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A Gene (*ccmA*) Required for Carboxysome Formation in the Cyanobacterium *Synechocystis* sp. Strain PCC6803

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A high-CO_2-requiring mutant, G7, of *Synechocystis* sp. strain PCC6803 capable of inorganic carbon transport but unable to utilize the intracellular inorganic carbon pool for photosynthesis was isolated. Transmission electron micrographs of the mutant indicated that the mutant does not have any carboxysomes. A clone (pHPG7) with a 7.5-kbp DNA insert that transforms the G7 mutant to the wild-type phenotype was isolated from a genomic library of wild-type *Synechocystis* sp. strain PCC6803. Complementation tests with subclones identified the mutation site in G7 within 208 bp. Sequencing of nucleotides in this region elucidated an open reading frame, designated *ccmA*, encoding a protein of 302 amino acids. Cloning and sequence analysis of the respective G7 gene revealed an A-to-G substitution that results in an Asp-to-Gly substitution in the deduced amino acid. The result indicated that the *ccmA* gene encodes a protein essential for the formation of carboxysomes. An open reading frame encoding a proline-rich protein of 271 amino acids was found downstream of the *ccmA* gene, but no *ccm*-like genes or *rbc* operon was found in this region.

Carboxysomes are small polyhedral bodies present in cyanobacterial cells and have most of the cell's ribulose 1,5-bisphosphate carboxylase oxygenase (RuBisCO) activity (4). They play an essential role in cyanobacterial photosynthesis. Cyanobacteria possess a CO_2-concentrating mechanism which involves active transport of inorganic carbon (C_i) driven by light energy (9, 10, 13, 18). The C_i accumulated by the transport system enters the carboxysomes, where HCO_3^- is dehydrated to CO_2 by catalysis with a low level of carbonic anhydrase (CA) activity and then fixed by RuBisCO. The significance of carboxysomes in cyanobacterial photosynthesis has been demonstrated by a number of studies. (i) High-CO_2-requiring (HCR) mutants which possess a C_i transport activity but are unable to utilize the intracellular C_i for photosynthesis have been isolated from *Synechococcus* sp. strain PCC7942 (12, 23, 28) and *Synechocystis* sp. strain PCC6803 (17). Transmission electron micrographs of a number of these mutants revealed rod-shaped carboxysomes or no detectable carboxysomes (7, 20). Five genes (*ccmK*, *ccmL*, *ccmM*, *ccmN*, and *ccmO*) responsible for the HCR phenotype in the mutants have been isolated (7, 10, 12, 17, 23, 25, 28). (ii) Mutants impaired in the *icfA* (*ccaA*) gene encoding carboxysomal CA are unable to utilize intracellular C_i for photosynthesis (8, 32). (iii) Introduction and expression of the human CA gene in the cytosol of wild-type (WT) *Synechococcus* sp. strain PCC7942 results in the leakage of CO_2 from the cells (22). These cells are unable to grow at air levels of CO_2. These results clearly demonstrated that the confinement of RuBisCO and CA in carboxysomes is essential for utilization of the intracellular C_i pool for photosynthesis.

Recently we have isolated an HCR mutant (G7) of *Synechocystis* sp. strain PCC6803, which does not have carboxysomes. Using this mutant, we cloned and sequenced a WT DNA fragment which, upon transformation, restored WT capabilities to the mutant. An open reading frame (ORF) (designated *ccmA*) encoding a protein of 302 amino acids was found in the region of the G7 mutation. This paper describes some characteristics of the G7 mutant and the *ccmA* gene and provides a restriction map of the region of the *ccmA* gene.

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MATERIALS AND METHODS

Mutagenesis. The HCR mutant (G7) was isolated from *Synechocystis* sp. strain PCC6803 following mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and ampicillin enrichment by a method described previously (14).

