MINI-REVIEW

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The Mrp system: a giant among monovalent cation/proton antiporters?

Abstract Mrp systems are a novel and broadly distributed type of monovalent cation/proton antiporter of bacteria and archaea. Monovalent cation/proton antiporters are membrane transport proteins that catalyze efflux of cytoplasmic sodium, potassium or lithium ions in exchange for external hydrogen ions (protons). Other known monovalent cation antiporters are single gene products, whereas Mrp systems have been proposed to function as hetero-oligomers. A *mrp* operon typically has six or seven genes encoding hydrophobic proteins all of which are required for optimal Mrp-dependent sodium-resistance. There is little sequence similarity of Mrp proteins to other antiporters but three of these proteins have significant sequence similarity to membrane embedded subunits of ion-translocating electron transport complexes. Mrp antiporters have essential roles in the physiology of alkaliphilic and neutralophilic *Bacillus* species, nitrogen-fixing *Sinorhizobium meliloti* and in the pathogen *Staphylococcus aureus*, although these bacteria contain multiple monovalent cation/proton antiporters. The wide distribution of Mrp systems leads to the anticipation of important roles in an even wider variety of pathogens, extremophiles and environmentally important organisms. Here, the distribution, established physiological roles and catalytic activities of Mrp systems are reviewed, hypotheses regarding their complexity are discussed and major open questions about their function are highlighted.

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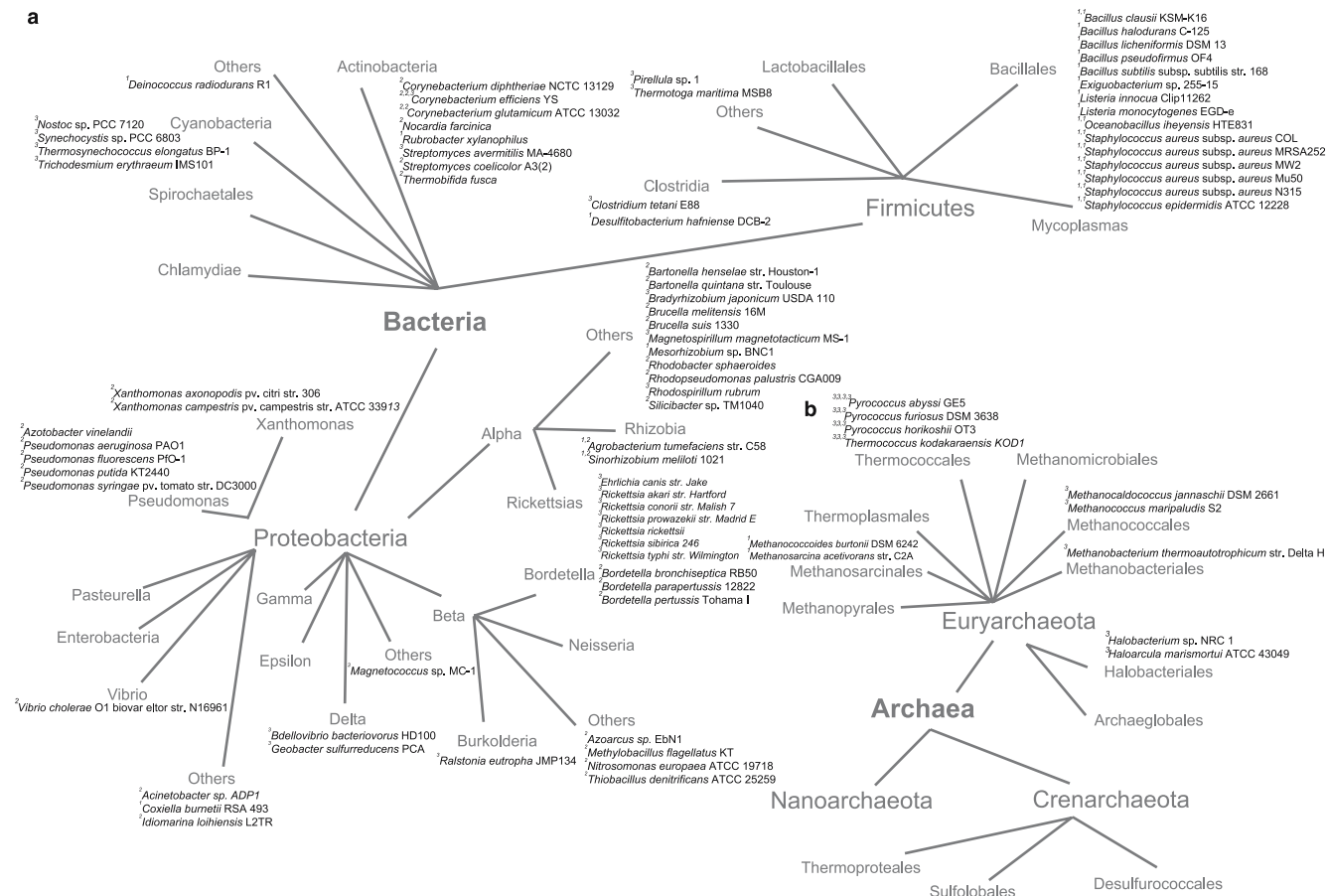Faculty of Life Sciences, Toyo University,
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Introduction

Mrp is a monovalent cation/proton antiporter system encoded by multi-cistronic operons that are annotated across a wide spectrum of prokaryotes (Fig. 1). Monovalent cation/proton antiporters are membrane transport proteins that catalyze the efflux of monovalent cations ions such as Na$^+$, K$^+$ and Li$^+$ outward in a coupled reaction that transports protons, H$^+$, inward. These antiporters are secondary active transporters that are energized by the electrochemical proton gradient, the Δp (inside negative and alkaline), that is generated by distinct primary proton pumps of the cells, e.g. proton-pumping complexes of the respiratory chain or other proton pumping oxidoreductases, the F-ATPase, or light-driven proton pumps (West and Mitchell 1974) (Fig. 2). Almost all prokaryotes express multiple, distinct monovalent cation/proton antiporters and many roles have been suggested for them, including essential roles in reducing the cytoplasmic concentration of toxic cations (e.g. Na$^+$ and Li$^+$) and in proton uptake that supports cytoplasmic pH homeostasis under alkaline conditions (Booth 1985; Padan and Schuldiner 1996; Padan and Krulwich 2000; Padan et al. 2001). Mrp antiporters are quite different from other antiporters and

Fig. 1 Distribution of putative *mrp* operons and gene clusters. Prokaryotic tree with genera in grey and organisms containing Mrp gene clusters among the bacteria (**a**) and archaea (**b**). This tree was adapted from http://www.ncbi.nlm.nih.gov/sutils/genom_tree.cgi and the gene information was obtained from NCBI. Superscripts next to the names of organisms indicate the whether that organism possesses one or more putative operon ([1,2]) or cluster ([3]) as described in the text and depicted in Fig. 3

they play major physiological roles in those bacteria in which their role has been probed.

