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# Absence of Light-Induced Proton Extrusion in a *cotA*-Less Mutant of *Synechocystis* sp. Strain PCC6803

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***cotA* of *Synechocystis* sp. strain PCC6803 was isolated as a gene that complemented a mutant defective in $CO_2$ transport and is homologous to *cemA* that encodes a chloroplast envelope membrane protein (A. Katoh, K. S. Lee, H. Fukuzawa, K. Ohyama, and T. Ogawa, Proc. Natl. Acad. Sci. USA 93:4006–4010, 1996). A mutant (M29) constructed by replacing *cotA* in the wild-type (WT) *Synechocystis* strain with the omega fragment was unable to grow in BG11 medium (~17 mM $Na^+$) at pH 6.4 or at any pH in a low-sodium medium (100 μM $Na^+$) under aeration with 3% (vol/vol) $CO_2$ in air. The WT cells grew well in the pH range between 6.4 and 8.5 in BG11 medium but only at alkaline pH in the low-sodium medium. Illumination of the WT cells resulted in an extrusion followed by an uptake of protons. In contrast, only proton uptake was observed for the M29 mutant in the light without proton extrusion. There was no difference in sodium uptake activity between the WT and mutant. The mutant still possessed 51% of the WT $CO_2$ transport activity in the presence of 15 mM NaCl. On the basis of these results we concluded that *cotA* has a role in light-induced proton extrusion and that the inhibition of $CO_2$ transport in the M29 mutant is a secondary effect of the inhibition of proton extrusion.**

The *cotA* gene of *Synechocystis* sp. strain PCC6803, a homolog of the *cemA* genes found in chloroplast genomes of higher plants, was cloned as a gene which complemented mutants defective in $CO_2$ transport (4). The gene encodes a hydrophobic protein of 247 amino acids (CotA). The exact function of CotA in $CO_2$ transport, however, remains unknown. The *cemA* genes in higher plants have been postulated to encode b-type heme-binding proteins (2, 9, 16, 20). Sasaki et al. have shown that the gene product in pea chloroplasts is localized in the inner envelope membrane (14). However, no data are available on the function of the *cemA* gene product (CemA). Both CemA and CotA contain four membrane-spanning domains, and their amino acid sequences are highly conserved, especially in the C-terminal regions (4). These results suggested that these chloroplast and cyanobacterial gene products may have a similar function, and elucidation of the role of CotA is an important step not only in the study of cyanobacterial physiology but also in clarifying the role of CemA in higher plants.

In this study, we constructed a mutant in which the *cotA* gene was completely deleted and demonstrated that the mutant does not extrude protons in the light. Light-induced proton extrusion has been found in a number of cyanobacterial strains. Kaplan et al. reported light-induced, sodium-dependent acidification of the medium by *Synechococcus* sp. and inferred that the acidification is mediated by $H^+$-ATPase (3). Scherer et al. (15) and Ogawa and Kaplan (7) observed a similar phenomenon for *Anabaena variabilis* and *Synechococcus* sp., respectively. They attributed the acidification to the efflux of protons produced as a result of $CO_2$ to $HCO_3^-$ conversion during $CO_2$ transport. Lockau and Pfeffer (5) reported on a plasma membrane-located proton pump, the activity of which is light and sodium dependent. However, no data were available on a gene(s) involved in light-induced proton extrusion. Physiological studies of the *cotA*-less mutant suggest that inhibition of $CO_2$ transport in the mutant is a secondary effect of the inhibition of proton extrusion. A possible role of *cotA* in light-induced proton extrusion will be discussed.

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## MATERIALS AND METHODS

**Growth conditions.** Cells of *Synechocystis* sp. strain PCC6803 were grown at 30°C in BG11 medium (17) buffered with 20 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES)–KOH (pH 7.0 to 8.0), *N*,*N*-bis(2-hydroxyethyl)glycine (bicine)–KOH (pH 8.5), or 2-(*N*-morpholino)ethanesulfonic acid (MES)–KOH (pH 6.4) during aeration with 3% (vol/vol) $CO_2$ in air. Low-sodium (low-$Na^+$) medium was prepared by adding NaCl (final concentration of 100 μM) to a modified BG11 medium in which all the sodium salts were replaced by potassium salts. Continuous illumination was provided by fluorescent lamps at 120 μmol of photosynthetically active radiation per $m^2$ per s (400 to 700 nm).

