Disruption of a Gene Encoding a Novel Thioredoxin-Like Protein Alters the Cyanobacterial Photosynthetic Apparatus†

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A gene that may encode a novel protein disulfide oxidoreductase, designated txlA (thioredoxin-like), was isolated from the cyanobacterium Synechococcus sp. strain PCC7942. Interruption of txlA near the putative thioredoxin-like active site yielded cells that grew too poorly to be analyzed. In contrast, a disruption of txlA near the C terminus that left the thioredoxin-like domain intact yielded two different mutant phenotypes. One type, designated txlXb, exhibited a slightly reduced growth rate and an increased cellular content of apparently normal phycobilisomes. The cellular content of phycobilisomes also increased in the other mutant strain, designated txlXg. However, txlXg also exhibited a proportionate increase in chlorophyll and other components of the photosynthetic apparatus and grew as fast as wild-type cells. Both the txlXb and txlXg phenotypes were stable. The differences between the two strains may result from a genetic polymorphism extant in the original cell population. Further investigation of txlA may provide new insights into mechanisms that regulate the structure and function of the cyanobacterial photosynthetic apparatus.

The conversion of light energy into chemical energy (ATP) and reducing power (NADPH) by oxygen-evolving photosynthetic organisms requires the coordinated activity of photosystem I (PSI) and photosystem II (PSII), along with their light-harvesting antenna pigments and intermediary electron transport components. NADPH is produced only by linear electron flow through both PSI and PSII, but ATP can be produced either by linear electron flow or by cyclic electron flow around PSI alone (24, 34). In cyanobacteria, PSI is excited by harvesting antenna pigments and intermediary electron transport components (PSI) and photosystem II (PSII), along with their light-harvesting capability. The cellular content of phycobilisomes also increased in the other mutant strain, designated txlXg. However, txlXg also exhibited a proportionate increase in chlorophyll and other components of the photosynthetic apparatus and grew as fast as wild-type cells. Both the txlXb and txlXg phenotypes were stable.

The differences between the two strains may result from a genetic polymorphism extant in the original cell population. Further investigation of txlA may provide new insights into mechanisms that regulate the structure and function of the cyanobacterial photosynthetic apparatus.

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MATERIALS AND METHODS

Strains and culture conditions. Synechococcus sp. strain PCC7942 was cultured on both liquid and solid BG-11 medium as described previously (5, 17). In liquid medium, cultures were grown at 32°C with a light intensity of 50 μmol of photons m−2 s−1 (from three 40-W incandescent bulbs) and bubbled with air enriched to 3% CO2. When appropriate, ampicillin was included at a concentration of 1.5 μg/ml, and spectinomycin was included at a concentration of 25 μg/ml in solid medium or 5 μg/ml in liquid medium.

Nucleic acid manipulations. All DNA and RNA techniques were performed according to standard procedures and as described previously (7, 18, 55). The
bysubcloningthe800-bp Eco strand-andgene-specificRNAprobeusedtodetect bp of upstream sequence, into pBluescript KS theendogenous oftheentireplasmidintothechromosome. Indoublehomologousrecombinants, wouldbebothampicillinandspectinomycinresistantbecauseoftheintegration terial genome will grow on spectinomycin. Single homologous recombinants nobacteria,onlythosecellsinwhichtheplasmidrecombinesintothecyanobac-
spp.,doublehomologousrecombinationisgenerallyfarmorecom-
plexes250 bp oftxlA into theBgl II site. The restriction sites underlined are as follows: bp 1, EcoRV; bp 452, Nco I; bp 701, Xho I; bp 718, EcoRV; bp 798 and 863, Xho I; and bp 1039, Bgl II. The amino acids in the hydrophobic region of the polypeptide (residues 14 to 31) are also under-
lined. The potential ribosome-binding site and the two cytidines of the putative active site are thickly underlined. 

The1-kbp Eco RV restriction fragment containingtxlA, into the
plasmid-borne txlA cassette, andtheremainderoftheplasmidwouldbelost.