How were Mrp systems discovered? And what is distinctive about them aside from their very wide distribution? Mrp antiporters were first discovered in work on alkaliphilic *Bacillus halodurans* C-125, in break-through studies by Kudo, Horikoshi and colleagues that identified the Mrp antiporter as the critical Na^+/H^+ antiporter for cytoplasmic pH homeostasis in alkaliphilic *Bacillus* (Kudo et al. 1990; Hamamoto et al. 1994). A cross-over from a cloned chromosomal DNA fragment containing the first three genes of the *mrp* operon was found to have corrected a point mutation in the *mrpA* gene of a non-alkaliphilic strain of *B. halodurans* C-125, simultaneously restoring wild-type levels of Na^+/H^+ antiport activity, a capacity for pH homeostasis and alkaliphily (Kudo et al. 1990; Hamamoto et al. 1994). Very modest sequence similarity between MrpA and any known monovalent cation/proton antiporters was found, but pronounced sequence similarity was noted between MrpA and membrane-embedded subunits of ion-translocating electron transport complexes. This sequence similarity

to subunits of the respiratory chain Complex I (Nuo or ND) has been extended to MrpC and MrpD proteins and described in detail now that the full six or seven genes of *mrp* operon homologues have been sequenced in many prokaryotes (Mathiesen and Hägerhäll 2002; Mathiesen and Hägerhäll 2003). In addition to this oddity, all the Mrp genes are predicted to encode integral membrane proteins with multiple membrane-spanning domains and studies of the full *mrp* operons of *Staphylococcus aureus* (Hiramatsu et al. 1998) and *Bacillus subtilis* (Ito et al. 2000) showed that all seven genes are required for the operon to confer significant Na^+-resistance. On the basis of this finding, the Mrp antiporter has been proposed to function as a hetero-oligomeric complex (Hiramatsu et al. 1998). If this is substantiated, it will make Mrp systems structurally distinct from other prokaryotic monovalent cation/proton antiporters; to date, these are single gene products that function as monomers or homo-oligomers (Gerchman et al. 2001; Safferling et al. 2003). A final distinction between the Mrp systems and other monovalent cation/proton antiporters is the finding that Mrp supports cholate-resistance in *B. subtilis*,
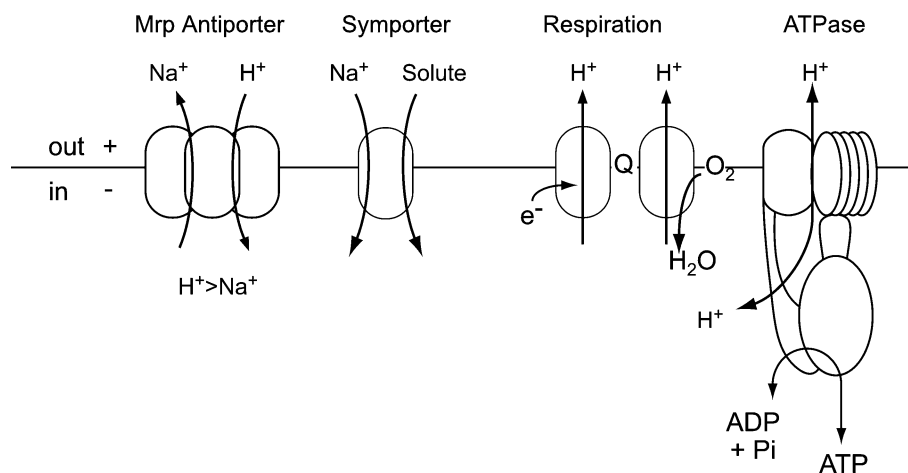
Fig. 2 Schematic representation of a hetero-oligomeric Mrp antiporter in a cytoplasmic membrane together with primary proton pumping complexes and Na^+/solute symporters. The Mrp antiporter is hypothesized to be a hetero-oligomeric complex (see text (Hiramatsu et al. 1998; Mathiesen and Hägerhäll 2003)). It catalyzes Na^+ efflux in exchange for a greater number of H^+, i.e. an electrogenic exchange. The antiport reaction is energized by the Δp (inside negative and alkaline) established by proton-pumping activities of the respiratory chain or ATPase, that are shown in a generic schematic form. For sustained Na^+/H^+ antiport under limiting Na^+ conditions, e.g. when antiport is serving the function of Na^+-dependent alkali-tolerance, Na^+ re-entry routes are important to ensure a steady supply of cytoplasmic substrate for Mrp (Booth 1985). Na^+/solute symporters are one of those re-entry routes, a major one in alkaliphiles (Krulwich 1995; Ito et al. 2004)

a novel conjoining of anion efflux with Na^+/H^+ antiport (Ito et al. 1999; Ito et al. 2000).

Because of these distinctive properties, Mrp antiporter systems have been classified in their own category, cation: proton antiporter-3 (CPA3), in the Transporter Classification system (Saier et al. 1999). In the first flurry of studies of full *mrp* loci, other operons were named: *pha*, for pH adaptation, in *Sinorhizobium meliloti* (Putnoky et al. 1998); *mnh*, for multi-subunit Na^+/H^+ antiporter, in *Staphylococcus aureus* (Hiramatsu et al. 1998); and *mrp/sha*, respectively multiple resistance and pH-related antiporter and sodium-hydrogen antiporter, in *Bacillus* (Ito et al. 1999; Kosono et al. 2000). We will use Mrp as the overall designation as we review the distribution, the roles and the catalytic properties of these systems, and summarize hypotheses in connection with their complexity.

Distribution and categories of *mrp* gene clusters

Putative operons and clusters of *mrp* gene homologues among Eubacteria (Fig. 1a) and Archaea (Fig. 1b), exhibit significant variations of the gene arrangements in different micro-organisms. These arrangements are depicted as including two sets of putative operons, Group 1 and Group 2, in Fig. 3a (and additional clusters of *mrp*-like genes in Fig. 3c). At present, there is almost no biochemical information about the individual Mrp proteins of any of these genes that would suggest a rationale for the different arrangements. In fact, direct evidence for a *mrp* operon has only been shown for Group 1 *Bacillus subtilis* (Ito et al. 1999) and *Bacillus pseudofirmus* OF4 *mrp* (Ito and Krulwich, unpublished data) for which *mrp* RNA corresponds to the size

expected for a single operon encompassing all the *mrp* genes. In the other putative Group 1 and 2 operons, as well as in many of the diverse mrp-like gene clusters shown in Fig. 3c, the location of promoter candidates and potential stem-loop terminators, gene spacing or overlaps have been taken as indications of an operon in annotation of genomes. The distinction between Group 1 and Group 2 *mrp* operons lies in the arrangement of their *mrpA* and *mrpB* genes (Fig 3b). Group 1 *mrp* operons contain separate *mrpA* and *mrpB* genes, but within that *mrpA* there is also a *mrpB* domain (Fig. 3b). By contrast, Group 2 *mrp* operons lack a separate *mrpB* gene but possess two *mrpB* domains within the *mrpA* gene (this larger *mrpA* is designated *mrpA'* in Fig. 3). Thus, both groups contain two *mrpB* coding regions but only in Group 1 operons is one of those encoded as a separate *orf*. By contrast, having one or more separate *mrpB* genes is a common theme among the diverse *mrp* gene clusters shown in Fig 3c. Some of the *mrpB* homologues in these clusters are longer and contain additional domains that are not found in Group 1 and Group 2 operons. For the purposes of Fig. 3, two criteria distinguish a *mrpA*/*mrpA'* gene from a *mrpD* gene: the presence of one or more *mrpB* domain(s); and a size range of ~800–1000 amino acid residues for MrpA (~800–900 for MrpA, ~1000 for MrpA') *vs.* ~400–500 amino acid residues for MrpD. The putative Group 1 and Group 2 *mrp* operons possess both *mrpA* genes and *mrpD* genes by these criteria. The clusters shown in Fig. 3c lack *mrpA* and possess only *mrpD*, sometimes in multiple copies. Perhaps a fusion of *mrpD* with one or more *mrpB* genes gave rise to a more derived gene, *mrpA* (Group 1)/*mrpA'* (Group 2).