**Transformation of *Synechocystis* sp. strain PCC6803.** The *cotA* gene was replaced by the omega fragment (10), which confers spectinomycin and streptomycin resistance (the $Sp^r/Sm^r$ cartridge). The plasmid containing the substituted gene was used to transform the wild-type (WT) cells of *Synechocystis* sp. strain PCC6803 into the $Sp^r/Sm^r$ mutant, by the protocol of Williams and Szalay (21).

**Silicone oil-filtering centrifugation.** Time courses of uptake of $^{14}CO_2$ and $H^{14}CO_3^-$ into the intracellular inorganic carbon ($C_i$) pool of the WT and mutant cells were determined by the silicone oil-filtering centrifugation method (19). Cells were harvested by centrifugation and resuspended in 20 mM tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES)–KOH buffer (pH 8.0) containing 15 mM (or 100 μM) NaCl at a chlorophyll (Chl) concentration of 20 μg/ml. $C_i$ uptake was initiated by the addition of $^{14}CO_2$ or $H^{14}CO_3^-$ (final concentrations of 25 and 120 μM, respectively; 2.0 MBq/μmol) in the light to the cell suspension layered on the silicone oil layer in a plastic tube (0.5 ml) and terminated by centrifugation. The sorbitol impermeable spaces of the WT and mutant cells and the pH of the cytoplasm were determined by the methods described by Heldt (1). The sorbitol impermeable spaces of the WT and mutant cells were 81 and 63 μl/mg of Chl, respectively. For the determination of the pH in the cytoplasm, the thylakoid space was assumed to be 10% of the sorbitol impermeable space.

Uptake of $^{22}Na^+$ into the WT and mutant cells was determined by the same method except that $Na^+$ uptake was initiated by the addition of $^{22}NaCl$ (final concentration of 100 μM or 15 mM; 4.7 or 0.47 MBq/μmol, respectively) in the light.

All the radioisotopes used in this study are the products of Dupont (Wilmington, Del.).

**Measurements of proton exchange and $O_2$ evolution.** The net proton exchange was measured at 30°C as described by Kaplan et al. (3). Cells were harvested by centrifugation and washed to achieve the required pH. Cells were suspended in the same buffer containing 100 μM NaCl, 15 mM KCl, or 15 mM NaCl at a Chl concentration of 14 μg/ml. Photosynthetic $CO_2$ fixation was inhibited by adding glyceraldehyde (11) to the cell suspension at a final concentration of 20 mM, and the pH of the external solution was monitored by a pH electrode with a meter (Inlar 423 and Delta 350; Mettler Toledo, Halstead Essex, United Kingdom) in a sample chamber used for the $O_2$ evolution measurement.

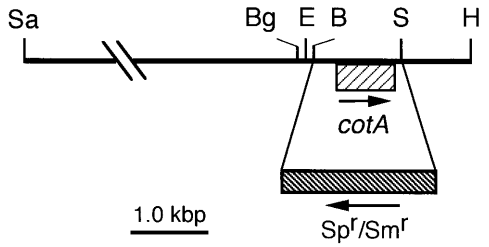


FIG. 1. Restriction map of a 6.9-kbp DNA insert in plasmid p*SC*. The 1.1-kbp *Bam*HI-*Spe*I fragment was replaced by the omega fragment ($Sp^r/Sm^r$ cartridge) (10) in plasmid p*MSC*. The *cotA* gene (▨) and the omega fragment (▧) are indicated; the arrows show the directions of transcription. B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hpa*I; S, *Spe*I; Sa, *Sal*I.

The rate of photosynthetic $O_2$ evolution was measured at 30°C with an $O_2$ electrode (Rank Brothers, Cambridge, United Kingdom). The suspension buffer was 20 mM TES-KOH buffer (pH 8.0) containing various concentrations of NaCl.

For all these measurements, the cell suspension was illuminated with white light from a 150-W halogen lamp guided to the sample chamber (tube) by a glass fiber (MHF-150L; Kagaku Kyoeisha Ltd., Osaka, Japan). The light intensity at the surface of the chamber (tube) was 4.0 mmol of photosynthetically active radiation per $m^2$ per s.