Growth conditions. WT and mutant cells were grown at 30°C in BG11 medium (30) buffered with 10 mM *N*-Tris(hydroxymethyl)-2-aminomethanesulfonic acid–KOH (pH 8) under 3% CO_2–97% air. The solid medium was BG11 supplemented with 1.5% agar, 5 mM sodium thiosulfite, and 20 mM buffer (the same as that described above). Continuous illumination was provided at 120 μmol of photosynthetically active radiation per m^2 per s by incandescent lamps for liquid culture or by fluorescent lamps for culture on solid medium.

Measurements of growth and gas exchange. Growth curves were determined from the rise in the optical density at 730 nm, measured with a Shimadzu Recording Spectrophotometer (UV200). CO_2 exchange of the WT and mutant cells was measured with an open gas analysis system described elsewhere (18).

Transmission electron microscopy. Cells were grown in 5%-CO_2-enriched air and then bubbled with air for 12 h under continuous illumination. Staining and sectioning of cells for transmission electron microscopy were done as described by Orus et al. (19).

Cloning and transformation. The genomic library of *Synechocystis* sp. strain PCC6803 constructed in pUC18 was a kind gift from J. G. K. Williams at Du Pont de Nemours & Co. (Wilmington, Del.). A complementation test was performed by the method of transformation reported by Dzelzkalns and Bogorad (5) and by a procedure described previously (15, 16).

Insertional inactivation. The cloned DNA fragment was interrupted by an aminoglycoside 3'-phosphotransferase gene, the Km^r cartridge C.K1 (6) originating from the bacterial

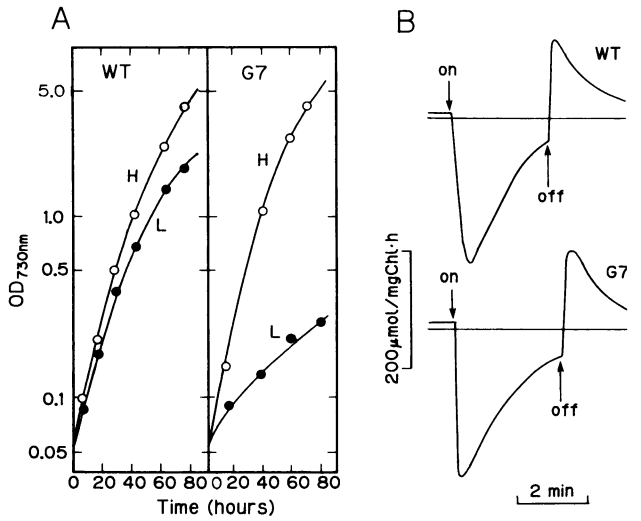

FIG. 1. (A) Growth curves of WT cells (left panel) and mutant G7 (right panel) under 3% CO_2 (closed symbols) and air (0.04% CO_2) (open symbols). OD_{730nm}, optical density at 730 nm; H, high CO_2 level; L, low CO_2 level. (B) CO_2 exchange profiles of low-CO_2-adapted WT and G7 mutant cells of *Synechocystis* sp. strain PCC6803 measured in the presence of 3% iodoacetamide. Cells were grown with 3% CO_2 in air and then bubbled with air for 15 h in the light. White light (1,380 microeinsteins per m^2 per s) was switched on and off as indicated by the arrows. For details of the gas exchange system, see the study of Ogawa et al. (18).

transposon Tn*5* (3), and the WT cells of *Synechocystis* sp. strain PCC6803 were transformed with a plasmid containing the modified DNA fragment according to a method reported by Williams and Szalay (31) and described previously (15, 16).

DNA manipulations. Unless otherwise stated, standard techniques were used for DNA manipulation (27). Nucleotides were deleted from the cloned DNA fragment by using a deletion kit (Takara Co., Tokyo) which contains exonuclease III and mung bean nuclease. The number of nucleotides deleted was determined by sequencing the DNA fragment after the deletion. A fragment of the *ccmA* gene of G7 (335 bp) was amplified by PCR (26) with the genomic DNA of the mutant as the template and cloned into TA vector (Invitrogen, San Diego, Calif.) for sequencing. The nucleotide sequences were determined by using a DuPont DNA analysis system (Genesis 2000). Both strands were sequenced, and no ambiguities were found.