Group 1 *mrp* operons, with seven genes in the order *mrpA–G*, are found in disparate Gram-positive and
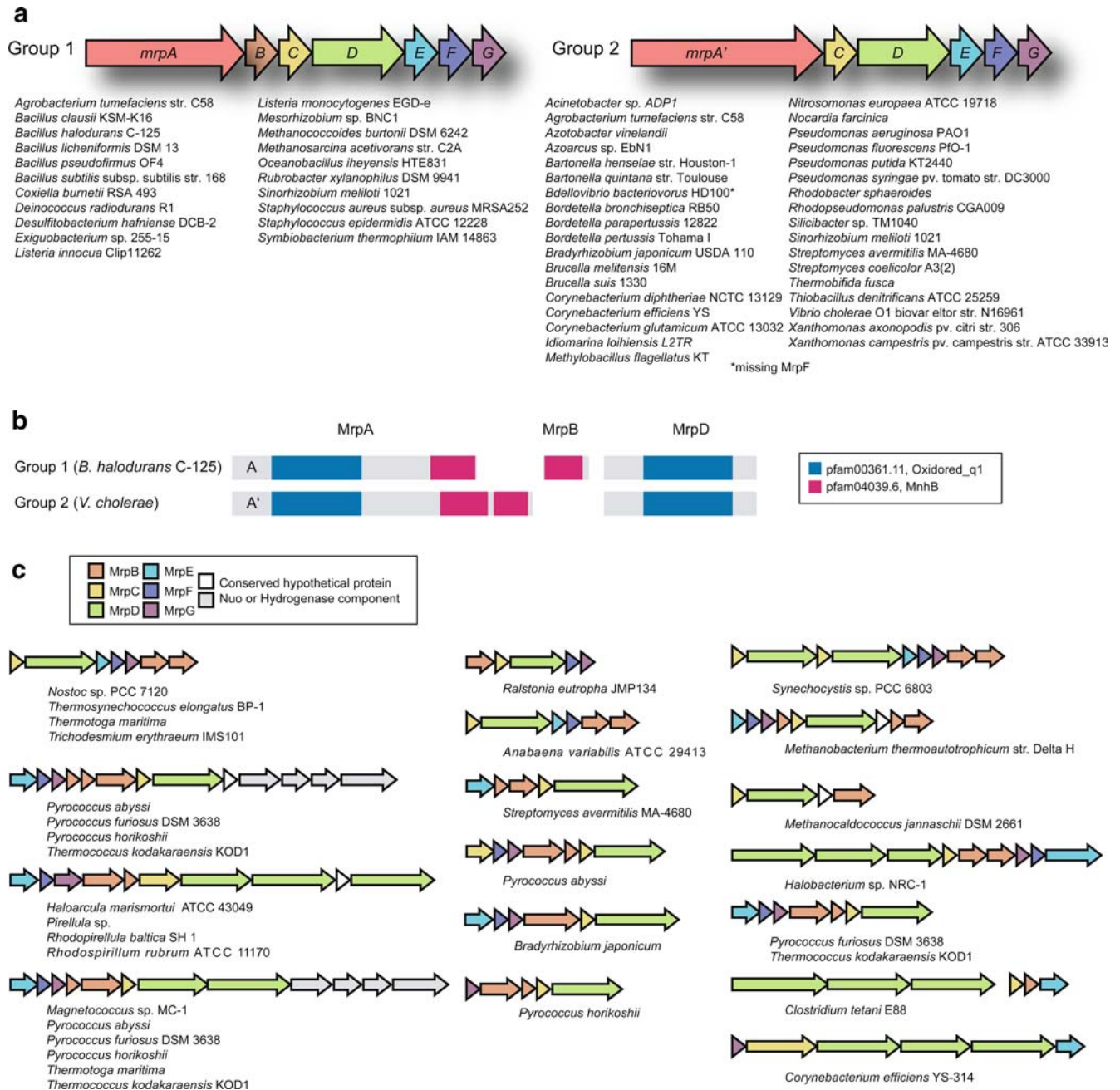
Fig. 3 Gene arrangements of putative *mrp* operons and clusters of *mrp*-like genes in diverse prokaryotes. **(a)** Group 1 operons includes organisms whose annotated *mrp* operons include 7 genes *mrpA-G* and whose *mrpA* gene contains a single *mrpB* domain. Group 2 includes organisms whose annotated *mrp* operons possess 6 genes, *mrpA′, C-G*, and whose *mrpA′* gene contains two *mrpB* domains. **(b)** Arrangements of domains in MrpA/MrpA′ MrpB and MrpD of putative Group 1 and 2 operons. A representative organism is noted on the left. The blue domain in MrpA/MrpA′ and MrpD, pfam00361.11, has homology to NuoL, HyfB, NuoM, and NuoN; the pink domain in MrpA/MrpA′ and MrpB, pfam04039.6, MnhB, has homology to MrpB. **(c)** Patterns and distribution of clusters of *mrp* homologues that overlap or are juxtaposed by less than 20 bp in diverse organisms. The gene information was obtained from NCBI

Gram-negative bacteria, including pathogens of plants (*Agrobacterium tumefaciens*, *Xanthomonas campestris*) and animals (*Coxiella burnetii*, *Listeria* species, and *S. aureus*), extremophiles (alkaliphilic *Bacillus* species, *Deinococcus radiodurans*), and also representatives from the archaea (*Pyrococcus* and *Thermococcus* species) (Fig. 3a). Group 1 includes the *mrp* operons for which most of the direct experimental studies have been reported to date, those of the alkaliphilic and neutralophilic *Bacillus* species, Pha of *S. meliloti* and Mnh of *S. aureus*. Each of the genomes of *S. aureus* strains contains two complete Group 1 *mrp* operons. The deduced products of one of these operons in each strain, e.g. Mnh, are closely related to Mrp protein sequences

from *Bacillus* species, while the predicted products of the second operon are distinctly different. This pattern is also seen in the moderately alkaliphilic marine bacterium *Oceanobacillus iheyensis*. *S. meliloti* and *A. tumefaciens* also have two complete *mrp* operons; but one has a Group 1 arrangement and the other has a Group 2 arrangement. There is thus far no information about the physiological roles of the two different Mrp systems in organisms that possess more than one.

