A New Rubisco-like Protein Coexists with a Photosynthetic Rubisco in the Planktonic Cyanobacteria * ‡ § ¶

Alyssa Carré-Mlouka 1, Annick Méjean 3, Philippe Quillardet 3, Hiroki Ashida 3, Yohtaro Saito 3, Akihiko Yokota 1, Isabelle Callebaut 4, Agnieszka Sekowska 2, Elke Dittmann 2 *, Christiane Bouchier 3, and Nicole Tandeau de Marsac 1 2

From the 1 Département de Microbiologie, Unité des Cyanobactéries (CNRS-URA 2172) and 11 Plate-forme Génomique-Pasteur Génomopole Île de France, Institut Pasteur, 28 rue du Docteur Roux, 75724 Paris Cedex 15, France, the 2 Nara Institute of Science and Technology (NAIST), Graduate School of Biological Sciences, 8916-5 Takayama, Ikoma, Nara 630-0101, Japan, the 3 Département de Biologie Structurale, Institut de Minéralogie et Physique des Milieux Condensés, CNRS UMR7590, Universités Paris 6 et 7, case 115, 4 place Jussieu, 75252 Paris Cedex 05, France, the 4 HKU-Pasteur Research Centre, Dexter HC Man Building, 8 Sassoon Road, Pokfulam, Hong Kong, China, and the 5 Institute of Biology, Humboldt University, Chausseestrasse 117, 10115 Berlin, Germany

Two genes encoding proteins related to large subunits of Rubisco were identified in the genome of the planktonic cyanobacterium Microcystis aeruginosa PCC 7806 that forms water blooms worldwide. The rbcL 1 gene belongs to the form I subfamily typically encountered in cyanobacteria, green algae, and land plants. The second and newly discovered gene is of the form IV subfamily and widespread in the Microcystis genus. In M. aeruginosa PCC 7806 cells, the expression of both rbcL 1 and rbcLIV is sulfur-dependent. The purified recombinant RbcLIV overexpressed in Escherichia coli cells did not display CO2 fixation activity but catalyzed enolization of 2,3-diketo-5-methylthiopentyl-1-phosphate, and the rbcLIV gene rescued a Bacillus subtilis MtnW-deficient mutant. Therefore, the Microcystis RbcLIV protein functions both in vitro and in vivo and might be involved in a methionine salvage pathway. Despite variations in the amino acid sequences, RbcLIV shares structural similarities with all members of the Rubisco superfamily. Invariant amino acids within the catalytic site may thus represent the minimal set for enolization, whereas variations, especially located in loop 6, may account for the limitation of the catalytic reaction to enolization. Even at low protein concentrations in vitro, the recombinant RbcLIV assembles spontaneously into dimers, the minimal unit required for Rubisco forms I–III activity. The discovery of the coexistence of RbcL 1 and RbcLIV in cyanobacteria, the ancestors of chloroplasts, enlightens episodes of the chaotic evolutionary history of the Rubiscos, a protein family of major importance for life on Earth.

In photosynthetic organisms, the ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) 4 enzyme enables the fixation of inorganic atmospheric carbon dioxide into organic matter for use as a source of energy after incorporation into cellular components (1, 2). Despite considerable efforts directed toward the crop improvement issue, the in vivo study of plant Rubisco has proven arduous, and it has not been possible to reconstitute flowering plant Rubisco from dissociated subunits in vitro (3).

Different types of Rubisco proteins have been described so far (3, 4). The form I enzyme, typically encountered in plants, eu- karyotic algae, and cyanobacteria, contains large (Rbcl) and small (RbcS) subunits that have been shown to assemble into a hexadecameric structure, (L2)4(S4)2. The catalytic site contains active amino acids from two neighboring large subunits, and studies of site-specific enzymes indicate that the small subunit is required for maximal catalysis and contributes to CO2/O2 specificity (5). Eukaryotic dinoflagellates, sulfur bacteria, and several chemoautotrophic bacteria synthesize, sometimes concomitantly with a form I enzyme, the form II Rubisco, which is constituted solely of large subunits (3). Crystallization of the Rhodospirillum rubrum form I Rubisco revealed a dimeric association (L2), and larger multiples of L2 associations have also been reported (2, 6).

The sequencing of the genome of various microorganisms has unveiled the presence of genes encoding distantly related large subunits of Rubiscos. This finding led to the definition of two new classes of Rubiscos (3). Archaeal members of form III proved to be an (L2)5 decamer (10, 11).

Members of the form IV subfamily (Rubisco-like proteins or RLPs) do not display Rubisco activity, but the two eubacterial

* This work was supported by the Institut Pasteur, the CNRS (URA 2172), the Ministère de l’Education Nationale, de la Recherche et de la Technologie (MENRT), and the University Paris 7-Denis Diderot. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Recipient of a Ph.D. fellowship from the MENRT. Present address: Laboratoire de Génétique et Cellule Biologique, Université Versailles-Saint Quen tin en Yvelines, 45 avenue des Etats-Unis, 78035 Versailles Cedex, France.

2 Present address: Laboratoire de Signalisation, Phosphoprotéome et Communautés Bactériennes, Institut de Génétique et Microbiologie, CNRS UMR8621, Bât. 409, Université Paris-Sud, 91405 Orsay Cedex, France.

3 To whom correspondence should be addressed. Tel.: 33-1-45-68-84-15; Fax: 33-1-40-61-30-42; E-mail: ntmarsac@pasteur.fr.

4 The abbreviations used are: Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; RLP, Rubisco-like protein; PCC, Pasteur Culture Collection; IPTG, isopropyl β-d-thiogalactopyranoside; LB, Luria-Bertani; RT, reverse transcription.
Two Proteins of the Rubisco Superfamily in Microcystis

To construct the *B. subtilis* mtnW deletion strain, a Smal-restricted spectinomycin resistance cassette (21) was used. Two DNA fragments were amplified by PCR, one upstream from the mtnW gene (nucleotides −731 to −138 relative to the translational start point of mtnW), amplified with the following primers: 5′-CCCGAATTCTTATTGCAAGCTGAGTCG-3′ (EcoRI site underlined) and 5′-TCCCCGGGCGCTTCGCTCCTTGATAAG-3′ (Smal site underlined) and the second one downstream from the mtnW gene (nucleotides −34 to +437 relative to the mtnW stop codon, amplified with the following primers: 5′-TCCCCGGGCGCTATGACGACTGAAAC-3′ (Smal site underlined) and 5′-GGCGATTCGATCTGATGATGC-3′ (BamHI site underlined). PCR products and the spectinomycin cassette were ligated and inserted into the EcoRI and BamHI sites of pUC19 (Roche Applied Science) producing plasmid pHPP7027. Prior to transformation in *B. subtilis*, this plasmid was linearized at its unique Scal site. Complete deletion of the gene was obtained by a double crossover event, giving strain BSHP7082 (trpC2 mtnW::spc).

