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# Cloning and sequence analysis of the gene encoding the low potential cytochrome *c* of *Synechocystis* PCC 6803

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**Abstract**

The gene for the low potential cytochrome *c* (*petK*) was isolated from a genomic library of *Synechocystis* PCC 6803. The nucleotide sequence of this gene contains two regions with sequence similarity to two regions in the gene for the high potential cytochrome $c_6$ of the organism. The sixth iron ligand can be identified with a conserved histidine. Experiments demonstrate the reduction of the low potential cytochrome by reduced ferredoxin II. The heme of the cytochrome is flanked by lysines which may be involved in orienting the ferredoxin near the site of electron donation.

*Key words:* Cytochrome *c*; Cyanobacterial fermentation; Photosynthesis; Ferredoxin; Hydrogenase

## 1. Introduction

A low potential cytochrome *c* with an alpha band at 549 or 550 nm has been recognized in a number of cyanobacteria and eukaryotic algae [1–5]. Holten and Myers, who first characterized this protein, suggested it might have a role in hydrogen metabolism [6]. While the first report dealt with a protein that was easily released in aqueous extracts of cells, there were subsequent reports, beginning with that of Krinner et al. [7], of a low potential cytochrome *c* which is tightly held to photosynthetic membranes [8,9]. Recently, Shen et al. have found this cytochrome in $O_2$ evolving photosystem II core particles and have shown that cytochrome depletion diminishes O2 evolution and cytochrome readdition restores this activity [10]. Cohn et al. [11] determined the complete amino acid sequence of the soluble, low potential cytochrome *c* from *Microcystis aeruginosa* and Shen et al. [10] published an N-terminal sequence of the cytochrome which is tightly bound to photosystem II of *Synechococcus vulcanus*. These sequences are quite similar.

The data in this paper allows inferences about the evolution of *c* type cytochromes and about the function of the low potential cytochrome *c* of cyanobacteria and algae.

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The nucleotide sequence data reported in this paper has been deposited in the GenBank database under the accession number U07021.

## 2. Materials and methods

### 2.1. Purification of the low potential cytochrome from Synechocystis PCC 6803

The cytochrome was purified as described by Cohn et al. [12] with one modification. After DEAE chromatography, the cytochrome was dialysed, concentrated and loaded on a 10% polyacrylamide SDS gel. After electrophoresis, the separated proteins on the gel were blotted onto a PVDF membrane (polyvinylidene difluoride, Applied Biosystems). A tight, pink band was used directly for N-terminal sequence analysis on an Applied Biosystems sequencer.

### 2.2. Genomic library screening and nucleotide sequencing

An oligonucleotide probe was designed based on residues 11 through 16 from the N-terminal amino acid sequence and based on a codon usage dictionary of *Synechocystis* PCC 6803 and was used to screen a genomic library of *Synechocystis* PCC 6803 DNA in the Bluescript SK(+) vector [13]. The oligonucleotide CCC (CT)C(GT) GA(TC) GAA GC was synthesized and phosphorylated with $^{32}P$ using the $T_4$ polynucleotide kinase. Plasmid DNA was isolated from positive colonies by the alkaline lysis method and screened further by dot blots and Southern blots [14]. The gene for the low potential cytochrome was found in a 1.5 kb DNA fragment. Primer-directed sequencing was used to determine the entire nucleotide sequence. Nucleotide sequences were aligned and analysed using the GCG Sequence Analysis Software package.

### 2.3. Assay of low potential cytochrome c reduction

Assays were carried out in anaerobic cuvettes which had been flushed with $O_2$-free argon and evacuated three times prior to the reaction. The side arm of the cuvette contained 0.3 μmol NADPH and 150 μmol glucose. The main compartment contained 300 μmol Tris buffer, pH 7.8, 0.1 ml of 7.5% ethanol, 0.5 μmol cytochrome *c*, 0.3 mg catalase, 1.5 mg glucose oxidase, 0.1 unit ferredoxin NADP oxidoreductase and ferredoxin as indicated. The NADPH, glucose oxidase, catalase and spinach ferredoxin NADP oxidoreductase were purchased from Sigma Chemical Co. Low potential cytochrome *c* was purified from *Microcystis aeruginosa* [11] and ferredoxins I and II were purified from *Aphanizomenon-flos aqua* [15]. Reaction rates were calculated from the absorbance change at 420 nm during the first 30 s of the reaction using an extinction of 133.5 A/mmol. An SLM DW2 spectrophotometer was used.


