1 and BSMRPNR, and BSMRPNF and BSMRPNB2. BSMRPNE1 (5′-GGAATCCAGCTGCGGCTGTCAAGTAT-3′) corresponded to the complementary sequence of bp 9563 to 9581 of the database entry GenBank accession no. Z93937 and additional nucleotides containing an *Eco*RI site. The restriction enzyme sites are underlined. BSMRPNR (5′-TTCTCATCAAGCTTGACCCGGGCGCTTCGAACTGCTGTAATGGA-3′) corresponded to the complementary sequence of bp 7154 to 7171 of the database entry GenBank accession no. Z93932, bp 10358 to 10337 of the database entry GenBank accession no. Z93937, and additional nucleotides containing a *Sma*I site in the middle of the sequence. BSMRPNF (5′-TCCATTAACAGCAGTTCGAAGCGTCCCGGGTCAAGCTTGATGAGAGAA-3′) corresponded to the complementary sequence of bp 10337 to 10359 of the database entry GenBank accession no. Z93937, bp 7171 to 7154 of the database entry GenBank accession no. Z93932, and additional nucleotides containing a *Sma*I site in the middle of the sequence. BSMRPNB2 (5′-CGCGGATCCATCAGCAAAACGGAATCT-3′) corresponded to the complementary sequence of bp 6313 to 6330 of the database entry GenBank accession no. Z93932 and additional nucleotides containing a *Bgl*II site. The two purified PCR products were used as a template for a second PCR with primers BSMRPNE1 and BSMRPNB2. The purified PCR product of this reaction was digested with *Eco*RI and *Bgl*II, and then cloned into *Eco*RI-*Bam*HI-digested pGEM11Zf(+) (Ap^r; Promega). The recombinant plasmid was digested with *Sma*I. A gene encoding Spc^r (20) was ligated to this linear plasmid, resulting in a recombinant plasmid containing fragments upstream and downstream of the *mrp* operon with a Spc^r gene between them instead of *mrp*. After isolation, the recombinant plasmid was digested with *Eco*RI and the linear plasmid was introduced into wild-type *B. subtilis*. Mutants with deletions in the *mrp* operon were identified by spectinomycin resistance (150 μg/ml) and confirmed by PCR and then sequencing for the strain used in the studies.

(ii) Mutants of the wild type (VK6) and JC112 (VK123) with polar disruptions in *mrpA*. PCR was performed on purified wild-type *B. subtilis* chromosomal DNA by using the PCR primers BSMRP1 and BSMRP2. The forward primer, BSMRP1 (5′-AGGAGGTCTTATCTTTGCAGCTC-3′), corresponded to the complementary sequence of bp 10449 to 10473 of the database entry GenBank accession no. Z93937 and is at the 5′ end of the region of interest (Fig. 1). The reverse primer, BSMRP2 (5′-GGCATAATCGCCATCAGGCCGCC-3′), corresponded to the complementary sequence of bp 11603 to 11581 of the same database entry. After 25 cycles of amplification, the purified PCR product (1,155 bp) was first ligated into *Hin*cII-digested pGEM11Zf(+) (Ap^r; Promega). The recombinant plasmid was identified by blue-white screening in *E. coli* DH5α.

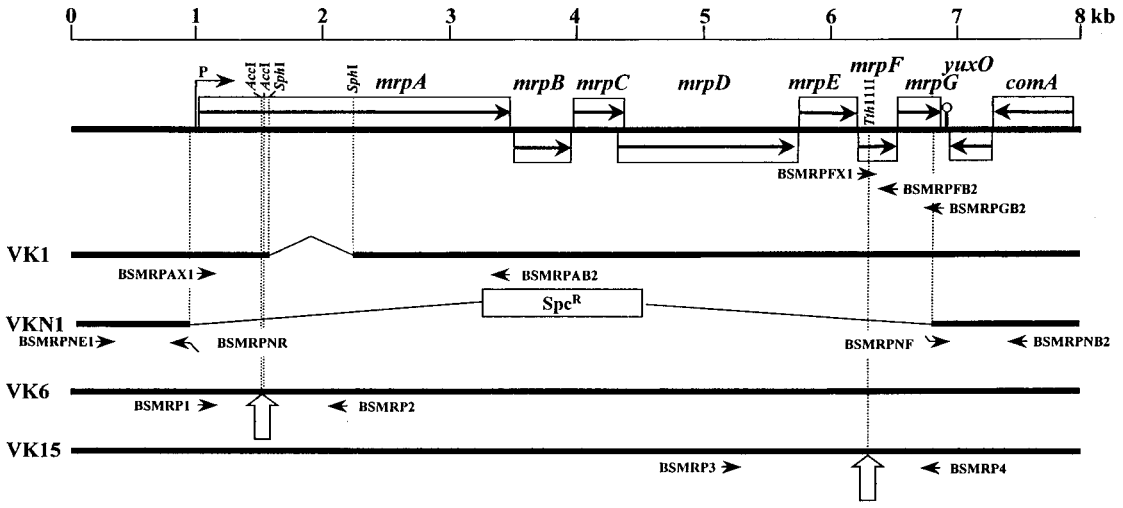

FIG. 1. Schematic diagram of the *mrp* locus showing the sizes of the predicted open reading frames, the site of the nonpolar mutation made in mutant strain VK1, and the sites of the disruptions made in mutant strains VKN1, VK6, and VK15. Seven open reading frames (*mrpABCDEFG*) are indicated, using homologues from other bacteria, especially the *R. meliloti pha* region, as part of the frame of reference. The open reading frames are shown as open boxes, with the direction of transcription indicated by the horizontal arrows within the boxes. An arrow emerging upward from the fragment upstream of *mrpA* and pointing in the direction of transcription indicates the putative promoter (P). The *Sph*I fragment deleted in VK1 to create a nonpolar in-frame deletion is indicated by the thin bent line flanked by dotted vertical lines. The sites disrupted by a spectinomycin resistance cassette in strains VK6 and VK15 are shown by the open arrows pointing upward. The replacement of the entire *mrp* locus with a spectinomycin resistance cassette is indicated for VKN1. The primers used to construct mutant strains and integration vectors in this work are shown by horizontal arrows (see Materials and Methods).

After isolation, the plasmid was digested with *Acc*I and a 46-bp *Acc*I-*Acc*I fragment was removed from the middle of *mrpA*. The plasmid was then treated with mung bean nuclease to generate blunt ends. A Spc^r gene (20) was ligated to this linear plasmid, resulting in a recombinant plasmid containing a fragment of *mrpA* with a small deletion in a region into which a Spc^r gene had been introduced. After isolation, the recombinant plasmid was digested with *Sca*I, whose site was located in the Ap^r gene, and the linear plasmid was introduced into wild-type *B. subtilis* and into the *tetA*(L) mutant strain JC112 by competent cell transformation (24). Mutants of each of these strains that were disrupted in the *mrpA* locus were identified by spectinomycin resistance (150 μg/ml) and confirmed by PCR analysis.