The mtnXYZ region (starting at nucleotide −31 relative to the mtnX translation start point and ending 3 bp after the stop codon of mtnZ) was amplified by PCR using a forward primer (5′-GGACTAGTCGATAGAATAATGGGGAAAGG-3′) introducing a SpeI cloning site at the 5′-end and a reverse primer (5′-GGCGATTCGATCTGATGATGC-3′) introducing a BamHI cloning site at the 3′-end of the fragment. This fragment was inserted into the SpeI and BamHI sites of the xylose-inducible pX plasmid (22) producing plasmid pHPP7015. Prior to transformation in *B. subtilis*, this plasmid was linearized at its unique Scal site. Complete integration of the plasmid was obtained by a double crossover event at the amyE locus in the BSHP7082 strain, giving strain BSHP7075 (trpC2 mtnW::spc amyE::mtnXYZ).

The replicative plasmid pDG148 (23) with its isopropyl β-D-thiogalactopyranoside (IPTG)-inducible promoter was used to clone the rbclIV gene from *M. aeruginosa* CCC 7806. The rbclIV gene was amplified from chromosomal DNA by PCR using the forward primer 5′-ACGCGTACGACAAGGAGGTACCTTCTTA-TGACTATAATTGTGCG-3′ (Sall site underlined and introduced ribosome binding site in bold) and the reverse primer 5′-ACATGCCATGACCTAGAAATCACTC-3′ (Sphl site underlined). The amplified fragment after digestion was inserted into the Sall and Sphl sites of pDG148, producing plasmid pHPP7032. The BSHP7075 strain was transformed with this plasmid giving strain BSHP7080 (trpC2 mtnW::spc amyEmt-nXYZ pDG148-rbclIV). All restriction enzymes used were from Roche Applied Science.

*B. subtilis* cells were transformed with plasmid DNA following the two-step protocol described previously (24). Transformants were selected on Luria-Bertani (LB) solid medium containing the appropriate antibiotics at the following concentrations: 100 µg/ml for spectinomycin, 5 µg/ml for chloramphenicol, or 5 µg/ml for kanamycin.

*Escherichia coli* Strains—The *E. coli* strains used were TG1 and XL1-blue (laboratory collection) for the construction of pHPP7032 and of pHPP7027 and pHPP7015, respectively; JM109 or GM48 (Promega Corp., Madison, WI) for the expression vector constructs; and BL21 or Rosetta(DE3)pLysS members of this family have been shown to play a role in sulfur metabolism. Indeed disruption of the RLP gene of *Chlorobium tepidum*, a green sulfur bacterium, provoked accumulation of sulfur inclusions (12). The mtnW (previously ykrW) gene of *Bacillus subtilis* encodes an enzyme of the methionine salvage pathway that permits recycling of methionyladenosine, a toxic end product of polyamine synthesis, into methionine through methylothioribose (13, 14). This metabolic route, first deciphered in *Klebsiella pneumoniae* (15), was also shown to occur in plants (16), yeast (17), and protozoal parasites (18). Although the bona fide function of Rubisco enzymes consists of a three-step sequential reaction (enolization, carboxylation/oxygenation, and final hydrolysis of the substrate ribulose 1,5-bisphosphate), 1H NMR experiments have shown that the *B. subtilis* MtnW RLP only catalyzed enolization of 2,3-diketo-5-methylthiopentyl-1-phosphate, a compound with structural similarities to ribulose 1,5-bisphosphate (19). Based on the fact that the form II Rubisco of *R. rubrum* retained the enolase function in the methionine salvage pathway of *B. subtilis*, form IV RLPs have been suggested to be the ancestors of the photosynthetic Rubisco (4, 19).

The planktonic *Microcystis* is one of the most common cyanobacterial genera found in water blooms worldwide. Sequence analysis of the partial genome of *Microcystis aeruginosa* PCC 7806 has unraveled the presence of two genes encoding large subunits of the Rubisco superfamily. The rbcLXS operon encodes a typical form I Rubisco. The second gene, *rbcLIV*, encodes a form IV Rubisco-like protein widespread in the *Microcystis* genus. This study reports the expression of the *rbcL* and *rbcLIV* genes and the functional and structural characterization of *RbcLIV*, a new form of Rubisco-like protein in cyanobacteria.

**Experimental Procedures**

Strains, Plasmids, and Media—Stock cultures of the *Microcystis* strains of the Pasteur Culture Collection (PCC) were maintained in BG11o (20) supplemented with 2 mM NaNO3 and 10 mM NaHCO3 at 22 °C under a light/dark regime (14 h/10 h). Light was provided by Osram Universal White fluorescent tubes under a photon flux density of 20 µmol/m2/s (LICOR LI-185B quantum radiometer/photometer equipped with a LICOR LI-190SB quantum sensor). Precultures were supplemented with 20 mM NaHCO3 and bubbled with 1% (v/v) CO2 in air at 28 °C under continuous light (30 µmol/m2/s). For transcription analyses, experimental cultures of 500 ml were obtained by dilution (1:10) of a preculture (OD750 0.5–0.6) were harvested by centrifugation (10,000 × g, 10 min, 25 °C). Cell pellets were washed and resuspended in the same volume of complete medium as described for precultures or in the same volume of sulfur-depleted medium prepared by replacing the sulfate salts with equimolar amounts of chloride ions. After a further incubation period of 15 h under the same conditions, cells were harvested by centrifugation (10,000 × g, 10 min, 25 °C). Cell pellets were immediately frozen in liquid nitrogen and kept at −80 °C until RNA extractions.

To construct the *B. subtilis* mtnW deletion strain, a Smal-restricted spectinomycin resistance cassette (21) was used. Two DNA fragments were amplified by PCR, one upstream from the mtnW gene (nucleotides −731 to −138 relative to the translational start point of mtnW), amplified with the following primers: 5′-CCCGAATTCTTATTGCAAGCTGAGTCG-3′ (EcoRI site underlined) and 5′-TCCCCGGGCGCTTCGCTCCTTGATAAG-3′ (Smal site underlined) and the second one downstream from the mtnW gene (nucleotides −34 to +437 relative to the mtnW stop codon, amplified with the following primers: 5′-TCCCCGGGCGCTATGACGACTGAAAC-3′ (Smal site underlined) and 5′-GGCGATTCGATCTGATGATGC-3′ (BamHI site underlined). PCR products and the spectinomycin cassette were ligated and inserted into the EcoRI and BamHI sites of pUC19 (Roche Applied Science) producing plasmid pHPP7027. Prior to transformation in *B. subtilis*, this plasmid was linearized at its unique Scal site. Complete deletion of the gene was obtained by a double crossover event, giving strain BSHP7082 (trpC2 mtnW::spc).

