JOURNAL OF BACTERIOLOGY, May 1991, p. 2751–2760
0021-9193/91/092751-10$02.00/0

Vol. 173, No. 9

Isolation and Characterization of a Sulfur-Regulated Gene Encoding a Periplasmically Localized Protein with Sequence Similarity to Rhodanese†

DAVID E. LAUDENBACH,[1]* DAVID EHRHARDT,[2] LAURA GREEN,[3] AND ARTHUR GROSSMAN[1]

Department of Plant Biology, The Carnegie Institution of Washington, 290 Panama Street, Stanford, California 94305[1]; Department of Biological Sciences, Stanford University, Stanford, California 94305[2]; and Division of Plant Industry, Commonwealth Scientific and Industrial Research Organisation, Canberra, ACT 2601, Australia[3]

Received 3 December 1990/Accepted 18 February 1991

During sulfur-limited growth, the cyanobacterium *Synechococcus* sp. strain PCC 7942 loses most of its photosynthetic pigments and develops an increased capacity to acquire sulfate. Sulfur deprivation also triggers the synthesis of several soluble polypeptides. We have isolated a prominent polypeptide of 33 kDa that accumulates specifically under sulfur-limiting conditions. This polypeptide was localized to the periplasmic space. The gene for this protein (designated *rhdA*) was isolated and discovered to lie within a region of the *Synechococcus* sp. strain PCC 7942 genome that encodes components of the sulfate permease system. The mRNA for the 33-kDa protein accumulates to high levels within an hour after the cells are deprived of sulfur and drops rapidly when sulfur is added back to the cultures. The amino acid sequence of the protein has similarity to bovine liver rhodanese, an enzyme that transfers the thiol group of thiosulfate to a thiophilic acceptor molecule, and a rhodaneselike protein of *Saccharopolyspora erythraea*. A strain in which *rhdA* was interrupted by a drug resistance marker exhibited marginally lower levels of rhodanese activity but was still capable of efficiently utilizing a variety of inorganic sulfur sources. The possible role of this protein in the transport of specific sulfur compounds is discussed.

The unicellular cyanobacterium *Anacystis nidulans* and the closely related *Synechococcus* sp. strain PCC 7942 undergo several changes during sulfur starvation. A specific response triggered by sulfur-limited growth is an increase in sulfate transport. This increase can be observed within 3 h after the cells have been deprived of a sulfur source. Kinetic measurements of transport have demonstrated that while the $K_{1/2}$ (concentration of sulfate at half-maximal transport rate) for transport remains the same, the V_{max} increases by approximately 20-fold (14, 18). This suggests that there is increased accumulation of a single transport system in *Synechococcus* sp. strain PCC 7942 under sulfur-limited growth conditions. This hypothesis was supported by experiments in which specific genes encoding components of the transport system were inactivated by interruption with a drug resistance marker (15, 24). These lesions resulted in mutated strains that were no longer able to grow on sulfate but could be rescued by growth on alternative sulfur sources, such as cysteine.

In addition to exhibiting an augmented level of sulfate transport, many cyanobacteria lose the majority of their pigmentation during sulfur-limited growth. The phycobilisome, the major light-harvesting complex in these organisms, is rapidly degraded (32). Additionally, there is a decrease in chlorophyll, an attenuation of the thylakoid membranes (41), and a shift in the ratios of the different carotenoids (10a). Granules composed of polyphosphate and lipid bodies containing β-hydroxybutyrate may also accumulate (1, 19, 25). Lastly, a number of polypeptides increase dramatically during the acclimation process (15); some increase to very high levels during sulfur stress. The series of physiological changes that *Synechococcus* sp. strain PCC 7942 experiences in a sulfur-limited environment terminate when the cells enter a dormant state, in which they can remain viable for an extended period of time.