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## 3. Results

### 3.1. Nucleotide sequence for the gene for low potential cytochrome c

A low potential cytochrome was isolated from the wild type strain of *Synechocystis* sp. PCC 6803 and an N-ter-minal sequence of residues 1 through 24 was determined. A genomic library was screened using a $^{32}$P-end labeled oligonucleotide probe corresponding to sequence of the resi- dues 11 through 16. Positive clones were isolated, and south- ern analysis indicated that the *petK* gene residue in an 1.5 kb *Sau*3A fragment which was sequenced by the dideoxy termination method using successive primers. The nucleotide sequence of the region within the 1.5 kb fragment revealed an open reading frame comprising the *petK* gene.

Fig. 1 shows the nucleotide sequence of *petK* and the deduced amino acid sequence of the low potential cytochrome *c*. The deduced amino acid sequence of residues 1 through 24 in the gene is identical to the N-terminal sequence determined on the protein. This protein is 135 amino acid residues in length with a mass of 15,662 and an isoelectric point of 4.47.

### 3.2. Similarity of the gene for low potential cytochrome c to the gene for cytochrome $c_6$

Earlier amino acid sequence work [11] suggested a resemblance between two regions of the low potential cytochrome *c* – one near the heme binding site and the other near the carboxy terminus – to two regions of the high potential cytochrome $c_6$ which functions in photosynthetic electron transfer in cyanobacteria and algae. Zhang, Pakrasi and Whitmarsh [17] have determined the nucleotide sequence for the gene *petJ* for cytochrome $c_6$ in *Synechocystis* PCC 6803. Fig. 2 shows an alignment of the two similar regions of these two genes. The sequence of the two genes are 47% identical in the heme binding regions and are 46% identical in the carboxy terminus regions.

```
        10                  30                  50
         .         .         .         .         .         .
caccatggtcgcagtgccaatgcggtggagttaaccgaaagcacccgcaccattcccctc
                       V  E  L  T  E  S  T  R  T  I  P  L

        70                  90                  110
         .         .         .         .         .         .
gatgaagccggtggcaccacaaccttaaccgcccgccagttcaccaacggccaaaagatt
D  E  A  G  G  T  T  T  L  T  A  R  Q  F  T  N  G  Q  K  I

       130                 150                 170
         .         .         .         .         .         .
tttgtggacacctgcacccaatgtcacctccagggtaaaaccaaaactaataataacgtt
F  V  D  T  C  T  Q  C  H  L  Q  G  K  T  K  T  N  N  N  V

       190                 210                 230
         .         .         .         .         .         .
agtttaggcttggcagacttggctggtgccgagccccctcggacaaccgtctggccttg
S  L  G  L  A  D  L  A  G  A  E  P  P  S  D  N  R  L  A  L

       250                 270                 290
         .         .         .         .         .         .
gtggaatttctcaagaatcccaagagttatgacggtgaagatgactattcggaattacat
V  E  F  L  K  N  P  K  S  Y  D  G  E  D  D  Y  S  E  L  H

       310                 330                 350
         .         .         .         .         .         .
cccaatatttcccgtcccgacatctacccagaaatgagaaactacaccgaagacgacatc
P  N  I  S  R  P  D  I  Y  P  E  M  R  N  Y  T  E  D  D  I

       370                 390                 410
         .         .         .         .         .         .
tttgatgtggctggttacaccctcattgctcccaaattagatgaacggtggggtggcacc
F  D  V  A  G  Y  T  L  I  A  P  K  L  D  E  R  W  G  G  T

       430                 450
         .         .         .         .
atctacttctagaaaaatagagaagtaaaccatcgaactccgtaa
I  Y  F
```

Fig. 1. The nucleotide sequence of the gene encoding the low potential cytochrome *c* of *Synechocystis* PCC 6803.