(iii) Mutant strain with a polar disruption of *mrpF* (VK15). The strategy was the same as in the construction of the polar *mrpA* mutants above, except that PCR primers BSMRP3 and BSMRP4 (Fig. 1) were used. The forward primer, BSMRP3 (5′-GTACTGTACTCTGTGCTGAGGATC-3′), corresponded to the complementary sequence of bp 8437 to 8414 of the database entry GenBank accession no. Z93932. The reverse primer, BSMRP4 (5′-AGCAAGAGAGGC TGATCTGTATATCCAGA-3′), corresponded to the complementary sequence of bp 6992 to 7020. The purified PCR product was ligated into pGEM11Zf(+), and a recombinant plasmid was isolated as described above. This plasmid was digested with *Tth*111I, whose site was located in the middle of *mrpF*. The digested plasmid was then treated with mung bean nuclease to generate blunt ends, and the same Spc^r gene was ligated into the disruption site. The introduction into wild-type *B. subtilis* and isolation and characterization of the mutants followed the procedures used for isolation of *mrpA* mutants.

(iv) Nonpolar mutant VK1 with a deletion in *mrpA*. An in-frame deletion in *mrpA* of wild-type *B. subtilis* BD99 was made as follows. PCR was performed on wild-type DNA using the PCR primers BSMRPAX1 and BSMRPAB2 (Fig. 1). The forward primer, BSMRPAX1 (5′-CTAGTCTAGAAAGGAGGTCTTATC TTTGCAGCTC-3′), corresponded to the complementary sequence of bp 10449 to 10473 of the database entry GenBank accession no. Z93937 and additional nucleotides containing an *Xba*I site. The reverse primer, BSMRPAB2 (5′-GAA GATCTCATTCATTCACCGCTTTCCCCTCCT-3′), corresponded to the complementary sequence of bp 12848 to 12871 of the same database entry and additional nucleotides creating a *Bgl*II site. The purified PCR product was cloned into *Hin*cII-digested pGEM9Zf(−) (Ap^r; Promega). This recombinant plasmid was digested with *Sph*I, and a 705-bp *Sph*I-*Sph*I fragment was removed from the middle of *mrpA*. The plasmid was ligated to itself, resulting in a recombinant plasmid containing a *mrpA* fragment with an in-frame deletion. For isolation of that fragment, the recombinant plasmid was digested with *Bgl*II and *Xba*I. The fragment was then cloned into *Bgl*II-*Xba*I-digested pDH88. The resulting plasmid, pDHA1, was integrated into the *mrpA* locus in the chromosome by a single crossover with chloramphenicol resistance for selection (11). To prepare strains that had lost the plasmid sequences, leaving a single mutant *mrpA* allele, several independent recombinants from the transformation with pDHA1 were grown under nonselective conditions (i.e., in the absence of chloramphenicol), and plated on LBK (Luria broth with KCl) plates. Colonies were screened for sensitivity to 100 mM Na^+, and such strains were further tested for chloramphenicol sensitivity, which would indicate loss of the plasmid. PCR analyses were used for initial confirmation of the deletion and were followed by sequencing.

Integration of selected *mrp* genes into the *amyE* loci of particular mutant strains under a p_{spac} promoter. Particular mutant strains that had been prepared as described above were constructed with one or more *mrp* genes integrated into the chromosomal *amyE* locus behind an IPTG-inducible p_{spac} promoter. Plasmid pDR67 was used for these constructions (14). This plasmid contains fragments of the front and back ends of the *amyE* gene flanking a chloramphenicol resistance (Cm^r) gene and also contains the p_{spac} promoter upstream of a multiple-cloning site. For construction of a plasmid that was carrying an intact *mrpA* gene, PCR was performed on wild-type DNA with the PCR primers BSMRPAX1 and BSMRPAB2 (Fig. 1). For construction of a plasmid that was carrying an intact *mrpF* gene, PCR was performed on wild-type DNA with the PCR primers BSMR PFX1 and BSMRPFB2 (Fig. 1). The forward primer BSMRPFX1 (5′-CTAGT CTAGAAAAAGCCATACAGGAGGTGAGCC-3′) corresponded to the complementary sequence of bp 7733 to 7757 of the database entry GenBank accession no. Z93932 and additional nucleotides containing an *Xba*I site. The reverse primer BSMRPFB2 (5′-GAAGATCTTAGCGGTTTCGATCATTTTCG-3′) corresponded to the complementary sequence of bp 7443 to 7465 of the same database entry plus additional nucleotides containing a *Bgl*II site. For construction of a plasmid that was carrying intact *mrpF* and *mrpG* genes together, PCR was performed on wild-type DNA with PCR primers BSMRPFX1 and BSMR PGB2 (Fig. 1). The reverse primer BSMRPGB2 (5′-GAAGATCTAGCAAGA GAGGCTGATCTGTATATCCAGATG-3′) corresponded to the complementary sequence of bp 6991 to 7022 of the database entry GenBank accession no. Z93932 plus additional nucleotides containing a *Bgl*II site. Each amplified fragment was cloned into *Xba*I-*Bgl*II-digested pDR67, yielding pDRA1 (*mrpA*), pDRF1 (*mrpF*), and pDRFG1 (*mrpFG*). Each plasmid DNA was linearized with *Nru*I and used to transform particular mutants to a Cm^r Amy$^-$ phenotype. The plasmids used in this study are listed together with the bacterial strains in Table 1; the ones used were all confirmed to have the correct sequence.

Northern analyses. Northern analyses were as described previously (7). An oligonucleotide probe was used. Oligonucleotide BSMRPA (5′-TCATTCACC GCTTTTCCCCTCCT-3′) corresponded to the complementary sequence of bp 12848 to 12871 of the database entry GenBank accession no. Z93937, a region downstream of the point of disruption of the polar *mrpA* mutants. The oligonucleotide was radiolabeled by incubation of [γ-^{32}P]ATP and T4 polynucleotide kinase (New England Biolabs) at 37°C for 30 min. Polynucleotide kinase was inactivated by heating to 65°C for 5 min. The radiolabeled oligonucleotide was separated from [γ-^{32}P]ATP by Microcon YM-3 centrifugal filter devices (Amicon). Additional analyses were conducted with a probe of 157 bp corresponding to a 5′ region of *mrpA* (sequence of bp 10450 to 10606 of GenBank accession no. Z93937).

