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A putative HCO_3^- transporter in the cyanobacterium *Synechococcus* sp. strain PCC 7942

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Abstract Cyanobacteria possess an inducible mechanism which enables them to concentrate inorganic carbon (Ci) within the cells. An inactivation library was used to raise the high-CO_2-requiring mutant of *Synechococcus* PCC 7942, IL-2, impaired in HCO_3^- transport. Analysis of the relevant genomic DNA detected several modifications, probably due to the single crossover recombination, leading to inactivation of ORF467 (designated *ictB*) in IL-2. IctB contains 10 trans-membrane regions and is homologous to several transport-related proteins from various organisms. Kinetic analyses of HCO_3^- uptake in the wild type and IL-2 suggested the presence of two or three HCO_3^- carriers exhibiting different affinities to HCO_3^-.

Key words: CO_2-concentrating mechanism; Crossover; Cyanobacteria; HCO_3^- uptake; *ict*B

1. Introduction

Photosynthetic microorganisms including cyanobacteria are capable of acclimating to a wide range of CO_2 concentrations. The process of acclimation is mediated via a syndrome of changes, at various cellular levels, including modulation of the expression of genes involved in the operation of the CO_2 concentrating mechanism (CCM) [1–5]. This mechanism enables photosynthetic microorganisms to raise the CO_2 level at the carboxylating sites and thus overcome the large (5- to 20-fold) difference between the K_m (CO_2) of their carboxylating enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (rubisco) and the concentration of dissolved CO_2 at equilibrium with air. In cyanobacteria, the components of the CCM include energy-dependent HCO_3^- transport and CO_2 conversion to HCO_3^- [3] and the highly organized carboxysomes where carbonic anhydrase catalyzes the formation of CO_2 from HCO_3^- in close proximity to rubisco [1–3]. The activity of the CCM increases dramatically following transfer from high to low CO_2 concentrations mainly due to changes in the inorganic carbon (Ci) transport capabilities and larger number of carboxysomes [3,6,7]. Some of the genes involved in the operation of the CCM were identified with the aid of high-CO_2-requiring mutants but there is little information on those directly involved in HCO_3^- uptake [3,4,8]. In this study we isolated a high-CO_2-requiring mutant of *Synechococcus* PCC 7942 impaired in HCO_3^- uptake and identified the mutation in ORF467, designated *ict*B (for inorganic carbon transport B).

2. Materials and methods

2.1. Growth conditions

Cultures of *Synechococcus* sp. strain PCC 7942 and mutant IL-2 thereof were grown at 30°C in BG_{11} medium supplemented with 20 mM HEPES-NaOH, pH 7.8, and 25 μg/ml kanamycin (in the case of the mutant). The medium was aerated with either 5% (v/v) CO_2 in air (high CO_2) or 0.0175% (v/v) CO_2 in air (by mixing air with CO_2-free air at a 1:1 ratio, low CO_2). *Escherichia coli* (strain DH5α) was grown on medium LB [9] supplemented with either kanamycin (50 μg/ml) or ampicillin (50 μg/ml) when required.

2.2. Measurements of photosynthesis and Ci uptake

The rates of Ci-dependent O_2 evolution were measured by an O_2 electrode as described elsewhere [10] and by a membrane inlet mass spectrometer (MIMS, [6,11]). The MIMS was also used for assessments of CO_2 and HCO_3^- uptake during steady state photosynthesis [6]. Ci fluxes following supply of CO_2 or HCO_3^- were determined by the filtering centrifugation technique [10]. High-CO_2-grown cells in the log phase of growth were transferred to either low or high CO_2 12 h before the experiments. Following harvest, the cells were resuspended in 25 mM HEPES-NaOH, pH 8.0, and aerated with air (Ci concentration was about 0.4 mM) under 100 μmol quanta m^{-2} s^{-1}). Aliquots were withdrawn and immediately placed in microfuge tubes kept under similar light and temperature. Tracers of ^{14}C-CO_2 or $^{14}C-HCO_3^-$ were injected, hardly affecting the Ci concentration, and the Ci uptake terminated after 5 s by centrifugation.

