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The Cyanobacterial Thioredoxin Gene Is Required for Both Photoautotrophic and Heterotrophic Growth[1]

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The gene encoding thioredoxin in the facultative heterotrophic cyanobacterium *Synechocystis* sp. PCC 6803 (*trxA*) has been cloned by heterologous hybridization using the corresponding gene *trxM* from the cyanobacterium *Anacystis nidulans* as a probe. The deduced amino acid sequence of *trxA* predicts a protein of relative molecular weight of 11,750 and has strong identity with its cyanobacterial counterparts and other *m*-type thioredoxins of photosynthetic eukaryotes. The *trxA* gene has been expressed in *Escherichia coli* as a functional protein of 12 kD and has been shown by western blot analysis to be the same size as in *Synechocystis*. The *trxA* gene is transcribed in *Synechocystis* as a single transcript of 450 nucleotides and accumulates to the same level under photosynthetic and heterotrophic growth conditions. The *trxA* gene was inactivated with a kanamycin-resistant cassette, and total segregation of the disrupted *trxA* gene was obtained only when the *trxM* gene from *A. nidulans* (E.D.G. Muller, B.B. Buchanan [1989] J Biol Chem 264: 4008–4014) was simultaneously expressed in *Synechocystis*. Our results suggest an essential role of thioredoxin not only when cells grow photosynthetically but also under heterotrophic conditions.

Thioredoxins are small ($M_r \approx 12$ kD), heat-stable proteins that are involved in disulfide/dithiol reactions and they serve as redox carriers in a wide range of enzymatic reactions in prokaryotic and eukaryotic cells (Holmgren, 1985; Buchanan, 1992). All thioredoxins have a cystine disulfide as their active site, and most have the amino acid sequence Trp-Cys-Gly-Pro-Cys conserved.

Thioredoxins are involved in light-dependent regulation of enzymes in all oxygen-producing photosynthetic organisms, from cyanobacteria to higher plants (Buchanan, 1980). Thioredoxin, reduced by Fd-thioredoxin reductase, in turn recognizes and reduces specific disulfide bonds of several enzymes, such as NADP-MDH or Fru-1,6-biphosphatase, triggering a change in their catalytic activity (Buchanan, 1980, 1992).

In higher plants, thioredoxins are classified as one of three different types, two of which are located in the chloroplasts (*m* and *f* types) and one located in the cytosol (*h* type) (Florencio et al., 1988; Buchanan, 1992). In cyanobacteria, however, only the *m*-type thioredoxin has been found, with the exception of a second, unusual thioredoxin in the cyanobacterium *Anabaena* 7120 (Alam et al., 1989; Gleason, 1992, 1995). The *m*-type thioredoxin seems to be essential for photosynthetic growth in cyanobacteria, since the inactivation of the corresponding gene in the photoautotrophic cyanobacterium *Anacystis nidulans* failed under all conditions tested (Muller and Buchanan, 1989).

Thioredoxin genes from both eukaryotes and prokaryotes have been cloned and sequenced (Höög et al., 1984; Kamo et al., 1989; Muller, 1991; Jacquot et al., 1992; Wedel et al., 1992; Rivera-Madrid et al., 1993; Reynolds et al., 1994), including the cyanobacterial genes from *A. nidulans* and *Anabaena* (Lim et al., 1986; Alam et al., 1989; Muller and Buchanan, 1989). In all of these thioredoxin sequences, the cystine disulfide active site is conserved.

Although it is known that most of the *m*-type thioredoxin is oxidized in the chloroplast after darkening and being rapidly reduced in the light (Crawford et al., 1989), little information exists about the expression of the thioredoxin *m*-type gene with respect to photosynthetic growth conditions. Recently, it was proposed that thioredoxin acts as a redox sensor in the chloroplast of the green alga *Chlamydomonas reinhardtii*. In this case, thioredoxin reduced in the light by Fd-thioredoxin reductase in turn reduced a disulfide regulatory site of a component of the translational activator complex that binds to the 5′ untranslated region of the *psbA* mRNA, which encodes for the D1 protein of PSII and increases its translation (Danon and Mayfield, 1994).

To study thioredoxin function we have chosen the unicellular heterotrophic cyanobacterium *Synechocystis* sp. PCC 6803, which can grow in the dark with Glc and a daily pulse of 5 min of light (heterotrophic growth), in the light with DCMU and Glc (photoheterotrophic growth), in the light with Glc (mixotrophic growth), or in the light (photoautotrophic growth) (Anderson and McIntosh, 1991). In spite of the fact that *Synechocystis* contains multiple copies of its chromosome, as do many other cyanobacteria (Rippka et al., 1979), it is a good organism for genetic analysis and has been used extensively to obtain mutants of the photosynthetic apparatus (Williams, 1988).

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Abbreviations: MDH, malate dehydrogenase; pBS, pBluescript II SK(+).

In this work we have analyzed the thioredoxin gene *trxA* of *Synechocystis* 6803 and shown that it is constitutively expressed under all of the growth conditions tested. Furthermore, we have been able to inactivate, to our knowledge for the first time, a photosynthetic thioredoxin gene (*trxA*) by introducing and expressing in *Synechocystis* the corresponding thioredoxin gene (*trxM*) from *A. nidulans* (Muller and Buchanan, 1989). Our results suggest that thioredoxin is essential for both photosynthetic and heterotrophic growth in *Synechocystis*.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

The *Escherichia coli* strain DH5α (BRL) was used for all plasmid constructions, and strains HB101 (Boyer and Roulland-Dussoix, 1969) and MC1061 (Sambrook et al., 1989) were used for gene library construction. All strains were grown in Luria broth (Sambrook et al., 1989) containing 100 μg/mL ampicillin. The HB101 derivative strain CM404, which harbors the conjugative plasmid pRK2013, was grown in Luria broth at 30°C (Mermet-Bouvier et al., 1993) and used for the mobilization of the RSF1010 derivative pFNT11 to the cyanobacterium *Synechocystis* sp. PCC 6803.