Determination of the cytoplasmic pH after a pH shift from 7.5 to 8.5. The pH of the cytoplasm was determined 10 min after a sudden shift in external pH as described previously (5). Briefly, cells were grown in, and then washed and equilibrated with, TTM medium at pH 7.5. Additions of various concentrations of choline chloride, KCl, or NaCl were made to the equilibration buffer as

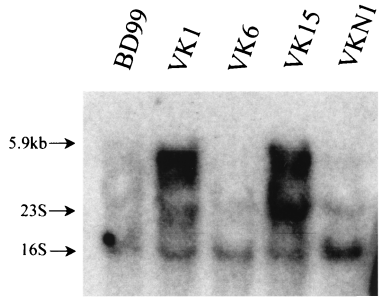

FIG. 2. Northern blot analysis of mRNA in the wild type (BD99), *mrpA* mutants VK1 and VK6, *mrpF* mutant VK15, and null mutant VKN1. The formaldehyde-agarose isolated RNA was probed with the end-labeled 5′ oligonucleotide probe described in Materials and Methods. The 23S and 16S rRNA bands are indicated. The top arrow indicates the position corresponding to 5.9 kb.

indicated. Then the external pH was rapidly adjusted to 8.5. After 10 min, the distribution of radiolabeled methylamine was used to assay the presence or absence of a transmembrane pH gradient, acid in; measurement of the precise external pH during the assay then allowed calculation of the cytoplasmic pH after corrections for binding that were conducted as described previously (5).

Solute efflux assays. For assays of Na^+ or cholate efflux, cells were grown to the mid-logarithmic phase in TKM medium (pH 7.0), harvested, washed twice with 50 mM potassium morpholinepropanesulfonate (MOPS) (pH 7.5), and resuspended to 20 mg of protein/ml in the same buffer. The cells were starved and loaded with 5 mM $^{22}NaCl$ (10 μCi/ml) or 20 μM sodium [^{14}C]cholate (1 μCi/ml) for 2 h. After starvation and loading, the cells in Na^+ efflux experiments were diluted 1:100 into 50 mM potassium–MOPS (pH 7.5) plus 5 mM NaCl. For cholate efflux experiments, 5 μl of cells was diluted into 500 μl of 50 mM potassium–MOPS (pH 7.5). The samples were vacuum filtered at various times onto Millipore HAWP 0.45-μm-pore-size filters, washed with 5 ml of buffer, dried, and counted by liquid scintillation. Where indicated, either 10 μM carbonyl cyanide *p*-chlorophenylhydrazone (CCCP) or 10 mM KCN was added for 15 min of preincubation to the cell suspension and was also included in the dilution buffer. Also, where indicated, 10 mM glucose was added to the dilution buffer. Protein concentrations in this and other assays were determined by the method of Lowry et al. (17) with egg white lysozyme as the standard.

RESULTS

Northern analyses of the wild type and potentially polar mutants with mutations in the *mrp* locus. Northern analyses were conducted to confirm the expectation that the *mrp* genes were expressed as an operon, VKN1 was a null strain for *mrp*, and VK6 was a polar *mrpA* mutant in contrast to VK1. As shown in Fig. 2, it proved difficult to visualize Northern data from the wild type, VK6, and VKN1, as well as VK1 and VK15. Under conditions of long exposures with both the oligonucleotide probe and DNA probes to 3′ and 5′ regions of *mrpA*, the wild type exhibited a band of 5.9 kb, which would correspond to the expected length of a transcript of all seven *mrp* genes. Under such conditions, VK6 exhibited a faint band at the predicted position for that construct. VK1 and VK15 evidently showed great overexpression of a species of the same size as each other and a little smaller than that of the wild type; the degree of overexpression made its measurement difficult. In other experiments, the size of the VK1 transcript was more clearly seen as predicted and that of VK15 was consistent with a polar *mrpF* mutation. In no instance was a band attributable to *mrp* observed in the null strain VKN1 (data not shown). In the particular experiment shown in Fig. 2, the oligonucleotide probe to a region at the 3′ end of *mrpA* was used and conditions were chosen such that the wild type was underexposed, with the 5.9-kb band just discernible in the wild type but not in VKN1 or even the polar VK6 mutant. This condition made it possible to see the distinction between the large top band and the ribosomal bands in VK1 and VK15; these highly expressed top bands were a little less than 5.9 kb and of similar size. Ultimately it will be of interest to investigate the mechanism of *mrp* overexpression in nonfunctional mutants and whether it is mediated by cytoplasmic Na^+ levels.

Na^+ sensitivity. As shown in Table 2, the null mutant VKN1 was exquisitely sensitive to growth inhibition by Na^+. All the other VK mutant strains, while not as sensitive as VKN1, exhibited pronounced Na^+ sensitivity relative to both the wild type and the moderately Na^+-sensitive *tetA*(L)-disrupted strain, JC112. TetA(L) is a multifunctional antiporter that catalyzes the efflux of a cobalt-tetracycline complex, Na^+, or K^+ in exchange for protons (3, 5, 6). The double mutant, VK123, in which a polar *mrpA* mutation identical to that in VK6 was introduced into JC112, exhibited the same Na^+ sensitivity as VK6. The Na^+ sensitivity in *mrp* mutants was particularly increased at pH 8.3, indicating that the Na^+ extrusion function of the operon is particularly important at elevated pH. Interestingly, VK1 was more sensitive than VK6 at pH 7.0. This suggests that one of the *mrp* genes downstream of *mrpA*, whose expression is reduced in polar VK6 relative to VK1, might catalyze the uptake of Na^+ at neutral pH, e.g., in symport with another solute. An inducible *mrpA* construct was introduced into the *amyE* loci of the null mutant (VKN1) as well as the polar (VK6) and nonpolar (VK1) mutants that had disruptions in their chromosomal *mrpA*. VKN1 exhibited no complementation, suggesting that one or more additional *mrp* gene products are required for *mrpA* function. VK1 was complemented almost to wild-type levels at pH 7.0 and also exhibited substantial complementation at pH 8.3. VK6 was also significantly complemented at both pH 7.0 and 8.3, although this complementation was lower than that observed with VK1. The significant complementation of VK6, with its reduced levels of *mrpB* to *mrpG*, by induced expression of *mrpA* in *trans* is important. It indicates that MrpA produced in stoichiometric excess with respect to the other *mrp* gene products is functional in Na^+ resistance. As shown below, the MrpA-dependent transport Na^+ activity in VK1/*mrpA* is elevated over that observed in the wild type, consistent with the same conclusion.