2.3. General DNA manipulations

Genomic DNA was isolated as described elsewhere [12]. Standard recombinant DNA techniques were used for cloning and Southern analyses [12,13] using the Random Primed DNA Labeling Kit or the DIG system (Boehringer, Mannheim, Germany). Sequence analysis was performed using the Dye Terminator cycle sequencing kit, ABI Prish (377 DNA sequencing Perkin Elmer). The genomic library used here was constructed using Lambda EMBL3/*Bam*HI vector kit (Stratagene, La Jolla, CA, USA).

2.4. Construction and isolation of mutant IL-2

A modification of the method developed by Dolganov and Grossman [14] was used to raise and isolate new high-CO_2-requiring mutants [4,15]. Briefly, genomic DNA was digested with *Taq*I and ligated within the *Acc*I site of the polylinker in a modified Bluescript SK plasmid. In the latter, we inactivated the gene conferring ampicillin resistance by the insertion (within the *Sca*I site) of a cartridge encoding kanamycin resistance (Kan^r, [8]). *Synechococcus* sp. strain PCC 7942 cells were transfected with the library [12]. Single crossover events conferred Kan^r and led to inactivation of various genes. The Kan^r cells were exposed to low CO_2 conditions for 8 h of adaptation followed by ampicillin treatment (400 μg/ml) for 12 h. Cells capable of adapting to low CO_2 and thus able to grow under these conditions were eliminated by this treatment. The high-CO_2-requiring mutant, IL-2, unable to divide under low CO_2 conditions, survived, and was

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The relevant DNA sequence is available in the GenBank (accession number U62616).

PII: S0014-5793(98)00662-0

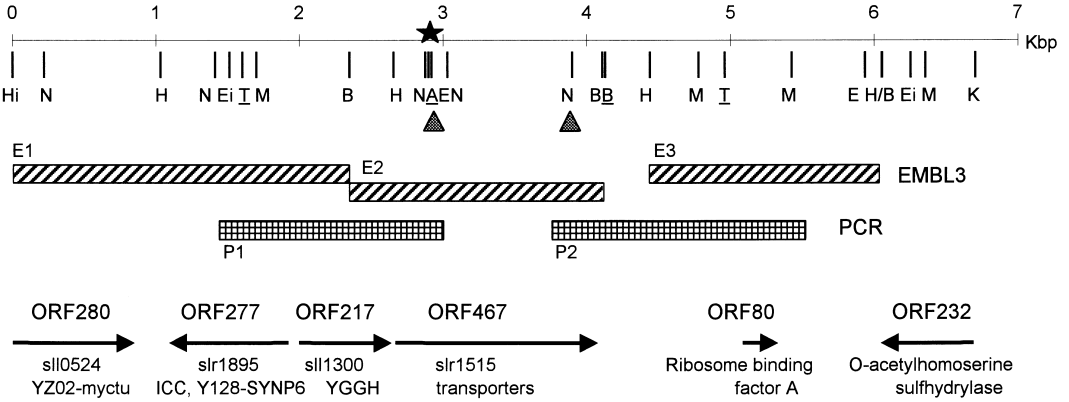

Fig. 1. A schematic map of the genomic region in *Synechococcus* sp. PCC 7942 where the insertion (indicated by a star) of the inactivation library fragment led to the formation of mutant IL-2. DNA sequence is available in the GenBank, accession number U62616. Restriction site are marked as: A, *Apa*I; B, *Bam*HI; Ei, *Eco*RI; E, *Eco*RV; H, *Hin*cII; Hi, *Hin*dIII; K, *Kpn*I; M, *Mfe*I; N, *Nhe*I; T, *Taq*I. Underlined letters represent the terminate position of the DNA fragments that were used as probes. Relevant fragments isolated from the EMBL3 library are marked E1, E2 and E3. P1 and P2 are fragments obtained by the PCR. Triangles indicate sites where a cartridge encoding Kan^r was inserted. Open reading frames are marked by an arrow and their similarities to other proteins are noted. Sll**** and slr**** are the homologous genes in *Synechocystis* PCC 6803 [23]; YZ02-myctu, accession no. Q10536; ICC, accession no. P36650; Y128-SYNP6, accession no. P05677; YGGH, accession no. P44648; ribosome binding factor A homologous to sll0754 and to P45141; *O*-acetylhomoserine sulfhydrylase homologous to sll0077 and NifS. ORF280 started upstream of the scheme presented here.

rescued following the removal of ampicillin and growth in the presence of high CO_2 concentration.