The mtnXYZ region (starting at nucleotide −31 relative to the mtnX translation start point and ending 3 bp after the stop codon of mtnZ) was amplified by PCR using a forward primer (5′-GGACTAGTCGATAGAATAATGGGGAAAGG-3′) introducing a SpeI cloning site at the 5′-end and a reverse primer (5′-GGCGATTCGATCTGATGATGC-3′) introducing a BamHI cloning site at the 3′-end of the fragment. This fragment was inserted into the SpeI and BamHI sites of the xylose-inducible pX plasmid (22) producing plasmid pHPP7015. Prior to transformation in *B. subtilis*, this plasmid was linearized at its unique Scal site. Complete integration of the plasmid was obtained by a double crossover event at the amyE locus in the BSHP7082 strain, giving strain BSHP7075 (trpC2 mtnW::spc amyE::mtnXYZ).

The replicative plasmid pDG148 (23) with its isopropyl β-D-thiogalactopyranoside (IPTG)-inducible promoter was used to clone the rbclIV gene from *M. aeruginosa* CCC 7806. The rbclIV gene was amplified from chromosomal DNA by PCR using the forward primer 5′-ACGCGTACGACAAGGAGGTACCTTCTTA-TGACTATAATTGTGCG-3′ (Sall site underlined and introduced ribosome binding site in bold) and the reverse primer 5′-ACATGCCATGACCTAGAAATCACTC-3′ (Sphl site underlined). The amplified fragment after digestion was inserted into the Sall and Sphl sites of pDG148, producing plasmid pHPP7032. The BSHP7075 strain was transformed with this plasmid giving strain BSHP7080 (trpC2 mtnW::spc amyEmt-nXYZ pDG148-rbclIV). All restriction enzymes used were from Roche Applied Science.

*B. subtilis* cells were transformed with plasmid DNA following the two-step protocol described previously (24). Transformants were selected on Luria-Bertani (LB) solid medium containing the appropriate antibiotics at the following concentrations: 100 µg/ml for spectinomycin, 5 µg/ml for chloramphenicol, or 5 µg/ml for kanamycin.

*Escherichia coli* Strains—The *E. coli* strains used were TG1 and XL1-blue (laboratory collection) for the construction of pHPP7032 and of pHPP7027 and pHPP7015, respectively; JM109 or GM48 (Promega Corp., Madison, WI) for the expression vector constructs; and BL21 or Rosetta(DE3)pLysS.
Two Proteins of the Rubisco Superfamily in Microcystis

(Invitrogen). Two liters of LB medium were inoculated at 0.5 OD750 in liquid cultures following standard procedures (25) and the control for DNA contamination consisted of the same reaction mixture except that SuperScript II reverse transcriptase was replaced by H2O. A total of 2 µl (0.5 µg of initial RNA) of the RT reaction mixture or 20 ng of genomic DNA as positive controls were used for subsequent PCR using the following oligonucleotide primers: 5′-CCCGCCTTTTAGCTAAGTGCT-3′ and 5′-GGGAAGCGTAGTCTTG-GGTTGA-3′ for rbcLp, 5′-GAAAGGCTACGCCAAAAGACG-3′ and 5′-TAAAGCCAGCAAAAGCCACTT-3′ for rbcLIV, and 5′-AATCCCTACCCCAATCGTT-3′ and 5′-CCTAGGAGGATAGTGCACCAGA-3′ for rnpB. Samples were taken at successive PCR cycles. Each sample was analyzed by gel electrophoresis on 1.5% (w/v) agarose in 1× Tris-borate-EDTA buffer, the gels were photographed under UV light as described above, and the gel image was quantified using the free software ImageJ (Millersville University).

Plasmid Constructs, Overexpression, and Purification of the Recombinant Proteins—The rbcLIV gene from M. aeruginosa PCC 7806 was amplified from chromosomal DNA, cloned in both the pET-43.1a(+) and pET-28a(+) systems by PCR amplification using a proofreading polymerase, and verified by sequencing. For cloning in the expression vector pET-28a(+) (Novagen, Darmstadt, Germany), the primers used were 5′-AACCCCGGGGGCAGCCATGACATTTCAGTT-GTTCGATCCACCGTAAGTGCT-3′ and 5′-GGGAAGCGTAGTCTTG-GGTTGA-3′ for rbcLp, and 5′-GAAAGGCTACGCCAAAAGACG-3′ and 5′-TAAAGCCAGCAAAAGCCACTT-3′ for rbcLIV, and 5′-AATCCCTACCCCAATCGTT-3′ and 5′-CCTAGGAGGATAGTGCACCAGA-3′ for rnpB. One microliter of cryolysate, 25 pmol of each primer, a 250 nM concentration of each dNTP, and the plasmid pET-28a(+), digested with Ndel and Sall, were ligated. The ligation mixture was transformed by electroporation in E. coli GM48 cells, and the construct was then transferred by electroporation to Rosetta(DE3)pLysS (Novagen, Darmstadt, Germany), a strain provided for “universal” translation. The recombinant plasmid pET-28a:rbcLIV was introduced into BL21(DE3). After IPTG induction, His-tagged RbcLIV was purified in one step on nickel-nitrilotriacetic acid resin (Novagen, Madison, WI) according to the manufacturer’s instruction.