An interesting set of observations on Na^+ sensitivity was made for VK15 and for complementation of both VK15 and VK6 by introduction of an inducible *mrpF* or *mrpFG* construct into the *amyE* locus. First, VK15 was just as sensitive to Na^+ as VK6 was. Induced expression of *mrpF* increased the Na^+ re-

TABLE 2. Na^+ resistance of *B. subtilis* strains with mutations in the *tetA*(L) and/or selected *mrp* genes

Strain	MIC (M)[a] of Na^+ at:	
	pH 7.0	pH 8.3
Wild type	1.30 ± 0.05	0.71 ± 0.03
JC112	0.80 ± 0.03	0.40 ± 0.02
VK123	0.27 ± 0.02	0.025 ± 0.01
VK1	0.15 ± 0.02	0.026 ± 0.01
VK1/*mrpA*	1.15 ± 0.04	0.45 ± 0.02
VK15	0.27 ± 0.03	0.026 ± 0.01
VK15/*mrpF*	0.45 ± 0.03	0.15 ± 0.02
VK15/*mrpFG*	0.65 ± 0.03	0.25 ± 0.02
VK6	0.29 ± 0.03	0.025 ± 0.01
VK6/*mrpA*	0.62 ± 0.04	0.15 ± 0.02
VK6/*mrpF*	0.46 ± 0.02	0.16 ± 0.01
VK6/*mrpFG*	0.45 ± 0.03	0.15 ± 0.02
VKN1	0.09 ± 0.03	0.024 ± 0.01
VKN1/*mrpA*	0.12 ± 0.02	0.035 ± 0.02
VKN1/*mrpFG*	0.14 ± 0.03	0.027 ± 0.02
VKN1/*mrpF*	0.092 ± 0.02	0.026 ± 0.02

[a] Minimal Na^+ concentration at which no growth was observed after 15 h. The values are means ± standard deviations.

sistance of VK6 at pH 7.0 and 8.3, and the increase was not augmented in the strain expressing *mrpFG* in *trans*. In VK15 itself, which has a functional chromosomal *mrpA*, the induced expression of *mrpF* in *trans* increased the Na^+ resistance, but in this strain there was an augmentation when *mrpFG* was induced instead of *mrpF*. These findings suggest that MrpF enhances Na^+ resistance both in the presence and in the absence of the putative antiporter-encoding MrpA whereas MrpG enhances only in association with a functional MrpA.

Work on an earlier Na^+ extrusion system of *B. subtilis*, the ATP-binding cassette-type *natAB* transport system for Na^+ extrusion, had indicated that this system was inducible by ethanol and other membrane perturbants and contributed to solvent resistance in part by excluding Na^+ that might leak in adversely. Several of the *mrp* mutants were examined for their sensitivity to ethanol. Although not shown, there was a qualitatively but not quantitatively consistent sensitivity of the mutants relative to the wild type.

pH homeostasis phenotypes. Earlier studies had shown that pH 7.5-equilibrated cells of JC112 were completely unable to regulate their cytoplasmic pH upon a sudden shift in the external pH to 8.5 in the presence of 100 mM K^+ or Na^+. These results indicated a major role for TetA(L) in pH homeostasis at that level of monovalent cation (5). Given the much greater Na^+ sensitivity of all the *mrp* mutants than of JC112, it seemed likely that any *mrp*-encoded antiporter activity might establish lower cytoplasmic Na^+ concentrations than could be accomplished by TetA(L) alone. Therefore, pH shift experiments were conducted at 100, 25, and 10 mM Na^+ or K^+ as well as in the absence of added Na^+ or K^+; in the latter experiments, choline chloride was substituted for the sodium and potassium salts. Although the results are not shown, none of the strains exhibited any capacity for pH homeostasis after the pH shift when the choline salts were used; i.e., after the shift, the cytoplasmic pH was the same as the new external pH. As shown in Table 3, the wild-type strain was capable of excellent pH homeostasis in the presence of only 10 mM Na^+, maintaining a cytoplasmic pH close to the preshift level. Significant but more modest acidification of the cytoplasm relative to the new external pH was observed in the presence of 10 mM K^+. The double mutant VK123, like the *tetA*(L) mutant JC112 (data not shown), exhibited neither Na^+- nor K^+-dependent pH homeostasis at any of the monovalent cation concentrations. By contrast, VKN1, VK1, VK6, and VK15 all showed a small deficit in K^+-dependent pH homeostasis. These strains also all exhibited significant and comparable deficits in cytoplasmic pH regulation relative to the wild type in the presence of 25 mM and, especially, 10 mM Na^+. Expression of *mrpA* in *trans* in VK1 but not in VKN1 restored a capacity for Na^+-dependent pH homeostasis that was comparable to that of the wild type.

Na^+ efflux. The apparent roles of MrpA in both Na^+ resistance and Na^+-dependent pH homeostasis were consistent with its being an Na^+/H^+ antiporter, as has been proposed for the homologues in alkaliphilic *Bacillus* strain C-125 (10) and *S. aureus* (12). To more directly assay such an activity, cells of the wild type, VK1, VK1/*mrpA*, VKN1, and VKN1/*mrpA* were partially energy depleted and loaded with 5 mM $^{22}Na^+$. The cells were then diluted into buffers containing 5 mM nonradioactive Na^+ in the presence or absence of other additions. The concentration of 5 mM Na^+ was chosen because it is well below the concentration at which the TetA(L) Na^+/H^+ antiporter activity is optimally active (8). Indeed, as shown in Fig. 3, wild-type *B. subtilis* exhibited fast efflux of Na^+ that was significantly stimulated by glucose addition and significantly inhibited by the addition of either cyanide or the protonophore CCCP. By contrast, Na^+ efflux from VK1 cells exhibited a

TABLE 3. Cytoplasmic pH measured in cells of wild type and various *mrp* constructs after a shift in the external pH from 7.5 to 8.5[a]

Concn of indicated salt added (mM)	Cytoplasmic pH of strain:															
	Wild type		VK1		VK1/*mrpA*		VK6		VK15		VK123		VKNI		VKN1/*mrpA*	
	NaCl	KCl	NaCl	KCl	NaCl	KCl	NaCl	KCl	NaCl	KCl	NaCl	KCl	NaCl	KCl	NaCl	KCl
100	7.5 ± 0.08	7.70 ± 0.07	7.6 ± 0.15	7.8 ± 0.17	7.5 ± 0.11	7.7 ± 0.04	7.60 ± 0.07	8.00 ± 0.09	7.7 ± 0.05	7.90 ± 0.10	8.5 ± 0.12	8.5 ± 0.13	7.6 ± 0.14	7.8 ± 0.16	7.5 ± 0.15	7.7 ± 0.16
25	7.50 ± 0.12	7.90 ± 0.11	8.2 ± 0.12	8.0 ± 0.15	7.5 ± 0.14	7.9 ± 0.11	7.90 ± 0.14	8.20 ± 0.11	8.00 ± 0.15	8.10 ± 0.15	8.5 ± 0.11	8.5 ± 0.12	8.3 ± 0.13	8.1 ± 0.14	8.2 ± 0.13	8.1 ± 0.15
10	7.50 ± 0.09	8.10 ± 0.13	8.4 ± 0.12	8.3 ± 0.15	7.8 ± 0.15	8.3 ± 0.12	8.50 ± 0.08	8.50 ± 0.10	8.50 ± 0.05	8.40 ± 0.15	8.5 ± 0.12	8.4 ± 0.15	8.5 ± 0.11	8.4 ± 0.13	8.5 ± 0.15	8.4 ± 0.14

[a] All strains were grown at pH 7.0 in TTM medium before being subjected to a sudden shift in the external pH to 8.5 as described under Materials and Methods. The values shown, means ± standard deviations, were measurements taken in duplicate from at least six independent experiments.