2.5. Cloning of the relevant genomic region impaired in mutant IL-2

DNA isolated from the mutant was digested with *Apa*I located on one side of the *Acc*I site in the polylinker; with *Bam*HI or *Eco*RI, located on the other side of the *Acc*I site; or with *Mfe*I that does not cleave the vector or the Kan^r cartridge. These enzymes also cleaved the genomic DNA. The digested DNA was self-ligated followed by transfection of competent *E. coli* cells (strain DH5α). Kan^r colonies carrying the vector sequences bearing the origin of replication, the Kan^r cartridge and part of the inactivated gene were then isolated. This procedure was used to clone the flanking regions on both sides of the vector inserted in the mutant. A 1.3-kbp *Apa*I and a 0.8-kbp *Bam*HI fragment isolated from the plasmids (one *Apa*I site and *Bam*HI site originated from the vector's polylinker) were used as probes to identify the relevant clones in an EMBL3 genomic library of the wild type, and for Southern analyses. The location of these fragments in the wild-type genome is shown in Fig. 1. The *Apa*I fragment is between positions 1600–2899, marked as T and A; the *Bam*HI fragment is between positions 4125–4957 marked as B and T. The 0.8-kbp *Bam*HI fragment hybridized with the 1.6-kbp *Hin*cII fragment (marked E3 in Fig. 1). The 1.3-kbp *Apa*I fragment hybridized with an *Eco*RI fragment of about 6 kbp. Interestingly, we could not clone this fragment from the genomic library in *E. coli*. Therefore, we used the *Bam*HI site (position 2348, Fig. 1) to split the EMBL3 clone into two clonable fragments of 4.0 and 1.8 kbp (E1 and E2, respectively, E1 started from a *Sau*3A site upstream of the *Hin*dIII site at the beginning of the scheme, Fig. 1). Confirmation that these three fragments were indeed located as shown in Fig. 1 was obtained by PCR using wild-type DNA as template, leading to the synthesis of fragments P1 and P2 (Fig. 1). Sequence analyses enabled comparison of the relevant region in IL-2 with the corresponding region in the wild type.

3. Results and discussion

3.1. Physiological analysis

IL-2 grew almost like the wild type in the presence of high CO_2 concentration but was unable to grow under low CO_2 (not shown). Analysis of the photosynthetic rate as a function of external Ci concentration revealed that the apparent photosynthetic affinity was 20 mM Ci, i.e. about 100 times higher than the concentration of Ci at equilibrium with low CO_2 conditions used here. We do not present the growth curves and the curves relating the photosynthetic rate to Ci concentration, in IL-2, since they were similar to those obtained with other high-CO_2-requiring mutants of *Synechococcus* PCC 7942 [16,17]. These data suggested that the poor photosynthetic performance resulted in inability of IL-2 to grow under low CO_2.

High-CO_2-requiring mutants showing such characteristics were recognized among mutants bearing aberrant carboxysomes [9,10,12,18,19] or defective in energization of Ci uptake [20,21]. All the carboxysome-defective mutants characterized to date were able to accumulate Ci within the cells like the wild type. However, they were unable to utilize it efficiently in photosynthesis due to low activation state of rubisco in mutant cells exposed to low CO_2 [10]. This was not the case in mutant IL-2 which possesses normal carboxysomes (not shown) but exhibited impaired HCO_3^- uptake (Table 1, Fig. 2). Measurements of ^{14}Ci accumulation indicated that HCO_3^- and CO_2 uptake were similar in the high-CO_2-grown wild type and the mutant (Table 1). Uptake of HCO_3^- by wild type cells increased by approximately 6-fold following exposure to low CO_2 conditions for 12 h. On the other hand, the same treatment resulted in only up to 2-fold increase in HCO_3^- uptake in IL-2. Uptake of CO_2 increased by approximately 50% following transfer of either the wild type or IL-2 from high- to low-

Table 1
The rate of CO_2 and of HCO_3^- uptake in *Synechococcus* sp. PCC 7942 and mutant IL-2 as affected by the concentration of CO_2 in the growth medium

	CO_2 uptake		HCO_3^- uptake	
	High CO_2	Low CO_2	High CO_2	Low CO_2
WT	31.6	53.9	30.9	182.0
IL-2	26.6	39.2	32.2	61.1

The unidirectional CO_2 or HCO_3^- uptake of cells grown under high CO_2 conditions or exposed to low CO_2 for 12 h is presented in μmol Ci accumulated within the cells mg^{-1} Chl h^{-1}. The results presented are the average of three different experiments, with four replicas in each experiment. The range of the data was within ±10% of the average.