## Sequence Similarities Between Parts of Two Synechocystis Cytochromes and Their Genes

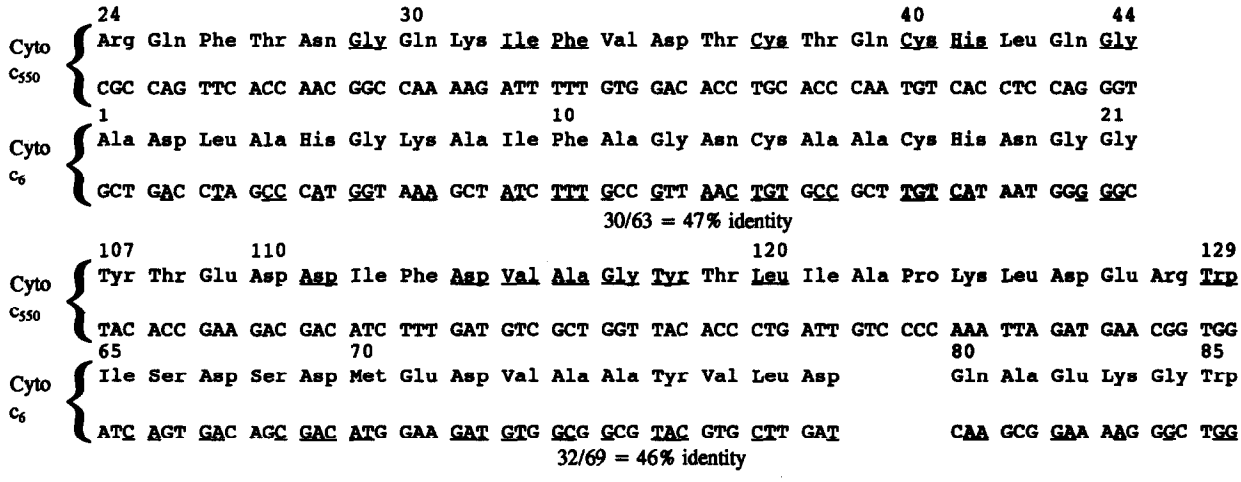


Fig. 2. Alignment of regions in the gene for low potential cytochrome *c* with similar regions in the gene for cytochrome $c_6$.

### 3.3. *Comparison of amino acid sequences of low potential cytochrome* cs

Fig. 3 contains the available amino acid sequences for several low potential cytochrome *cs* of cyanobacteria. The sequence of the cytochrome from *A. flos-aqua* is incomplete and lacks a crucial residue at position 92. The *Synechocystis* and *Microcystis* sequences show a histidine at position 92. Hoganson et al. [9] have published an EPR spectrum of a low potential cytochrome *c* from *A. nidulans* and we have obtained a similar spectrum from the pure cytochrome of *M. aeruginosa*. These two spectra show the characteristics of a cytochrome or heme with two histidines as the fifth and sixth ligands to iron. The conserved histidine residues in positions 41 and 92 in the two complete sequences are the fifth and sixth ligands to the iron. Fig. 3 shows the two regions of these cytochromes that are similar to regions of cytochrome $c_6$. Between these two regions of sequence similarity there is a sequence that is highly conserved. Of the 137 positions describing the total sequence, 49 show conserved residues giving an overall conservation of 36%. In the central conserved region, 27 of the 56 residues are conserved giving 48% conservation here. Generally, conserved basic residues are not found close to the heme on

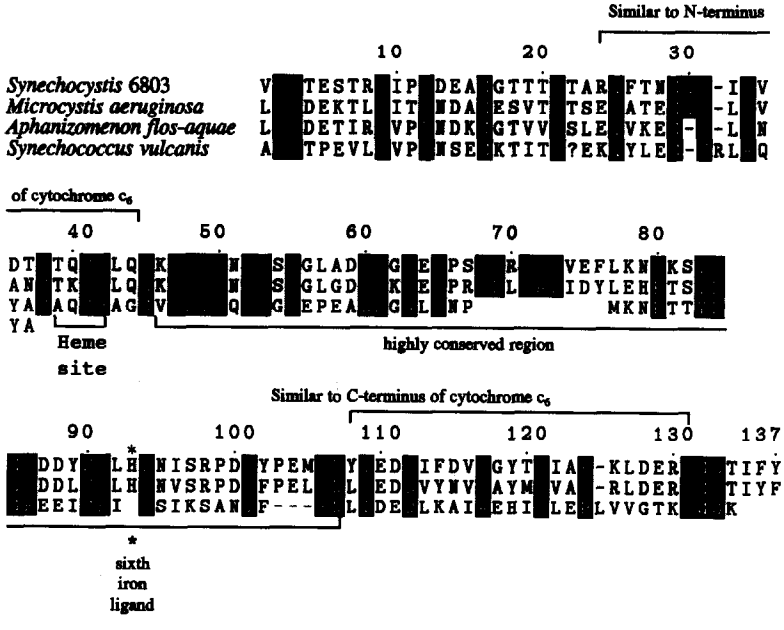


Fig. 3. Alignment of amino acid sequences of low potential cytochrome *c* from several genera of cyanobacteria.