For cloning in the expression vector pET-43.1a(+) (Novagen, Darmstadt, Germany), the rbcLIV gene from M. aeruginosa PCC 7806 was amplified from chromosomal DNA by PCR using the forward primers 5′-AATGACTATAATTTGCAGATTACGC-3′ (no restriction site) and the reverse primer 5′-GGGATTTACCCCACCTGATTA-3′ (BamHI site underlined). The PCR product was digested with BamHI (Invitrogen) and ligated to pET-43.1a(+) digested with BamHI and PshAI (Amersham Biosciences). The ligation mixture was transformed in E. coli JM109 by a heat shock procedure (Promega Corp.), and the construct was transferred by electroporation to E. coli BL21 (Novagen, Darmstadt, Germany). Two liters of LB medium were inoculated at A600 = 0.1 with E. coli cells containing the recombinant plasmid pET-43.1arbcLIV. Upon reaching A600 = 0.5, IPTG was added to a final concentration of 1 mM to induce gene expression for 4 h. The
cells were harvested by centrifugation (8,000 \( \times \) g, 15 min, 4 °C) and kept at –20 °C until purification of the protein. The cell pellet was resuspended in 25 mM Tris-HCl buffer, pH 8.3, 5 mM dithiothreitol and sonicated on ice. After centrifugation (17,000 \( \times \) g, 15 min, 4 °C), the soluble supernatant was adjusted to a concentration of 800 mM \((NH_4)_2SO_4\) and loaded on a HiPrep 16/10 Butyl FF column (Amersham Biosciences) pre-equilibrated in 25 mM Tris-HCl buffer, pH 8.3. A step gradient of decreasing \((NH_4)_2SO_4\) concentration (800-0 mM \((NH_4)_2SO_4\) in 25 mM Tris-HCl, pH 8.3) at a flow rate of 5 ml/min was applied, and the NusA-RbcLIV fusion protein (100 kDa) was eluted at 100 mM \((NH_4)_2SO_4\). Digestion with 1 unit of thrombin protease (Novagen, Madison, WI)/mg of protein was performed overnight in 1× buffer (Novagen, Madison, WI) at room temperature. The sample was desalted and concentrated using an Amicon Ultra-15 centrifugal filter device (Millipore) prior to separation of the cleaved NusA (58 kDa) and RbcLIV proteins (42 kDa) on a Mono Q HR 10/30 column (Amersham Biosciences) using an increasing gradient of \((NH_4)_2SO_4\) (0–500 mM in 25 mM Tris-HCl, pH 8.3) at a flow rate of 1 ml/min. RbcLIV and NusA were eluted at 75 and 500 mM \((NH_4)_2SO_4\), respectively. Purified RbcLIV was finally applied to a Superdex 75 HR 10/30 column (Amersham Biosciences) in 25 mM Tris-HCl, pH 8.3, 150 mM \((NH_4)_2SO_4\) at a flow rate of 0.2 ml/min and was applied to a concentration of \(100 \mu g/ml\) of 10 μg/ml in the absence or presence of denaturant (8 M urea). A blank spectrum of buffer alone was subtracted from all spectra.

Sedimentation equilibrium experiments were carried out in a Beckman XL-A analytical ultracentrifuge with an AN60Ti rotor at 20 °C in 25 mM Tris-HCl (pH 8.2), 150 mM \((NH_4)_2SO_4\). Different protein concentrations (75, 125, 250, 500, and 1000 μg/ml) were investigated at two speeds: 13,000 and 15,000 rpm for the lower protein concentrations, and 12,000 and 15,000 rpm for the higher protein concentrations (density, 1.003). Each speed was maintained until reaching equilibrium (over 20 h). The sedimentation profiles were recorded using absorbance optics simultaneously at 230 and 235 nm for 75 and 125 μg of protein/ml, 235 and 280 nm for 250 μg of protein/ml, 280 nm for 500 μg of protein/ml, and 260 nm for 1000 μg of protein/ml. The base line was measured at 55,000 rpm. Sedimentation equilibrium data were evaluated with the programs provided by Beckman and fitted with model ideal1, assuming a unique molecular species and using a partial specific volume of the protein (v) of 0.744 ml/g as calculated from protein sequence.

**RESULTS**

* M. aeruginosa PCC 7806 Genome Carries Two Rubisco Large Subunit Genes—BLAST comparison analysis revealed that two different amino acid sequences encoded in the *M. aeruginosa* PCC 7806 genome displayed similarity to RbcL of form I. A 471-residue putative protein presented up to 92% identity with RbcL of *Synechocystis* sp. PCC 6803. The corresponding gene was named *rbcL_1*. Genes with similarity to *rbcX* and *rbcS*, often in operon structures with cyanobacterial *rbcL* genes, were found downstream. The other open reading frame is bordered by genes not related to Rubisco. This open reading frame is
shorter (386 amino acids) and more similar to the RLP members of form IV subfamily than to the other three Rubisco subfamilies. The form IV subfamily consists of sequences from very diverse Gram-negative and -positive bacteria and Archaea. The M. aeruginosa PCC 7806 RLP shows up to 40% identity to Bacillus RLPs in amino acid sequences placing it near Bacillus RLPs in phylogenetic tree (see supplemental Fig. S1). The M. aeruginosa PCC 7806 RLP gene was designated rbcLIV.

Both RbcL1 and RbcLIV are simultaneously synthesized in M. aeruginosa PCC 7806 cells (data not shown). Members of form IV RLPs have been proposed to play a role in sulfur metabolism (12–14, 19); the possibility of a sulfur regulation of the IV RLPs have been proposed to play a role in sulfur metabolism.

transcripts are 22-fold more abundant in sulfur-depleted than in sulfur-replete cultures. Conversely a 5-fold decrease of the rbcL1 transcript abundance is observed under the same conditions.

Oligonucleotide primers designed on strain PCC 7806 rbcL1 and rbcLIV sequences were used to test by PCR whether other strains of the Microcystis genus also contained two rbcL genes. Indeed a rbcL1 product was amplified for all the strains tested, and a rbcLIV product was amplified for 22 of the 25 Microcystis strains of the PCC. The results for each strain are listed in supplemental Table S2. Some sequence divergence may have prevented amplification for the three strains PCC 9804, PCC 9805, and PCC 10025. The presence of counterparts of rbcLIV was also investigated by BLAST comparison in all the available cyanobacterial genome data bases. In each of them, a unique sequence displayed 22–24% identity to RbcLIV and more than 90% identity to RbcLs of form I Rubiscos. This renders the planktonic water bloom former of the genus Microcystis rather unique in this respect.

The Recombinant RbcLIV Protein Catalyzes an Enolase Reaction—To determine the enzyme activity of the M. aeruginosa PCC 7806 RbcLIV, the corresponding gene was cloned in the pET-28a(+) system, and the purified His-tagged protein was tested in in vitro assays. The M. aeruginosa PCC 7806 RbcLIV does not catalyze carboxylation of ribulose 1,5-bisphosphate (data not shown) but is capable of 2,3-diketo-5-methylthiopentyl-1-phosphate enolization (Fig. 2). The K_m value for the substrate was calculated as 13 mM and was similar to that for the Bacillus counterpart. To confirm that M. aeruginosa PCC 7806 RbcLIV can function in vivo, we performed a complementation test using a B. subtilis RLP-deficient mutant (mtnW mut). Making use of the methionine salvage pathway, wild type B. subtilis can grow on a medium with methylthioadenosine as the sole source of sulfur, but a mtnW mutant cannot (Fig. 3). After transformation, the M. aeruginosa PCC 7806 rbcLIV gene rescues the growth of the mtnW mutant on methylthioadenosine to some extent (Fig. 3). The observed inefficient rescue of the mtnW mutant by the rbcLIV gene from M. aeruginosa PCC 7806 might be due to a low level of expression of the rbcLIV gene in the mutant. These results demonstrate that M. aeruginosa PCC 7806 RbcLIV can catalyze the 2,3-diketo-5-methylthiopentyl-1-phosphate enolase reaction both in vitro and in vivo.

