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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 others (*mrp*-like gene clusters) (13). Group 1 and group 2 Mrp antiporters have been shown to have roles in alkaline pH homeostasis and Na^+ resistance (5, 8, 9), sporulation (9), symbiotic nitrogen fixation (11), pathogenesis (10), arsenite resistance (8), and bile salt resistance (7). Cyanobacterial Mrp-like clusters have been reported to be involved in salt stress tolerance (1, 2) and CO_2 deficiency-induced expression (1, 17, 18). However, the molecular properties of cyanobacterial Mrp systems have not yet been reported.

Aphanothece halophytica is a halotolerant cyanobacterium, isolated from the Dead Sea, which accumulates betaine and can grow under a wide range of salinity conditions (0.25 to 3.0 M NaCl) as well as at alkaline pH (6, 16). Previous studies have shown that *A. halophytica* has an NhaP-type Na^+/H^+ antiporter (CPA1), Ap-NhaP1, with novel ion specificity (15), and two NapA1-type Na^+/H^+ antiporters (CPA2) of similar size, Ap-NapA1-1 and Ap-NapA1-2 (19). Although the Na^+/H^+ exchange activity of Ap-NhaP1 was high over a wide pH range, Ap-NapA1-1 and Ap-NapA1-2 exhibited strongly pH-dependent Na^+/H^+ and Li^+/H^+ exchange activities, with higher activities at alkaline pH.

From shotgun sequencing of the whole genome, we found in this study that *A. halophytica* contains an *mrp*-like gene cluster. It consists of eight open reading frames (ORFs) which seem to be one large transcriptional unit. Each of the ORFs starts with ATG. The entire cluster consists of 5,668 bp (accession number AB507743). A homology search revealed that eight ORFs encode the proteins Ap-MrpC, Ap-MrpD1, Ap-MrpD2, Ap-MrpE, Ap-MrpF, Ap-MrpG, Ap-MrpA, and Ap-MrpB, as shown in Fig. 1. The organization of the *A. halophytica mrp*-like gene cluster is similar to that of the marine costal cyanobacterium *Synechococcus* sp. strain PCC 7002 but different from other cyanobacterium *mrp* clusters. One unique feature is an apparent *mrpD* duplication. The *mrpD* repeat also occurs 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 pseudofirmus* 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-*mrp* system is the clustering together with Ap-*bicA* and Ap-*napA1-2* (Fig. 1). BicA is a medium-affinity, high-flux Na^+-dependent inducible HCO_3^- 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 *Synechococcus* sp. strain PCC 7942 has only the *sbtA* gene, which encodes a high-affinity, low-flux Na^+-dependent HCO_3^- 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′-AAGGATCCAGTTTGTCATT AGTCATT-3′) and reverse (5′-TGGGATCCTTAAAGTA AACCACGATAG-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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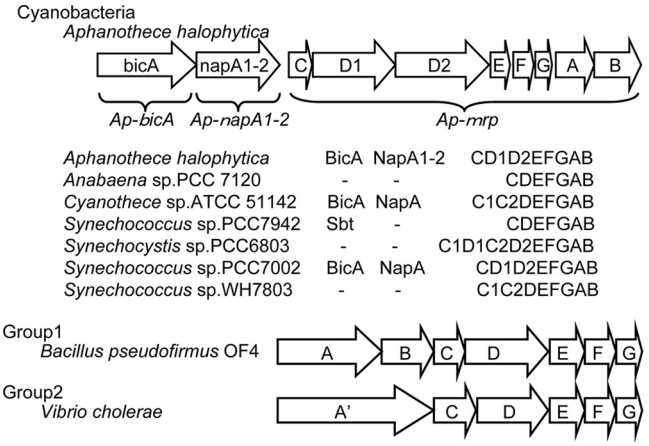

FIG. 1. Gene arrangements of *mrp*-like gene clusters. Gene arrangements of the *mrp* region of *A. halophytica* and several cyanobacteria are shown in the upper portion of the figure. Genetic information for *Anabaena* sp. strain PCC 7120, *Cyanothece* sp. strain ATCC 51142, *Synechococcus* sp. strain PCC 7942, *Synechocystis* sp. strain PCC6803, *Synechococcus* sp. strain PCC 7002, and *Synechococcus* sp. strain WH7803 was obtained from Cyanobase (http://genome.kazusa.or.jp/cyanobase/). Arrangements of the group 1 *mrp* from *Bacillus pseudofirmus* OF4 (accession number EF468713) and group 2 *mrp* from *Vibrio cholerae O395* (EF546428) are shown in the bottom portion of the figure.

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 in the presence of 0.2 M NaCl (Fig. 2A). By contrast, cells transformed with pApMrp were able to grow under these conditions (Fig. 2A). Figure 2B shows that the growth of cells transformed with vector alone was severely inhibited in the presence of 4 mM LiCl, whereas the cells transformed with pApMrp were able to grow under the same conditions.