Plasmid derivatives of pBS used were pFNT1, which contains a 1.3-kb *Acc*I *Synechocystis* DNA fragment that includes part of the *trxA* gene, and pFNT4 and pFNT8, which contain a 4-kb *Sma*I and a 1.8-kb *Hin*cII chromosomal DNA fragment, respectively. Both fragments include the whole *trxA* gene. Plasmid pFNT11 was constructed by purifying by electrophoresis the 0.85-kb *Hin*dIII-*Eco*RI fragment of pLI720 that contains the *trxM* gene from *Anacystis nidulans* and the *tac* promoter (Muller and Buchanan, 1989). The ends were filled in with the Klenow fragment of DNA polymerase I, and the fragment was inserted into a *Sma*I site of the pFC1 plasmid (Mermet-Bouvier and Chauvat, 1994).

Growth Conditions

Synechocystis sp. PCC 6803, SFNT1+, SFNT1−, and SFNT3 (*trxA* mutants) were cultured at 30°C in BG11, a standard cyanobacterial growth medium containing nitrate as a nitrogen source (Rippka et al., 1979) and bubbled with a continuous stream of 1.5% (v/v) CO_2 in air. For the *trxA* mutants SFNT1+ and SFNT1−, the medium was supplemented with kanamycin to a final concentration of 500 μg/mL. For SFNT3 the medium was supplemented with kanamycin and chloramphenicol at 500 and 40 μg/mL final concentration, respectively. Constant illumination was provided with a total photon flux density of 50 μE m^{-2} s^{-1}. For heterotrophic growth tubes wrapped in three layers of aluminum foil and containing the same medium described above, supplemented with 10 mM Glc were innoculated with cells at an initial concentration of A_{665} = 0.02 to 0.03. The culture was illuminated for 5 min every 24 h with white light (50 μE m^{-2} s^{-1}). The doubling time of the culture in these conditions was about 34 h. Where indicated in other growth conditions, Glc was added to a final concentration of 10 mM. DCMU was used at 5 μM final concentration. When sodium thiophosphate was used, it was added to the cultures to a final concentration of 300 μM. For plate cultures, BG11 liquid medium was supplemented with 1% (w/v) agar, and kanamycin and chloramphenicol were used at 500 and 40 μg/mL, respectively, when required.

Cloning Techniques

All DNA manipulations and *E. coli* transformations were performed following standard procedures (Sambrook et al., 1989). DNA fragments were purified from the agarose gels using the GeneClean Kit (Bio 101, Inc., La Jolla, CA). Total *Synechocystis* DNA was isolated as described by Cai and Wolk (1990) by supplementing the culture with penicillin G at 100 μg/mL 20 h before the extraction. For Southern analysis DNA was digested and fragments were electrophoresed in 0.7% agarose gels using the Tris-borate-EDTA buffer system (Sambrook et al., 1989). DNA was transferred to Z-probe membranes (Bio-Rad) using a vacuum, and Southern blot hybridizations were performed as described by Ausubel et al. (1992). Colony hybridization was carried out following the method described by Sambrook et al. (1989). Homologous and heterologous hybridizations were performed at 65 and 55°C, respectively, in 5× SSC (0.15 M NaCl in 1.5 mM sodium citrate), 5× Denhardt's solution (0.02% [w/v] Ficoll, PVP, and BSA in water), 1% SDS, 25 mM potassium phosphate buffer, pH 7.4, and 50 μg/mL salmon sperm DNA (Sambrook et al., 1989). DNA probes were ^{32}P labeled with a nick translation kit (Boehringer Mannhein) or a random-primer kit (Pharmacia) using [α-^{32}P]dCTP. Two chromosomal libraries were constructed by ligating size-fractionated DNA fragments (1.3-kb *Acc*I and 4-kb *Sma*I) from *Synechocystis* sp. PCC 6803 into pBS. These were used to transform *E. coli* HB101 and MC1061, respectively, and screened by colony hybridization.

DNA Sequence Determination and Analysis

Different regions of the cloned 1.3-kb *Acc*I and 4-kb *Sma*I DNA fragments were subcloned in pBS and sequenced by the dideoxy chain-termination method (Sanger et al., 1977) using Sequenase 2.0 (United States Biochemical). Computer sequence analysis was carried out using the Genetics Computer Group software package (Devereux et al., 1984). Amino acid sequences were compared using the FASTA program (Pearson and Lipman, 1988), and alignments were produced, using default parameters, by the PileUp program of the Genetics Computer Group and optimized manually.