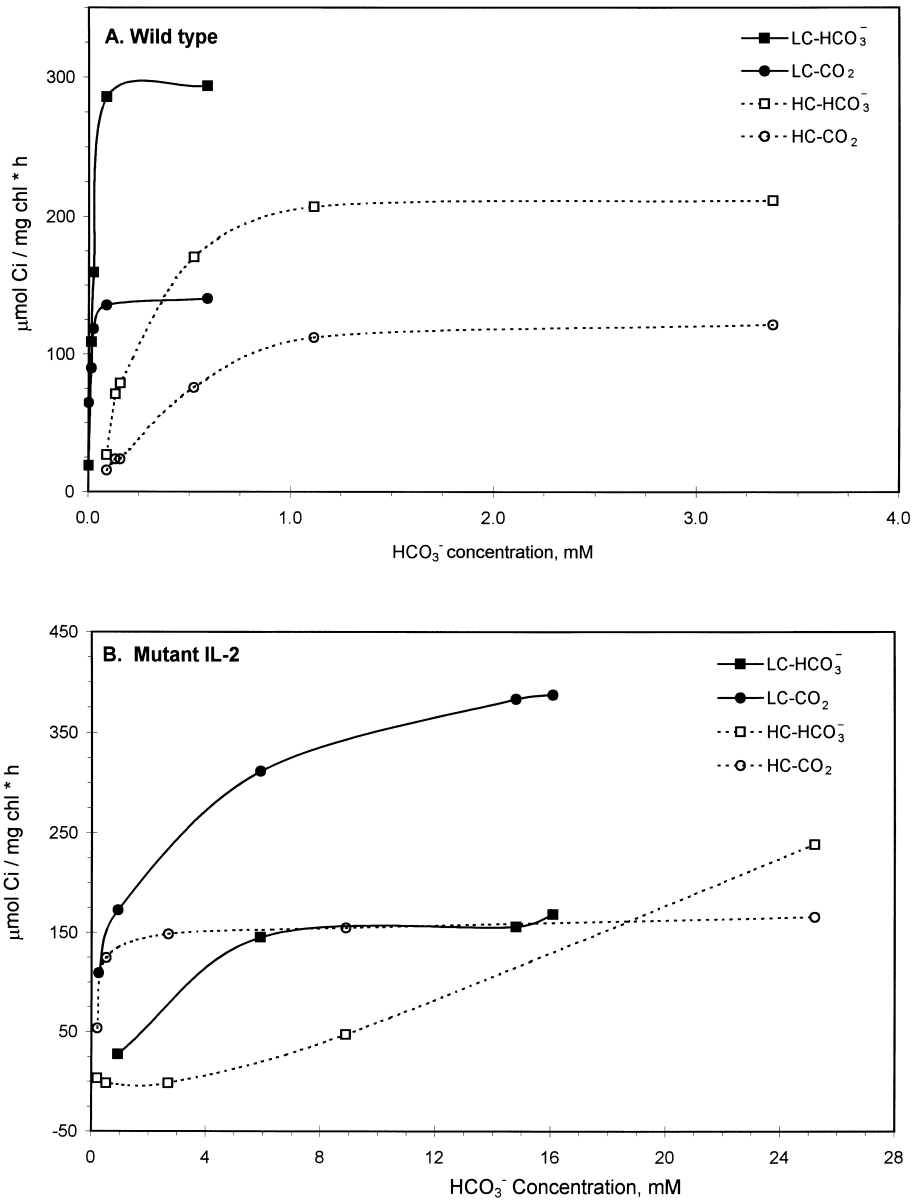

Fig. 2. The rates of CO_2 and of HCO_3^- uptake by *Synechococcus* PCC 7942 (A) and mutant IL-2 (B) as a function of external Ci concentration. The rates were assessed from measurements during steady state photosynthesis using a membrane inlet mass spectrometer (MIMS) [6,7,22].

CO_2 conditions. These data provided the first indication that HCO_3^- transport but not CO_2 uptake was impaired in mutant IL-2.