Group 2 *mrp* operons, containing six genes that include the larger *mrpA′*, are dominated by Gram-negative bacteria but the group contains a wide variety of bacterial types, both phylogenetically and physiologically (Fig. 3a). All organisms listed in Fig. 3a have complete gene sets except for *Bdellovibrio bacteriovorans*, which lacks an apparent *mrpF*. Among its Gram-positive representatives are operons from several high G + C organisms (*Thermobifida fusca*, *Corynebacterium* species). Group 2 *mrp* operons are also found in photo- and chemo-autotrophs as well as several nitrogen-fixing bacteria, free-living and symbiotic. Both plant (*Xanthomonas*, *Agrobacterium*) and animal pathogens (*Brucella melitensis*, *Pseudomonas aeruginosa*, *Vibrio cholerae*, *Acinetobacter*) are again represented.

Additional organisms possess clusters of *mrp* homologues in their genomes in a different gene order from those in the putative operons of Groups 1 and 2 (Fig. 3c). Many of them have *mrpEFG* at the beginning of the cluster. The clusters that are included in Fig. 3c have *mrp* gene homologues that either overlap or are less than 20 bp apart, but no functional roles have yet been demonstrated for any of them. In some of the clusters, the *mrp*-like genes are intermixed with genes of unknown relatedness that are indicated by white filling in the figure. Almost all clusters have apparent *mrpB* duplications, and some have the variant, longer *mrpB* gene and/or duplications of *mrpD*. The *mrpD* repeats exhibit the usual close similarity to genes of the NADH:oxidoreductase. In a group of largely extremophilic organisms, the *mrp* gene homologues are just upstream of *mbh* genes that encode a membrane-bound hydrogenase whose activity has been demonstrated in the hyperthermophilic archaeon, *Pyrococcus furiosus* (Silva et al. 2000; Sapra et al. 2003) (Fig. 3c).

It is worth noting that Mrp antiporter systems are thus far absent from some entire groups of bacteria and archaea, including the enteric bacteria, lactobacilli, streptococci, spirochetes, chlamydiae, chrenarchaea and nanoarchaea (Fig. 1). Clearly Mrp systems are not ubiquitous among organisms in which Na^+-resistance and alkali-tolerance are known to depend upon monovalent cation/proton antiporters. *Escherichia coli*, whose monovalent cation/proton antiporters are among the most extensively characterized (Padan and Schuldiner 1996; Padan et al. 2001; Shijuku et al. 2001; Lewinson et al. 2004), uses NhaA for Na^+ resistance and pH homeostasis up to about pH 9, and the multidrug and Na^+ $(K^+)/H^+$ antiporter MdfA to extend alkali-toler-

ance above pH 9 (Lewinson et al. 2004; Padan et al. 2004). It will be interesting to unravel the added competitive value conferred by Mrp on the many organisms in which it is found and the relationship of that added value to the ecological challenges, metabolic profile, and use of Na^+ for bioenergetic work such as solute uptake in those organisms.

Catalytic properties and physiological roles of Mrp antiporters

The functional studies conducted to date on individual Mrp systems from the Group 1 cluster (Fig. 3a) include limited assays of cation and proton fluxes in whole cells and in everted membrane vesicles. Information from these assays is supplemented by inferences drawn from physiological experiments on Na^+- or K^+-sensitivity, Na^+ exclusion from whole cells or the cation-dependence of Mrp-dependent pH homeostasis. Together these studies indicate that: the Pha system of *S. meliloti* is a K^+/H^+ antiporter (Putnoky et al. 1998); the Mrp systems of alkaliphilic *Bacillus* species, *B. halodurans* C-125 and *B. pseudofirmus* OF4 (Hamamoto et al. 1994; Ito et al. 2000) and the Mnh system of *S. aureus* (Hiramatsu et al. 1998) are Na^+/H^+ antiporters and; the Mrp (Sha) system of *B. subtilis* catalyzes both Na^+/H^+ (Ito et al. 1999; Kosono et al. 1999) and K^+/H^+ antiport (Ito et al. 1999). Mrp-dependent antiport in alkaliphilic *Bacillus* spp. and in *B. subtilis* can be energized by an imposed transmembrane potential (Hamamoto et al. 1994; Ito et al. 1999; Ito et al. 2000) as well as by an imposed transmembrane ΔpH (Hiramatsu et al. 1998; Ito et al. 2001), i.e. Mrp-dependent monovalent cation/proton antiport behaves as expected for a secondary antiport energized by the Δp. The energization by an imposed transmembrane electrical potential is consistent with an electrogenic monovalent cation/proton exchange that involves net movement of charge during each antiport turnover, e.g. a Na^+/H^+ antiport for which the stoichiometry of H^+:Na^+ transported per turnover is more than 1 (Fig. 2). An electrogenic Na^+/H^+ antiport can utilize the energy of the transmembrane potential component of the Δp, the $\Delta\Psi$ (inside negative in whole cells or right-side-out membrane vesicles) which is a useful feature for antiporters supporting alkaline pH homeostasis (MacNab and Castle 1987). No detailed biochemical characterization of a Mrp antiporter has yet been conducted in membrane vesicles, e.g. measuring kinetic parameters, nor are there any data yet reported for a purified, reconstituted Mrp system.

As described in the Introduction, the first role established for a Mrp antiporter was in cytoplasmic pH homeostasis of alkaliphilic *B. halodurans* C-125 at highly alkaline external pH, studies that also showed a Mrp role in Na^+-resistance (Hamamoto et al. 1994; Kitada et al. 2000; Krulwich et al. 2001). The Na^+/H^+ antiport activity is attributed to MrpA since the point mutation that leads to a non-alkaliphilic phenotype was in *mrpA*

(Hamamoto et al. 1994; Kitada et al. 2000). The finding that a mutant in MrpC is also non-alkaliphilic (Seto et al. 1995) raises the possibility that additional *mrp* genes play critical roles even if MrpA contains the cation and proton translocation pathways. A requirement for multiple *mrp* genes in the extreme alkaliphiles is consistent with our inability to recover mutants of genetically accessible *B. pseudofirmus* OF4 when attempts are made to disrupt any one of several *mrp* genes. The major Na^+/H^+ antiporter of alkaliphilic *Bacillus* species is likely to be required throughout their pH range. Na^+-specific monovalent cation/proton antiport is required for the alkaliphile to maintain a cytoplasmic pH of 8.2 to 9.5 at external pH values from 10.5 to 11.2 (Sturr et al. 1994). In addition, the alkaliphile's use of $Na^+/$ solute symport (co-transport) for all ion-coupled solute systems at both near-neutral and highly alkaline pH may result in a requirement for robust Mrp activity, dependent upon multiple Mrp proteins, under all pH conditions (Krulwich 1995; Krulwich et al. 1998).

