An Mrp-Like Cluster in the Halotolerant Cyanobacterium *Aphanothece halophytica* Functions as a Na⁺/H⁺ Antiporter

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The *mrp* homolog gene cluster *mrpCD1D2EFGAB* (*Ap-mrp*) was found in a halotolerant cyanobacterium, *Aphanothece halophytica*, amplified, and expressed in *Escherichia coli* mutant TO114. *Ap-mrp* complemented the salt-sensitive phenotype of TO114 and exhibited Na⁺/H⁺ and Li⁺/H⁺ exchange activities, indicating that *Ap-Mrp* functions as a Na⁺/H⁺ antiporter.

Bacterial *mrp* (multiple resistance and pH adaptation) loci encode highly unusual multisubunit cation/proton antiporters (CPAs) (13). Mrp systems are widespread among bacteria and archaea and, because of their unique complexity, are classified as the separate CPA3 family (12). They are known as group 1, group 2, and group 3 Mrp-like clusters (13). One unique feature is an apparent *mrpD* duplication. The *mrpD* repeat unit is repeated in *Synechocystis* sp. strain PCC 6803, but in that strain, the repeated unit is *mrpC* *mrpD*. Each protein in the Ap-Mrp cluster shows significant similarity (≥60%) with the corresponding protein of cyanobacteria but low homology to the proteins of group 1 and group 2 clusters (data not shown). Moreover, *Bacillus pseudomureus* OF4 *MrpA* (Bp-MrpA) possesses an oxidoreductase domain and a C-terminal MrpB homolog sequence. Cyanobacterial *MrpA* does not contain an oxidoreductase domain and only contains a sequence homologous to MrpB. Therefore, *MrpB* might be a more appropriate name for cyanobacterial *MrpA*, although we have used “*MrpA*,” following the naming convention of Blanco-Rivero et al. (1). The fact that homology between Ap-MrpD1 and Ap-MrpD2 is higher than any other MrpD suggests that a duplication of Ap-MrpD occurred after the evolution of the Ap-Mrp system. Another interesting aspect of the Ap-mp system is the clustering together with *Ap-bicA* and *Ap-napA1-2* (Fig. 1). *BicA* is a medium-affinity, high-flux Na⁺-dependent inducible HCO₃⁻ transporter (18). Among the organisms shown in Fig. 1, *Synechococcus* sp. strain PCC 7002 and *Cyanothece* sp. strain ATCC 51142 also have *bicA* and *napA* genes, whereas *Synecococcus* sp. strain PCC 7942 has only the *sbta* gene, which encodes a high-affinity, low-flux Na⁺-dependent HCO₃⁻ transporter. The implications of the tripartite clustering of transporter genes *bicA*, *napA1-2*, and *mrp* are discussed below.

Until now, no functional properties of cyanobacterial Mrp systems have been demonstrated. To characterize the molecular properties of Ap-Mrp, the Ap-Mrp cluster gene was amplified by PCR using specific forward (5′-AAGGATCCATGGTTGACATG-3′) and reverse (5′-CCGGATCCCTAAAGCTTGCAC-3′) primers. The amplified DNA fragment was ligated into the BamHI restriction site of the pBSK+ plasmid (Stratagene, CA) and sequenced. The DNA sequence was confirmed to be the same as that obtained using shotgun sequencing. The resulting plasmid, pApMrp, was expressed in *Escherichia coli* host cells (TO114), which lack Na⁺/H⁺ antiporter genes (*nhaA*, *nhaB*, and *chaA*) (4). Figure 2A shows that the *E. coli* TO114 cells transformed with pApMrp could grow at a rate similar to that of