Nucleotide sequence accession number. The sequence reported in this paper has been deposited in the GenBank data base (accession no. D26444).

RESULTS AND DISCUSSION

Growth responses to air and CO_2 uptake activity. Growth rates of the G7 mutant and the WT cells were similar when the cultures were aerated with 3% CO_2 in air (Fig. 1A; open circles). The mutant grew significantly more slowly in air than the WT (Fig. 1A; closed circles). The CO_2 exchange profiles of air-adapted WT and G7 cells measured in the presence of iodoacetamide, an inhibitor of CO_2 fixation, are shown in Fig. 1B. Upon illumination with white light, the rate of CO_2 uptake of the WT cells increased to a maximal level and then declined to a steady-state level. The maximal rate of CO_2 uptake of the mutant was as high as that of the WT. Thus, there is no significant difference between the WT and the G7 mutant in CO_2 uptake activity. It is therefore concluded that the G7 mutant is impaired in its ability to utilize the intracellular C_i pool for photosynthesis.

Absence of carboxysomes. In contrast to the WT cells of *Synechocystis* sp. strain PCC6803, most of which showed one carboxysome per sectioned cell, no polyhedral body was observed in the G7 population. An extensive examination was made by using a transmission electron microscope with a large number of G7 grids, but neither normal nor aberrant carboxysomes were ever seen. On the basis of these observations, we concluded that G7 is a carboxysomeless mutant and that a gene required for carboxysome formation is impaired in the mutant. The mutant had a large number of electron-dense inclusions of cyanophycin, a polymer of L-arginine and L-aspartic acid whose clear function is nitrogen storage (1). These inclusions have been repeatedly reported to increase under various stress conditions. A typical cell of WT *Synechocystis* sp. strain PCC6803 with a central carboxysome is presented in Fig. 2A, and a representative example of the G7 cells is shown in Fig. 2B.

Complementation of the G7 mutation. A clone that transforms the G7 mutant to the WT phenotype, pHPG7, contains a 7.5-kbp DNA insert (a restriction map is shown in Fig. 3). Several fragments obtained by digestion with *Xba*I and *Nhe*I were subcloned into pUC119 at the *Xba*I site. Complementation tests with the subclones indicated that the mutation site in G7 is located within 1.4 kbp between the *Xba*I and *Nhe*I sites on the left side of the map (Fig. 3). A 6.8-kbp *Xba*I fragment was subcloned into pUC119, and nucleotides were deleted from the *Xba*I site on the left side. Deletion of 403 bp did not have any effect on the ability of the clone to complement the mutant, while further deletion of 208 bp led to loss of the complementing ability. This result identified the site of the mutation within the 208-bp stretch of nucleotides.

The nucleotide sequence within the mutation region is shown in Fig. 4, together with the sequences of amino acids encoded by ORFs. There exists an ORF encoding a protein of 302 amino acids (designated *ccmA*), which covers the 208-bp region. The *ccmA* gene extends from bases 235 to 1140, as numbered from the *Xba*I site. A Shine-Dalgarno (SD) (29) sequence (GAAG) was found upstream of the postulated ATG codon of the *ccmA* gene. A hairpin structure in the sequence downstream of *ccmA* (bases 1319 to 1355) may be involved in transcription termination.

Sequence analysis of a *ccmA* fragment of the G7 mutant (346 bp from bases 335 to 680) revealed an A-to-G substitution at base 419 within the *ccmA* gene. This change resulted in an Asp-to-Gly substitution in the deduced amino acid sequence (Fig. 4). The mutation was located within the region expected from the results of the complementation test.