In neutralophilic *B. subtilis*, the Mrp system has a role in Na^+-resistance and in both Na^+- and K^+-dependent alkaline pH homeostasis (Ito et al. 1999). Expression of *mrp* genes is up-regulated by alkaline shock (Wiegert et al. 2001) and elevated cytoplasmic $[Na^+]$ (Ito et al. 2000). Conversely, a *mrpA/shaA* mutant of *B. subtilis* changes its use of the diverse ECF (extracytoplasmic function) σ factors in a growth phase-dependent manner, including increased σ^W- and σ^M-dependent transcription in transition phase (Kosono et al. 2004) that is consistent with a complex stress reaction. In contrast to the central role of Mrp in pH homeostasis of alkaliphilic *Bacillus* species, Mrp is not the dominant antiporter in this process in *B. subtilis*; that role belongs to the multifunctional (tetracycline-divalent metal)$^+$ $(Na^+)(K^+)/H^+$ antiporter TetL (Cheng et al. 1996; Ito et al. 1999). Instead, the *B. subtilis* Mrp system plays a dominant role in Na^+-resistance in this organism as indicated by the profoundly Na^+-sensitive phenotypes of *mrp/sha* mutants in each individual gene (Ito et al. 1999; Kosono et al. 1999; Ito et al. 2000). This is probably why *mrp* is listed among the essential genes of *Bacillus subtilis* as examined on LB medium (Kobayashi et al. 2003) even though Mrp point mutants, a full *mrp* null strain, as well as single gene deletions are all viable in *B. subtilis* (Ito et al. 2000). LB contains levels of NaCl that are toxic for *mrp* mutants (Ito et al. 1999; Kosono et al. 1999). A sporulation defect that has also been noted in *mrp/sha* mutants of *B. subtilis* (Kosono et al. 2000; Yoshinaka et al. 2003; Kosono et al. 2004) may be secondary to Na^+ toxicity at a critical point in sporulation. It will be of interest to compare the activity *vs*. pH profile of alkaliphile and *B. subtilis* Mrp to determine whether a limited efficacy of *B. subtilis* Mrp in the alkaline end of its growth range correlates with its relatively small role in alkaline pH homeostasis compared to alkaliphile Mrp. Similarly, profiles of the activity and apparent K_m of *S. aureus* Mnh in *E. coli* vesicles may provide inferences about the

role that makes this Mrp system essential in this alkaline- and salt-tolerant pathogen (Vijaranakul et al. 1997; Hiramatsu et al. 1998; Ji et al. 2001).

The other transport substrate thus far reported for a Mrp system has been mentioned, i.e. a capacity for cholate efflux by the *B. subtilis* Mrp system (Ito et al. 1999; Ito et al. 2001). An *mrp* null strain of *B. subtilis*, from which the entire *mrp* operon is deleted, exhibits significantly reduced resistance to growth inhibition by added cholate that is complemented by introduction of the *mrpF* gene into the chromosomal *amyE* locus under control of an IPTG-inducible promoter (Ito et al. 2000). Reduced cholate efflux was observed in starved whole cells of the mutant relative to the wild type. This defect, too, is complemented significantly by re-introduction of *mrpF*. Homology has been noted between MrpF and Na^+-coupled bile transporters (Ito et al. 1999) and between MrpF and a region of voltage-gated Na^+ channels (Mathiesen and Hägerhäll 2003). However, no experimental evidence for MrpF-mediated coupling between Na^+ and cholate fluxes has been found (Ito et al. 1999). If efflux of bile salts turns out to be a general property of Mrp systems, it would be one of the potential benefits of this system to bacteria for which bile salts are a challenge (Hase and Barquera 2001; Hung and Mekalanos 2005), e.g. *Vibrio cholerae*, which also has multiple Na^+/H^+ antiporters as well as a respiration-coupled Na^+ pump (Hase and Barquera 2001; Dzioba et al. 2002; Herz et al. 2003).

The other Mrp antiporter system for which a role is indicated is the K^+/H^+ antiporter of the Mrp (Pha) system whose mutational loss results in K^+-sensitivity and loss of the capacity for nitrogen fixation (Putnoky et al. 1998). Perhaps the connection of the antiport to nitrogen fixation depends upon an indirect effect on $[K^+]$ or pH, but an interesting alternative possibility is that ammonium, the product of nitrogen fixation, is an additional cytoplasmic substrate of this Mrp antiporter system. Some other K^+ translocating membrane transporters have a capacity for NH_4^+ translocation as well (Buurman et al. 1991; Wei et al. 2003).

Genomic inferences and mutant phenotypes suggest a possible Mrp complex

Two sets of data contribute to the hypothesis that Mrp antiporters function as a hetero-oligomer containing multiple Mrp proteins (if not all). One set of data includes the experimental observations that all or most of these hydrophobic membrane proteins are required for full Mrp-dependent Na^+-resistance conferred by Mnh, as shown by deletion analysis of a recombinant plasmid containing the full operon and expressed in antiporter-deficient *E. coli* (Hiramatsu et al. 1998) and by *B. subtilis* Mrp, as assessed by a panel of non-polar in-frame deletions in each gene (Ito et al. 2000).

The other set of observations encompasses similarities in sequence between several Mrp proteins and membrane-embedded protein subunits of NADH:quinone oxidoreductase ion-translocating complexes and others, e.g. Ech (Sauter et al. 1992), Coo (Fox et al. 1996), Mbh (Sapra et al. 2000; Silva et al. 2000), Fpo (Baumer et al. 2000), and *E. coli* hydrogenase Hyf (Künkel et al. 1998) and Hyc (Andrews et al. 1997). For example, the deduced sequence of MrpA from *B. halodurans* C-125 and that of NuoL of the respiratory chain complex I from *Escherichia coli* exhibit 32% identity and 48% similarity over 494 amino acids. These similarities are presumed to relate to the common roles of these membrane proteins in ion translocation within their particular settings (Friedrich and Weiss 1997; Friedrich and Scheide 2000; Steuber et al. 2000; Stolpe and Friedrich 2004). Of note in connection with the hypothesis of a hetero-oligomeric Mrp complex are the indications that the subunits of the energy-coupled NADH:quinone oxidoreductase (Complex I) to which MrpA, MrpD and MrpC exhibit sequence similarity are all part of the membrane-embedded arm of the L-shaped Complex I (Leonard et al. 1987), where they are proposed to be part of the same or contiguous sub-domains or modules (Friedrich and Weiss 1997; Sazanov et al. 2000; Friedrich 2001; Yagi and Matsuno-Yagi 2003). Mathiesen and Hägerhäll hypothesize that a multi-subunit Mrp antiporter complex module was recruited to an ancestral complex I, with NuoL/ND5 (Nuo is bacterial, ND is mitochondrial) being the counterpart of MrpA; NuoN/ND2 of MrpD and NuoK/ND4L of MrpC (Mathiesen and Hägerhäll 2002; Mathiesen and Hägerhäll 2003; Steuber 2003).

Two *caveats* should be noted. First, in spite of the requirement for all of the Mrp proteins for full Mrp-dependent resistance to Na$^+$, sensitive assays of the amount of actual antiport activity supported by individual Mrp proteins have not been conducted. Further, no experimental evidence has yet been reported for an active, purified hetero-oligomeric Mrp complex.

What is the role of the multiple Mrp proteins?