The V_{max} of HCO_3^- uptake, estimated by the MIMS [7,22] at steady state photosynthesis (Fig. 2A), were 220 and 290 µmol HCO_3^- mg^{-1} Chl h^{-1} for high and low-CO_2-grown wild type, respectively, and the corresponding $K_{1/2}$ (HCO_3^-) were 0.3 and 0.04 mM HCO_3^-. These estimates are in close agreement with those reported earlier [7]. In high-CO_2-grown mutant IL-2, on the other hand, the HCO_3^- transporting system was apparently inactive. The curve relating the rate of HCO_3^- transport to its concentration did not resemble the expected saturable kinetics (observed in the wild type), but was closer to a linear dependence as expected in a diffusion mediated process (Fig. 2B). It was essential to raise the concentration of HCO_3^- in the medium to values as high as 25 mM in order to achieve rates of HCO_3^- uptake similar to the V_{max} depicted by the wild type.

The estimated V_{max} of CO_2 uptake by high-CO_2-grown wild type and IL-2 were 130–150 µmol CO_2 mg^{-1} Chl h^{-1} and the $K_{1/2}(CO_2)$ values were around 5 µM (Fig. 2), indicating that CO_2 uptake was far less affected by the mutation in IL-2. Mutant cells that were exposed to low CO_2 for 12 h showed saturable kinetics for HCO_3^- uptake suggesting the involvement of a carrier. However, the $K_{1/2}$ (HCO_3^-) was 4.5 mM HCO_3^- (i.e. 15- and 100-fold lower than in high- and in low-CO_2-grown wild type, respectively) and the V_{max} was approximately 200 µmol HCO_3^- mg^{-1} Chl h^{-1}. These data may indicate the presence of a low affinity HCO_3^- transporter that became apparent following inactivation of a high affinity HCO_3^- uptake in the mutant. The activity of the 'low affinity' transporter resulted in the saturable transport kinetics ob-

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Wild type          GGGCT-AGCCGCGATCGCGGCCTATTGGGCCC
IL-2 ApaI side     GGGCT-AG--G-GATCGC-GCCTATTGGGCCC
IL-2 BamHI side    GGGCTCA-----GATCGC-GCCTATTGGGCCC
IctB               G  L   A  A  I  A  A  Y  W  A  L
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Fig. 3. The sequences of the relevant region of *ictB* in *Synechococcus* PCC 7942 and in mutant IL-2. This region was duplicated in the mutant due to the single crossover event. Compared with the wild type, one additional nucleotide and deletion of six nucleotides were found in the *Bam*HI side, and 4 nucleotides were deleted in the *Apa*I side (see Fig. 1). These resulted in stop codons in IctB after 168 or 80 amino acids in the *Bam*HI and *Apa*I sides, respectively. The sequence of IctB presented here starts from the 69th amino acid.

served in the low-CO_2-exposed mutant. Further, these data demonstrated that the mutant was able to respond to the low CO_2 signal.

The reason for the discrepancy between the data obtained by the two methods used, with respect to HCO_3^- uptake in wild type and mutant cells grown under high-CO_2 conditions, is not fully understood. It might be related to the fact that in the MIMS method HCO_3^- uptake is assessed as the difference between net photosynthesis and CO_2 uptake [6,7,22]. Therefore, at Ci concentrations below 3 mM where the mutant did not exhibit net photosynthesis, HCO_3^- uptake was calculated as nil (Fig. 2). On the other hand, the filtering centrifugation technique, as used here, measured the unidirectional HCO_3^- transport close to steady state, i.e. isotopic equilibrium exchange. A comprehensive discussion of the validity of some of the assumptions made and of the difficulties involved in the assessment of the kinetic parameters for CO_2 and HCO_3^- transport by these methodologies is beyond the scope of the present paper. Nevertheless, the data obtained by both methods clearly indicated severe inhibition of HCO_3^- uptake in mutant cells exposed to low CO_2. It is interesting to note that while the characteristics of HCO_3^- uptake changed during acclimation of the mutant to low CO_2, CO_2 transport was not affected (Fig. 2). We conclude that the high-CO_2-requiring phenotype of IL-2 stemmed from a lesion in a HCO_3^- transporter rather than from acclimation to low CO_2.