Insertional Mutagenesis of the trxA Gene of *Synechocystis* and Mutant Selection

To mutate the *trxA* gene, a 1.3-kb *Xmn*I-*Kpn*I fragment from pFNT4 was cloned in an *Eco*RV-*Kpn*I pBS plasmid, and both of them end-filled. Then, a 1.3-kb fragment containing a kanamycin-resistant gene from Tn*5* (Elhai and Wolk, 1988) was cloned into an *Rsa*I site of the *trxA* gene in both orientations, generating pFNT3+ and pFNT3−. Plas-

mids containing the mutated region of the *trx*A gene were used to transform *Synechocystis* wild type by the method described by Chauvat et al. (1986). After 10 to 12 d, kanamycin-resistant transformants appeared on plates grown photoautotrophically, mixotrophically, heterotrophically, and photoheterotrophically. The transformants were grown for several segregation rounds, each for 10 to 12 d in the same culture medium with increasing amounts of kanamycin. Thereafter, total DNA from eight clones selected from each growth condition (four resulting from the transformation with pFNT3+ and four with pFNT3−) was obtained and analyzed by the Southern blot method using the probe of 1.2-kb *Kpn*I-*Acc*I (Fig. 1A).

Conjugation of SFNT1+ (*trxA* Mutant)

The complete segregation of the *trxA* gene was obtained by conjugation of *Synechocystis* strain SFNT1+ with the *E. coli* strain CM404, which harbors the pFNT11 plasmid, using the method described by Mermet-Bouvier et al. (1993). A culture of the CM404 strain grown to a concentration of 5×10^7 cells mL^{-1} to 6×10^7 cells mL^{-1} (corresponding to the early stationary stage of growth) was mixed with the same amount of exponentially growing SFNT1+ cells. The mixed cells were spread over a filter on BG11 medium supplemented with 5% Luria broth and incubated in the light (50 $\mu E\ m^{-2}\ s^{-1}$) at 30°C for 24 h. The filter was transferred to a plate containing only BG11 medium and incubated for 24 h, and exconjugants were then selected by adding kanamycin (50 $\mu g/mL$) and chloramphenicol (20 $\mu g/mL$) under agar. Selection was carried out by growing the exconjugants in BG11 medium containing kanamycin (50 $\mu g/mL$) and chloramphenicol (20 $\mu g/mL$) at 30°C in the light (50 $\mu E\ m^{-2}\ s^{-1}$). After 10 to 12 d, total DNA was isolated from five clones and analyzed by the Southern blot method using the probe of 1.2-kb *Kpn*I-*Acc*I shown in Figure 1A.

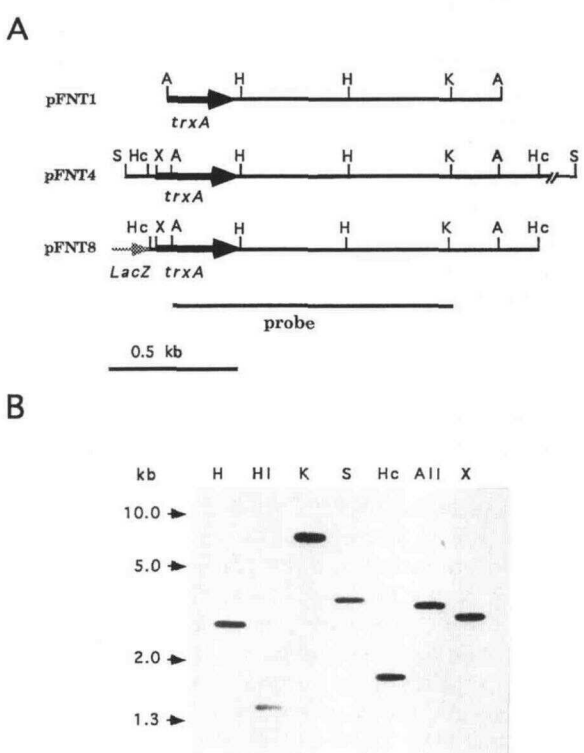

Figure 1. Identification of the thioredoxin gene *trxA* in the cyanobacterium *Synechocystis* 6803. A, Restriction map of the plasmid contructions used, containing the whole (pFNT4 and pFNT8) or part (pFNT1) of the *trxA* gene. B, Hybridization of total *Synechocystis* genomic DNA using the probe indicated in A. A, *Acc*I; H, *Hae*II; K, *Kpn*I; S, *Sma*I; Hc, *Hin*cII; HI, *Hin*fI; AII, *Ava*II; X, *Xmn*I.

RNA Isolation and Northern Blot Analysis

Total RNA was extracted from 35 mL of *Synechocystis* 6803 cultures grown to the mid-exponential phase (A_{665} = 0.5–0.6) under different growth conditions. In the case of heterotrophic growth, RNA was isolated after 7 d, when the culture reached the same A_{665} as above. The cells were harvested by centrifugation at 6,000*g* for 4 min, placed in 1 mL of resuspension buffer (0.3 M Suc and 10 mM sodium acetate, pH 4.5), transferred to an Eppendorf tube, pelleted at 12,000*g* for 30 s, and then frozen in liquid nitrogen. Extraction of RNA, separation on formaldehyde gels, transfer to nylon membranes (Hybond N, Amersham), prehybridization, and hybridization were carried out using the methods described by Reyes and Florencio (1994).

The DNA used as a probe was radioactively labeled with a random-primer kit (Pharmacia) using [α-^{32}P]dCTP. As a control for sample loading, all of the filters were stripped and reprobed with a 450-bp *Hin*dIII-*Bam*HI fragment from pAV1100 that contains the constitutively expressed RNase P RNA gene from *Synechocystis* 6803, which has a very stable transcript (Vioque, 1992; Reyes and Florencio, 1995). Relative transcript levels were quantified with Instantimager Electronic Autoradiography (Packard Instruments, Downers Grove, IL).