Insertional inactivation of the *ccmA* gene. There exists an ORF (ORF125) in the complementary sequence (bases 581 to 195) which overlaps with a part of the *ccmA* sequence and covers the site of the G7 mutation (Fig. 3). To confirm that the inactivation of the *ccmA* gene caused an HCR phenotype, the gene was inactivated by insertion of the Km^r cartridge at the *Bal*I site which is within the *ccmA* gene but outside ORF125 (Fig. 3). The Km^r mutant thus obtained showed an HCR phenotype (data not shown), indicating that the *ccmA* gene is essential for the cells to grow under low-CO_2 conditions. It appears unlikely that ORF125 is functioning as a gene because of the unusual codon usages. The codon usages of *Synechocystis* sp. strain PCC6803 calculated from the data obtained for 13,506 codons indicated that the usages of TCA for Ser, AGG for Arg, and CCA for Pro are very low (3.9, 3.3, and 5.2%, respectively, of the total codon usage for each amino acid).

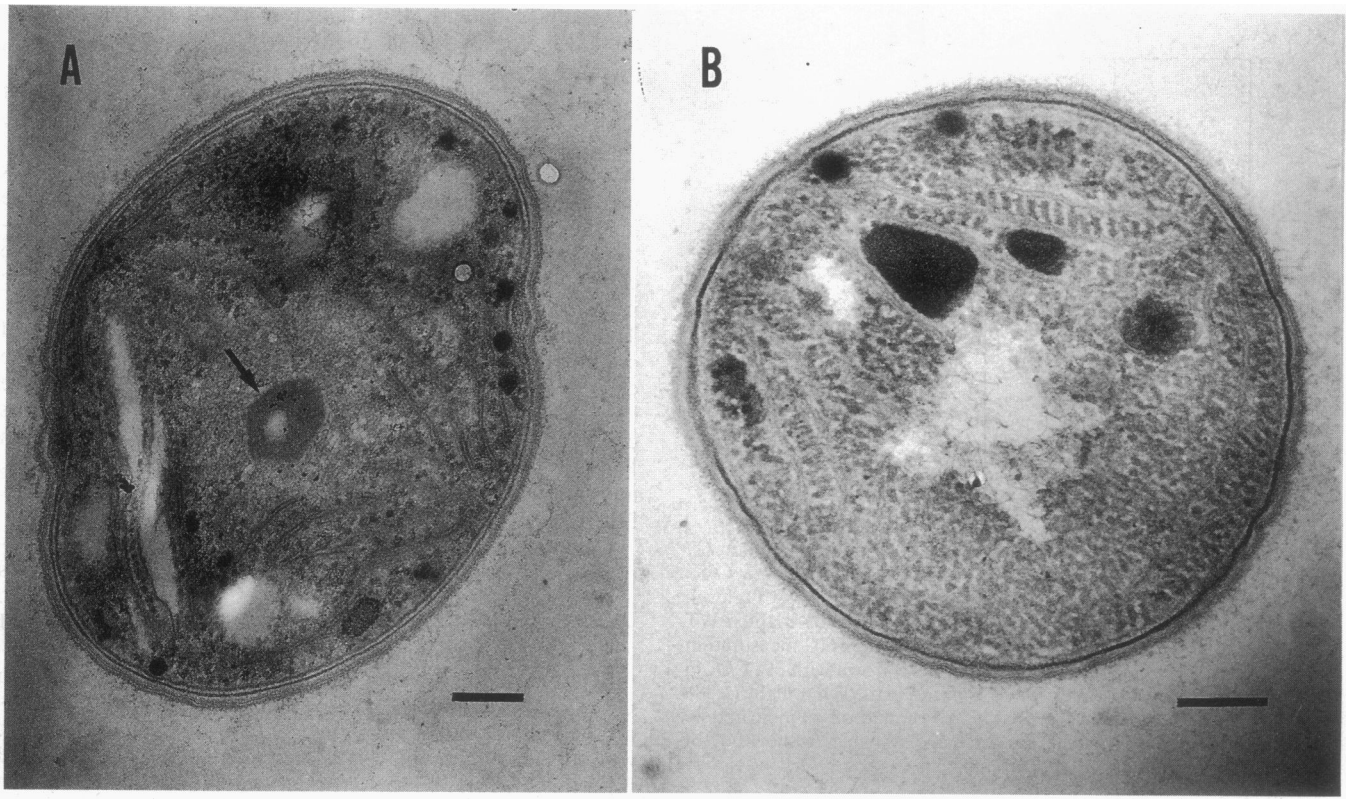

FIG. 2. Electron micrographs of WT (A) and G7 mutant (B) cells of *Synechocystis* sp. strain PCC6803. A carboxysome in the WT cell is indicated by the arrow. Bars, 0.5 μm.

The usages of these codons in ORF125 were 26.3, 28.6, and 36.4%, respectively, which are unusually higher than the average values. Such unusual codon usages were not observed for the *ccmA* gene.

Region of the *ccmA* gene. There is an *Xba*I site within the *rbcL* gene and another *Xba*I site 22 kbp upstream of the *rbcL* gene (2, 17). Since the distance between the two *Xba*I sites in pHPG7 is 6.8 kbp, these sites cannot be identical to the sites in the *rbc* region. It is therefore evident that the *ccmA* gene is not located in the 5′-flanking region of the *rbc* operon. We have recently cloned the *ccmM* and *ccmN* genes from *Synechocystis* sp. strain PCC6803 (17) and showed that these genes are not located within the 22-kbp stretch