3.2. Genomic analysis

Since IL-2 is impaired in HCO_3^- transport, it was used to identify and clone the relevant genomic region presumably involved in the high affinity HCO_3^- uptake. Fig. 1 presents a schematic map of the genomic region in *Synechococcus* sp. PCC 7942 where the insertion of the inactivating vector by a single crossover recombination event (indicated by a star) led to the formation of mutant IL-2. Sequence analysis (GenBank, accession no. U62616) identified several open reading frames (identified in the legend of Fig. 1), some are similar to those identified in *Synechocystis* PCC 6803 [23]. Comparison of the DNA sequence in the wild type with those in the two repeated regions (due to the single crossover) in mutant IL-2, identified several alterations in the latter. This included a deletion of 4 nucleotides in the *Apa*I side and a deletion of 6 nucleotides but the addition of one bp in the *Bam*HI side (Fig. 3). The reason(s) for these alterations is not known, but they occurred during the single cross recombination between the genomic DNA and the super coiled plasmid bearing the insert in the inactivation library. The high-CO_2-requiring phenotype of mutant JR12 of *Synechococcus* sp. PCC 7942 also resulted from deletions of part of the vector and of a genomic region, during a single crossover event, leading to a lesion in purine biosynthesis under low CO_2 [24].

The alterations depicted in Fig. 3 resulted in frame shifts which led to inactivation of both copies of ORF467 in IL-2. Insertion of a Kan^r cartridge within the *Eco*RV or *Nhe*I sites in ORF467, positions 2919 and 3897, respectively (indicated by the triangles in Fig. 1), resulted in mutants capable of growing in the presence of kanamycin under low CO_2 conditions, though significantly (about 50%) slower than the wild type. Southern analyses of these mutants clearly indicated that they were merodiploids, i.e. contained both the wild-type and the mutated genomic regions. We were unable to isolate a fully segregated mutant of *Synechococcus* sp. PCC 7942 where all the chromosomal copies of the wild type contained the Kan^r cartridge. Similar results were obtained with *Synechocystis* PCC 6803 where attempts (kindly performed by Professor T. Ogawa, Nagoya University) to isolate a fully segregated mutant bearing a transposon-inactivated slr1515 (highly homologous to ORF467) were not successful. It is tempting to suggest that a functional ORF467 of *Synechococcus* sp. PCC 7942 and slr1515 of *Synechocystis* PCC 6803 were essential and that complete segregation was lethal. However, since the inactivation library enabled the isolation of a mutant that does not possess a normal ORF467, we must conclude that the failure to isolate a fully segregated mutant stemmed from segregation problems rather than lethality.

3.3. HCO_3^- transporters in Synechococcus sp. PCC 7942

The protein encoded by ORF467 contains 10 putative transmembrane regions and is inner-membrane-located. It is highly homologous to several transport-engaged and oxidation-reduction proteins including the Na^+/pantothenate symporter of *E. coli* (accession no. P16256). Na^+ ions are essential for HCO_3^- uptake in cyanobacteria and the possible involvement of a Na^+/HCO_3^- symport has been discussed [3,25,26]. The sequence of the fourth transmembrane contains a region which is similar to the DCCD binding motif in subunit C of ATP synthase with the exception of the two outermost positions, replaced by conservative changes in ORF467. The large number of transport proteins that are homologous to the gene product of ORF467 suggests that it is also a transport protein, possibly involved in HCO_3^- uptake. We designated ORF467 as *ict*B (for inorganic carbon transport B [27]).

Sequence similarity between *cmpA*, encoding a 42-kDa polypeptide which accumulates in the cytoplasmic-membrane of low-CO_2-exposed *Synechococcus* PCC 7942 [28], and *nrtA* involved in nitrate transport [29], raised the possibility that CmpA may be the periplasmic part of an ABC-type transporter engaged in HCO_3^- transport ([21], Omata, personal communication). The role of the 42-kDa polypeptide, however, is not clear since inactivation of *cmpA* did not affect the ability of *Synechococcus* PCC 7942 [30] and *Synechocystis* PCC 6803 [21] to grow under air level of CO_2 but lowered growth under 20 ppm CO_2 in air [21]. It is possible that *Synechococcus* sp. PCC 7942 contains three different HCO_3^- carriers: the one encoded by *cmp*; IctB; and the one expressed in mutant IL-2 cells exposed to low CO_2 whose identity is yet to be elucidated. These transporters enable the fulfillment of the vital task of inorganic carbon supply under various environmental conditions.

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