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The fluorescence emission spectra at 77 K of cyanobacterial cultures were
measured with a Photon Technologies International (New Brunswick, N.J.) sin-
gle-beam fluorometer as described previously (16, 19). The background fluo-
cescence spectrum measured from sterile medium was subtracted from each sample spectrum, but no corrections were made for the spectral sensitivity of the instru-
ment. The pairs of curves in each panel of Fig. 3 were normalized to each other
at 650 nm.

A post hoc statistical analysis of the data was performed with either the Tukey honestly significant difference test of the SYSTAT package (1986; L. Wilkinson, Evanston, Ill.), when the number of values (n) was equal, or the Scheffe test of
theSTATVIEW package (version 4.01; Abacus Concepts, Berkeley, Calif.),
when n differed. For each characteristic (i.e., doubling time, PC/Chl ratio, phy-
cyanin [PC] per cell), Chl per cell, and rates ofO 2 evolution), these analyses
were performed separately for each characteristic. All strains within group A
weresignificantlydifferentfromthosewithin group B, and the level of signifi-
cance for those differing from the wild type is indicated in Tables 2, 3, and 4. For
txlA::ptxlX analysis of both PC per cell and Chl per cell did not allow for
classification into either group A or B group.

RESULTS

The txlA (thioredoxin-like) gene was cloned and sequenced because it lies immediately downstream of and in the opposite
orientation from nblA, a gene required for the degradation of PBS in nutrient-deprived *Synechococcus* sp. strain PCC7942 (18). We began investigating the function of *txlA* because antisense *txlA* transcripts (originating from nblA) are produced in nutrient-deprived cells, suggesting that changes in the expression of *txlA* may be involved in acclimation to nutrient-deficient conditions (16).

**Sequence analysis.** A restriction map of the region containing *txlA* is shown in Fig. 1A, and the DNA sequence between the EcoRV and BglII sites is presented in Fig. 1B. The *txlA* open reading frame initiates with a methionine that is preceded by a potential ribosome-binding site. The predicted molecular mass of TxlA is 21 kDa, with an isoelectric point (pI) of 4.2. The predicted protein is largely hydrophilic except for a domain of 18 hydrophobic residues (residues 14 to 31, underlined in Fig. 1B) near the N terminus. The TxlA sequence was used to search the GenBank database (Release 76). The 37 N-terminal and 44 C-terminal residues had no detectable similarity to any sequence in the database, but the central 110-N-terminal and 44 C-terminal residues had no detectable similarity to any sequence in the database, but the central 110-N-terminal and 44 C-terminal residues had no detectable similarity to any sequence in the database. Sequence alignments to other thioredoxins and thioredoxin-like proteins (Trxs) of *Synechococcus* sp. strain PCC7942 (46) show that the central region of TxlA is 29% identical and 60% conserved relative to TrxM (Fig. 1C). TxlA exhibits comparable levels of similarity to other thioredoxins and to the thioredoxin-like domains of PDI (data not shown).

**Genetic analysis.** To investigate the function of *txlA*, the *txlA* region was cloned into pUC118, and a spectinomycin resistance cassette called Ω (see Materials and Methods) was inserted in vitro into the *txlA* open reading frame in two different positions (Fig. 1A). In the first of these constructs, designated ptxlAXN, more than half of the thioredoxin-like region of the *txlA* open reading frame was removed and replaced with the Ω cassette. In the second construct, designated ptxlX, the Ω cassette was inserted into the Xhol site, which would cause a truncation of TxlA near the C terminus. If the normal function of TxlA requires that its thioredoxin-like domain be intact, replacement of normal *txlA* with the *txlANX* disruption should abolish TxlA activity. In contrast, the *txlX* disruption leaves the thioredoxin-like domain intact; it is less likely to have a severe effect on any PDO-like activity of TxlA.