Enzyme Assays

All of the enzyme assays were performed using cell-free extracts of *E. coli* DH5α transformed with the plasmids pFNT8 or pBS. The extracts were prepared in 50 mM Tris-HCl buffer, pH 7.9, containing 0.1 mM EDTA and 1 mM PMSF.

The activation of corn NADP-MDH by thioredoxins was carried out as previously described (Crawford et al., 1989). The NADP-MDH was preincubated for 5 min with *E. coli* extracts in 0.2 mL (final volume) of a solution containing 20 μmol of Tris-HCl buffer, pH 7.9, and 2 μmol of DTT. An aliquot of the preincubation mixture (0.1 mL) was added to a 1-mL cuvette containing 100 μmol of Tris-HCl buffer, pH 7.9, and 0.25 μmol of NADPH in 0.85 mL. The reaction was started by the addition of 50 μL of 50 mM oxalacetic acid; NADP-MDH activity was followed by measuring the oxidation of NADPH at 340 nm. One unit of NADP-MDH activity corresponds to the formation of 1 μmol of $NADP^+$ per min.

Protein concentrations were determined by the method of Bradford (1976) using ovalbumin as the standard.

PAGE and Western Blot Procedures

Proteins from cell-free extracts were separated by SDS-PAGE according to the method described by Laemmli (1970) using 15% acrylamide slab gels. Western blot analysis was carried out as described by Navarro et al. (1995) using corn type-*m* thioredoxin antibodies.

RESULTS

Cloning of the *trxA* Gene

The thioredoxin gene *trxA* from *Synechocystis* sp. PCC 6803 was cloned by digestion of total genomic DNA with different restriction enzymes and subsequent heterologous hybridization with a 0.8-kb *Bam*HI-*Hin*dIII fragment from the plasmid pLI720, which contains the thioredoxin gene *trxM* from *A. nidulans* (Muller and Buchanan, 1989), as a probe. A 1.3-kb *Acc*I fragment hybridized with the probe. Total *Synechocystis* sp. PCC 6803 DNA was then digested with *Acc*I and fragments of approximately 1.3 kb were purified and ligated to pBS plasmids to construct a *Synechocystis* gene library, which was screened in *E. coli* HB101. Out of the 200 clones tested by colony hybridization with the probe mentioned above, 2 were positive. Plasmids from 1 of the positive clones (pFNT1) were isolated and the 1.3-kb *Acc*I insert was partially sequenced, confirming the existence of part of the thioredoxin gene *trxA* of *Synechocystis* 6803, which contains an *Acc*I internal restriction site. Based on the restriction map of pFNT1, a new genomic DNA hybridization was performed, using as a probe the 1.2-kb *Kpn*I-*Acc*I fragment indicated in Figure 1A.

Southern blot analysis showed that a single copy of the *trxA* gene exists in *Synechocystis* 6803 (Fig. 1B). The 4-kb *Sma*I fragment, which hybridized with the probe, was selected to construct a new chromosomal library and screened by colony hybridization in *E. coli* MC1061 using the same probe (Fig. 1A). Plasmid pFNT4 (Fig. 1A) obtained from the positive clone that appeared in the colony hybridization was used to sequence the *trxA* gene and its flanking regions, revealing an open reading frame of 321 bp (Fig. 2). The sequence AAGGA, which is similar to the Shine-Dalgarno sequence and could constitute a ribosome-binding site, was found 9 nucleotides upstream of the first codon of the *trxA* gene. A sequence (TCTAAT) that matches 5 out of 6 nucleotides of the *E. coli* −10 consensus sequence (TATAAT), lies 34 nucleotides upstream of the translation start of the *trxA* gene. A sequence (TCAACA) similar to the *E. coli* −35 consensus sequence (TTGACA) lies 17 nucleotides upstream of the −10 sequence indicated above (Hawley and McClure, 1983), and is a putative promoter region similar to those found in other *Synechocystis* 6803 genes (Mohamed et al., 1993). In the 3′ downstream region of the *trxA* gene, inverted repeats (nucleotides 601–618) that could form a stem-loop structure and act as a transcriptional terminator were also found.

The open reading frame codes for a polypeptide of 107 amino acid residues (M_r 11,750), which contains the active-site amino acid sequence Trp-Cys-Gly-Pro-Cys conserved in thioredoxin proteins. A search of the SWISS-PROT and EMBL/GenBank databases revealed strong sequence identities of the *Synechocystis* protein with other previously sequenced cyanobacterial thioredoxins (Fig. 3), such as *Anabaena* 7119 (83%) (Lim et al., 1986) and *A. nidulans* (78%) (Muller and Buchanan, 1989), as well as with thioredoxins from other photosynthetic sources, such as red and green algae (63–70%) (Jacquot et al., 1992; Reynolds et al., 1994) and higher plant *m*-type thioredoxins (about 52%) (Wedel et al., 1992). Homology was also found with bacterial thioredoxins such as *E. coli* (50%) (Höög et al., 1984) and with *f*- and *h*-type thioredoxins (33–39%) (Kamo et al., 1989; Rivera-Madrid et al., 1993). Taken together these data indicate unambiguously that the cloned gene corresponds to the *Synechocystis* sp. PCC 6803 thioredoxin gene.