Recently, we have isolated a cluster of genes that are activated during conditions of sulfur deprivation. Included in this cluster are genes that encode the sulfate transport system (24), a permease system that resembles the periplasmic transport systems of the enteric bacteria. The system is composed of a sulfate-binding protein, two proteins that probably form a channel in the inner cell membrane, and a nucleotide-binding protein that hydrolyzes ATP, thereby generating energy for the transport of the nutrient through the channel and into the cell against a concentration gradient (3, 4, 6). The nucleotide-binding protein gene is transcribed divergently from the three genes encoding the other components of the transport system. Additional genes in this region encode proteins of other permease systems and regulatory components that might function in controlling the activities of genes required for the acclimation process.

The work presented in this article demonstrates that a prominent 33-kDa polypeptide that accumulates to high levels in cells experiencing sulfur-limited growth is localized to the periplasmic space and encoded by a gene (designated *rhdA*) adjacent to genes encoding the sulfate permease. RNA transcribed from this gene accumulates only during sulfur-limited growth and is rapidly degraded when sulfur is added back to the growth medium. The deduced amino acid sequence encoded by *rhdA* has some similarity to the enzyme rhodanese. Since we have not been able to establish a clear phenotype for a strain of *Synechococcus* sp. strain PCC 7942

* Corresponding author.
† Carnegie Institution of Washington publication 1089.

in which *rhdA* has been inactivated, its role in the acclimation process is uncertain.

MATERIALS AND METHODS

Reagents. All chemicals were of reagent grade. DNA restriction and modification enzymes were from Pharmacia, United States Biochemical Corporation, or Boehringer Mannheim. Reagents for dideoxy chain termination sequencing of DNA fragments were from United States Biochemical Corporation.

Growth conditions. Axenic cultures of *Synechococcus* sp. strain PCC 7942 were grown in liquid and solid (1.0% agar) BG-11 (2) medium buffered to pH 8.0 with 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid). Liquid cultures were grown at 29°C in 50-ml culture tubes bubbled with 3% CO_2. Illumination was from incandescent bulbs at 60 microEinsteins/m^2/s. When appropriate, the growth medium was supplemented with spectinomycin (25 μg/ml) and ampicillin (1 μg/ml). *Escherichia coli* strains DH5α (Bethesda Research Laboratories) and JM109 were used as hosts for the plasmid libraries and constructions.

Purification of the 33-kDa protein. Cells were broken in a French pressure cell at 16,000 lb/in^2 in 20 mM HEPES, pH 8.0. The membranes were pelleted by centrifugation at 40,000 × *g* for 30 min. The 33-kDa protein remained with the membrane fraction. The membranes were then resuspended in 20 mM HEPES (pH 8.0)–100 mM NaCl and centrifuged again for 30 min at 40,000 × *g*. Under these conditions, the 33-kDa protein was released from the membranes and remained in the supernatant fraction. The supernatant was decanted into a second tube and brought to 50% saturation with ammonium sulfate. After 1 h on ice, the ammonium sulfate-treated sample was centrifuged at 10,000 × *g* for 10 min, and the supernatant was made 70% ammonium sulfate. Most of the protein was present in the pellet of the 50 to 70% ammonium sulfate fraction. The pellet was resuspended in 50 mM Tris-HCl, pH 9.0, dialyzed against the same buffer, and then passed through a QAE Sepharose G-50-150 column equilibrated to pH 9.0 with 50 mM Tris-HCl. Under these conditions, the 33-kDa protein did not bind to the column.

SDS-PAGE. Protein samples were precipitated by the addition of trichloroacetic acid to 10%. The samples were prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (14) and resolved by electrophoresis in a linear 12 to 18% polyacrylamide gradient gel with the buffer system of Laemmli (22). The protein bands were visualized by staining with Coomassie brilliant blue G-250.

Preparation of antibody and Western immunoblot analysis. Purified 33-kDa protein was resolved by SDS-PAGE, and the protein band was excised from the gel, electroeluted, and used to inject rabbits by the schedule of Chua and Blomberg (10). Immunoglobulin G (IgG) was purified by chromatography over DEAE Affigel Blue (Bio-Rad) as described by the manufacturer, concentrated by ammonium sulfate precipitation (25 g/ml), and stored at 4°C in 10 mM Tris-HCl (pH 8.0)–100 mM NaCl–0.02% sodium azide. For Western blot analyses, the proteins were transferred from polyacrylamide gels to nitrocellulose by the method of Towbin et al. (38) and stained immunologically with monospecific antibodies raised against the 33-kDa protein and protein A-conjugated horseradish peroxidase (Boehringer Mannheim).