**Growth curves.** Growth curves were determined from the rise in the optical density at 730 nm with a Shimadzu recording spectrophotometer (model UV-2200). The specific growth rates ($\mu$) are expressed per day. (To convert the growth rates to doublings per day, divide ln 2 (0.693) by $\mu$.)

**Other methods.** Unless otherwise stated, standard techniques were used for DNA manipulations (13). Pigments in the cells were extracted with methanol, and the Chl concentration in the extract was determined (8).

## RESULTS

**Construction of mutant (M29) lacking the *cotA* gene.** The *cotA* gene in the p*SC* plasmid, constructed by inserting a 6.9-kbp *Sal*I-*Hpa*I fragment of *Synechocystis* sp. strain PCC6803 into the pKY184 vector (18), was replaced by the omega fragment (10) to produce the p*MSC* plasmid (Fig. 1). The p*MSC* plasmid was used to transform the WT cells of *Synechocystis* sp. strain PCC6803 to $Sp^r/Sm^r$ resistance through homologous recombination. The transformant showed a mutant phenotype and was unable to grow in low-$Na^+$ medium. The segregation of the modified gene(s) in the mutant cells was complete, as confirmed by the PCR method (12) with genomic DNA of the mutant as a template (data not shown). The mutant thus constructed was named as M29.

**Growth characteristics of WT and mutant cells.** The growth rates of the WT and M29 cells were determined in standard (BG11) and low-$Na^+$ media buffered at various pHs during aeration with 3% (vol/vol) $CO_2$ in air and are plotted as a function of the pH of the growth media (Fig. 2). The WT cells showed 80% of the growth rate at pH 8.5 in the standard medium even at pH 6.4. In low-$Na^+$ medium, the WT cells were unable to grow at pH 6.4, and the growth rate at pH 7.2 was half the rate at pH 8.5. The M29 mutant grew well at pHs above 7.6 in standard medium but hardly grew at pHs below 7.2. In low-$Na^+$ medium, the mutant did not grow at any pH examined. With the $CO_2$ concentrations used in these experiments, the growth of cells was not limited by the supply of the carbon source. This finding was confirmed by the result obtained with the *ndhB*-less mutant, M55, which does not have $C_i$ transport activity (6). The M55 mutant grew as fast as the WT in low-$Na^+$ medium at 3% (vol/vol) $CO_2$ in air but did not grow with air even in the standard medium (data not shown). Thus, the inability of M29 to grow under low-sodium conditions is not due to an insufficient supply of $C_i$.

The growth rates of the WT and mutant cells plotted as a function of the sodium concentration in the medium are shown in Fig. 3. The mutant required more than 5 mM sodium for growth (curve B) whereas the WT grew well at 100 $\mu$M NaCl (curve A). These results suggest that translocation of the ion(s) essential for growth of the cells is affected by the sodium concentration in the medium.

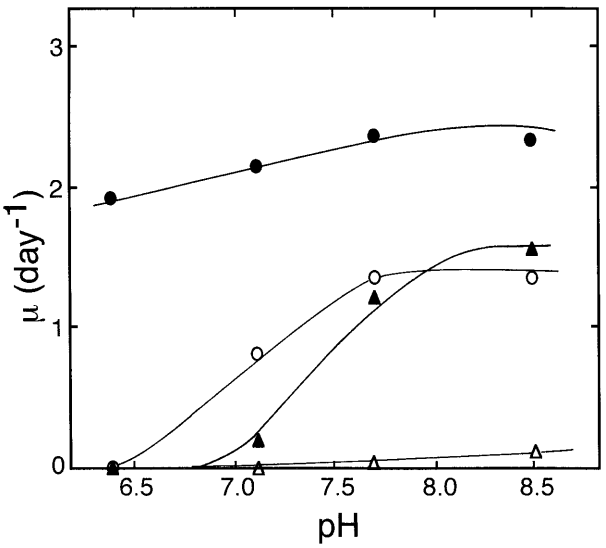


FIG. 2. Growth rates ($\mu$) of WT (circles) and M29 (triangles) cells as a function of pH of the medium (closed symbols for BG11 medium and open symbols for low-$Na^+$ medium). Cultures were aerated with 3% (vol/vol) $CO_2$ in air.