Because enolization of 2,3-diketo-5-methylthiopentyl-1-phosphate is a key step in the methionine salvage pathway in bacteria (14), a BLAST search for putative counterparts of the genes encoding other enzymes of this pathway in bacteria was conducted using the genome sequence of M. aeruginosa PCC 7806 presently available. With the exception of speD, all the genes have been found (see supplemental Table S3). The methionine salvage pathway is thus most likely to be present in M. aeruginosa PCC 7806.

The Recombinant RbcLIV Is a Dimer—Because the expression of the RbcLIV protein is low with the pET-28a(+) system, the M. aeruginosa PCC 7806 rbcLIV gene was cloned in the pET-43.1a(+) system. The resulting NusA-RbcLIV protein of 100 kDa was recovered in the soluble fraction and purified (data not shown). The pure RbcLIV devoid of NusA was then used for further biochemical studies.

The secondary structure composition of the RbcLIV protein was estimated by deconvolution of the circular dichroism spec-
Two Proteins of the Rubisco Superfamily in Microcystis

Amino Acid Sequence Comparison and Three-dimensional Structure Modeling—An amino acid alignment of the form IV RLPs was performed and compared with the corresponding part of the amino acid sequences of form I RbcLs of *Synechococcus* sp. PCC 6301 and *M. aeruginosa* PCC 7806. As shown in Table 1, the Rubisco catalytic motif (Gly, Asp, Lys) is well conserved among members of the type IV subfamily with the exception of *Rhodopseudomonas palustris* (Rpa-rlp2) and *R. rubrum* (Rru), which are the first examples of RLP sequences lacking Glu. Four other residues, Lys, His, Gly, and Arg, known to be involved in the carboxylation reaction or the binding of ribulose 1,5-bisphosphate are universally present among proteins of the I–IV subfamilies. *M. aeruginosa* PCC 7806 RbcL, shares with all *Bacillus* sequences the presence of a Gly instead of a Gly in position 60, a Leu instead of a His in position 327, and an Ala in position 380 instead of a Gly. In addition, *M. aeruginosa* PCC 7806

![FIGURE 3. Complementation of the B. subtilis mtnW mutant by introduction of the M. aeruginosa PCC 7806 rbcL gene. Growth in the presence of 1 mm IPTG of wild type (filled squares), M. aeruginosa (open circles), and M. roseus (asterisks).](image)

**TABLE 1** Conservation of amino acids involved in catalysis and ribulose 1,5-bisphosphate binding in form I Rubisco proteins within the sequences of *Synechococcus elongatus* PCC 6301 form I (Syn; gi:38927, CA26972), *M. aeruginosa* PCC 7806 form I (Mae-I) and form IV (Mae-IV), and other putative bacterial form IV Rubiscos and RLPs

| Subunit I | Subunit II |
|-----------|------------|
| C | R | C |
| Glu | Thr | Asn |
| Syn | K | DC | K |
| Mae-I | Glu | Thr | Asn |
| IV | K | DC | K |
| Asf-1 | Glu | Thr | Asn |
| 2 | 177 | 177 | G104L, D104,F104,K104,D104,E104 | H394R,255, G222,D161,H257, V161, V254, S228, G241, G402, G404 |
| Mlo | Glu | Val | Gly |
| Cte | Glu | Gln | Gly |
| Rpa-rlp2 | Glu | Gln | Gly |
| Rpa-rlp1 | Glu | Cys | Asn |
| Rru | Glu | Met | Asn |
| Bbr | Glu | Thr | Asn |
| Rhu | Glu | Thr | Asn |
| Bcf | Glu | Thr | Asn |
| Bth | Gln | Ser | Lys |
| Gka | Gln | Ser | Lys |
| Bli | Gln | Ser | Lys |
| Ban | Gln | Ser | Lys |
| Bce | Gln | Ser | Lys |
| Bau | Gln | Ser | Lys |
| Mae-IV | Glu | Thr | Asn |
| Glu | Thr | Asn |
| 2 | 2 | 177 | G104L, D104,F104,K104,D104,E104 | H394R,255, G222,D161,H257, V161, V254, S228, G241, G402, G404 |
| Glu | Thr | Asn |
| Glu | Thr | Asn |
| Glu | Thr | Asn |
| Glu | Thr | Asn |
| Glu | Thr | Asn |
| Glu | Thr | Asn |
| Glu | Thr | Asn |
| Glu | Thr | Asn |
| Glu | Thr | Asn |
| Glu | Thr | Asn |

Note: Identical amino acids are shown in bold. C are the residues involved in catalysis; R are those that bind to ribulose 1,5-bisphosphate. Asf-1, *Archeaeoglobus fulgidus* DSM 4304 rbcL-1 (gi:2648975; AA868661); *S. sinorhizobium mellitii* 1021 (gi:1540252; CA58779); *Mlo, Mesorhizobium loti* MAFF303099 (gi:14036595; BAA53192); *Cte, Chlorobium limicola* f. thiosulfatophilum (gi:13173182; AAK14332); *Gka, Geobacillus kaustophilus* K334 S379 (gi:776878; ZP_00268971); *Bbr, Burkholderia bronchiseptica* RB50 (gi:74876441; ZP_00268971); *Bif, Bordetella bronchiectatica* RB50 (gi:33567621; CAE31534); *Bf, Burkholderia fungorum* LB400 (gi:89788861; ZP_00284840); *Bcl, Bacillus clausii* AAK14332; *Bce, Bacillus cereus* ESX (gi:7974924; AAU16474); *Bau, B. subtilis subsp. subtilis* strain 168 (gi:2653739; CAB13252). Accession numbers are from GenBank.
Two Proteins of the Rubisco Superfamily in Microcystis

RbcL IV has Lys, Pro, and Ser in common with other Bacillus sequences, except Bacillus clausii, in the Asn^123, Arg^295, and Lys^334 positions, respectively. To get deeper insights into the M. aeruginosa PCC 7806 RbcL IV protein, alignments of its sequence with those of forms I and II Rubiscos was refined using the hydrophobic cluster analysis method, and its three-dimensional structure was modeled on the basis of the alignment of its sequence with that of the RLP from C. tepidum (36). As shown in Figs. 4 and 5 and Table 1, Lys^201, Asp^203, and Glu^204 from the Rubisco catalytic motif (Lys^157, Asp^159, and Glu^160) in M. aeruginosa PCC 7806 RbcL IV are conserved, but Lys^334 of the photosynthetic Rubiscos is substituted by a serine residue in Bacillus and M. aeruginosa PCC 7806 sequences (Table 1 and Fig. 4). Lys^334 has been proposed to play an important role, along with Mg^2+, in the stabilization of one reaction intermediate arising from the reaction of carboxylation/oxygenation of enol-ribulose 1,5-bisphosphate (for a review, see Ref. 37). A change in amino acid residue at that position may thus have major consequences on the function of the enzyme. The secondary structural content of the three-dimensional structure model (33% α-helices and 20% β-strands) was consistent with those obtained from deconvolution of the circular dichroism spectrum.