Immunocytochemistry. Both sulfur-starved and unstarved cells were embedded in 2% agarose at 65°C, which, after cooling, was cut into cubes (1 by 1 by 1 mm). The cubes were placed for 1 h in electron microscopy-grade glutaraldehyde in 50 mM sodium cacodylate, pH 7.4, at 4°C. Samples were dehydrated for 15 min each in 30, 50, 70, and 95% ethanol and then three times for 15 min each in 100% ethanol. All of the ethanol incubations were performed at −20°C except for the 30% ethanol incubation, which was done at 0°C. The cells were then infiltrated with Lowicryl K4M (Chemische Werke Lowi) under nitrogen at −20°C as follows: (i) 30-min incubation in 1:1 Lowicryl K4M-ethanol, (ii) 30-min incubation in 100% Lowicryl K4M, and (iii) 16-h incubation in 100% Lowicryl K4M. The cubes were then placed into gelatin capsules and covered with fresh Lowicryl. The resin was polymerized by placing the capsule 10 cm from a short-wavelength UV light source for 4 h at −20°C and then cured for 3 days at room temperature. Thin sections were cut with a diamond knife on an LKB Ultramicrotome III and floated onto Formvar-coated copper slot grids. The grids were placed in 50 mM NH_4Cl for 12 min to block unreacted free aldehydes and then for 30 min in 5% normal goat serum in phosphate-buffered saline (PBS).

Antibodies raised against the 33-kDa protein were diluted 1:200 in 2% normal goat serum in PBS with 2.5% saponin and incubated for 6 h at room temperature. Excess antibody was removed from the sections by four washes (12 min each) in PBS. The washed sections were incubated for 1 h in goat anti-rabbit IgG conjugated to 10-nm gold particles (Janssen Pharmaceutica, Beerse, Belgium) at a dilution of 1:300 in 30% normal goat serum–PBS–2.5% saponin. The sections were washed three times in PBS and three times in water and then poststained with osmium tetroxide vapors for 15 min at room temperature before the samples were viewed with a Philips 410 transmission electron microscope (TEM) operating at 80 kV.

Osmotic shock procedure. Cells (300 ml, at approximately 6 × 10^8/ml) were used to prepare a periplasmic fraction by osmotic shock (7). The cells were harvested at 3,500 × *g* for 5 min and resuspended in 10 ml of 40% sucrose containing 30 mM Tris-HCl, pH 7.5, and 2 mM EDTA. Following 30 min of gentle agitation at room temperature, the cells were pelleted by centrifugation at 8,000 × *g* for 10 min and resuspended in 5 ml of ice-cold 30 mM Tris-HCl, pH 7.5. The suspension was incubated on ice for 10 min, the cells were pelleted at 6,000 × *g* for 5 min, and the supernatant was saved and brought to 3 mM $MgCl_2$, 1 mM benzamidine-HCl, 1 mM phenylmethylsulfonyl fluoride, and 1 mM ε-aminocaproic acid.

Amino-terminal sequencing. After purification as described above, the 33-kDa polypeptide was resolved by preparative electrophoresis (22) on a 7.5 to 15% polyacrylamide gradient gel and electroeluted from the gel in a bicarbonate buffer system (17). The protein was subjected to automated Edman degradation on an Applied Biosystems model 470A gas-phase microsequencer.