**$O_2$ evolution.** $O_2$ evolution of the WT at 100 $\mu$M NaCl was as high as that at 15 mM NaCl (Fig. 4, curve A). In contrast, the level of activity in the M29 mutant was low at 100 $\mu$M NaCl and increased as the sodium concentration was raised to attain a maximum level above 1 mM NaCl (curve B). The results are consistent with the growth characteristics of the WT and mutant cells (Fig. 3), although growth of the mutant required higher concentrations of sodium.

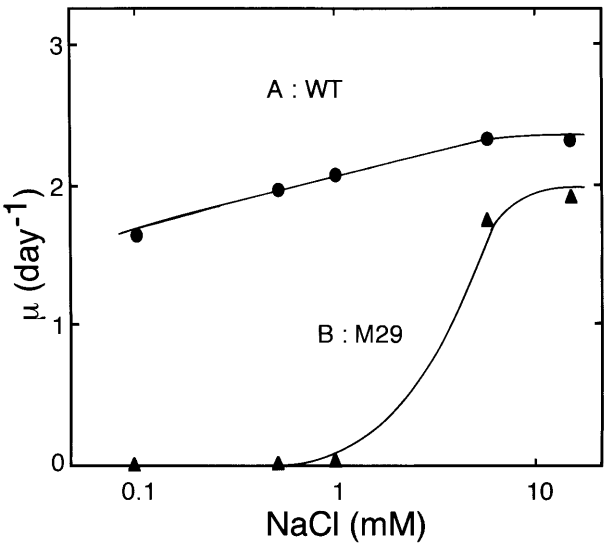


FIG. 3. Growth rates ($\mu$) of WT (circles) and M29 (triangles) cells as a function of sodium concentration in BG11 medium (pH 8.0) in which all sodium salts were replaced by potassium salts. Cultures were aerated with 3% (vol/vol) $CO_2$ in air.

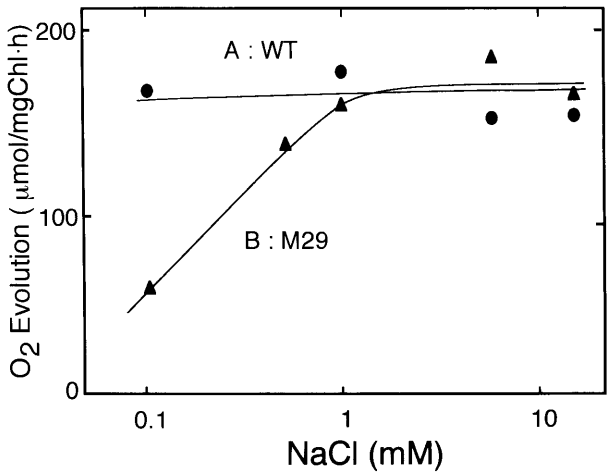


FIG. 4. Rates of $O_2$ evolution of WT (circles) and M29 (triangles) cells as a function of sodium concentration in the sample solution. Cells were suspended in 20 mM TES-KOH buffer (pH 8.0) containing various concentrations of NaCl.

**$^{22}$Na uptake, $C_i$ transport, and intracellular pH.** Figure 5A shows the time courses of sodium uptake by the WT and M29 cells in light at the extracellular NaCl concentrations of 15 mM and 100 μM. There was no significant difference between sodium uptake in the WT and the mutant. Thus, the sodium uptake system is not impaired in the mutant. The rate of sodium uptake at 15 mM NaCl was about 60 times the rate at 100 μM NaCl.

Since *cotA* was cloned as a gene which complemented mutants defective in $CO_2$ transport, the gene product was postulated to encode a component of the $CO_2$ transport system (4). The deletion of the *cotA* gene, however, did not abolish $CO_2$ transport completely; M29 still possessed about 51 and 17% of the WT activity at 15 mM and 100 μM NaCl, respectively (Fig. 6B). The $HCO_3^-$ transport was also reduced in the mutant and was 77 and 62% of the WT activity at 15 mM and 100 μM NaCl, respectively (Fig. 6B). On the basis of these results, we conclude that the observed inhibition of $CO_2$ transport (and $HCO_3^-$ transport in M29) in the mutants described previously (4) and herein is a secondary effect which also affects the growth of the cells under low-sodium conditions.