Plasmids ptxlANX and ptxlX were introduced into wild-type *Synechococcus* sp. strain PCC7942, and transformants were selected on plates containing spectinomycin. The results of three separate transformations of wild-type *Synechococcus* sp. strain PCC7942 cultures with ptxlANX or ptxlX are summarized in Table 1. Two types of transformants were observed in each case. One colony type appeared blue and smaller than normal (blue colonies), while the other appeared similar to the wild type (green colonies). This would be expected if one type (i.e., blue colonies) resulted from the disruption of *txlA* by double homologous recombination, while the other (i.e., green colonies) resulted from single homologous integration of the plasmid into the genome generating both an intact and a disrupted copy of *txlA*. Following the introduction of ptxlANX, all of the green colonies were ampicillin resistant (single homologous recombinants), while all of the blue colonies were ampicillin sensitive (double homologous recombinants), as predicted. The blue colonies, designated *txlANX*b, grew very slowly on solid medium and died much more quickly than wild-type cells. We were only able to grow the *txlANX*b mutant in liquid medium twice. In both cases, a very long (7-day) lag period was observed, and the phenotypes of the two cultures were different. Therefore, the growth of *txlANX*b was greatly impaired by a loss of TxlA activity, and it is likely that the mutant strain only grew in liquid medium after the generation of secondary suppressor mutations. Hence, the *txlANX*b mutant was considered unreliable for further analysis.

In contrast to the results with ptxlANX, transformation with plasmid ptxlX yielded a much higher ratio of green to blue colonies (Table 1). All of the blue colonies were ampicillin sensitive, as expected for double homologous recombinants. However, approximately 60% of the green colonies were unexpectedly ampicillin sensitive; that is, they were also likely to be the consequence of double homologous recombination events. Hence, in this case, the true ratio of single to double recombinants was not equal to the ratio of green to blue colonies. If the ratio of single to double recombinants were calculated based on ampicillin sensitivity, it was approximately 0.1, which is more similar to the results for ptxlANX. Ampicillin-sensitive strains with the blue phenotype were designated *txlX*b, and ampicillin-sensitive strains with the green phenotype were designated *txlX*g. As shown in Southern analyses of genomic DNA (Fig. 2A), the 1.5-kbp *EcoR* and 3.5-kbp *PstI* fragments of wild-type cells increased in size to 3.5 and 5.5 kbp, respectively, in both the *txlX*b and *txlX*g mutants. The probe used included the region shown in Fig. 1A plus 800 bp upstream of nblA. These results demonstrated that both *txlX*b and *txlX*g contained the predicted disruption of *txlA* by the 2.0-kbp *HindIII* fragment inserted into the *XhoI* site. Longer exposures confirmed that no unincorporated copies of *txlA* could be detected in either *txlX*b or *txlX*g. Both the *txlX*b and *txlX*g phenotypes were stable, and their growth on plates and in liquid medium was much faster than that of *txlANX*b.

The growth of the *txlX*b strains might be expected to surpass that of *txlANX*b if a truncated *txlA* polypeptide synthesized in the *txlX*b strains (which includes the thioredoxin-like region) possesses some TxlA activity. Northern (RNA blot) analysis of RNA from both *txlX*b and *txlX*g confirmed that no uninterrupted copies of *txlA* were detected in either *txlX*b or *txlX*g. Both the *txlX*b and *txlX*g RNAs lacked a putative open reading frame and were unstable, and their growth on plates and in liquid medium was much faster than that of *txlANX*b.