upstream of *rbcL*. In *Synechococcus* sp. strain PCC7942, five *ccm* genes (*ccmK*, *-L*, *-M*, *-N*, and *-O*) have been mapped in the 5′-flanking region of *rbcL* (7, 10, 25). However, the present and previous results obtained for *Synechocystis* sp. strain PCC6803 suggest that the distance between the *ccm* genes and the *rbc* operon has no physiological significance.

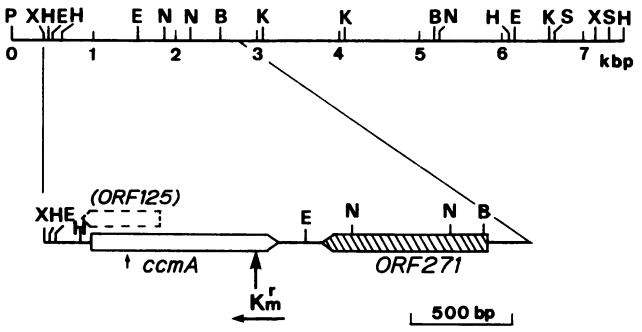

FIG. 3. Restriction map of the 7.5-kbp DNA insert in plasmid pHPG7 that complemented the G7 mutant. The 906-bp ORF (*ccmA*) is indicated by the open bar, and the 813-bp ORF (ORF271) is indicated by the hatched bar. The pointed ends denote the directions of the ORFs. ORF125 in the complementary sequence overlapping the *ccmA* gene is shown by the dashed bar. This ORF may be not functioning as a gene, as described in the text. The Km^r cartridge was inserted at the position indicated, and its direction is shown by the horizontal arrow. B, *Bam*HI; H, *Hin*dIII; K, *Kpn*I; N, *Nhe*I; P, *Pst*I; E, *Eco*RI; S, *Spe*I; X, *Xba*I.

The nucleotide sequence in the downstream region of the *ccmA* gene revealed another ORF (from bases 2187 to 1375 on the complementary sequence, ORF271) which encodes a protein of 271 amino acids with 50 proline residues (the deduced amino acid sequence is shown in Fig. 4). Homology search by the computer program of Pearson and Lipman (21) indicated that the protein is homologous to the extensin precursor of maize (23.7% identity for a 173-amino-acid overlap). It appears plausible that the protein encoded by ORF271 is a constituent of the cell wall. No *ccm*-like genes were present in this region or in the region upstream of the *ccmA* gene. It appears that the *ccmA* gene is not clustered with other *ccm* genes and that the transcript for *ccmA* is synthesized as a monocistronic mRNA. This can be clarified by Northern (RNA) analysis, which will be done in future.