A major question with respect to Mrp antiporter systems is the role of the six or seven Mrp proteins, especially since other highly kinetically competent Na$^+$/H$^+$ antiporters are encoded in monocistronic gene loci and appear to assemble without dedicated chaperones or assembly factors (Padan and Schuldiner 1996; Padan et al. 2004). All the Mrp proteins are predicted to be very hydrophobic integral membrane proteins that span the membrane multiple times (Krulwich et al. 1998; Krulwich et al. 1999), but this does not rule out roles for some of the Mrp proteins in expression, assembly or stability. A requirement for one or more *mrp* gene products for expression of the others is not likely since each of the non-polar in-frame deletions of *mrp* genes in *B. subtilis* leads to increased expression of the other genes (Ito et al. 2000), consistent with the conclusion that a defect in Na$^+$ exclusion accompanies deletion of each gene and that the elevated cytoplasmic Na$^+$ increases expression of the remaining genes. This leaves assembly and stability effects to be directly tested through combined mutagenesis, activity and Western analyses.

Additional possibilities for the novel involvement of multiple genes in monovalent cation/proton antiport activity have also been raised. The first suggestion is that in addition to demonstrated Δ*p*-energized secondary antiporter activity, Mrp possesses a novel primary antiport option in which the Mrp complex itself uses redox energy to directly energize monovalent cation/proton antiport (see the description of the CPA3 group monovalent cation/proton antiporters (http://saier-14–64.ucsd.edu/tcdb/index.php?tc = 2.A.63). If Mrp has such a primary energization mode, some Mrp proteins in addition to those containing the cation and proton pathways could have essential roles in the redox activities, explaining the complexity of *mrp* operons. A primary energization mode for Mrp antiport also addresses an energetic problem of cytoplasmic pH homeostasis at extremely high pH. As noted by Skulachev (Skulachev 1988), the low total Δ*p* at very high pH makes it challenging to use a Δ*p*-dependent secondary antiport for pH homeostasis. If Mrp functions as a primary redox-driven antiporter, energy from down-hill electron transport would energize outward Na$^+$ pumping, producing an electrochemical Na$^+$ gradient. Partial dissipation of the ΔΨ, the electrical component of that gradient, could then catalyze uptake of a smaller number of H$^+$ than the Na$^+$ ions pumped. The overall antiport activity would thus achieve redox-dependent establishment of active Na$^+$ efflux, generation of a ΔΨ (negative in) and net acidification of the cytoplasm.

The idea of a primary Mrp antiporter mode was tested by examining whether expression of the *B. pseudofirmus* OF4 enhanced non-fermentative growth of a respiratory-deficient (Δ*nuo*Δ*ndh*, the two NADH:quinone oxidoreductase-encoding genes) strain of *E. coli* (Wallace and Young 1977). This strain is impaired in respiration-dependent ΔΨ generation. Initial data indicated that the presence of a *Bacillus* Mrp system does enhance non-fermentative growth and oxygen uptake as well as specifically increasing the rate of L-malate oxidation (Ito et al. 2001; Swartz et al. 2005). This effect has now been definitively shown not to reflect a primary electron transport capacity of the Mrp system itself. Rather, the beneficial effect on this mutant is an indirect effect of Mrp, whose expression leads to induction of the L-malate:oxidoreductase of this particular, fragile *E. coli* host, resulting in more efficient flow of electrons to the ΔΨ-generating terminal oxidases (Swartz et al. 2005). There is no support for a primary Mrp antiport mode, but abundant support for secondary antiport, as shown in fluorescence assays and assays in which Mrp-dependent antiport is energized by an imposed transmembrane electrical potential (Hamamoto et al. 1994; Hiramatsu et al. 1998; Ito et al. 1999; Ito et al. 2000; Ito et al. 2001).

What might multiple Mrp proteins contribute to secondary Na^+/H^+ antiport? Perhaps the Mrp proteins or a subset of Mrp proteins have independent catalytic activities of their own, so that a hetero-oligomeric Mrp complex is part of a consortium of transporters that are each advantaged in some way by working in concert. This hypothesis is consistent with the suggestion that MrpA and MrpD are distinct monovalent cation/proton antiporters (Mathiesen and Hägerhäll 2002), with indications that MrpF of *B. subtilis* has cholate transport activity (Ito et al. 1999; Ito et al. 2000) and with sequence similarities of one or more other Mrp proteins to membrane transporters. Mathiesen and Hägerhäll (2003) noted similarity between a region of MrpB and cation translocating δ-endotoxin from *Bacillus thuringiensis* (Bravo 1997) and motif algorithms (Henikoff and Henikoff 1996; Henikoff et al. 1997) suggest a region of similarity between MrpG and Na^+/solute symporters (see Fig. 2). A speculative possibility is that under specific conditions, catalytic activities of one or more Mrp proteins are synergistic for monovalent cation/proton antiport although these proteins do not themselves participate in the antiport reaction directly, e.g. they might help meet the challenge of Na^+ recycling under conditions of limiting $[Na^+]$, enhance proton capture on the alkaline external surface, and relieve transient membrane depolarization during acute changes in the load on the antiport. We note that physical associations have been suggested for eukaryotic Na^+/H^+ exchanger (antiporter) NHE1 and both carbonic anhydrase II and cytoskeleton elements (Li et al. 2002; Orlowski and Grinstein 2004).

Conclusions and investigative challenges

The Mrp antiporter system is found across an enormous phylogenetic range of bacteria and archaea, including numerous pathogenic and environmentally important organisms. Physiological roles have been demonstrated in alkali-, Na^+-, and K^+-resistance that secondarily impact processes such as sporulation and nitrogen fixation in particular species (Putnoky et al. 1998; Kosono et al. 2000). The physiological roles of Mrp systems have so far been attributed to their activity as secondary Na^+/H^+ and/or K^+/H^+ antiporters. Consideration should be given to the possibility of additional Mrp substrates (e.g. NH_4^+) as well as distinct catalytic activities as part of a Mrp complex (e.g. bile salt efflux). Clarification of the physiological roles of Mrp antiporters in more organisms may provide clues to such additional substrates or activities, but detailed biochemical and genetic characterization of individual Mrp systems are also needed. The novel possibility that the active Mrp antiporter is a hetero-oligomeric complex has been a persistent suggestion (Hiramatsu et al. 1998; Mathiesen and Hägerhäll 2003) that will ultimately be confirmed or negated by biochemical work. Finally, the regulation of *mrp* gene expression is yet to be directly explored in spite of interesting indications of regulatory effects of alkaline pH (Wiegert et al. 2001), elevated cytoplasmic $[Na^+]$ (Ito et al. 2000) and growth phase (Kosono et al. 2004). Insights of broad interest will emerge with answers to questions such as: does Mrp function as a hetero-oligomer, i.e. is it a giant among monovalent cation/proton antiporters?; and, if so, does the complex possess more than one catalytic activity?; are there ecological, metabolic and/or bioenergetic features that are shared by the prokaryotes that possess Mrp systems as compared to those that do not?; are there general functional differences between Group 1 and Group 2 Mrp systems?; are there kinetic properties of particular Mrp systems that are clearly adaptive to the major challenges of their individual hosts?; and are the regulatory features of *mrp* gene expression similar across a wide array of hosts or do they reflect the particular roles of individual Mrp systems in ecologically diverse hosts?