**Physiological analysis.** The growth and pigmentation of *txlX*b and *txlX*g in liquid medium are summarized in Table 2. The growth rate of *txlX*g was similar to that of the wild type, but *txlX*b grew more slowly. Whole-cell absorbance spectra were used to monitor the pigmentation of the cells, since the *A_{680}* reflects Chl content and the *A_{630}* reflects PC (the major phycobiliprotein component of the PBS) content. The elevated PC/Chl absorbance ratio and blue appear-

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>No. of green colonies/no. of blue colonies (ratio)</th>
<th>No. of Amp&lt;sup&gt;r&lt;/sup&gt; colonies/total (%) Amp&lt;sup&gt;r&lt;/sup&gt;</th>
<th>Blue colonies</th>
<th>Green colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>ptxlANX</td>
<td>1 104/1,215 (0.083)</td>
<td>4/4 (100)</td>
<td>36/36 (100)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 18/400 (0.045)</td>
<td>36/36 (100)</td>
<td>36/36 (100)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 15/470 (0.032)</td>
<td>36/36 (100)</td>
<td>36/36 (100)</td>
<td></td>
</tr>
<tr>
<td>ptxlX</td>
<td>1 ND</td>
<td>1/4 (25)</td>
<td>2/3 (67)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 216/588 (0.367)</td>
<td>2/3 (67)</td>
<td>36/36 (100)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 55/184 (0.30)</td>
<td>14/36 (40)</td>
<td>14/36 (40)</td>
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</tr>
</tbody>
</table>

*ND, not determined.*
The mutant strains exhibited a normal size, PC-to-allophycocyanin ratio, polypeptide composition, and physical attachment to the thylakoid membranes. Furthermore, PBS isolated from both txlXb and txlXg exhibited normal fluorescence emission spectra, indicating that energy transfer within the PBS was normal. Finally, the PBS from the mutant strains were shown to be identical to those of the wild type in their susceptibility to proteolysis by trypsin, either when purified or when still attached to the thylakoid membrane. These data (16) demonstrated that the phycobiliproteins and PBS in both txlXb and txlXg were structurally and functionally indistinguishable from those of the wild type and that the increased PC content per cell in the mutants reflected an increased number of apparently normal PBS per cell.

We also examined the energetic connection of the light-harvesting antennae pigments (both PBS and Chl) to the photosynthetic reaction centers by fluorescence emission spectroscopy of whole cells frozen in liquid nitrogen (77 K). As shown in Fig. 3, four main emission peaks are produced when wild-type cells are excited either with 440-nm (absorbed primarily by Chl) or 570-nm (absorbed primarily by PBS) wavelength light. The peak at 650 nm is emitted from PC within the PBS, the peak at 685 nm is due to emissions from both the PBS and the terminal energy acceptors (at about 680 nm [27, 28]) and the Chl antenna of PSI (CP43, at about 686 nm [22, 33, 49, 52]). The peak at 695 nm is emitted from the PSII reaction center and its CP47 antennae Chl (22, 33, 49, 52), and the peak at 715 nm is emitted from the Chl of PSI (49). In cells excited with 570-nm light, the emission peaks at 650, 685, and 695 nm dominate because PBS transfer energy mainly to PSI (Fig. 3A). In cells excited with 440-nm light, the emission peak at 715 nm is more pronounced because Chl transfers light energy mainly to PSI (Fig. 3B). Also shown are state transitions, which reflect a short-term mechanism for redistributing absorbed light energy between the two reaction centers. If the cells are exposed to light that primarily excites PSI (440 nm) before freezing, relatively more of the absorbed light energy is directed to PSI (state 1; Fig. 3, dark traces). If the cells are exposed to light absorbed primarily by PSI (570 nm) before freezing, relatively more of the absorbed light energy is directed to PSI (state 2; Fig. 3, light traces).

In the mutant txlXg, the state transitions and fluorescence emission spectra appeared normal (Fig. 3E and F). This suggests that despite the increased pigment levels in these cells, the stoichiometry of PSI and PSI, as well as energy transfer between the pigments and photosystems, was normal. Therefore, the reaction centers of both PSI and PSII, along with other components of the photosynthetic apparatus, probably increased proportionately with PBS and Chl in the txlXg mutant. The txlXb mutant also showed relatively normal fluorescence emissions and state transitions when excited with 440-nm