Properties of a putative protein encoded by the *ccmA* gene. The *ccmA* gene product consists of 177 hydrophobic, 77 hydrophilic, and 48 neutral amino acids. The calculated molecular weight of this putative protein is 32,442. The hydropa-

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   1 TCTAGAGCTAATCGAATCACCAAAAGCTTACGTCAGCCAACATTTGGGCGATTTGAATTCTGCTCCCACCTACCAAAATTTTGAAGAAGCCATTGCCCATTCAAGCCAGCTATACGATTT
 121 CTCTCCCCAGGAAATTGTTGCAGATTTACACCCTGATTATTTCAGTCATCAATATGCTGAAAACCAAGCTTTTAACCCTCCCTGTCCCAACCTATCGAATAGAGGAAGTTTGTCATGATC
                                                                                                    SD ccmA> M  I
 241 GTCGTCATGAAAGTCGGCACCCCAGAAATAGAAATTGAGCGCATTGGTAAGGAGTTTGCCGAACGGGGCCTCACCCCCGAAAAGATTGTTGGTAAGCACAAAGTTGTGATGGGGTTGGTG
     V  V  M  K  V  G  T  P  E  I  E  I  E  R  I  G  K  E  F  Ag E  R  G  L  T  P  E  K  I  V  G  K  H  K  V  V  M  G  L  V
 361 GGGGAAACAGCCGGACTTGATCCTTTGCAAATCCAAGAGGTTAGCCCCTGGATTGAAGATGTATTACGGGTGGAACAACCTTTTAAACGGGCTAGTTTGACTTTCCGCCATGGGGAATAC
     G  E  T  A  G  L  D  P  L  Q  I  Q  E  V  S  P  W  I  E  DG V  L  R  V  E  Q  P  F  K  R  A  S  L  T  F  R  H  G  E  Y
 481 AGTGAAGTAATCGTTTCTACCCCCGCTGGCCCCGTGGCGATCGGCAAAAACCATCCGGTGGCTGTGGTAGCAGGCCCCTGTTCGGTGGAAAATGAAACCATGATTGTGGAAACGGCCCGT
     S  E  V  I  V  S  T  P  A  G  P  V  A  I  G  K  N  H  P  V  A  V  V  A  G  P  C  S  V  E  N  E  T  M  I  V  E  T  A  R
 601 CGAGTTAAGGCGGCCGGGGCCCAGTTTTTACGGGGCGGCGCCTTCAAACCCCGTACCTCTCCCTACGCTTTCCAAGGTCATGGGGAAAGTGCTCTAGGTCTGTTGGCAGCAGCTAAGGAA
     R  V  K  A  A  G  A  Q  F  L  R  G  G  A  F  K  P  R  T  S  P  Y  A  F  Q  G  H  G  E  S  A  L  G  L  L  A  A  A  K  E
 721 GCCACTGGTTTGGGCATTATCACCGAAGTGATGGATGCGGCGGATTTAGAAATCATTGCGGAAGTGGCGGATGTAATCCAAGTGGGTGCCCGCAATATGCAGAATTTCTCCATGCTGAAA
     A  T  G  L  G  I  I  T  E  V  M  D  A  A  D  L  E  I  I  A  E  V  A  D  V  I  Q  V  G  A  R  N  M  Q  N  F  S  M  L  K
 841 AAGGTAGGGGCCCAGGATAAGCCAGTGCTGTTAAAGCGGGGCATGGCGGCCACCATTGATGAATGGTTAATGGCGGCAGAATACATTCTCGCGGCGGGTAATCCCAACGTGATTCTTTGT
     K  V  G  A  Q  D  K  P  V  L  L  K  R  G  M  A  A  T  I  D  E  W  L  M  A  A  E  Y  I  L  A  A  G  N  P  N  V  I  L  C
 961 GAGCGGGGCATTCGCACCTTTGACGGCCATTACACCCGCAATTGCCTTGATTTGTCGGTGTTGCCTGTCCTGCGCTCCCTTACGCACCTGCCCATTATGATCGACCCCAGCCACGGCACC
     E  R  G  I  R  T  F  D  G  H  Y  T  R  N  C  L  D  L  S  V  L  P  V  L  R  S  L  T  H  L  P  I  M  I  D  P  S  H  G  T
1081 GGTAAATCCGAGTATGTGCCCTCCATGGCCATGGCGGCGATCGCGCGGGGACAGACTCCTTGATGATTGAAGTGCATCCCAACCCGGCTAAAGCTATGTCCGATGGGCCCCAATCTTTGA
     G  K  S  E  Y  V  P  S  M  A  M  A  A  I  A  R  G  Q  T  P  *
1201 CCCCCGACCGCTTCGACGAAACCATGAAGCAATTGGCGGTTATTGGGCAAACTGTAGGACGTTGGCCTAAAGTAGCGGCCCTGGCCTAGGAATTCGCGCTGTAAAACTAAAACAGATTAA
1321 ACCAGGCCCGACGGAAAAACCAGCGGGCCTTTTTTGTTGG