References

Andrews SC et al. (1997) A 12-cistron *Escherichia coli* operon (hyf) encoding a putative proton-translocating formate hydrogenlyase system. Microbiology 143(Pt 11):3633–3647

Baumer S, Ide T, Jacobi C, Johann A, Gottschalk G, Deppenmeier U (2000) The $F_{420}H_2$ dehydrogenase from *Methanosarcina mazei* is a Redox-driven proton pump closely related to NADH dehydrogenases. J Biol Chem 275:17968–17973

Booth IR (1985) Regulation of cytoplasmic pH in bacteria. Microbiol Rev 49:359–378

Bravo A (1997) Phylogenetic relationships of *Bacillus thuringiensis* delta-endotoxin family proteins and their functional domains. J Bacteriol 179:2793–2801

Buurman ET, Teixeira de Mattos MJ, Neijssel OM (1991) Futile cycling of ammonium ions via the high affinity potassium uptake system (Kdp) of *Escherichia coli*. Arch Microbiol 155:391–395

Cheng J, Guffanti AA, Wang W, Krulwich TA, Bechhofer DH (1996) Chromosomal *tetA*(L) gene of *Bacillus subtilis*: regulation of expression and physiology of a *tetA*(L) deletion strain. J Bacteriol 178:2853–2860

Dzioba J, Ostroumov E, Winogrodzki A, Dibrov P (2002) Cloning, functional expression in *Escherichia coli* and primary characterization of a new Na^+/H^+ antiporter, NhaD, of *Vibrio cholerae*. Mol Cell Biochem 229:119–124

Fox JD, Kerby RL, Roberts GP, Ludden PW (1996) Characterization of the CO-induced, CO-tolerant hydrogenase from *Rhodospirillum rubrum* and the gene encoding the large subunit of the enzyme. J Bacteriol 178:1515–1524

Friedrich T (2001) Complex I: a chimaera of a redox and conformation-driven proton pump? J Bioenerg Biomembr 33:169–177

Friedrich T, Scheide D (2000) The respiratory complex I of bacteria, archaea and eukarya and its module common with membrane-bound multisubunit hydrogenases. FEBS Lett 479:1–5

Friedrich T, Weiss H (1997) Modular evolution of the respiratory NADH:ubiquinone oxidoreductase and the origin of its modules. J Theor Biol 187:529–540

Gerchman Y, Rimon A, Venturi M, Padan E (2001) Oligomerization of NhaA, the Na$^+$/H$^+$ antiporter of *Escherichia coli* in the membrane and its functional and structural consequences. Biochemistry 40:3403–3412

Hamamoto T et al. (1994) Characterization of a gene responsible for the Na$^+$/H$^+$ antiporter system of alkalophilic *Bacillus* species strain C-125. Mol Microbiol 14:939–946

Hase CC, Barquera B (2001) Role of sodium bioenergetics in *Vibrio cholerae*. Biochim Biophys Acta 1505:169–178

Henikoff JG, Henikoff S (1996) Blocks database and its applications. Methods Enzymol 266:88–105

Henikoff JG, Pietrokovski S, Henikoff S (1997) Recent enhancements to the Blocks Database servers. Nucleic Acids Res 25:222–225

Herz K, Vimont S, Padan E, Berche P (2003) Roles of NhaA, NhaB, and NhaD Na$^+$/H$^+$ antiporters in survival of *Vibrio cholerae* in a saline environment. J Bacteriol 185:1236–1244

Hiramatsu T, Kodama K, Kuroda T, Mizushima T, Tsuchiya T (1998) A putative multisubunit Na$^+$/H$^+$ antiporter from *Staphylococcus aureus*. J Bacteriol 180:6642–6648

Hung DT, Mekalanos JJ (2005) Bile acids induce cholera toxin expression in *Vibrio cholerae* in a ToxT-independent manner. Proc Natl Acad Sci USA 102:8028–8033

Ito M et al (2004) MotPS is the stator-force generator for motility of alkaliphilic *Bacillus*, and its homologue is a second functional Mot in *Bacillus subtilis*. Mol Microbiol 53:1035–1049

Ito M, Guffanti AA, Oudega B, Krulwich TA (1999) *mrp*, a multigene, multifunctional locus in *Bacillus subtilis* with roles in resistance to cholate and to Na$^+$ and in pH homeostasis. J Bacteriol 181:2394–2402

Ito M, Guffanti AA, Wang W, Krulwich TA (2000) Effects of nonpolar mutations in each of the seven *Bacillus subtilis* mrp genes suggest complex interactions among the gene products in support of Na$^+$ and alkali but not cholate resistance. J Bacteriol 182:5663–5670

Ito M, Guffanti AA, Krulwich TA (2001) Mrp-dependent Na$^+$/H$^+$ antiporters of *Bacillus* exhibit characteristics that are unanticipated for completely secondary active transporters. FEBS Lett 496:117–120

Ji Y et al. (2001) Identification of critical staphylococcal genes using conditional phenotypes generated by antisense RNA. Science 293:2266–2269

Kitada M, Kosono S, Kudo T (2000) The Na$^+$/H$^+$ antiporter of alkaliphilic *Bacillus* sp. Extremophiles 4:253–258

Kobayashi K et al (2003) Essential *Bacillus subtilis* genes. Proc Natl Acad Sci USA 100:4678–4683

Kosono S, Morotomi S, Kitada M, Kudo T (1999) Analyses of a *Bacillus subtilis* homologue of the Na$^+$/H$^+$ antiporter gene which is important for pH homeostasis of alkaliphilic *Bacillus* sp. C-125. Biochim Biophys Acta 1409:171–175

Kosono S, Ohashi Y, Kawamura F, Kitada M, Kudo T (2000) Function of a principal Na$^+$/H$^+$ antiporter, ShaA, is required for initiation of sporulation in *Bacillus subtilis*. J Bacteriol 182:898–904

Kosono S, Asai K, Sadaie Y, Kudo T (2004) Altered gene expression in the transition phase by disruption of a Na$^+$/H$^+$ antiporter gene (shaA) in *Bacillus subtilis*. FEMS Microbiol Lett 232:93–99

Krulwich TA (1995) Alkaliphiles: 'basic' molecular problems of pH tolerance and bioenergetics. Mol Microbiol 15:403–410

Krulwich TA, Ito M, Gilmour R, Hicks DB, Guffanti AA (1998) Energetics of alkaliphilic *Bacillus* species: physiology and molecules. Adv Microb Physiol 40:401–438

Krulwich TA, Guffanti AA, Ito M (1999) pH tolerance in *Bacillus*: alkaliphile *vs.* non-alkaliphile. In: Mechanisms by which bacterial cells respond to pH. Novartis Found Symp 221, Wiley, Chichester, pp 167–182

Krulwich TA, Ito M, Guffanti AA (2001) The Na$^+$-dependence of alkaliphily in *Bacillus*. Biochim Biophys Acta 1505:158–168

Kudo T, Hino M, Kitada M, Horikoshi K (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

Künkel A, Vorholt JA, Thauer RK, Hedderich R (1998) An *Escherichia coli* hydrogenase-3-type hydrogenase in methanogenic archaea. Eur J Biochem 252:467–476