Table 2. Growth characteristics

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mean doubling time (h) ± SD</th>
<th>Mean PC/Chl ratio ± SD</th>
<th>Mean content per cell ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PC (10⁻¹⁰ mg)</td>
<td>Chl (10⁻¹¹ mg)</td>
</tr>
<tr>
<td>Wild type</td>
<td>8.0 ± 0.33 (n = 7), A</td>
<td>1.28 ± 0.04 (n = 19), A</td>
<td>1.33 ± 0.24 (n = 12), A</td>
</tr>
<tr>
<td>txlXb</td>
<td>10.1 ± 0.57 (n = 8), B**</td>
<td>1.55 ± 0.08 (n = 23), B***</td>
<td>1.68 ± 0.2 (n = 15), B**</td>
</tr>
<tr>
<td>txlXg</td>
<td>7.5 ± 0.28 (n = 3), A</td>
<td>1.28 ± 0.03 (n = 10), A</td>
<td>1.75 ± 0.2 (n = 10), B***</td>
</tr>
</tbody>
</table>

* Cells were grown with 3% CO₂ in air and 50 μmol of photons m⁻² s⁻¹. The PC/Chl ratio was calculated as (A₆₂₀ - A₅₇₀)/(A₆₈₀ - A₅₇₀). A and B indicate the statistically distinct groups within each column, and the significance of differences from the wild-type values is indicated as follows: *, P < 0.05; **, P < 0.01; ***, P < 0.001.
light (Fig. 3D; the relative intensity of the PSII and PSI emissions is within the range observed for the wild type). However, when ttxXB was excited with 570-nm light (Fig. 3C), the 685-nm peak was much larger relative to the other peaks, and its position was shifted toward the blue end of the spectrum. The positions of this peak were 684.75 ± 0.43 nm (n = 3) in wild-type cells, 685.25 ± 0.53 nm (n = 5) in ttxXg, and 683.45 ± 0.65 nm (n = 5) in ttxXB. The differences between ttxXB and both the wild type and ttxXg were statistically significant (P < 0.05, Scheffe’s f test). Similar changes in the emission maximum near 685 nm have previously been observed when emissions from the PBS terminal energy acceptors (near 680 nm) increase relative to those from PSI antennae Chl (near 686 nm), reflecting a decreased efficiency of energy transfer from the PBS to PSI (21, 30, 58). These data suggest that the excess PBS present in the ttxXB strain are not able to transfer harvested light energy to the photosystems. In addition, state transitions were observed in ttxXB excited with 570-nm light (Fig. 3C), but in state 2, ttxXB showed a relatively greater increase in PSI and smaller decrease in PSII emissions than wild-type cells.

The rate of O₂ evolution was used to measure the photosynthetic capacity of PSII in the wild-type and ttxX mutant strains. As shown in Table 3, the rates of O₂ evolution on a Chl basis in both ttxXB and ttxXg were nearly identical to that of wild-type cells. The same was true even at very low light intensities (data not shown). These observations suggest that there were no major differences in the efficiency of PSI or in the stoichiometries of Chl, PSI, and PSII among the strains.

However, since ttxXg has more Chl per cell, its rate of O₂ evolution per cell was almost 30% higher than that of the wild-type or ttxXB strain (Table 3). These data support the suggestion from fluorescence emission spectra that the increased cellular pigmentation of ttxXg was accompanied by an increase in the reaction centers and the rest of the photosynthetic apparatus. The elevated photochemical capacity of ttxXg per cell might explain why its growth rate is faster than that of ttxXB and similar to that of wild-type cells (Table 2).