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GGTAGGATGATGTTCGGGTGGTTTCTTCTTCTACTGCTGGGAGGGGGA  48

AAAGTGTGAGGTGAACTGTGAGAAAAGATGAATAAATTCTAAGTTTCA  96

CGATATTTTTCCATACAGGGGTCAACAATTGGTTATGGTAGTATTCTA 144

ATCAGCCGATCACGAGGTTTAGAAGGATTTCCAGTATGAGTGCTACCC 192
                                    M  S  A  T  P
CTCAAGTTTCCGACGCAAGTTTTAAAGAGGATGTATTAGACAGCGAGT 240
  Q  V  S  D  A  S  F  K  E  D  V  L  D  S  E  L
TACCCGTGCTCGTAGACTTTTGGGCTCCCTGGTGTGGCCCTTGTCGCA 288
  P  V  L  V  D  F  W  A  P  W  C  G  P  C  R  M
TGGTAGCCCCCGTCGTTGATGAAATCTCACAACAGTACGAAGGTAAGG 336
  V  A  P  V  V  D  E  I  S  Q  Q  Y  E  G  K  V
TCAAAGTCGTTAAGTTAAACACCGACGAGAACCCCAATACCGCTAGTC 384
  K  V  V  K  L  N  T  D  E  N  P  N  T  A  S  Q
AGTATGGCATCCGCAGTATTCCCACCCTAATGATTTTCAAGGGGGGCC 432
  Y  G  I  R  S  I  P  T  L  M  I  F  K  G  G  Q
AGAGAGTGGATATGGTGGTGGGAGCCGTGCCCAAAACCACCCTGGCCA 480
  R  V  D  M  V  V  G  A  V  P  K  T  T  L  A  S
GCACCCTAGAAAAATATCTTTAAGCGCTGGCTTCCCTGCTTTGTGACG 528
  T  L  E  K  Y  L

GTTTCCCTCCGGATCGTGACCCTTCCTAGGACACGGTCTTTTTTTTGC 576

CTTTTTATCCCCCCTGTTGGCTATGCCCAAATTATTTTGGGCTAAATA 624

TCGGAGTTTCGTTTTTTCGTTCTAATGGGCTGATCCACCCCCCAACTT 672

TTAGAGTTCACCAGCATGGCTAATTCGCCAATCACTTCCAGGGCGTTT 720

ATATTTCCCAGCCGGACGAATTTCCCCGA                     749
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Figure 2. Nucleotide and deduced amino acid sequence of the gene coding for thioredoxin (*trxA*) in *Synechocystis* 6803. Nucleotides are numbered consecutively. Putative promoter sequence regions are in boldface. Putative ribosome binding sites and potential stem-loop structures in the 3′ downstream region are underlined.

Expression of the Cloned *trxA* Gene in *E. coli*

To express the *trxA* gene of *Synechocystis* 6803 in *E. coli*, a 1.8-kb *Hin*cII internal fragment of the insert contained in pFNT4 was purified and used to construct a new pBS derivative plasmid, pFNT8, which placed the *trxA* gene under the control of the promoter of *LacZ* (Fig. 1A). Cultures of the strains DH5α (pFNT8) and DH5α (pBS), corresponding to the DH5α *E. coli* strain transformed with either pFNT8 or pBS, were grown in Luria broth. Total soluble protein of cell-free extracts was separated by SDS-PAGE and transferred to nitrocellulose filters for western blot analysis. As shown in Figure 4, antibodies against corn *m*-type thioredoxin cross-reacted with the thioredoxin expressed in *E. coli*, as well as with the native thioredoxin from *Synechocystis* sp. 6803 (Fig. 4, lanes 1 and 2), but no reaction was observed with proteins of the same size in the crude extract of the *E. coli* strain transformed with pBS plasmid (Fig. 4, lane 3). To confirm that *trxA* was expressed in *E. coli*, we assayed the activation of corn NADP-MDH on

Figure 3. Comparison of the thioredoxin sequences from different sources. Identical amino acids in the majority of the sequences are shaded and those identical in all sequences are marked by asterisks (*). Thioredoxin amino acid sequences were obtained from the EMBL/GenBank. Accession numbers: *Griffithsia pacifica*, X76611; *Porphyra yezoensis*, X76612; *Anabaena* 7119, M14736; *Anacystis nidulans*, J04475; *Synechocystis* 6803, X80486; *Chlamydomonas reinhardtii*, X78821; *Cyanidium caldarium*, Z21723; *Spinacia oleracea m*, X51462; *Pisum sativum*, X76269; *Zea mays*, L40975; *Escherichia coli*, M54881; and *Spinacia oleracea f*, X14959). The transit peptide sequences of higher plant thioredoxins were removed for clarity.

reduction by thioredoxin (Crawford et al., 1989) using *E. coli* crude extracts of DH5α (pFNT8) and DH5α (pBS). The results of these assays showed that the crude extract from the strain DH5α (pFNT8) gave a 30-fold greater activation of corn NADP-MDH (1.2 ± 0.02 units mg^{-1} protein) than the extract from strain DH5α (pBS) (0.04 ± 0.015 units mg^{-1} protein). Taking into account that the DH5α strain has its own thioredoxin, the increase of activity found in DH5α (pFNT8) indicates that the *trxA Synechocystis* gene is expressed in *E. coli*.

trxA Transcript Accumulation in *Synechocystis* 6803 Wild-Type Cells Grown under Different Conditions

Total RNA isolated from *Synechocystis* cells grown photoautotrophically, photoheterotrophically, mixotrophically, or heterotrophically was subjected to northern blot analysis using as a probe the 0.4-kb *Hin*cII-*Hae*II fragment of pFNT4 containing the *trxA* gene. As shown in Figure 5A, a single transcript of 450 nucleotides appears. The accumulation of the *trxA* transcript was similar in all growth conditions, suggesting a constitutive expression of *trxA* gene in *Synechocystis*.