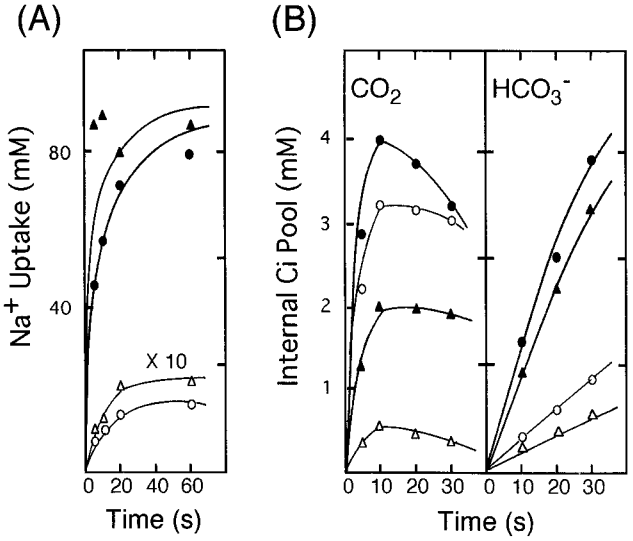


FIG. 5. (A) Time courses of $^{22}Na^+$ uptake by WT (circles) and M29 (triangles) cells in light. Cells were suspended in 20 mM TES-KOH buffer (pH 8.0) containing 100 μM (open symbols) or 15 mM (closed symbols) NaCl. The $^{22}$NaCl solution was added to the cell suspension in the light in an amount to give the above concentrations of NaCl. (B) Time courses of uptake of $CO_2$ and $HCO_3^-$ into the intracellular $C_i$ pool of WT (circles) and M29 (triangles) cells. Cells were suspended in 20 mM TES-KOH buffer (pH 8.0) containing 100 μM (open symbols) or 15 mM (closed symbols) NaCl. The concentrations of $CO_2$ and $HCO_3^-$ were 25 and 120 μM, respectively.

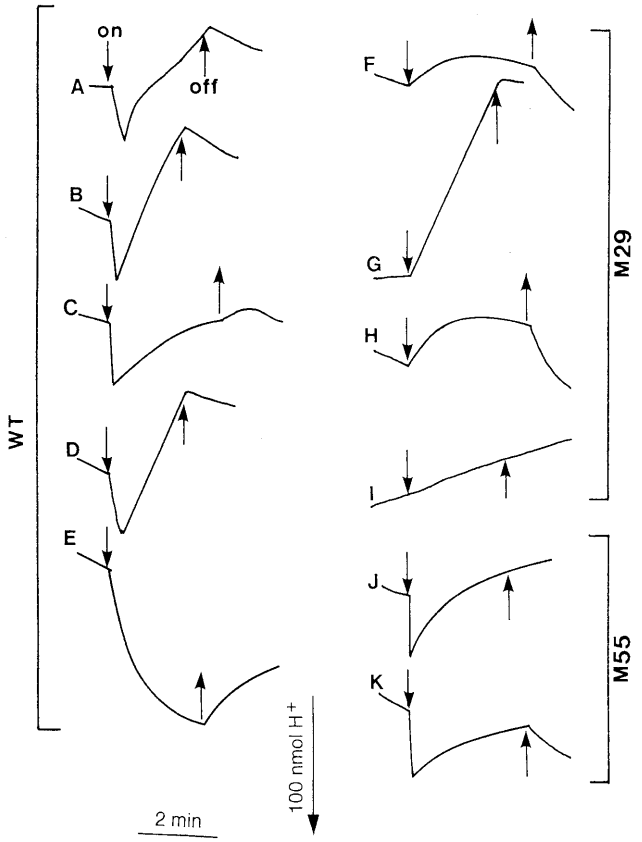


FIG. 6. Changes in pH of cell suspensions with (on) and without (off) light. WT (profiles A to E), M29 (profiles F to I), and M55 (profiles J and K) cells were suspended in 0.2 mM MES, HEPES, bicine-KOH buffer (pH 7.9 for profiles A to C, F to H, J and K; pH 6.5 for profiles D, E, and I) containing 15 mM KCl (profiles A and F) or 15 mM NaCl (profiles B to E, G to K). Photosynthetic $CO_2$ fixation of the cells was inhibited by 20 mM glyceraldehyde in profiles C, E, and K.