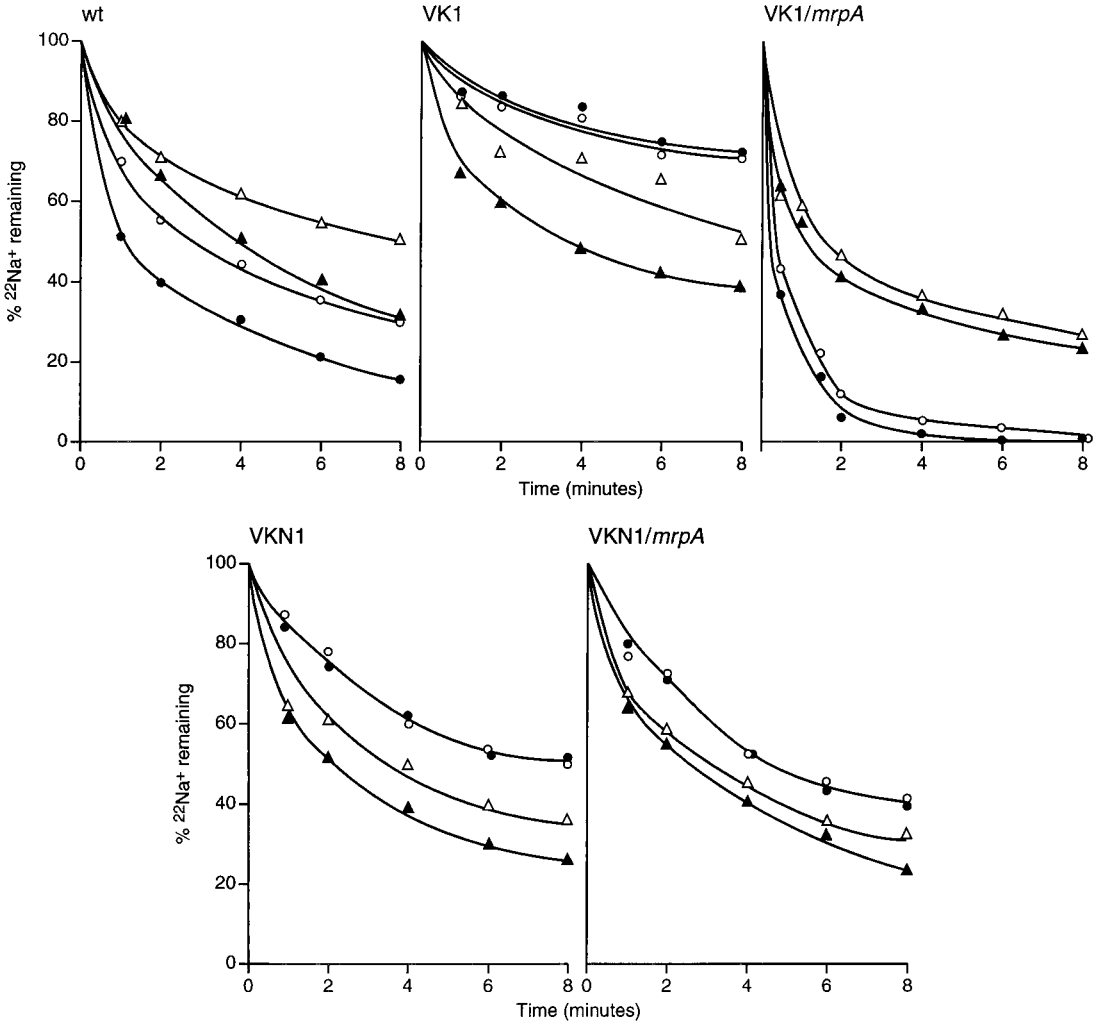

FIG. 3. Efflux of Na^+ from cells of wild type (wt), VK1, VK1/*mrpA*, VKN1, and VKN1/*mrpA*. The cells were washed, energy depleted, and loaded with 5 mM $^{22}NaCl$ as described in Materials and Methods. Efflux was initiated by diluting the suspension 100-fold into buffer (containing 5 mM NaCl) and no further additions (○), buffer containing 10 mM glucose (●), buffer containing glucose plus 10 mM cyanide (△), or buffer containing glucose plus 10 μM CCCP (▲). Samples were taken at various times, filtered, and washed, and the radioactivity was determined by liquid scintillation counting.

much slower Na^+ efflux which was not stimulated by glucose but was enhanced by both cyanide and CCCP; most probably, in the only partially starved cells, the cytoplasmic Na^+ concentration slightly exceeded the external Na^+ concentration after loading, and the stimulation by cyanide and CCCP represents a stimulation of a leak of cytoplasmic Na^+ down its concentration gradient once the electrical potential component of the proton motive force, positive out, is dissipated. In the *mrpA*-complemented VK1, the Na^+ efflux was faster than in the wild-type cells, in both the absence and presence of added glucose. Cyanide and CCCP both inhibited efflux significantly but not completely. The Na^+ efflux pattern of VKN1 was similar to that of VK1 except for a slightly faster Na^+ efflux in VKN1; this, again, could be explained if the complete deletion of VKN1 removes a Na^+-coupled uptake protein as well as Na^+ efflux systems. Restoration of *mrpA* to VKN1 increased the efflux rate slightly, but that efflux rate was still stimulated rather than inhibited by both cyanide and CCCP.

MrpF-dependent resistance to cholate and efflux of Na^+ and cholate. The determinations of Na^+ resistance in *mrpF*-complemented VK6 indicated that there was an enhancement of Na^+ resistance when MrpF expression was strongly induced in this $MrpA^-$ mutant at pH 7.0. Concomitant expression of MrpG did not increase the enhancement. These results suggested that MrpF might be a transporter that catalyzed Na^+ efflux independently of MrpA. The greater Na^+ sensitivity of VKN1 relative to VK1 or VK6 (Table 2) was similarly consistent with there being an *mrp*-encoded Na^+ efflux system in addition to MrpA. Since sequence similarity was noted between MrpF and several eukaryotic Na^+-coupled bile acid transporters (9, 15, 26, 28) in BLAST analyses (1), the possibility was raised that the Na^+ and a bile salt type of compound might be cosubstrates for an efflux system which could be energized by the proton motive force. Cholate was used as the probe to assess this hypothesis. The cholate resistance of various *mrp* mutant strains was determined in comparison to the wild type and to each other, with and without induced expression of *mrpA* or *mrpF* in *trans*. The differences among the strains examined with respect to growth inhibition by cholate were not sufficient to affect the MIC in the same pronounced manner as was observed among the strains for Na^+ resistance. However, at particular concentrations of cholate, e.g., 0.08% (wt/vol), significant and highly reproducible differences were observed. As shown in Fig. 4, VKN1, VK1, VK6, and VK15 all exhibited