Insertional Mutagenesis of the *Synechocystis trxA* Gene

To generate *Synechocystis* mutant strains lacking thioredoxin, we constructed the plasmids pFNT3+ and pFNT3− (Fig. 6A), which contain the *trxA* gene interrupted with a kanamycin-resistance cassette in both orientations (see "Materials and Methods"). *Synechocystis* cells were transformed with each of the plasmids, and transformants appeared at a frequency of 10^{-8}, after several rounds of segregation (described in "Materials and Methods") for cultures grown photoautotrophically, photoheterotrophically, mixotrophically, and heterotrophically. Southern blot analysis of the transformants (see "Materials and Methods") showed that no more than 40 to 50% segregation, with respect to wild type, of inactivated *trxA* gene was obtained under all of the conditions tested. Thereafter, mutant strains SFNT1+ (pFNT3+) and SFNT1− (pFNT3−)

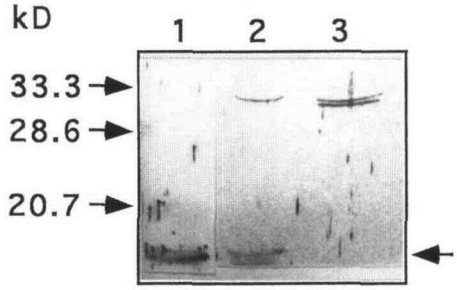

Figure 4. Expression of the thioredoxin gene (*trxA*) in *E. coli*. Western blot analysis of the result of SDS-PAGE of total soluble protein extracts from strains *Synechocystis* (lane 1); *E. coli* DH5α (pFNT8) (lane 2); and DH5α (pBS) (lane 3). Sixty micrograms of protein was loaded in each lane. The arrow on the right indicates thioredoxin protein.

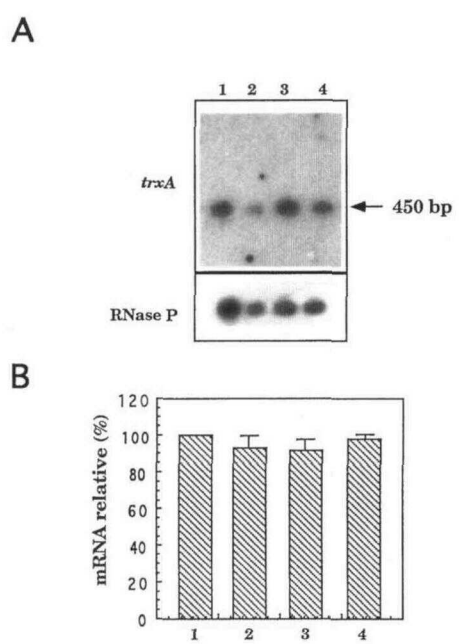

Figure 5. Analysis of the thioredoxin (*trxA*) transcript in *Synechocystis* 6803 cells. A, Northern blot analysis of 15 μg of total RNA isolated from each culture: photoautotrophic (lane 1), mixotrophic (lane 2), photoheterotrophic (lane 3), and heterotrophic (lane 4) growth were carried out. The hybridization with the RNase P RNA gene as a probe was used as a control of RNA loading. B, Relative levels of *trxA* transcript values are averages of two hybridizations and are expressed as percentages of the control.

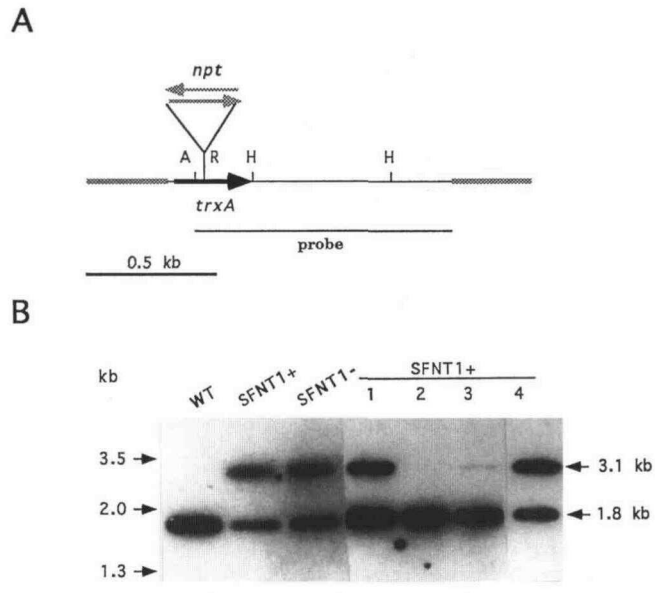

Figure 6. Southern blot analysis of SFNT1+ and SFNT1− genomic thioredoxin (*trxA*) gene disruption. A, Restriction map of the plasmids pFNT3+ and pFNT3− containing the *trxA* gene of *Synechocystis* with the insertion of a kanamycin-resistant gene (*npt*) in both orientations, as indicated by arrows. A, *Acc*I; R, *Rsa*I; H, *Hae*II. B, Southern blot analysis of total DNA digested with *Hin*cII, from wild-type, SFNT1+, and SFNT1− strains grown photoautotrophically, and from SFNT1+ cells grown in photoautotrophic (lane 1), photoheterotrophic with (lane 2) or without thiophosphate (lane 3), and heterotrophic (lane 4) conditions.