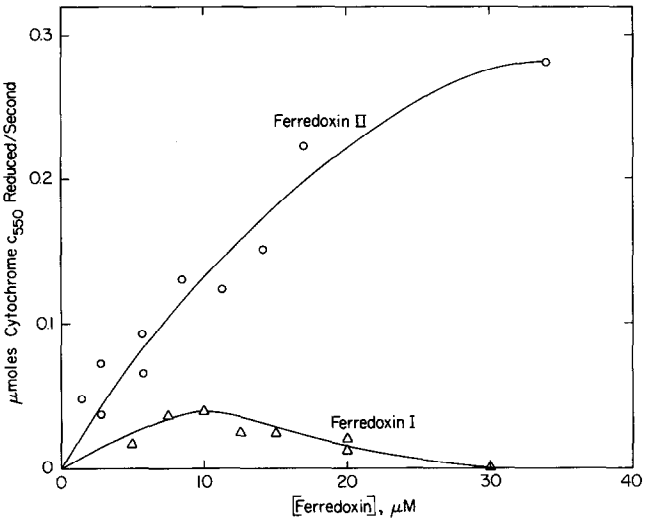


Fig. 4. Measurements of the effects of ferredoxins on the rate of reduction of low potential cytochrome *c*.

the N-terminal side of *c* type cytochromes. Thus the conserved lysine in position 31 of all of the low potential cytochrome *c* sequences is of special interest.

### 3.4. *Enzymatic reduction of low potential cytochrome* c

We have constructed a model system for the enzymatic reduction of the low potential cytochrome *c*. Since this cytochrome is autooxidized very rapidly, these experiments were performed under anaerobic conditions. While the pyruvate ferredoxin oxidoreductase is the likely source of electrons for reduction of ferredoxin by in vivo fermentation, we used NADPH and ferredoxin NADP oxidoreductase to reduce ferredoxin in these experiments. Reduced ferredoxin NADP oxidoreductase does not reduce low potential cytochrome *c*. As seen in Fig. 4, if electrons are transferred from NADPH through the ferredoxin NADP oxidoreductase to ferredoxin II, there is rapid reduction of the cytochrome. It is important to note that ferredoxin I from several sources did not reduce this cytochrome.

The unique lysine at position 31 and the lysine at position 48 of the low potential cytochrome may aid in aligning the acidic ferredoxin with the heme site on the generally acidic cytochrome.

## 4. Discussion

The low potential cytochrome *c* of cyanobacteria and algae resembles in its redox properties only one other *c* type cytochrome – the low potential cytochrome $c_3$ of *Desulfovibrios*. Cytochrome $c_3$ mediates the transfer of electrons from reduced ferredoxin generated by the phosphoroclastic oxidation of pyruvate to the enzyme hydrogenase [17]. This enables the anaerobic, heterotrophic *Desulfovibrios* to sustain themselves by a hydrogen producing fermentation. There is no sequence similarity between the cyanobacterial cytochromes and cytochrome $c_3$.

The gene sequence presented here does show striking similarity to parts of the sequence of the gene for the high potential cytochrome $c_6$ from the same organism. It seems that one of these genes was duplicated and that parts of the duplicate copy were used in the assembly of the second cytochrome gene. One might guess that a gene used in anaerobic fermentation was the precursor of the gene used in oxygenic photosynthesis. Krogmann [18] has reviewed the tentative evidence that this low potential cytochrome participates in hydrogen production and Asada et al. [19] have added new observations that the low potential cytochrome can transfer electrons from hydrosulfite to hydrogenase.

Shen et al. [10] have established that a bound form of the low potential cytochrome has a structural role in photosystem II of cyanobacteria. They have found this cytochrome is present in photosystem II particles in amounts stoichiometric with other constituents of photosystem II [20]. The bound cytochrome is released by high concentrations of salt or by detergents. In contrast, Holton and Myers [1,5], Ho et al. [21], and Cohn et al. [11] have found the soluble form of the cytochrome was released in aqueous extracts of cells and was often present in 2-to-3-fold higher concentration than cytochrome $c_6$. The bound form of the cytochrome may be a constitutively expressed component of the photosynthetic apparatus and the soluble cytochrome may, in some species, be induced by anaerobic conditions just as hydrogenase is induced. The soluble cytochrome may function in dark, anaerobic fermentation [22] and the membrane-bound cytochrome may function in light driven uptake of hydrogen [23,24].

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