Cloning, insertional inactivation, and sequence analysis. Genomic DNA was extracted by the method of Tandeau de Marsac et al. (37). For Southern hybridizations, DNA fragments were transferred from agarose gels to nitrocellulose filters as described by Maniatis et al. (26). Radiolabeling of the DNA fragments was performed by primer extension of random oligonucleotides (13), and DNA-DNA hybridizations were performed by the method of Conley et al. (11). The DNA sequence encoding the 33-kDa polypeptide was discovered in a λEMBL-3 clone of *Synechococcus* sp. strain PCC 7942 that had been isolated previously by homology to the *cysA* locus of *Salmonella typhimurium* (21). The gene was determined to encode the 33-kDa protein by compari-

sons of the open reading frame with the amino-terminal sequence of the isolated protein. A restriction map of the region was generated, as shown in Fig. 4B, and the DNA fragments were subcloned into M13mp18 or M13mp19. Synthetic oligonucleotides were used as sequencing primers when no suitable restriction sites for subcloning were available. The DNA sequence was determined by the dideoxy chain termination method (30) with the modified bacteriophage T7 DNA polymerase (Sequenase; United States Biochemical). Ambiguities in the G+C-rich regions were resolved by substituting dI for dG in the sequencing reactions.

RNA isolation and Northern (RNA blot) analysis. RNA was isolated as described by Conley et al. (11) with the following modifications. After the second precipitation, the RNA was resuspended in sterile 100 mM EDTA, pH 8.0, and the solution was brought to 0.4 g of CsCl per ml, layered over a cushion of 5.7 M CsCl in 0.1 M EDTA, pH 7.0, and centrifuged in a swinging-bucket rotor (Tst41.14) for 16 h at 132,000 × *g*. The RNA pellet was drained, resuspended in TE (10 mM Tris, 1 mM EDTA [pH 7.6]), extracted with phenol, precipitated with ethanol, resuspended in sterile water, and stored at −80°C.

RNA species were resolved by electrophoresis in a 1.5% agarose gel under denaturing conditions (11). The RNA was transferred to nitrocellulose filters, and Northern hybridizations were performed as described previously (11). The sizes of the hybridizing bands were estimated by using RNA size markers ranging from 1.77 to 0.23 kb (Bethesda Research Laboratories).

Sulfate transport assay. Sulfate transport assays were performed as previously described (14).

Rhodanese and thiosulfate reductase assays. Rhodanese (thiosulfate-cyanide sulfur transferase; EC 2.8.1.1) activity was estimated from colorimetric measurements of the formation of thiocyanate from cyanide and thiosulfate (35). The reaction mixture contained 50 mM sodium thiosulfate, 50 mM potassium cyanide, 100 μl of crude enzyme extract, and 0.1 M Tris-HCl, pH 8.6, in a final volume of 2.0 ml. The mixture was preincubated at 30°C for 5 min, and the reaction was initiated by the addition of sodium thiosulfate. The reaction was stopped after 30 min by the addition of 500 μl of 38% formaldehyde, and the formation of thiocyanate was monitored after the addition of 2.5 μl of ferric nitrate reagent [100 g of $Fe(NO_3)_3 \cdot 9H_2O$ and 200 μl of 65% HNO_3 per 1,000 ml] by the formation of the red-brown acidic iron-thiocyanate complex. The sample was centrifuged for 3 min at 14,000 × *g*, and the A_{460} was measured in a Beckman DU-62 spectrophotometer. Spontaneous rates of thiocyanate formation were determined by omitting the crude enzyme extract from the reaction mixture. The A_{460} of the crude enzyme extract was measured by adding 500 μl of 38% formaldehyde to the assay mixture prior to the addition of sodium thiosulfate. Rates determined at various times following the initiation of the reaction remained linear for at least 20 min.

Thiosulfate reductase activity was estimated by the pararosaniline procedure described by Koj (20). A standard reaction mixture contained 50 mM Tris-HCl, pH 9.0, 20 mM sodium thiosulfate, 10 mM glutathione, and 100 μl of crude enzyme extract in a final volume of 1 ml. Solution A, containing 100 mM Tris-HCl, pH 9.0, and 40 mM sodium thiosulfate, and solution C, containing 20 mM glutathione, were incubated separately for 5 min at 37°C. The pH of solution C was adjusted to 9.0 by the addition of NaOH; solution C was then combined with solution A, and the reaction was initiated by the addition of crude enzyme extract. After incubation at 37°C for an appropriate time period (the reaction was linear for at least 20 min), the reaction was quenched by the addition of an equal volume of 0.23 M mercuric chloride. The resulting precipitate was pelleted by centrifugation for 5 min at 14,000 × *g*, and 0.5 ml of the supernatant was removed to estimate the sulfite content (20). A 2-ml amount of 0.04% pararosaniline hydrochloride in 0.72 M HCl and 2 ml of 0.2% formaldehyde were added to the supernatant, and after a 5-min incubation at room temperature, the A_{570} was determined. Control values were obtained by adding mercuric chloride to the reaction mixture before the addition of glutathione.