(Fig. 6B) were selected as representative transformants to continue subsequent analysis.

It has been reported previously that thioredoxin is essential for photosynthetic growth in the photoautotrophic cyanobacterium *A. nidulans* (Muller and Buchanan, 1989) and is also involved in sulfate reduction (Schmidt, 1988; Russell et al., 1990), because the *Synechococcus* 7942 mutants of sulfate transport were able to grow like the wild-type strain by supplementing the culture medium with sodium thiophosphate (Laudenbach and Grossman, 1991). Since *Synechocystis* 6803 is capable of growing without an operational PSII (photoheterotrophic and heterotrophic growth conditions), it was thought that inactivation of the *trxA* gene in all of the chromosome copies could be made possible by growing this cyanobacterium under the aforementioned conditions and in the presence of sodium thiophosphate in the culture medium. The higher segregation of interrupted *trxA* gene in SFNT1+, grown during 10 to 12 d in the different growth conditions, appeared in photoautotrophic and heterotrophic (Fig. 6B, lanes 1 and 4) rather than in photoheterotrophic growth conditions (Fig. 6B, lane 2), whereas the addition of sodium thiophosphate had no effect (Fig. 6B, lane 3). The fact that segregation of the mutated *trxA* gene was not possible under any of the conditions tested suggests that thioredoxin is essential not only for photosynthetic but also for heterotrophic growth.

Further analysis of the strain SFNT1+ also showed that its doubling time of growth was higher than in the wild-type strain under photosynthetic growth conditions (18 versus 9–10 h). All of these results point to an involvement of thioredoxin in other metabolic processes, in addition to its function in photosynthetic metabolism.

Transfer of the *trxM* Gene from *A. nidulans* to the *trxA* Mutant Strain SFNT1+

The existence of plasmids that can be transferred efficiently by conjugation from an *E. coli* donor to *Synechocystis* 6803 (Mermet-Bouvier et al., 1993; Mermet-Bouvier and Chauvat, 1994), allowing stable replication in this cyanobacterium, provides a way to introduce into the SFNT1+ strain an external gene coding for thioredoxin in order to segregate its own interrupted *trxA* gene. A derivative pFC1 plasmid (Mermet-Bouvier and Chauvat, 1994), pFNT11, containing the *trxM* gene from *A. nidulans* under the control of *tac* promoter (Fig. 7A), was transferred by conjugation to the SFNT1+ strain (see "Materials and Methods"). About 200 colonies per plate appeared, at a frequency of 10^{-5}, and the Southern blot analysis of the conjugants (see "Materials and Methods") using the 1.2-kb probe (Fig. 1A) showed complete segregation of the inactivated *trxA* gene in all of the clones analyzed (not shown). In Figure 7B, lanes T3, the SFNT3 strain is shown as the representative conjugant, which was completely segregated for the interrupted *trxA* gene, whereas SFNT1+ was only 40% segregated (lanes T1). The hybridization of the same filter with the 0.8-kb *Bam*HI-*Eco*RI fragment from pLI720 (which con-

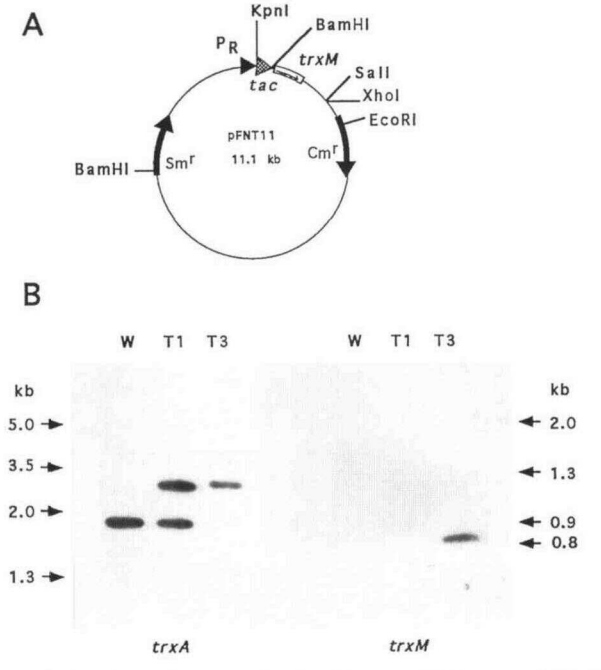

Figure 7. Southern blot analysis of wild-type, SFNT1+, and SFNT3 genomic DNA. A, Restriction map of the plasmid pFNT11 containing the *trxM* gene from *A. nidulans* under the control of the *tac* promoter. B, Southern blot analysis of total DNA from wild-type (W), SFNT1+ (T1), and SFNT3 (T3) strains hybridized with the *trxA* gene from *Synechocystis* (probe indicated in Fig. 1A) or with the *trxM* gene from *A. nidulans*.

tains the *trxM* gene from *A. nidulans*) as a probe (Fig. 7B), indicated that SFNT3 contained the thioredoxin gene from this cyanobacterium. Total RNA from SFNT3, SFNT1+, and wild-type *Synechocystis* 6803, analyzed by slot-blot analysis using the *Bam*HI-*Eco*RI fragment mentioned above, confirmed the expression of the *trxM* gene in the SFNT3 strain (data not shown). These results showing that complete segregation of the interrupted *trxA* gene can be achieved only in the presence of a functional thioredoxin gene clearly indicated that a functional thioredoxin gene is required for *Synechocystis* growth.