**DISCUSSION**

This study is the first report describing a microorganism containing both a typical photosynthetic form I Rubisco and a form IV RLP that catalyzes enolization of 2,3-diketo-5-methylthiopentyl-1-phosphate, a key substrate in the methionine salvage pathway (14). In addition, the genome of M. aeruginosa PCC 7806 carries putative orthologs of the genes involved in this pathway in phylogenetically distant bacteria. To the best of our knowledge, this constitutes the first straightforward evidence for such a metabolic pathway in photosynthetic bacteria. Although a C. tepidum mutant defective for a form IV RLP has revealed impairment of sulfur metabolism (12, 38), data so far obtained indicate that RLP in this organism plays a distinct physiological role from that proposed for MtnW in Bacillus. Taken together, our results argue in favor of a role of RbcL IV in Microcystis similar to that of MtnW in Bacillus. However, whether the methionine salvage pathway is functional in Microcystis remains to be demonstrated.

A methionine salvage pathway has not been previously reported in cyanobacteria, but such a pathway is most likely a general property of Microcystis as indicated by the occurrence of the RLP gene rbcL IV in a number of strains belonging to this genus. This may reflect adaptation of Microcystis cellular metabolism to environmental conditions that cells encounter during their life cycle, in particular during water bloom periods and surface scum formation with exposure to high light intensity, O2 concentration, and temperature. The selective advantage represented by the capacity to recycle methylthioribose, a toxic by-product of polyamine synthesis excreted by bacteria (13), and simultaneously to consume oxygen could protect cells against oxidative stress and contribute to the success of Microcystis in colonizing aquatic ecosystems worldwide (39). The current absence of genes encoding RLPs in the cyanobacterial genomes publicly available indicates that, in this respect, Microcystis is rather unique in this phylum, but it is worth noting that none of these other cyanobacteria share a similar life style. Sequencing of new genomes of cyanobacteria, in particular those forming mats at the surface of which cells are exposed to atmospheric oxygen and intense sunlight in a way similar to Microcystis cells in surface scums, may reveal the presence of new RLP orthologs.

The *bona fide* function of the Rubisco enzyme consists of a three-step sequential reaction: 1) enolization, 2) carboxylation/oxygenation, and 3) hydrolysis of the substrate ribulose 1,5-bisphosphate. The first event in catalysis corresponds to the activation of the enzyme through carbamylation of Lys^201* (i.e. binding of CO2 to the ε amino group). In this reaction, chelation of metal ions, such as Mg^2+, by Asp^203 and Glu^204 cations enables entry and correct positioning of the substrate, ribulose 1,5-bisphosphate, into the catalytic pocket. The C-3 proton of the substrate is subsequently abstracted by the Lys^201 carboxylate with Lys^175 and His^294 acting as general bases promoting conversion of ribulose 1,5-bisphosphate into the intermolecular enediol (for reviews, see Refs. 37 and 40). The Microcystis and B. subtilis form IV RLPs represent a related enzyme, sharing with Rubiscos some degree of substrate specificity, because 2,3-diketo-5-methylthiopentyl-1-phosphate is structurally similar to ribulose 1,5-bisphosphate. These RLPs, however, allow solely enolization (this study and Ref. 19).

Biochemical and structural studies have shown that the secondary structure of the Microcystis RbcL IV is much better conserved than is its primary structure with content in α-helices and β-strands consistent with those observed within crystal structures of Rubiscos of different bacterial families (see supplemental Fig. S4 and Table S5) (10, 36, 41, 42). At least some elements of the tertiary structure of RbcL IV are also similar to what is observed for other members of forms I and III Rubiscos in which two tryptophan residues (Trp^31 and Trp^285 in the Microcystis amino acid sequence) are present at equivalent positions (Fig. 4 and Ref. 10). Pure Microcystis RbcL IV spontaneously assembles into dimers whatever the protein concentration (see supplemental Fig. S7) and possesses an enolase activity (Fig. 2) indicating that this oligomerization state represents the functional unit. The form IV RLPs and the form II Rubiscos, despite functional divergence, share the characteristics of associating into stable dimers *in vitro*, although the existence of higher degrees of oligomerization *in vivo* cannot be dispelled (36, 42). In addition Rubisco forms I and III assemble into L^8^S^8^ octamers and (L^2^)^5^ decamers, respectively, but the minimal unit necessary for the carboxylase/oxygenase activity in these enzymes is also a dimer with a catalytic site constituted of amino acids from two neighboring subunits (3, 10, 43). Taken together these observations argue in favor of the similarity of the mechanisms driving enolization in all forms of Rubiscos and RLPs.

The three-dimensional model we have constructed provides a structural basis for understanding the particular catalytic activity of the Rubisco and RLP enzymes by attempting to identify the set of active residues specifically involved in the enolization reaction. Indeed after a sensitive analysis allowing the accurate alignment of RbcL IV with known three-dimensional templates (the sequences of RbcL IV and three-dimensional...
templates share less than 30% identity, a level for which automatic alignment procedures do not furnish accurate alignments in many places), we have shown that despite a common structural core with 
\textit{bona fide} Rubisco, RbcLIV possesses singularities within its active site that may explain the differences observed in the catalytic activity of the enzyme (Fig. 4). These

![Alignment of the sequences of \textit{M. aeruginosa} PCC 7806 RbcLIV (Mae-IV) and \textit{B. subtilis} MtnW (Bsu) with those of spinach form I Rubisco, \textit{R. rubrum} form II Rubisco, and RLP from \textit{C. tepidum}, the three-dimensional structures of which were experimentally solved (Protein Data Bank identifiers 8RUC, 9RUB, and 1TEL, respectively). Amino acid numbers on top of the sequence alignment refer to their position in the 1TEL sequence. The observed regular secondary structures of 1TEL and 9RUB are shown above and below their sequences, respectively. Identical amino acids are shown in white on a black background, similarities are boxed, and gray shading is used for positions occupied by hydrophobic amino acids. The amino acids depicted in the three-dimensional view of Fig. 5 are shown with asterisks. This figure was prepared using ESPript (48).}