To test whether the inability of the mutant to grow under acidic conditions is due to a failure to control the intracellular pH, the uptake of $^{14}$C-labeled 5,5-dimethyloxazolidine-2,4-dione was determined in WT and mutant cells suspended in BG11 medium buffered at pH 6.5. The pHs in the cytoplasm of the mutant were 6.9 in the dark and 7.5 in the light, which were essentially the same as pHs 6.8 and 7.5, respectively, obtained with the WT. Thus, the pH homeostasis is functioning normally in the mutant.

**Net proton movements.** The profiles of net proton movements determined in the WT and M29 cells are shown in Fig. 6. When the WT cells suspended in 0.2 mM MES, HEPES, bicine-KOH buffer (pH 7.9) containing 15 mM KCl (curve A) or NaCl (B) were illuminated, there was an acidification ($H^+$ extrusion or $OH^-$ uptake) followed by an alkalization ($H^+$ uptake or $OH^-$ extrusion) of the medium. In contrast, acidi-

fication was not observed for the M29 mutant with illumination under the same conditions, and only alkalization of the medium was observed (profiles F and G). Both acidification and alkalization were stimulated by 15 mM sodium (profiles A, B, F, and G), but the profiles obtained with 15 mM KCl and 100 μM NaCl were the same as those without NaCl (data not shown). Inhibition of photosynthetic $CO_2$ fixation by glyceraldehyde (11) significantly reduced the rate of alkalization (profiles C and H), indicating that the hydroxyl ion produced as a result of bicarbonate utilization is extruded in the light. Glyceraldehyde did not have any effect on light-induced acidification. The profile of net proton movement of the WT cells obtained at pH 6.5 (profile D) was the same as that obtained at pH 7.9 (profile B). However, the addition of glyceraldehyde completely abolished the alkalization at pH 6.5 (profile E). Proton movement was hardly observable for the M29 mutant at pH 6.5 in either the absence (profile I) or presence of glyceraldehyde. It is evident that M29 is a mutant which is unable to extrude protons in the light. The M55 mutant, which does not have $C_i$ transport activity (6), showed profiles of proton movement similar to that of the WT (profiles J and K). These profiles indicate that the observed proton movement is not a phenomenon accompanied by $C_i$ transport.

## DISCUSSION

The present study clearly demonstrated that the mutant (M29) in which the *cotA* gene was completely deleted did not show light-induced proton extrusion (Fig. 6). Although light-induced acidification of the medium has been observed for a number of cyanobacterial strains, there were no data available on the gene(s) involved in this phenomenon. *cotA* is the first gene found to be involved in light-induced proton extrusion.

The extrusion of protons will produce $\Delta\psi$ and $\Delta$pH across the cytoplasmic membrane to drive the transport of other ions. $C_i$ transport was inhibited in the M29 mutant (Fig. 5B), indicating that transport is at least partially coupled with proton extrusion. However, we were not able to specify other transporting processes coupled with the observed light-induced proton extrusion. The growth characteristics of the mutant cannot be explained by the inhibition of $C_i$ transport since 3% $CO_2$ in air (vol/vol) was bubbled during the growth rate determination. It is considered that the supply of the carbon source does not limit cell growth under these conditions. pH homeostasis was normal in the mutant at external pH 6.5, and there was no significant difference between sodium uptake in the WT and in the mutant (Fig. 5A). Therefore, at present we have to ascribe the inability of the mutant to grow in low-$Na^+$ medium or in standard medium below pH 7.0 to reduced activities of transport processes which are coupled to the light-induced proton extrusion in the WT. Some transport processes may be dependent on the pH and the sodium concentration.

The light-induced proton exchange was observed for the M55 mutant (Fig. 6, profiles J and K), which does not have active $C_i$ transport and an NADPH-mediated photosystem-1 cyclic electron pathway (6). Thus, the acidification of the medium is not due to the efflux of protons produced during $CO_2$ uptake, and a cyclic electron flow of this type is not needed for energizing the proton exchange. The mechanism of proton extrusion dependent on the *cotA* gene product is not known. It is unlikely that the gene product is $H^+$-ATPase because of the absence of an ATP-binding motif in the deduced amino acid sequence. The gene product could be another type of $H^+$ transporter or a component involved in the energization or regulation of the transporting system.

## ACKNOWLEDGMENTS

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