To prepare crude enzyme extracts, cells from a 40-ml culture of *Synechococcus* sp. strain PCC 7942 were collected by centrifugation, washed with 5 mM Tris-HCl (pH 8.6)–2 mM $MgCl_2$, and resuspended in 4 ml of wash buffer. The cells were broken by passage through a French pressure cell at 16,000 lb/in^2, and the extract was used directly in enzyme assays.

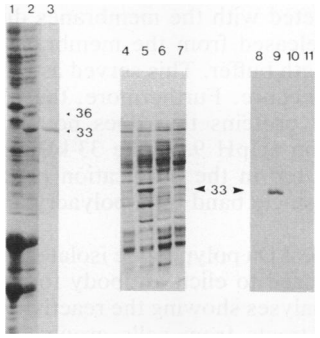

FIG. 1. Occurrence of the 33-kDa polypeptide in sulfur-deprived cells. Soluble polypeptides from cells grown on complete medium (lane 1) or for 24 h in medium lacking sulfur (lane 2) were electrophoresed in a 12 to 18% polyacrylamide gradient gel. Purified protein (lane 3) was isolated as described in Materials and Methods. Proteins were also isolated from sulfur-sufficient (lane 4), sulfur-deprived (lane 5), nitrogen-deprived (lane 6), and phosphorus-deprived (lane 7) cells. Western blot analysis of soluble proteins from sulfur-sufficient cells (lane 8) and cells deprived of sulfur (lane 9), nitrogen (lane 10), and phosphorus (lane 11) was also done. The arrows show the positions of the sulfur-regulated proteins with apparent molecular masses of 33 and 36 kDa. The molecular mass markers used to determine the masses of the different proteins were: phosphorylase *b*, 92.5 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 45 kDa; bovine carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; and lysozyme, 14.4 kDa.

RESULTS

The major polypeptides that accumulate when *Synechococcus* sp. strain PCC 7942 is deprived of sulfur are shown in Fig. 1 (compare lanes 1 and 2). Two of the major soluble polypeptides synthesized during sulfur stress, indicated with arrows in Fig. 1, have molecular masses of 36 and 33 kDa. Generally, the 33-kDa polypeptide becomes the most prominent protein in sulfur-deprived cells. To probe the function of this protein, we purified it from *Synechococcus* sp. strain PCC 7942 after starving the cells for 24 h and used it for the preparation of monospecific antibodies. While the details of the protein purification are described in Materials and Methods, the characteristics of the polypeptide most important in its purification were its behavior under different salt conditions and its basic isoelectric point. At low ionic strength,

the protein pelleted with the membranes during centrifugation and was released from the membrane fraction with a high-ionic-strength buffer. This served as the first step in the purification procedure. Furthermore, the 33-kDa protein is one of the few proteins that does not bind to an anion-exchange column at pH 9.0. The 33-kDa protein obtained after this final step in the purification procedure could be visualized as a single band on a polyacrylamide gel (Fig. 1, lane 3).

The purified 33-kDa polypeptide isolated from a polyacrylamide gel was used to elicit antibody formation in rabbits. Western blot analyses showing the reactivity of the antibody with protein extracts from cells grown with and without sulfate are presented in Fig. 1 (stained, soluble proteins in lanes 4 and 5, Western blot analyses in lanes 8 and 9). The antibodies reacted with the 33-kDa soluble polypeptide, which was present in protein extracts from sulfur-starved cells but not in extracts from sulfur-sufficient cells. The 33-kDa protein was not immunologically detected in protein extracts of cells starved for other nutrients, such as nitrogen (Fig. 1, lanes 6 and 10) and phosphorus (Fig. 1, lanes 7 and 11). Hence, the increased accumulation of this protein is a specific response to sulfur deprivation and is not a general stress response.