1321 TGGTCCGGGCTGCCTTTTTGGTCGCCCGGAAAAAAACAACCTACCACGCCTGATAACCCCAACTAGAAGGTCTTTTCGGAAGTGTAGAGGGTCCCCTCATTCATGATTCGCTTACCAAAG
                                                                                                    *  Q  P  Q  D  E  L  F  A  K  V  D  G  L  P  T  L  V  L  R  I  T  E
1441 GTAGGACAGTAGCCGCTCCCCTCTCTGGTTGCCAAGGGGTCCCCGTCCAACCCCGTCCGCCCGGTACATTTACGATCGCCGAGCCGGATCCCCGTACCAAGAGAAGTGGCAGGGTGACCC
     M  R  D  D  A  L  P  S  V  L  P  E  G  P  A  P  Q  P  L  R  A  M  Y  I  S  A  A  R  G  L  P  M  T  R  K  V  T  G  S  P
1561 TAGTTAAACATCCGCTATAAGGTTGCAAACCCAAACTAGAAGGCCGCCAACGGTGACGAGAACTATCTTGAATGCGTGAAGGCGGTGGACCCCTTACCACAGGTCATTTAACCACCTTGC
     D  I  Q  L  R  Y  E  L  T  Q  T  Q  D  E  P  P  Q  W  Q  E  Q  Y  F  K  R  V  E  A  V  Q  P  I  T  D  L  L  N  T  S  R
1681 CCCACCGTGTCGTGCCCCAGCTCCGCCGGATTTTGATTGCCCTAATCTTCGTCCCCCGTCTCCGCCATTTAGCCCCCATCACCTGTTATAGAGCGGTGGTATCCCATCCCCAACTGGCAG
     P  P  V  A  R  P  R  P  P  P  R  F  S  V  P  N  S  A  P  P  L  P  P  L  D  P  T  T  S  L  I  E  G  G  Y  P  L  P  Q  G  D
1801 CCCCGCCCCGTTTTATCGGTTCCGTGGCCCCATTCTGCCAAGACCGCCGCCACAGTCCCCAACATCTAATCACCCTAGTCCTTACCGTTATATCCAAAGGGGACCCCGAGGACCTCGAAC
     P  R  P  L  I  A  L  A  G  P  Y  S  P  E  P  P  P  T  L  P  Q  L  N  T  P  D  P  I  A  I  Y  T  E  G  P  A  G  P  A  Q
1921 ATTTAGTCCCCCTGCGTGGGAAAGACCCCCGTGGTGATCCCCTTATAATCCTAATGCCCCTCCCCCTCGTCGTCGATCGAAAAACGGGTTTAGGAGGTTGAGGTCTCCTACCAGGTATAA
     L  D  P  P  R  V  R  E  P  P  V  V  L  P  I  N  P  N  R  P  P  P  A  A  A  L  K  K  G  L  D  E  L  E  L  P  H  D  M  N
2041 GTACCGGGTTGTCTACGCGAGGTGACACGGGTAGTTTAAGTGGCGGTGGCCCTTTCCCCTCGGAAACTGTAAGAGTTTATTGTCGGGACCCGGTGGCCGGACATCGTAGGCCAATCTCTA
     M  A  W  C  I  R  E  V  T  G  M  L  N  V  A  V  P  F  P  S  G  K  V  N  E  F  L  L  G  P  G  G  A  Q  L  M  R  N  S  I
2161 GACGCGGTGAAGGTTTAACCCTAGGTGGAGTCGCCATTACGCCGGTTGACGGGGGCACAAAGGTTGTTGGCTTACTTAGGGGCATTTAGAGACCGGTACCGACTACAGATTTGTTTGTGC
     Q  A  V  E  L  N  E  D  V <ORF271
2281 GAGAAATCGAATTGCCCAAATCGGGCTGGTCCTACGGGTAGAAGTTGCCCTTAAACGGCGATACAATCCCCAGTTGCGAAAGTGGTGCCCGTCTCCGCTAAACCGAAGAACAC
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FIG. 4. Nucleotide sequence of the region affected by the G3 mutation. Nucleotides are numbered from the *Xba*I site on the left side of the map shown in Fig. 3. Complementary sequence is shown from bases 1321 to 2393, and these numbers are underlined. The deduced amino acid sequences of the ORFs (*ccmA* and ORF271) are presented in single-letter code. The mutation in the G7 mutant is shown at base 419 by a small italic letter for the nucleotide and a capital italic letter for the amino acid. For the *ccmA* gene, a possible ribosome binding site (29) is labeled SD and underlined. An inverted repeat downstream of *ccmA* (underlined) may be involved in transcription termination. The inverted repeat for ORF271 is also underlined.

