Inactivation of *ccmO* in *Synechococcus* sp. Strain PCC 7942 Results in a Mutant Requiring High Levels of CO_2

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Inactivation of *ccmO* in *Synechococcus* sp. strain PCC 7942 resulted in a mutant which possesses aberrant carboxysomes and a normal inorganic carbon uptake capability but a reduced ability to photosynthetically utilize the internal inorganic carbon pool. Consequently, it exhibits low apparent photosynthetic affinity for extracellular inorganic carbon and demands high levels of CO_2 for growth.

Cyanobacterial mutants which cannot grow under different levels of CO_2 are being used for the elucidation of the physiological and molecular bases of the inorganic carbon (C_i)-concentrating mechanism (1–4, 6–14, 16–18, 20). Some of these mutants show a total lack of carboxysomes or possess aberrant forms of these bodies, which contain most of the rubisco and carbonic anhydrase in the cell (7, 17). These mutants are useful for testing predictions made by a quantitative model which ascribes a central role to the structural organization of the carboxysomes in the functioning of the C_i-concentrating mechanism and CO_2 fixation in cyanobacteria (19).

In *Synechococcus* sp. strain PCC 7942, the lesions in all of the CO_2-dependent mutants (identified so far) have been mapped in the genomic region of the *rbc* operon, encoding the large and small subunits of rubisco (4, 8, 18). Friedberg et al. (4) identified two open reading frames (ORFs) in the 5'-flanking region of *rbc*: ORFI (designated *ccmN* in reference 18) and ORFII (designated *ccmO* in this report). Modifications of *ccmN* (4), *ccmK*, *ccmL*, and *ccmM* (located upstream of *ccmN* [18]) resulted in mutants requiring high levels of CO_2, suggesting that some of the genes involved in the ability of this organism to grow under low levels of CO_2 are clustered in this region. On the other hand, insertion of *nptII* (conferring kanamycin resistance [Km^r]) near the C terminus of *ccmO*, located immediately upstream of *rbc*, resulted in Km^r mutants capable of growing under high and low CO_2 concentrations. It was therefore suggested that this ORF is not involved in growth under low levels of CO_2 (4). In the present study, we tested the possibility that the insertion at the C terminus did not result in functional inactivation of the ORF by inserting *nptII* near its N terminus.

A *Cla*I-*Kpn*I fragment (2.7 kbp) was isolated from a genomic library of *Synechococcus* sp. strain PCC 7942. The *Cla*I site is located 2,521 bp upstream of the initiation codon of *rbc*, and *Kpn*I is located 180 bp downstream of the N terminus of *rbc* (see reference 4, which provides the relevant sequence). This fragment was subcloned into the respective sites of a modified Bluescript SK plasmid (digested with *Eco*RV and *Sma*I and blunt ligated). A 1.3-kbp *Hinc*II fragment bearing *nptII* (see reference 4) was inserted into the *Ava*I site (originally located 1.08 kbp upstream of *rbc*) after the protruding ends were filled. *Synechococcus* sp. strain PCC 7942 cells were transformed with

the resulting 4.0-kbp *Cla*I-*Kpn*I fragment. The transformation resulted in Km^r mutants requiring high levels of CO_2, one of which, designated N1, was chosen for further analysis.

Confirmation that the Km^r cartridge was inserted in the desired *Ava*I site was obtained by sequence and Southern analyses. The genomic DNA from N1 was digested with *Hinc*II and *Sac*I, and fragments of 2.0 to 3.0 kbp were isolated and ligated within the *Hinc*II and *Sac*I sites of Bluescript SK. Competent cells of *Escherichia coli* (DH5α) were transfected with the recombinant plasmids obtained and then selected for Km resistance. Sequence analysis (Sequenase kit; United States Biochemical Corp.) with the aid of synthetic oligonucleotides (Biotechnology General, Rehovot, Israel) homologous to the 5' and 3' ends of the Km^r cartridge confirmed that it was inserted in the desired site of the genome of N1.

Southern analyses (Fig. 1) were performed on genomic DNA isolated from the wild type and the mutant, digested with *Hinc*II and *Cla*I. A 1.3-kbp *Hinc*II fragment (bearing *nptII*) and a genomic 1.2-kbp *Hinc*II fragment were used as probes. The *nptII* probe hybridized only with DNA fragments from N1, exhibiting bands of 2.5 kbp (*Hinc*II) and 2.5 and 6.7 (*Cla*I) kbp. The genomic *Hinc*II probe hybridized with fragments of 1.2 (*Hinc*II) and 8 (*Cla*I) kbp in DNA from the wild type and with fragments of 2.5 kbp (*Hinc*II) and 2.5 and 6.7 (*Cla*I) kbp in DNA from N1. The sequence and Southern data clearly indicated that the cloned fragment was inserted into the desired *Ava*I site via a double-crossover recombination event and that complete segregation (i.e., replacement of the original with the modified region) was obtained. Electron micrographs of the wild type and mutant N1 (prepared as described in reference 15) clearly indicated the absence of normal carboxysomes in mutant N1 (Fig. 2).