To examine the antiporter activity of Ap-Mrp directly, everted membrane vesicles were prepared and antiporter activities were monitored by measuring lactate-induced fluorescence quenching (Q) and salt-induced fluorescence dequenching ($\triangle Q$) as described previously (4, 15). As shown in Fig. 3A, dequenching ($\triangle Q \times 100/Q$) was observed upon addition of NaCl in Ap-Mrp-expressing cells but not in control cells, indicating that Ap-Mrp has Na^+/H^+ antiporter activity. 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 of 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.*

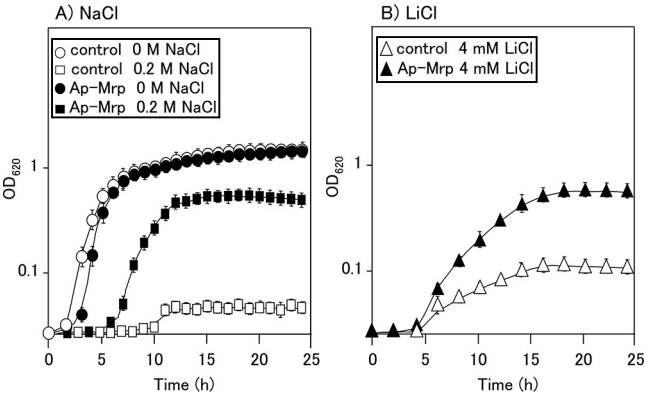

FIG. 2. Effects of NaCl and LiCl on the growth of *E. coli* cells expressing Ap-Mrp in LB medium containing 30 mM KCl and the indicated concentrations of NaCl (A) or LB medium containing 30 mM KCl and 4 mM LiCl (B). Control TO114 cells and transformant TO114 cells expressing Ap-Mrp were transferred to the growth medium containing the indicated salts at pH 7.2. Each value represents the average of three independent measurements of the optical density at 620 nm (OD_{620}).

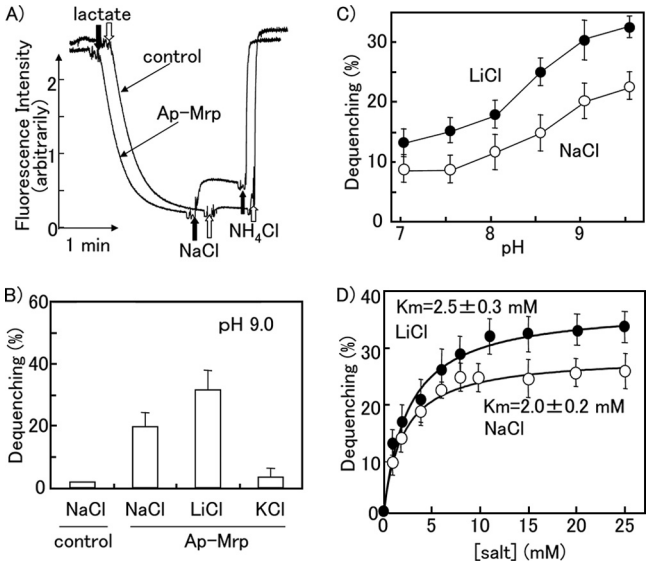

FIG. 3. Cation-proton exchange activities measured by the acridine orange fluorescence quenching method. Control TO114 cells and transformed TO114 cells expressing Ap-Mrp were grown in LBK medium at pH 7.5, from which everted membrane vesicles were prepared. (A) Changes of acridine orange fluorescence upon addition of NaCl. (B) The Na^+/H^+, Li^+/H^+, and K^+/H^+ exchange activities of TO114 cells expressing Ap-Mrp. For the experiments shown in panels A and B, the pH was 9.0. (C) pH dependence of Na^+/H^+ and Li^+/H^+ exchange activities of Ap-Mrp. The final concentration of salts was 10 mM. Each value represents the average of three independent measurements. (D) Plots based on the Michaelis-Menten equation.

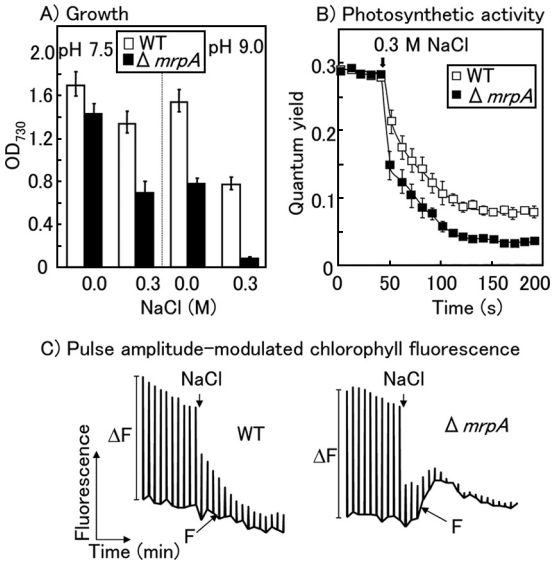

FIG. 4. Effects of disruption of the MrpA subunit of *Synechococcus* sp. strain PCC 7942 on growth and photosynthetic activity. Wild-type and *mrpA*-disrupted mutant *Synechococcus* sp. strain PCC 7942 cells were grown in BG11 medium containing 0 or 0.3 M NaCl at pH 7.5 or 9.0. (A) The optical density at 730 nm (OD_{730}) after 6 days of growth was measured. (B and C) Wild-type and Syn7942-Δ*mrpA* cells were grown in BG11 medium until the OD_{730} reached 1.5, and then NaCl was added into the medium to a final concentration 0.3 M. Photosynthetic electron transport activity was measured using a pulse amplitude-modulated fluorometer. Each value represents the average of three measurements.

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-Δ*mrpA* 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-Δ*mrpA* cells exhibited a rapid decrease (within 10 s) of photosynthetic electron transport activity ($\triangle F$) after salt shock. Moreover, Fig. 4C shows that the levels of *F* in Syn7942-Δ*mrpA* 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 *mrp*? Of course further studies are required to answer this. But one plausible explanation is the coupling of HCO_3^- uptake and Na^+ circulation. At alkaline pH, HCO_3^- is the major component of inorganic carbon. Since it is presumed that BicA and SbtA are Na^+-induced Na^+/HCO_3^- symporters (18), the role of BicA or SbtA would become important for CO_2 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 HCO_3^- 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 CO_2.

Until now, four Na^+/H^+ antiporter genes have been functionally characterized in *A. halophytica*. Ap-NhaP1 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 suggests 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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