The monospecific antibodies were used to localize the 33-kDa protein in the cell. Figure 2 shows electron micrographs of both unstarved (Fig. 2A) and starved (Fig. 2B) cells. When *Synechococcus* sp. strain PCC 7942 was grown on complete salt medium, there were three or four prominent, concentric rings of thylakoid membranes in the cytoplasm. The cytoplasm of sulfur-starved cells was less densely stained, and the internal photosynthetic membranes were reduced to a single layer. As reported previously (19, 32), both the light-harvesting phycobiliproteins and chlorophyll molecules were extremely diminished in bleached cells. Both starved and unstarved cells were immunocytochemically stained to define the site of function of the 33-kDa protein. Unstarved cells showed little affinity for the antibody, with low levels of randomly distributed colloidal gold particles over the entire grid (results not shown). In sulfur-starved cells, the colloidal gold particles were concentrated at the cell periphery (Fig. 2C), suggesting that the protein is either in the periplasmic space or associated with the outer or inner membrane of the cell envelope.

To further define the location of the 33-kDa protein, cells were subjected to osmotic shock treatment, which causes the release of periplasmic proteins (3, 7). Total soluble proteins from both sulfur-sufficient and sulfur-deficient cells are shown in Fig. 3, lanes 1 and 2, respectively. In this experiment, a short period of sulfur starvation (approximately 3 h) accounted for the low level of both the 33- and 36-kDa polypeptides in soluble protein extracts from starved cells. Both the 36- and 33-kDa proteins, indicated with arrows, were released from the cells after osmotic shock treatment and were only present in preparations of shock-released proteins from sulfur-deprived cells. Immunological analyses of both total soluble and osmotic shock-released proteins (Fig. 3, lanes 5 to 8) confirmed that the 33-kDa polypeptide was released from the cells after the treatment. Sometimes a higher-molecular-mass species of approximately 35 kDa in extracts from starved cells reacted with the antibody, as shown in Fig. 3, lane 6. This species, which was not detected in the periplasmic protein fraction (Fig. 3, lane 8), may represent low levels of unprocessed precursor present in the cell. The data presented in Fig. 3 support the immunocytochemical results and suggest that the 33-kDa protein resides in the periplasmic space. It may be loosely associated with the cell envelope since it can be removed from membrane preparations by high-ionic-strength buffers.

To help isolate and identify the gene encoding the 33-kDa polypeptide, the amino terminus of the isolated protein was determined. This sequence is shown in line C of Fig. 4. Previously, we had discovered a region of the *Synechococcus* sp. strain PCC 7942 genome that was transcriptionally activated during sulfur stress (Fig. 4, line A). This region encodes the sulfate transport system of *Synechococcus* sp. strain PCC 7942 plus other genes that might be involved in the acquisition and utilization of other sulfur compounds. Details describing the organization and function of these genes have been published previously (15) or in the accompanying article (24). The amino acid sequence deduced from one of these genes, designated *rhdA*, was identical to the amino-terminal sequence of the isolated 33-kDa polypeptide. A restriction map of the *rhdA* gene showing the orientation of the transcript and the sequencing strategy is presented in Fig. 4B.

The sequence of the *rhdA* gene and the deduced amino acid sequence of the gene product were determined by sequencing both strands of the DNA (Fig. 5). A potential ribosome-binding site (GAGG, underlined in Fig. 5) preceded the initiator methionine by seven nucleotides. The deduced protein sequence establishes the presence of a putative leader peptide which terminates after Ala-37. The presence of a putative leader sequence supports the observations concerning the subcellular location of the protein in the periplasmic space. The molecular mass of the mature protein, as determined from the deduced amino acid sequence, is 31 kDa, which is close to the apparent molecular mass of 33 kDa estimated from polyacrylamide gel electrophoresis. The calculated pI of the mature protein is 9.1; the basic pI explains why the protein passes through a QAE Sepharose column at pH 9.0.