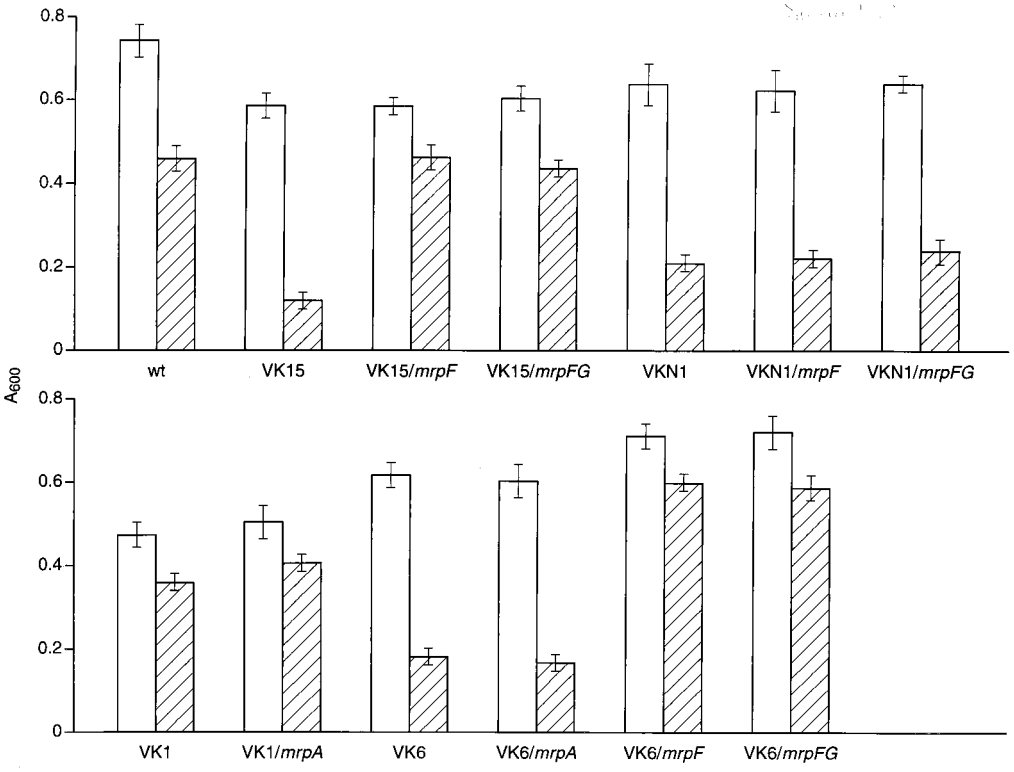

FIG. 4. Effect of cholate on the growth of wild-type (wt) *B. subtilis* and several uncomplemented and complemented strains with mutations in the *mrp* gene. Cells were grown in TKM medium (pH 7.0) in the presence (hatched bars) or absence (open bars) of 0.08% (wt/vol) cholate. The absorbance at 600 nm (A_{600}) was determined after 6 h of shaking at 30°C. The results represent the mean of at least six determinations; standard deviations are shown as error bars.

somewhat less growth than the wild-type strain at pH 7.0 in the absence of added cholate; this is likely to have resulted in part from inevitable contamination of the media with Na^+. In addition, and especially in the strains strongly expressing *mrpA* or *mrpF*, overexpression of a hydrophobic protein may account for some of this small growth deficit. As expected, the nonpolar mutant VK1 showed no increase in cholate inhibition of growth relative to the wild type and the *mrpA* status of the strain was similarly not a significant factor in cholate sensitivity. In contrast, the cholate sensitivity of VKN1 and of the polar VK6 mutant was more similar to that of VK15, i.e., significantly greater than the sensitivity of the wild type. The same difference in sensitivity was not observed when taurocholate was used instead of cholate (results not shown). The *mrpA* status was again irrelevant with respect to significant effects on cholate sensitivity in VK6. Expression of *mrpF* or of *mrpFG* restored a comparable level of resistance to both VK6 and VK15 that was even greater than that of the wild type. Neither *mrpF* nor *mrpFG*, however, restored cholate resistance to the *mrp*-null strain VKN1. These findings were consistent with the function of MrpF as an Na^+-coupled cholate efflux system whose functional expression requires at least low levels of one or more additional *mrp* gene products but not of *mrpA*.

Efflux assays were undertaken to further document such an MrpF-dependent activity. MrpF-dependent efflux of $^{22}Na^+$ was examined in VK6 cells with and without *mrpF* expression from the *amyE* locus. VK6 was used to eliminate the contribution of MrpA to the Na^+ efflux, even though a modest level of residual MrpF function may exist in VK6. The efflux was measured in cells that were partially energy depleted and loaded with $^{22}Na^+$ in either the presence or absence of 0.08% (wt/vol) cholate. As shown in Fig. 5, VK6/*mrpF* exhibited significantly faster Na^+ efflux than VK6 did. No stimulation of Na^+ efflux was observed in the presence of cholate. Efflux of cholate was monitored in cells of VK15, with and without *mrpF* expression from the *amyE* locus. The cells were preloaded with 20 μM [^{14}C]cholate in the presence or absence of 5 mM Na^+. As shown in Fig. 6, cholate efflux was significantly fast-

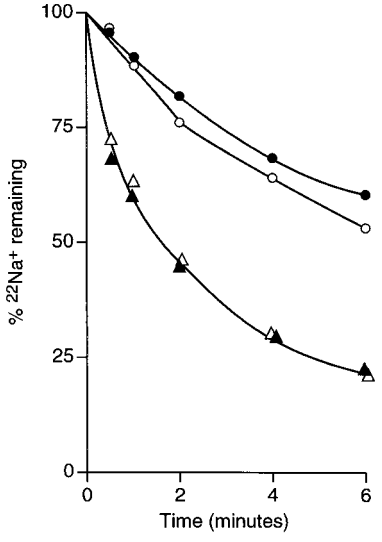

FIG. 5. Efflux of Na^+ from cells of VK6 and VK6/*mrpF* in the presence or absence of cholate. The cells were starved and loaded with ^{22}NaCl, as described in Materials and Methods, in the absence (open symbols) or presence (solid symbols) of 0.08% (wt/vol) cholate. Efflux of Na^+ from VK6 (○, ●) and VK6/*mrpF* (△, ▲) was assayed in the presence of glucose, as in Fig. 3.

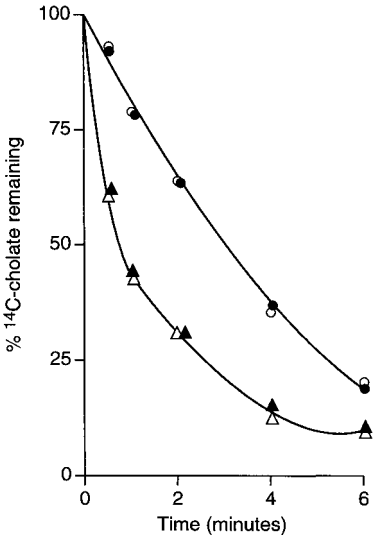

FIG. 6. Efflux of cholate from cells of VK15 and VK15/*mrpF*. The cells were energy depleted and loaded with 20 μM [^{14}C]cholate as described in Materials and Methods. Half of the cells were also loaded with 5 mM nonradioactive NaCl (solid symbols), and half had no further additions (open symbols). Cholate efflux was measured by sampling, as described in the legend to Fig. 3, from the assay mixtures with VK15 (○, ●) and VK15/*mrpF* (△, ▲).

er in VK15/*mrpF* than in VK15. No effect of Na^+ addition was observed.