Leonard K, Haiker H, Weiss H (1987) Three-dimensional structure of NADH: ubiquinone reductase (complex I) from *Neurospora* mitochondria determined by electron microscopy of membrane crystals. J Mol Biol 194:277–286

Lewinson O, Padan E, Bibi E (2004) Alkalitolerance: a biological function for a multidrug transporter in pH homeostasis. Proc Natl Acad Sci USA 101:14073–14078

Li X, Alvarez B, Casey JR, Reithmeier RA, Fliegel L (2002) Carbonic anhydrase II binds to and enhances activity of the Na$^+$/H$^+$ exchanger. J Biol Chem 277:36085–36091

MacNab RM, Castle AM (1987) A variable stoichiometry model for pH homeostasis in bacteria. Biophys J 52:637–647

Mathiesen C, Hägerhäll C (2002) Transmembrane topology of the NuoL, M and N subunits of NADH:quinone oxidoreductase and their homologues among membrane-bound hydrogenases and bona fide antiporters. Biochim Biophys Acta 1556:121–132

Mathiesen C, Hägerhäll C (2003) The 'antiporter module' of respiratory chain Complex I includes the MrpC/NuoK subunit – a revision of the modular evolution scheme. FEBS Lett 5459:7–13

Orlowski J, Grinstein S (2004) Diversity of the mammalian sodium/proton exchanger SLC9 gene family Eur. J Physiol 447:549–565

Padan E, Krulwich TA (2000) Sodium stress. In: Storz G, Hengge-Aronis R (eds) ASM Press, Washington, DC, pp117–130

Padan E, Schuldiner S (1996) Bacterial Na$^+$/H$^+$ antiporters – molecular biology, biochemistry and physiology. In: Konings WN, Kaback HR, Lolkema J (eds) The Handbook of Biological Physics, vol II. Transport processes in membranes. Elsevier Science, Amsterdam, pp 501–531

Padan E, Venturi M, Gerchman Y, Dover N (2001) Na$^+$/H$^+$ antiporters. Biochim Biophys Acta 1505:144–157

Padan E, Tzubery T, Herz K, Kozachkov L, Rimon A, Galili L (2004) NhaA of *Escherichia coli*, as a model of a pH-regulated Na$^+$/H$^+$ antiporter. Biochim Biophys Acta 1658:2–13

Putnoky P et al (1998) The *pha* gene cluster of *Rhizobium meliloti* involved in pH adaptation and symbiosis encodes a novel type of K$^+$ efflux system. Mol Microbiol 28:1091–1101

Safferling M et al (2003) TetL tetracycline efflux protein from *Bacillus subtilis* is a dimer in the membrane and in detergent solution. Biochemistry 42:13969–13976

Saier MH Jr et al (1999) Phylogenetic characterization of novel transport protein families revealed by genome analyses. Biochim Biophys Acta 1422:1–56

Sapra R, Verhagen MF, Adams MW (2000) Purification and characterization of a membrane-bound hydrogenase from the hyperthermophilic archaeon *Pyrococcus furiosus*. J Bacteriol 182:3423–3428

Sapra R, Bagramyan K, Adams MW (2003) A simple energy-conserving system: proton reduction coupled to proton translocation. Proc Natl Acad Sci USA 100:7545–7550

Sauter M, Bohm R, Bock A (1992) Mutational analysis of the operon (hyc) determining hydrogenase 3 formation in *Escherichia coli*. Mol Microbiol 6:1523–1532

Sazanov LA, Peak-Chew SY, Fearnley IM, Walker JE (2000) Resolution of the membrane domain of bovine complex I into subcomplexes: implications for the structural organization of the enzyme. Biochemistry 39:7229–7235

Seto Y, Hashimoto M, Usami R, Hamamoto T, Kudo T, Horikoshi K (1995) Characterization of a mutation responsible for an alkali-sensitive mutant, 18224, of alkaliphilic *Bacillus* sp. strain C-125. Biosci Biotechnol Biochem 59:1364–1366

Shijuku T, Saito H, Kakegawa T, Kobayashi H (2001) Expression of sodium/proton antiporter NhaA at various pH values in *Escherichia coli*. Biochim Biophys Acta 1506:212–217

Silva PJ et al. (2000) Enzymes of hydrogen metabolism in *Pyrococcus furiosus*. Eur J Biochem 267:6541–6551

Skulachev VP (1988) In: The sodium world. Membrane Bioenergetics. Springer-Verlag, Berlin, pp 293–326

Steuber J (2003) The C-terminally truncated NuoL subunit (ND5 homologue) of the Na^+-dependent complex I from *Escherichia coli* transports Na^+. J Biol Chem 278:26817–26822

Steuber J, Schmid C, Rufibach M, Dimroth P (2000) Na^+ translocation by complex I (NADH:quinone oxidoreductase) of *Escherichia coli*. Mol Microbiol 35:428–434

Stolpe S, Friedrich T (2004) The *Escherichia coli* NADH:ubiquinone oxidoreductase (complex I) is a primary proton pump but may be capable of secondary sodium antiport. J Biol Chem 279:18377–18383

Sturr MG, Guffanti AA, Krulwich TA (1994) Growth and bioenergetics of alkaliphilic *Bacillus firmus* OF4 in continuous culture at high pH. J Bacteriol 176:3111–3116

Swartz TH, Ito M, Hicks DB, Nuqui M, Guffanti AA, Krulwich TA (2005) The Mrp Na^+/H^+ antiporter increases the activity of the malate:quinone oxidoreductase of an *Escherichia coli* respiratory mutant. J Bacteriol 187:388–391

Vijaranakul U, Nadakavukaren MJ, Bayles DO, Wilkinson BJ, Jayaswal RK (1997) Characterization of an NaCl-sensitive *Staphylococcus aureus* mutant and rescue of the NaCl-sensitive phenotype by glycine betaine but not by other compatible solutes. Appl Environ Microbiol 63:1889–1897

Wallace BJ, Young IG (1977) Aerobic respiration in mutants of *Escherichia coli* accumulating quinone analogues of ubiquinone. Biochim Biophys Acta 461:75–83

Wei Y et al. (2003) Mutational loss of a K^+ and NH_4^+ transporter affects the growth and endospore formation of alkaliphilic *Bacillus pseudofirmus* OF4. J Bacteriol 185:5133–5147

West IC, Mitchell P (1974) Proton/sodium ion antiport in *Escherichia coli*. Biochem J 144:87–90

Wiegert T, Homuth G, Versteeg S, Schumann W (2001) Alkaline shock induces the *Bacillus subtilis* sigma^W regulon. Mol Microbiol 41:59–71

Yagi T, Matsuno-Yagi A (2003) The proton-translocating NADH-quinone oxidoreductase in the respiratory chain: the secret unlocked. Biochemistry 42:2266–2274

Yoshinaka T, Takasu H, Tomizawa R, Kosona S, Kudo T (2003) A *shaE* deletion mutant showed lower Na^+ sensitivity compared to other deletion mutants in the *Bacillus subtilis* sodium/hydrogen antiporter (Sha) system. J Biosci Bioeng 95:306–309