The abundance of the 33-kDa protein would suggest that the transcript accumulates to high levels in sulfur-stressed cells. Northern blot analyses showing the presence of the transcript in sulfur-starved cells are presented in Fig. 6A. Many transcripts in cyanobacteria give a smeared banding pattern because the transcripts are rapidly degraded. The 1.1-kb transcript from the *rhdA* gene was distinct and very abundant. This is probably due both to a high rate of transcription and to an increased stability of the mRNA (unpublished data). Although we have not mapped the 3′ end of the transcript, a potential stem-loop structure in the RNA, underlined with arrows in Fig. 5, is located 36 nucleotides downstream of the termination codon. This structure may play a role in either transcription termination or stabilizing the transcript by limiting nucleolytic processes.

The mRNA for the 33-kDa polypeptide, detected only in cells deprived of sulfur, attained high levels even after 1 h of starvation for sulfur (Fig. 6A, compare lanes 1 and 2). These levels were maintained for over 12 h (Fig. 6A, lane 6). During this starvation period, the levels of other transcripts, such as those encoding the light-harvesting protein phycocyanin, dropped rapidly, and such transcripts were barely detectable after 12 h (Fig. 6B).

The sequence of the RhdA protein, as deduced from the gene sequence, was compared with sequences in the GenBank and NBRF data bases. As shown in Fig. 7, there is similarity between the *rhdA* gene product of *Synechococcus* sp. strain PCC 7942, bovine liver rhodanese (29), and a rhodaneselike protein from *Saccharopolyspora erythraea* (12). Rhodanese catalyzes the transfer of sulfur from a