DISCUSSION

The name *mrp* is proposed for the group of genes whose analysis was initiated in this study because of the multiple resistance and pH homeostasis-related functions of this locus. MrpA is a strong candidate for a secondary Na^+/H^+ antiporter which probably also has some K^+/H^+ antiport capacity. A mutant with a nonpolar mutation in *mrpA* (VK1) is highly sensitive to Na^+ and exhibits a defect in Na^+-dependent pH homeostasis at moderate concentrations of cation. This mutant exhibits no proton motive force-dependent Na^+ efflux from cells preloaded with 5 mM Na^+ and diluted into energization buffer containing the same Na^+ concentration. Induction of *mrpA* in the *amyE* locus from the p_{spac} promoter restores close to wild-type levels of Na^+ resistance, Na^+-dependent pH homeostasis, and even faster protonophore-sensitive Na^+ efflux than the wild type. Moreover, since *mrpA* expression in *trans* in the polar VK6 mutant also complemented significantly, MrpA is apparently catalytically competent in stoichiometric excess over the other *mrp* gene products. Together, these results are consistent with MrpA being an Na^+/H^+ antiporter that can be energized by the proton motive force and that can function independently of a fixed complex with other *mrp* gene products. This would make MrpA function similar to other prokaryotic Na^+/H^+ antiporters, three of which have rigorously been shown to catalyze antiport when purified and reconstituted alone in proteoliposomes (6, 22, 25). In studies of the *S. aureus* homologue that were conducted entirely in *E. coli*, Hiramatsu et al. (12) noted the requirement of multiple genes for antiport function and proposed that the antiporter functions as a multisubunit entity. The current finding that MrpA raises antiporter activity upon overexpression in the wild type and, most impressively, in the polar VK6 mutant with low levels of other *mrp* genes is difficult to reconcile with a strict dependency on a particular stoichiometric complex. The present studies do not rule out the possibility, however, that under particular conditions MrpA can also function within a specific stoichiometric relationship to other *mrp* gene products in a membrane complex. Moreover, it is conceivable that under these circumstances there is a primary mode of energization via electron transport through the complex. A more complete mutational and biochemical analysis of this complicated locus is needed to fully resolve this important issue.

It is likely that at least one other *mrp* gene product is needed as a chaperone or assembly factor for catalytic *mrp* gene products, e.g., MrpA and MrpF. This would account for the lack of complementation of VKN1. Expression of *mrpA* from an IPTG-inducible promoter would have obviated the need for any otherwise necessary transcriptional regulator. This level of overexpression might also have allowed the assembly of significant amounts of active MrpA even at lower levels of a chaperone or assembly factor (i.e., as in VK6) than are essential for significant activity when *mrpA* expression occurs at normal low levels. In VK15, which has an intact *mrpA* gene but no *mrpFG* function, the Na^+ resistance was enhanced more by *mrpFG* expression than by *mrpF* expression. No difference was observed when the *mrpA* gene was absent. This suggests that MrpG enhances MrpA stability, assembly, or function when *mrpA* is expressed in its normal chromosomal setting. We hypothesize that MrpG may play chaperone or assembly roles for MrpA and perhaps for other *mrp*-encoded structural gene products.

MrpF also appears catalytically competent when expressed from an IPTG-inducible promoter in the VK15 mutant as well as in VK6, which has no MrpA and greatly reduced levels of all other *mrp* gene products. MrpF-dependent Na^+ efflux and cholate efflux were evident, but coupling of the two fluxes was not demonstrated. It may be difficult to demonstrate cholate-dependent Na^+ efflux, because additional Na^+ efflux systems are present in the biological membranes. Because of the low concentration of cholate used, it is similarly unlikely that Na^+-dependent cholate efflux could be demonstrated without developing a much more purified system. It is of interest that a soil bacterium such as *B. subtilis* has a cholate efflux system and, as assessed from the genome project annotations, may have more than this one (YocS; GenBank accession no. Z99114). Whether this relates to the need to extrude some endogenous substrate that has a related structure or to the presence of cholate-like animal or plant products in the natural environment is unknown. Other bacterial extrusion systems for cholate have been reported (18, 19), and one of them is induced by Na^+, although the cation has not been shown to be a substrate (18).

Both MrpA and MrpF may contribute to the Na^+ resistance role. If MrpF-dependent Na^+-cholate efflux is coupled to H^+ uptake, they may both contribute to pH homeostasis as well. Clarification of the relative contributions, under different conditions, will require nonpolar mutations in each gene. Elucidation of the roles of the other *mrp* gene products, including the hypothesized chaperone role for MrpG, will similarly require additional, single nonpolar mutations in those other genes.

Thus far, all the *B. subtilis mrp* gene functions are linked by a relationship to Na^+, and by far the dominant phenotype associated with all the mutations in this locus is Na^+ sensitivity. After clarification of the full panoply of functions of this locus, it will be of interest to examine the differences between the *B. subtilis* operon and those of other organisms in which the dominant physiological role is different or predominantly related to a different cation, e.g., Na^+-specific pH homeostasis in *Bacillus* strain C-125 (10) and K^+ sensitivity in *R. meliloti* (23). The *mrp* locus is the third locus of *B. subtilis* that has been

shown to play a role in Na^+ resistance. *tetA*(L) plays a dominant role in Na^+ (K^+)-dependent pH homeostasis in *B. subtilis* (5) and also plays a significant role in Na^+ exclusion in a range of Na^+ above 25 mM, but the present studies indicate that Mrp function is a crucial adjunct to TetA(L) for the purposes of Na^+ resistance. Another Na^+ extrusion system, the ATP-binding cassette-type *natAB* of *B. subtilis*, is probably more specialized, becoming important primarily under circumstances when the membrane integrity and/or the proton motive force are reduced (4). Preliminary experiments indicate, though, that *mrp* also plays a role under such circumstances.

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ADDENDUM IN PROOF

While this article was under review, Kosono et al. (S. Kosono, S. Morotomi, M. Kitada, and T. Kudo, Biochim. Biophys. Acta **1409:**171–175, 1999) reported the properties of a *mrpA* mutant that was probably equivalent to the polar *mrpA* mutant (VK6) described in this study; these investigators found sensitivity to Na^+ similar to that observed in VK6.

REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. F. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Andrews, S. C., B. C. Berks, J. McClay, A. Ambler, M. A. Quail, P. Golby, and J. R. Guest.** 1997. A 12-cistron *Escherichia coli* operon (*hyf*) encoding a putative proton-translocating formate hydrogenlyase system. Microbiology **143:**3633–3647.
3. **Cheng, J., A. A. Guffanti, and T. A. Krulwich.** 1994. The chromosomal tetracycline-resistance locus of *Bacillus subtilis* encodes a Na^+/H^+ antiporter that is physiologically important at elevated growth pH. J. Biol. Chem. **269:** 27365–27371.
4. **Cheng, J., A. A. Guffanti, and T. A. Krulwich.** 1997. A two gene ABC-type transport system involved in Na^+ extrusion by *Bacillus subtilis* is induced by ethanol and protonophore. Mol. Microbiol. **23:**1107–1120.
5. **Cheng, J., A. A. Guffanti, W. Wang, T. A. Krulwich, and D. H. Bechhofer.** 1996. Chromosomal *tetA*(L) gene of *Bacillus subtilis*: regulation of expression and physiology of a *tetA*(L) deletion strain. J. Bacteriol. **178:**2853–2860.
6. **Cheng, J., D. B. Hicks, and T. A. Krulwich.** 1996. The purified *Bacillus subtilis* tetracycline efflux protein TetA(L) reconstitutes both tetracycline-cobalt/H^+ and $Na^+(K^+)/H^+$ exchange. Proc. Natl. Acad. Sci. USA **93:** 14446–14451.
7. **Dimari, J. F., and D. Bechhofer.** 1993. Initiation of mRNA decay in *Bacillus subtilis*. Mol. Microbiol. **7:**705–717.
8. **Guffanti, A. A., and T. A. Krulwich.** 1995. Tetracycline/H^+ antiport and Na^+/H^+ antiport catalyzed by the *Bacillus subtilis* TetA(L) transporter expressed in *Escherichia coli*. J. Bacteriol. **177:**4557–4561.
9. **Hagenbuch, B., B. Stieger, M. Foguet, H. Lubbert, and P. J. Meier.** 1991. Functional expression cloning and characterization of the hepatocyte Na^+/bile acid cotransport system. Proc. Natl. Acad. Sci. USA **88:**10629–10633.
10. **Hamamoto, T., M. Hashimoto, M. Hino, M. Kitada, Y. Seto, T. Kudo, and K. Horikoshi.** 1994. Characterization of a gene responsible for the Na^+/H^+ antiporter system of alkalophilic *Bacillus* species strain C-125. Mol. Microbiol. **14:**939–946.
11. **Henner, D. J.** 1990. Inducible expression of regulatory genes in *Bacillus subtilis*. Methods Enzymol. **185:**223–228.
12. **Hiramatsu, T., K. Kodama, T. Kuroda, T. Mizushima, and T. Tsuchiya.** 1998. A putative multisubunit Na^+/H^+ antiporter from *Staphylococcus aureus*. J. Bacteriol. **180:**6642–6648.
13. **Horton, R. M.** 1996. *In vitro* recombination and mutagenesis of DNA. Methods Mol. Biol. **67:**141–149.
14. **Ireton, D. D., Z. Rudner, K. J. Siranosian, and A. D. Grossman.** 1993. Integration of multiple developmental signals in *Bacillus subtilis* through Spo0A transcription factor. Genes Dev. **7:**283–294.
15. **Jacquemin, E., B. Hagenbuch, B. Stieger, A. W. Wolkoff, and P. J. Meier.** 1994. Expression cloning of a rat liver Na^+-independent organic anion transporter. Proc. Natl. Acad. Sci. USA **91:**133–137.
16. **Kudo, T., M. Hino, M. Kitada, and K. Horikoshi.** 1990. DNA sequences required for the alkalophily of *Bacillus* sp. strain C-125 are located close together on its chromosomal DNA. J. Bacteriol. **172:**7282–7283.
17. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
18. **Ma, D., D. N. Cook, M. Alberti, N. G. Pon, H. Nikaido, and J. E. Hearst.** 1995. Genes *acrA* and *acrB* encode a stress-induced efflux system of *Escherichia coli*. Mol. Microbiol. **16:**45–55.
19. **Mallonee, D. H., and P. B. Hylemon.** 1996. Sequencing and expression of a gene encoding a bile acid transporter from *Eubacterium* sp. strain VPI 12708. J. Bacteriol. **178:**7053–7058.
20. **Murphy, E., L. Huwyler, and M. D. D. Bastos.** 1985. Transposon *Tn*554: complete nucleotide sequence and isolation of transposition defective and antibiotic-sensitive mutants. EMBO J. **4:**3357–3365.
21. **Oudega, B., G. Koningstein, L. Rodriguez, M. deSalas Ramon, H. Hilbert, A. Disterhoft, T. M. Pohl, and T. Weitzenegger.** 1997. Analysis of the *Bacillus subtilis* genome: cloning and nucleotide sequence of a 62 kb region between 275− (*rrnB*) and 284− (*pai*). Microbiology **143:**2769–2774.
22. **Pinner, E., E. Padan, and S. Schuldiner.** 1994. Kinetic properties of NhaB, a Na^+/H^+ antiporter from *Escherichia coli*. J. Biol. Chem. **269:**26274–26279.
23. **Putnoky, P., A. Kerezt, T. Nakamura, G. Endre, E. Grosskopf, P. Kiss, and A. Kondorosi.** 1998. The *pha* cluster of *Rhizobium meliloti* involved in pH adaptation and symbiosis encodes a novel type of K^+ efflux system. Mol. Microbiol. **28:**1091–1101.
24. **Spizizen, J.** 1958. Transformation of biochemically deficient strains of *Bacillus subtilis* by deoxyribonucleate. Proc. Natl. Acad. Sci. USA **44:**1072–1078.
25. **Taglicht, D., E. Padan, and S. Schuldiner.** 1991. Overproduction and purification of a functional Na^+/H^+ antiporter coded by *nhaA*(*ant*) from *Escherichia coli*. J. Biol. Chem. **266:**11289–11294.
26. **Thanassi, D. G., L. W. Cheng, and H. Nikaido.** 1997. Active efflux of bile salts by *Escherichia coli*. J. Bacteriol. **179:**2512–2518.
27. **Weidner, U., S. Geier, A. Ptock, T. Friedrich, H. Leif, and H. Weiss.** 1993. The gene locus of the proton-translocating NADH:ubiquinone oxidoreductase in *Escherichia coli*. J. Mol. Biol. **233:**109–122.
28. **Wong, M. H., P. Oelkers, A. L. Craddock, and P. A. Dawson.** 1994. Expression cloning and characterization of the hamster ileal sodium-dependent bile acid transporter. J. Biol. Chem. **269:**1340–1347.
29. **Yagi, T.** 1993. The bacterial energy-transducing NADH-quinone oxidoreductases. Biochim. Biophys. Acta **1141:**1–17.