Mutant N1 was unable to grow at air levels of CO_2 in either solid or liquid medium (see growth conditions in reference 4). In the presence of 5% (vol/vol) CO_2, on the other hand, its growth rate was identical to that of the wild type. Its maximum rate of photosynthesis (measured with an O_2 electrode [5]) at saturating extracellular C_i concentrations was similar to that of the wild type, but its apparent photosynthetic affinity for extracellular C_i was approximately 200-fold lower [$K_{1/2(C_i)}$ = 0.1 and 24 mM C_i in wild type and N1, respectively]. The mutant was able to accumulate C_i within the cells (measured by the filtering centrifugation technique [5]) as efficiently as the wild type. On the other hand, the accumulation of acid-stable ^{14}C (photosynthetic products) was 10-fold lower in the mutant, suggesting that the low apparent photosynthetic affinity for C_i in N1 is most probably attributable to a defect in its ability to photosynthetically utilize the internal C_i pool.

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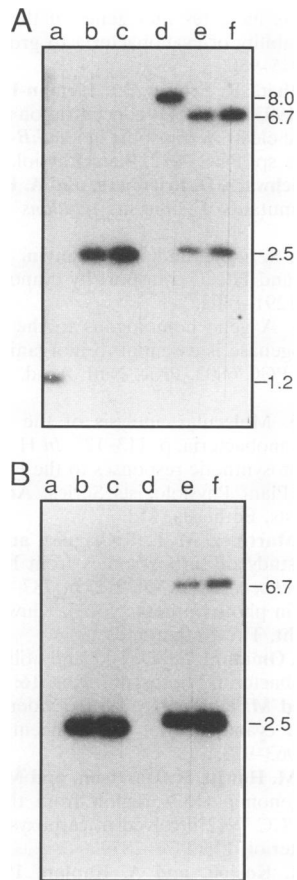
FIG. 1. Southern analysis of the genomic DNA from mutant N1 (two clones) and the wild type. A 1.2-kbp *Hinc*II fragment from the genomic DNA containing *ccmO* and the 3' end of *ccmN* and a 1.3-kbp *Hinc*II fragment containing *nptII* were used as probes in panels A and B, respectively. Lanes: (a, d) genomic DNA from the wild type; (b, c, e, f) genomic DNA from the mutant N1. Restriction enzymes used were *Hinc*II (lanes a, b, and c) and *Cla*I (lanes d, e, and f). The size (in kilobase pairs) of the different bands is given on the right.

These findings are similar to those reported for other mutants of *Synechococcus* sp. strain PCC 7942 requiring high levels of CO_2 which contain defective carboxysomes (1, 4, 7–9, 11, 14, 17, 18, 20).

Measurements of the activation state of rubisco and of the pool size of ribulose-1,5-bisphosphate in some of the mutants which possess aberrant carboxysomes indicated that their requirement for high levels of CO_2 probably stems from a low state of activation of the enzyme when the mutant cells are exposed to low levels of CO_2 (20). The data provide strong support for the suggestion that proper organization of the carboxysomes is essential for the efficient utilization of C_i in cyanobacterial photosynthesis.

The nature, role, and mode of regulation of the gene cluster *ccmK-L-M-N-O* involved in the ability of *Synechococcus* sp. to grow under low levels of CO_2 are not known, but modification of these genes resulted in mutants which contain defective carboxysomes. Very little information is available on the biogenesis of the carboxysomes. Analysis of different mutants with lesions in the *ccm* genes (4, 15, 18) indicated various types of structural modifications. It is important to note, however, that these modifications are also observed, though rarely, in
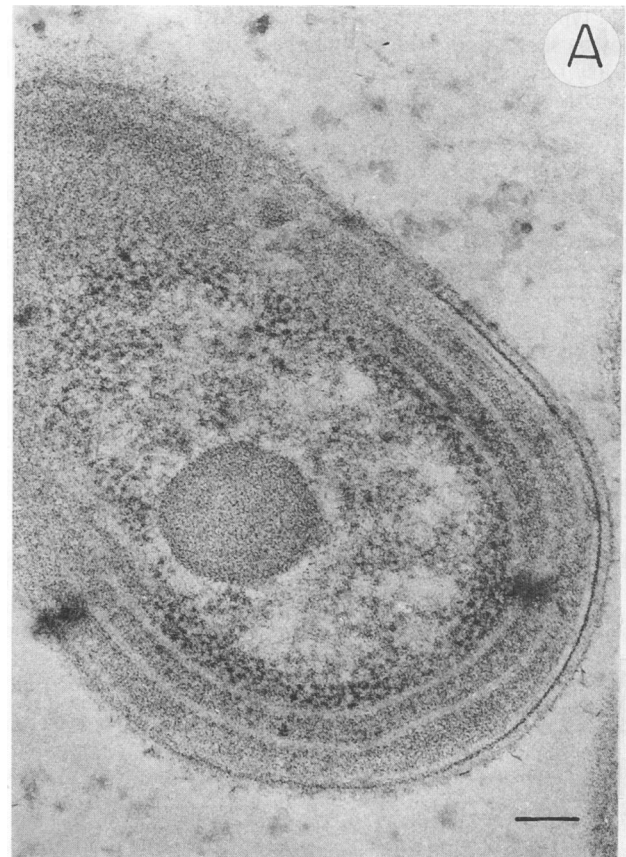

FIG. 2. Electron micrographs of the wild type (A) and mutant N1 (B) showing a normal carboxysome in the wild type but no carboxysomes in the mutant. Bars, 0.1 μm.

wild-type cells. The various types of carboxysomes observed may be stages in their development so that the preponderance of any one form may represent retarded stages in the development of the carboxysomes.

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