thy index of the protein, according to Kyte and Doolittle (11), indicated five hydrophobic domains (Fig. 5). The amino acid sequence of this putative protein was subjected to a homology search by the computer program developed by Pearson and Lipman (21), but no homologous genes were found in the data base.

Carboxysome is composed of at least 12 structural proteins (24), and the *ccmA* gene product could be one of them. Lack of this protein might result in the failure to assemble of other structural proteins. It is also possible that the *ccmA* gene encodes a nonstructural protein involved in carboxysome maturation. The exact role of the *ccmA* gene product in carboxysome formation is not yet clear.

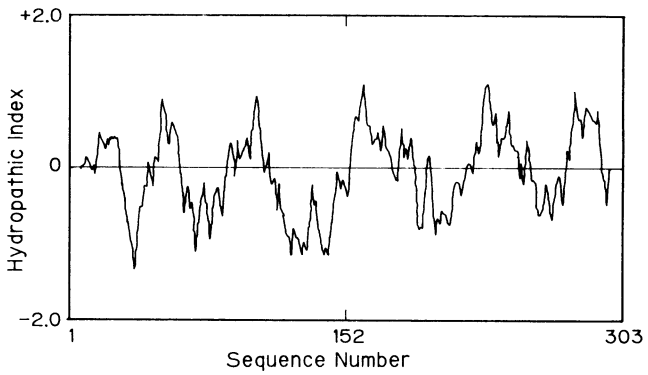

FIG. 5. Hydropathy profile of the *ccmA* gene product, determined by the method of Kyte and Doolittle (11). A window of 19 amino acids was used for the analysis.

ACKNOWLEDGMENTS

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