**DISCUSSION**

The very poor growth of the ttxANXb mutant suggests that txlA plays an important, although perhaps not absolutely essential, role in the metabolism of the cyanobacterium *Synechococcus* sp. strain PCC7942. It is possible that the ttxXb and ttxX mutants displayed better growth because the removal of only the C-terminal 34 codons did not prevent TxlA from performing part of its normal function. Hence, the thio-
Both strains synthesized truncated TxlA and were functionally integrated (Fig. 3, Table 3). Intact of the photosynthetic apparatus, with which the “excess” PBS content was accompanied by a proportionate increase in the efficiency of harvested light energy to the photosynthetic apparatus. If disruption of txlA is the consequence of a spontaneous mutation occurring in homologous recombinants, respectively, it is not likely that the two strains. Since the frequencies of both the txlXb and txlXg phenotypes are relatively high (80 and 20% of the double mutants (Table 4). The presence of full-length TxlA in the txlXb strain converted it to the phenotype of txlXg. One possible explanation for these results is that the interaction of truncated and full-length TxlA polypeptides in the txlXb:ptxlA strain produced a similar effect as when the truncated TxlA polypeptide interacted with another protein in mutant txlXg. Both the txlXb and txlXg phenotypes could result from the same mutation in txlA if this second protein differed between the two strains. Since the frequencies of both the txlXb and txlXg phenotypes are relatively high (80 and 20% of the double homologous recombinants, respectively), it is not likely that either is the consequence of a spontaneous mutation occurring after disruption of txlA. It is more likely that the putative second-site locus was polymorphic in the original population of mutagenized cells. Since each cyanobacterial cell carries multiple copies of the chromosome, it is also possible that such a polymorphism could reside within a single cell.

The phenotypes of the txlX mutants show that the C-terminal domain is important for some aspect of TxlA’s role in the cell. It is possible that the disruption of txlA in the txlX strains was directly responsible for the production of excess PBS and that the slow growth of txlXb simply reflected the diversion of resources from growth into the production of excess PBS. The proportionate increase in cellular photosynthetic capacity observed in txlXg might then provide the extra biosynthetic capacity needed to attain a normal growth rate despite the higher PBS content. However, the growth rates of the wild-type, txlXb, and txlXg strains under carbon-limited conditions (i.e., during growth on air instead of air enriched with 3% CO2) were indistinguishable (doubling time approximately 14 h; data not shown). The rates of O2 evolution in the wild-type and txlXb strains during carbon-limited growth were also indistinguishable [approximately 5.5 μmol of O2 (mg of Chl)−1 min−1]. Under these conditions, txlXb still had normal levels of Chl per cell, elevated levels of PC per cell, and abnormal fluorescence emission spectra (data not shown). Therefore, the production of excess PBS did not slow the growth of txlXb on air supplemented with CO2, making it seem unlikely that the expenditure of energy required to synthesize excess PBS was alone responsible for retarding its growth on CO2-enriched air.

It is possible that the changes in cellular pigmentation observed in the txlX mutants are indirect, occurring in response to an underlying change in cellular metabolism. The observation that txlXb does not grow more slowly than the wild type on air that is not enriched with CO2 suggests that txlXb is unable to adjust its metabolism to match the faster growth rate of the wild type under carbon-rich conditions. This could reflect a number of defects, such as improper regulation of carbon metabolism or an inability to properly modulate the pathways of photosynthetic electron transport, which differ during carbon-limited and carbon-rich growth. The similarity of TxlA to thioredoxins and other PDOs also suggests that it could play a role in the synthesis, monitoring, or redox regulation of electron transport pathways. If disruption of txlA perturbed the normal flow of electrons, cyanobacteria might respond by adjusting the photosynthetic apparatus, just as they modulate the structure and function of the photosynthetic apparatus to suit the metabolic demands of different environmental conditions (see the introduction). An even more direct link between TxlA and the regulation of the cyanobacterial photosynthetic apparatus may be implied if the antisense txlA mRNA produced in nutrient-deprived Synechococcus sp. strain PCC7942 cells is found to regulate TxlA expression (16, 18).

While these data do not allow us to precisely define the function of txlA, they strongly suggest that TxlA can influence the structure and function of the cyanobacterial photosynthetic apparatus. Further physiological and biochemical characterization of TxlA, txlXb, and txlXg not only should reveal the function of TxlA, but may also provide new molecular insights into the regulation of the cyanobacterial photosynthetic apparatus.
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