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
\textbf{1TEL} & 1KNAEV...DEYLVETLQ...GDTNTALA...CGS...T & 1MTVI...DEK...AFTG...C...G...
\textbf{Mae-IV} & 1MDENRS...LTLTEP...GATDXTKE...AENGL...V...
\textbf{Bsu} & 1MNQ...L...DEYLVETLQ...GDTNTALA...CGS...T & 1MTVI...DEK...AFTG...C...G...
\textbf{8RUC} & 1MS...EV...DEYLVETLQ...GDTNTALA...CGS...T & 1MTVI...DEK...AFTG...C...G...
\textbf{9RUB} & 1HDQ...YNV...DEYLVETLQ...GDTNTALA...CGS...T & 1MTVI...DEK...AFTG...C...G...
\hline
\textbf{1TEL} & 50 51...52...53...54...55...56...57...58...59...60...61...62...63...64...65 & 60 61...62...63...64...65...66...67...68...69...70...71...72...73...74...75...76...77...78...79...80...81...82...83...84...85...86...87...88...89...90...91...92...93...94...95...96...97...98...99...100
\textbf{Mae-IV} & 51 52...53...54...55...56...57...58...59...60...61...62...63...64...65 & 60 61...62...63...64...65...66...67...68...69...70...71...72...73...74...75...76...77...78...79...80...81...82...83...84...85...86...87...88...89...90...91...92...93...94...95...96...97...98...99...100
\textbf{Bsu} & 52 53...54...55...56...57...58...59...60...61...62...63...64...65 & 60 61...62...63...64...65...66...67...68...69...70...71...72...73...74...75...76...77...78...79...80...81...82...83...84...85...86...87...88...89...90...91...92...93...94...95...96...97...98...99...100
\textbf{8RUC} & 53 54...55...56...57...58...59...60...61...62...63...64...65 & 60 61...62...63...64...65...66...67...68...69...70...71...72...73...74...75...76...77...78...79...80...81...82...83...84...85...86...87...88...89...90...91...92...93...94...95...96...97...98...99...100
\textbf{9RUB} & 54 55...56...57...58...59...60...61...62...63...64...65 & 60 61...62...63...64...65...66...67...68...69...70...71...72...73...74...75...76...77...78...79...80...81...82...83...84...85...86...87...88...89...90...91...92...93...94...95...96...97...98...99...100
\hline
\end{tabular}
\end{table}

\textbf{FIGURE 4.} Alignment of the sequences of \textit{M. aeruginosa} PCC 7806 RbcLIV (Mae-IV) and \textit{B. subtilis} MtnW (Bsu) with those of spinach form I Rubisco, \textit{R. rubrum} form II Rubisco, and RLP from \textit{C. tepidum}, the three-dimensional structures of which were experimentally solved (Protein Data Bank identifiers 8RUC, 9RUB, and 1TEL, respectively). Amino acid numbers on top of the sequence alignment refer to their position in the 1TEL sequence. The observed regular secondary structures of 1TEL and 9RUB are shown above and below their sequences, respectively. Identical amino acids are shown in white on a black background, similarities are boxed, and gray shading is used for positions occupied by hydrophobic amino acids. The amino acids depicted in the three-dimensional view of Fig. 5 are shown with asterisks. This figure was prepared using ESPript (48).
differences, although not identical, match up with those observed in the structure inferred by the crystals of *C. tepidum* RLP, the only other RLP for which structural data are available (Fig. 5 and see Ref. 36). Presumably, and if the participation of all the RLP sequences in methionine recycling are confirmed, the catalytic site and the universally conserved residues Lys175 and His294 may represent the minimal set required for enolization of the substrate whether the latter is ribulose 1,5-bisphosphate for Rubiscos or 2,3-diketo-5-methylthiopentyl-1-phosphate for *Microcystis* and *B. subtilis* form IV RLPs, the enolases of the methionine salvage pathway.

An important structural difference between the form I/II and form IV subfamilies lies in loop 6 in which the Lys334 of the photosynthetic Rubiscos is generally substituted by another amino acid residue (Table 1 and Figs. 4 and 5). Loop 6 shifts from a retracted (open) to an extended (closed) conformation following entry of the substrate into the catalytic pocket. The closed conformation, observed in the form I Rubisco from spinach, allows covering of the ligand and subsequent hydrolysis. In contrast, in the form II Rubisco from *R. rubrum*, which does not catalyze the overall carboxylation reaction, loop 6 extends away from the active site in the complex with ribulose 1,5-bisphosphate, and this open conformation is held through crystal packing forces (44). Mutation of Lys334 causes for a loss of Rubisco form I carboxylase/oxygenase activity (37), and accordingly, the *Microcystis* RbcL IV could not catalyze the carboxylase reaction in our assay. Thus, in the RLP sequences, the substitution of the loop 6 Lys334 by another residue (a serine in *B. subtilis* and *Microcystis*) may be directly involved in the limitation of the catalysis to enolization as the resulting conformation would not allow stabilization of the reaction intermediate arising from carboxylation/oxygenation of the enediol. The effect of other amino acid variations within the active site is also worth investigation.

Of particular importance is the finding of the coexistence of both the photosynthetic Rubisco and a form IV RLP in a cyanobacterium in light of the chaotic phylogeny of the Rubisco superfamily. Indeed bacterial phylogenies based on Rubisco large subunit sequences conflict with those relying on other genes such as those of 16S and 23S ribosomal RNAs. Several explanations have been proposed including multiple independent horizontal transfers between cyanobacteria and α- or γ-proteobacteria at different periods during evolution or ancient gene duplication following selective loss of one of the copies (45, 46). The recent discovery of the form III Rubisc and form IV RLP has rendered the evolutionary study of Rubisco even more arduous. As more RLP sequences become available, the picture that emerges is that form III Rubisco possessing bona fide carboxylase/oxygenase function is more closely related to forms I and II Rubiscos than to form IV RLPs (12, 19). The form IV enzymes would therefore constitute a distinct branch in the phylogeny of the Rubisco superfamily. The robustness of this hypothesis is worth being tested in light of refined phylogenetic analyses.

The presence of an RLP encoding an enolase involved in sulfur metabolism in *Microcystis*, a cyanobacterium that is derived from organisms that were once the ancestors of the chloro-
plasts, underscores fundamental implications on the evolutionary history of the Rubisco superfamily and modulates previous discussions on this topic. Indeed the current hypothesis concerning the methionine salvage pathway was that bacteria that emerged early (such as *B. subtilis*) would function with a RLP, whereas organisms that appeared later in evolution, including cyanobacteria, would use a phylogenetically unrelated enzyme, an enolase/phosphatase (4). In addition correlate regulation by sulfur of both form I Rubisco and form IV RLP in *Microcystis* may reflect the initial relatedness of the reaction catalyzed by both enzymes and in itself opens new fascinating insights into mechanisms of regulation in cyanobacteria. This is most likely to be different from the control in *B. subtilis* (47), the only other organism for which regulation by sulfur of a RLP gene, *mtnW*, has been evidenced and relies on S-box riboswitches not found in *Microcystis*.

The present work puts forward clues to assess the catalytic relationships between *bona fide* Rubisco enzymes and RLPS. Although the availability of a primitive type of enzyme offers the rare opportunity to dissect the different steps of a catalytic mechanism, our study also provides the first evidence of the missing link between RLPS and Rubiscos and sheds light on the as yet still obscure evolution of this family of proteins.