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TO114 cells transformed with an empty vector in LBK (Luria broth with KCl instead of NaCl) medium at pH 7.2 and 37°C. However, TO114 cells transformed with vector alone could not grow under these conditions (Fig. 2A). By contrast, cells transformed with pApMrp were able to grow under these conditions, whereas the cells transformed with pApMrp were able to grow in the presence of 0.2 M NaCl (Fig. 2A). Changes of acridine orange fluorescence upon addition of NaCl and LiCl were determined to be 2.0 and 2.5 mM. These values are slightly higher than those for the Vibrio cholerae group 2 Mrp system (14) and much higher than the apparent $K_m$ values for Na$^+$ antiporter. Dequenching was also observed upon addition of LiCl, but not upon addition of KCl (Fig. 3B) or divalent cations such as Ca$^{2+}$ and Mg$^{2+}$ (data not shown). Dequenching by NaCl and LiCl increased with an increase in pH (Fig. 3C). As shown in Fig. 3D, from the dependence of NaCl and LiCl concentrations on the dequenching, the apparent $K_m$ values for Ap-Mrp for Na$^+$ and Li$^+$ were determined to be 2.0 and 2.5 mM. These values are slightly higher than those for the Vibrio cholerae group 2 Mrp system (3) and much higher than the apparent $K_m$ for Na$^+$ from group 1 Mrp systems (14). The apparent $K_m$ value of Ap-Mrp for Na$^+$ (2.0 mM) is slightly higher than that of Ap-NapA1-1 (0.8 mM) but similar to that of Ap-NapA1-2 (1.8 mM) and would be low enough compared with the internal Na$^+$ concentration, at high salinity as demonstrated previously (15, 19). The apparent $K_m$ value for Li$^+$ (2.5 mM) is significantly higher than those for Ap-NapA1-1 (0.05 mM) and Ap-NapA1-2 (0.3 mM). Anyway, these results clearly demonstrate that a cyanobacterial Mrp, Ap-Mrp, functions as a Na$^+/H^+$ and Li$^+/H^+$ antiporter. To our knowledge, this is the first report demonstrating that the Mrp-like cluster classified as “others” also functions as a Na$^+/H^+$ antiporter.

The knockout of the mrp gene is a useful method to study the physiological function of Mrp. Since transformation of A.
halophytica has not been reported, we used a freshwater cyanobacterium, Synechococcus sp. strain PCC 7942, for which the Mrp system has not been studied. The mrpA gene of Synechococcus sp. strain PCC 7942 (Syn7942-mrpA) was disrupted by insertion of the spectinomycin resistance gene (spc). Complete segregation of Syn7942-mrpA was confirmed by PCR amplification (data not shown). The growth rates of wild-type and Syn7942-ΔmrpA cells were almost the same when they were grown in BG11 medium at pH 7.5, but more severe inhibition was observed for the mutant cells when the cells were grown at alkaline pH (pH 9.0) or at high salinity (Fig. 4A). Here, we provide a functional explanation of how a protective function of a Na+/H+ antiporter (Ap-Mrp) can be achieved at “0 mM Na+.” Under these conditions, no driving force for the proposed uptake of H+ against the pH gradient (with the inside more acidic) would be present. It seems much more likely that the physiological function of the Mrp transporters is restricted to the export of Na+ ions driven by the electrochemical membrane potential. At high pH, such a function is impaired, and therefore cells lacking an Mrp system are much more sensitive toward salt stress.

Figure 4B shows that the Syn7942-ΔmrpA cells exhibited a rapid decrease (within 10 s) of photosynthetic electron transport activity (ΔF) after salt shock. Moreover, Fig. 4C shows that the levels of F in Syn7942-ΔmrpA cells significantly increased upon the salt shock compared with the wild-type cells, suggesting the accumulation of reductants due to the inhibition of electron transport. These data suggest a direct or close link between photosynthetic activity and Ap-Mrp at alkaline pH, namely, Ap-Mrp might utilize the reductants directly or via the electrochemical membrane potential produced by photosynthesis. Obviously, further studies are required to understand the molecular mechanisms of Ap-Mrp.

What is the physiological role of tripartite clustering of transporter genes bicA, napA1-2, and mrpA? Of course further studies are required to answer this. But one plausible explanation is the coupling of HCO3− uptake and Na+ circulation. At alkaline pH, HCO3− is the major component of inorganic carbon. Since it is presumed that BicA and SbtA are Na+−induced Na+/HCO3− symporters (18), the role of BicA or SbtA would become important for CO2 uptake at alkaline pH. In addition, at alkaline pH, protection of intracellular pH increase is important and might be carried out by Ap-Mrp and Ap-NapA1-2. Upon the transport of H+ into the cell, Na+ is extruded to the cell exterior. Extracellular Na+ could promote the uptake of HCO3− into the cell, which could then be used for photosynthesis. Thus, cooperation between Ap-BicA, Ap-NapA1-2, and Ap-Mrp would be beneficial for growth under conditions of alkaline pH, high salinity, and low CO2.

Until now, four Na+/H+ antiporter genes have been functionally characterized in A. halophytica. Ap-NhA1P1 exhibited very high exchange activity over a wide range of pH values (15). By contrast, three Na+/H+ antiporters, including Ap-NapA1-1, Ap-NapA1-2 (19), and Ap-Mrp, have high exchange activities at alkaline pH. These facts suggest that Na+/H+ antiporters are important for alkaline pH tolerance as well as for high-salinity stress tolerance.

Nucleotide sequence accession number. Data for the Ap-mrp region are available in the DDBJ databases under accession number AB507743.

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