DISCUSSION

The gene encoding the *m*-type thioredoxin of *Synechocystis* 6803 has been cloned and sequenced. A comparative analysis of the predicted amino acid sequence (Fig. 3) indicates that the *Synechocystis* thioredoxin belongs to a conserved group of thioredoxins that includes those from cyanobacteria (identity higher than 75%), eukaryotic red and green algae (identity higher than 60%), and higher plants (identity around 53%). In this regard, the cyanobacterial thioredoxin gene could be considered to be of the same lineage as the corresponding *m*-type thioredoxin gene of photosynthetic eukaryotes. In the red algae *Porphyra yezoensis*, *Porphyra purpurea*, and *Griffithsia pacifica*, thioredoxin genes are plastid-encoded, the *trxA* gene of *P. yezoensis* is hybridized with both nuclear and plastid DNA of *G. pacifica* (Reith and Munholland, 1993; Reynolds et al., 1994), and the antibodies against corn *m*-type thioredoxin recognize *Synechocystis* thioredoxin, supporting the theory that a cyanobacteria-like endosymbiont gave origin to the photosynthetic plastids (Hartman et al., 1990; Reynolds et al., 1994).

Analysis of the thioredoxin amino acid sequences presented in Figure 3 shows possible amino acid signatures that are present only in *m*-type photosynthetic thioredoxin, such as the cluster KVVK (positions 54–57) and NTDE/D (positions 59–62). The active-site WCGPC is absolutely conserved in all of the thioredoxins analyzed so far. In addition, two regions relatively well conserved in all of these proteins, YGIRSIPT (positions 70–77) and GAVPK (positions 92–96), could also be important for the structure function of this protein, as could the sequence DFWAP, which precedes the active site and is found in most of the *m*-type thioredoxins (Fig. 3).

Although many thioredoxin genes have been cloned and sequenced, little is known about their expression in photosynthetic organisms. Northern blot analysis of the *trx*A gene shows a monocistronic transcript similar in size to the corresponding transcript of *A. nidulans* (Muller and Buchanan, 1989). Transcriptional analysis in different growth conditions indicated a pattern of accumulation similar to that of a constitutive gene, such as the RNase P RNA gene (Vioque, 1992; Reyes and Florencio, 1995). However, transfer of *Synechocystis* cells from light to dark reduces dramatically the amount of *trxA* transcript (F. Navarro and F.J. Florencio, unpublished results). In this regard, it has been shown that in *Chlamydomonas reinhardtii*, reduced thioredoxin is required to translate the transcript of the *psbA* gene, which encodes the D1 protein of PSII in a process that involves the reduction of a disulfide bridge of one of the proteins of the RNA-binding complex to facilitate binding to the leader sequence of the *psbA* mRNA (Danon and Mayfield, 1994). Thioredoxin may have a key role in the transducing signal between chloroplast redox state and its functionality in this system. Thus, in the light, reducing power generated by photosynthesis reduces thioredoxin, which in turn could modulate the binding of the mRNA translational activator complex, increasing the translation of several chloroplast-encoded proteins.

It was previously reported that the *trxA* gene is essential for photosynthetic growth in the obligate photoautotrophic cyanobacterium *A. nidulans* (Muller and Buchanan, 1989). However, we expected that the ability of *Synechocystis* 6803 to grow heterotrophically would accommodate the disruption of *trxA* if the corresponding protein were essential only under photosynthetic conditions. Although we obtained double recombinants, it was not possible to get a complete segregation of the disrupted gene, and the incompletely segregated *trxA* mutants, such as SFNT1+, grew slowly compared with the wild-type strain. These results are in contrast with those of experiments carried out with *A. nidulans*, in which only single recombinants were obtained, probably due to differences in transformation and recombination systems between the strains. In addition, efforts were made to increase the segregation of the SFNT1+ strain by adding thiophosphate to bypass the requirement of thioredoxin for sulfate reduction, or by adding, in the light, Glc with or without DCMU. However, under these conditions, as in heterotrophic growth, no further segregation of *trxA* was obtained. The failure to increase segregation of the interrupted *trxA* gene under various growth conditions, the similar frequency of appearance of kanamycin-resistant transformants under all conditions tested, and the fact that the segregation of the interrupted *trxA* gene did not increase even after direct rescue of kanamycin-resistant transformants in photoheterotrophic and heterotrophic conditions all suggest that thioredoxin is essential not only for photosynthetic but also for heterotrophic growth. However, the introduction of a foreign gene coding for thioredoxin, the *trxM* gene from A. *nidulans*, using a derivative pFC1 plasmid allowed the total segregation of the disrupted *trxA* gene from *Synechocystis* 6803, which confirmed that thioredoxin is necessary for the growth of this cyanobacterium.

Our results as well as other reports of functions carried out by thioredoxins, such as that of hydrogen donor for ribonucleotide reductase (Holmgren, 1985), suggest that thioredoxin is essential not only for photosynthetic growth but also for functions that take place in the absence of photosynthesis. Furthermore, the total segregation of the disrupted *trxA* gene will allow the construction of *Synechocystis* strains carrying a thioredoxin gene under the control of promoters that could be externally regulated, such as temperature-dependent promoters, providing the opportu-

nity to obtain more information regarding the function of this protein.

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