Acknowledgments—We are very grateful to A. M. Castets, S. Ferris, C. Pichon, L. Frangeul, A. Marcel, P. Glaser, and S. Cole for the sequencing of the *Microcystis* genome. We express our gratitude to P. England, R. Nageotte, and A. Chaffotte from the “Plate-forme de Biophysique des macromolécules et de leurs interactions” for valuable discussions and technical expertise. We thank A. Danchin and J. F. Humbert for support and helpful discussions, S. Gribaldo for the phylogenetic tree construction, B. Quest for constructing the recombinant plasmid pET-28a(+)TEVIRBcl10, and T. Coursin for technical help. We also thank F. R. Tabita for the gift of RbCl form I antibodies.

REFERENCES

1. Hartman, F. C., and Harpel, M. R. (1994) *Annu. Rev. Biochem.* 63, 197–234
2. Tabita, F. R. (1988) *Microbiol. Rev.* 52, 155–189
3. Tabita, F. R. (1999) *Photosynth. Res.* 60, 1–28
4. Ashida, H., Danchin, A., and Yokota, A. (2005) *Res. Microbiol.* 156, 611–618
5. Spreitzer, R. J. (2003) *Arch. Biochem. Biophys.* 414, 141–149
6. Andersson, I., Knight, S., Schneider, G., Lindqvist, Y., Lundqvist, T., Brändén, C.-I., and Lorimer, G. H. (1989) *Nature* 337, 229–234
7. Watson, G. M. F., Yu, J.-P., and Tabita, F. R. (1999) *J. Bacteriol.* 181, 1569–1575
8. Ezaki, S., Maeda, N., Kishimoto, T., Atomi, H., and Imanaka, T. (1999) *J. Biol. Chem.* 274, 5078–5082
9. Finn, M. W., and Tabita, F. R. (2003) *J. Bacteriol.* 185, 3049–3059
10. Kitano, K., Maeda, N., Fukui, T., Atomi, H., Imanaka, T., and Mikki, K. (2001) *Structure (Lond.*) 9, 473–481
11. Maeda, N., Kanai, T., Atomi, H., and Imanaka, T. (2002) *J. Biol. Chem.* 277, 31656–31662
12. Hanson, T. E., and Tabita, F. R. (2001) *Proc. Natl. Acad. Sci. U. S. A.* 98, 4397–4402
13. Sekowska, A., and Danchin, A. (2002) *BMC Microbiology* 2, 1–14
14. Sekowska, A., Dénervaud, V., Ashida, H., Michoud, K., Haas, D., Yokota, A., and Danchin, A. (2004) *BMC Microbiology* 4, 1–17
15. Hellbronn, J., Wilson, J., and Berger, B. J. (1999) *J. Bacteriol.* 181, 1739–1747
16. Wang, S. Y., Adams, D. O., and Lieberman, M. (1982) *Plant Physiol.* 70, 117–121
17. Marchitto, K. S., and Ferro, A. J. (1985) *J. Gen. Microbiol.* 131, 2153–2164
18. Sufrin, J. R., Meshnick, S. R., Spiess, A. J., Garofalo-Hannan, J., Pan, X.-Q., and Bacchi, C. I. (1995) *Antimicrob. Agents Chemother.* 39, 2511–2515
19. Ashida, H., Saito, Y., Kojima, C., Kobayashi, K., Ogawasara, N., and Yokota, A. (2003) *Science* 302, 286–290
20. Castenholz, R. W. (2001) in *Berger’s Manual of Systematic Bacteriology* (Boone, D. R., Castenholz, R. W., and Garrity, M. G., eds) 2nd Ed., Vol. 1, pp. 473–487, Springer Verlag, New York
21. Murphy, U. (1985) *Mol. Gen. Genet.* 200, 33–39
22. Kim, L., Mock, A., and Schumann, W. (1996) *Gene (Amst.*) 181, 71–76
23. Stragier, P., Bonamy, C., and Karmazyn-Campelli, C. (1988) *Cell* 52, 697–704
24. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, pp. 1.21–1.28, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
25. Smidt, R. (1970) *Nature* 227, 680–685
26. Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997) *Nucleic Acids Res.* 25, 3389–3402
27. Kelley, L. A., MacCallum, R. M., and Sternberg, M. J. (2000) *J. Mol. Biol.* 299, 499–522
28. Sh, J., Blundell, T. L., and Mizuguchi, K. (2001) *J. Mol. Biol.* 310, 243–257
29. Gaboriaud, C., Bissery, V., Benchetrit, T., and Mornon, J. P. (1987) *FEBS Lett.* 224, 149–155
30. Callebaut, I., Labesse, G., Durand, P., Poupon, A., Canard, L., Chemli, J., Henriassat, B., and Mornon, J. P. (1997) *Cell. Mol. Life Sci.* 53, 621–645
31. Salis, A., and Blundell, T. L. (1993) *J. Mol. Biol.* 234, 779–815
32. Eisenberg, D., Lüthy, R., and Bowie, J. U. (1997) *Methods Enzymol.* 277, 396–404
33. Sippl, M. J. (1993) *Proteins* 17, 355–362
34. Li, H., Sawaya, M. R., Tabita, F. R., and Eisenberg, D. (2005) *Structure (Comb.*) 13, 779–789
35. Spreitzer, R. J., and Salvucci, M. E. (2002) *Annu. Rev. Plant Biol.* 53, 449–475
36. Hanson, T. E., and Tabita, F. R. (2003) *Photosynth. Res.* 78, 231–248
37. Mur, L. R., Skulberg, O. M., and Utken, H. (1999) in *Toxic Cyanobacteria in Water—A Guide to Their Public Health Consequences, Monitoring and Management* (Chorus, T., and Bartram, J., eds) pp. 15–40, E & FN Spon, London
38. Roy, H., and Andrews, T. J. (2000) in *Photosynthesis* (Leegood, R. C., Sharkey, T. D., and von Caemmerer, S., eds) pp. 53–83, Kluwer Academic Publishers, Dordrecht, The Netherlands
39. Newman, J., and Gutteridge, S. (1994) *Structure (Lond.*) 2, 495–502
40. Schneider, G., Knight, S., Andersson, I., Brändén, C.-I., and Lorimer, G. (1998) *Science* 276, 7023–7026
41. Deibl, S. F., and Palmer, J. D. (1996) *Mol. Biol. Evol.* 13, 873–882
42. Watson, G. M. F., and Tabita, F. R. (1997) *FEBS Microbiol. Lett.* 146, 13–22
43. Grundy, F. J., and Henkin, T. M. (1998) *Mol. Microbiol.* 30, 737–749
44. Gouet, P., Robert, X., and Courcelle, E. (2003) *Nucleic Acids Res.* 31, 3320–3323