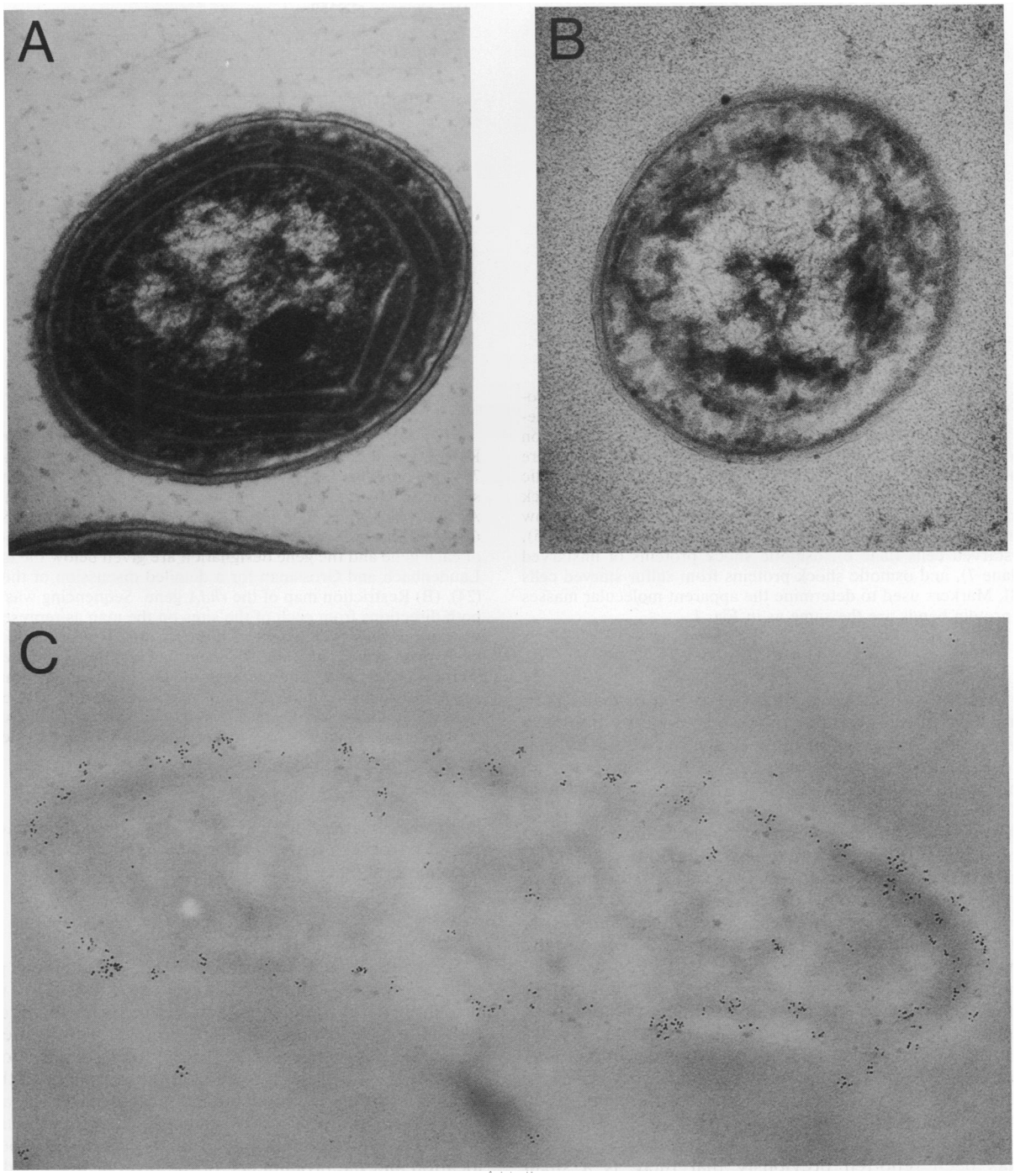

FIG. 2. Electron micrographs of cells grown in the presence and absence of sulfur. Cells were grown either in sulfur-replete medium (A) or for 24 h in medium devoid of sulfur (B). Immunocytochemistry with colloidal gold particles was used to localize the 33-kDa protein in cells grown in sulfur-deficient (C) medium (24-h starvation period). Magnification: (A and B) ×76,000; (C) ×43,000.

sulfur-containing compound such as thiosulfate to a thiophilic acceptor molecule such as cyanide (43). The similarity to bovine liver rhodanese was approximately 26% and was present throughout the molecule. Furthermore, the two proteins were similar in size, and the active site of rhodanese (Cys-247) is conserved in RhdA (27, 42). Additionally, the RhdA polypeptide is approximately 21% identical to the rhodaneselike protein (designated Orf2) of *S. erythraea* (12).

To test the function of the 33-kDa protein in the utilization of sulfur-containing compounds, we interrupted the *rhdA* gene by in vitro cartridge mutagenesis. The region of the gene between the *Sma*I sites was deleted (Fig. 4B) and replaced with a spectinomycin resistance gene from pHP45Ω (28). Wild-type *Synechococcus* sp. strain PCC 7942 was transformed with this construct, and the resulting spectinomycin-resistant transformants were restreaked to single col-

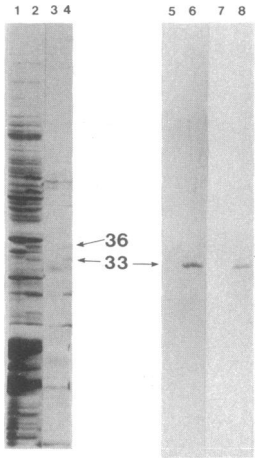

FIG. 3. Immunological analysis of soluble and periplasmic proteins. Total soluble protein from sulfur-sufficient and sulfur-deprived cells and proteins of the periplasmic space were resolved on a 12 to 18% polyacrylamide gradient gel. Protein profiles shown are from unstarved cells (lane 1), sulfur-starved cells (lane 2), osmotic shock proteins from unstarved cells (lane 3), and osmotic shock proteins from sulfur-starved cells (lane 4). Lanes 5 to 8 show Western blots of soluble proteins from unstarved cells (lane 5), sulfur-starved cells (lane 6), osmotic shock proteins of unstarved cells (lane 7), and osmotic shock proteins from sulfur-starved cells (lane 8). Markers used to determine the apparent molecular masses of the protein bands are the same as in Fig. 1.

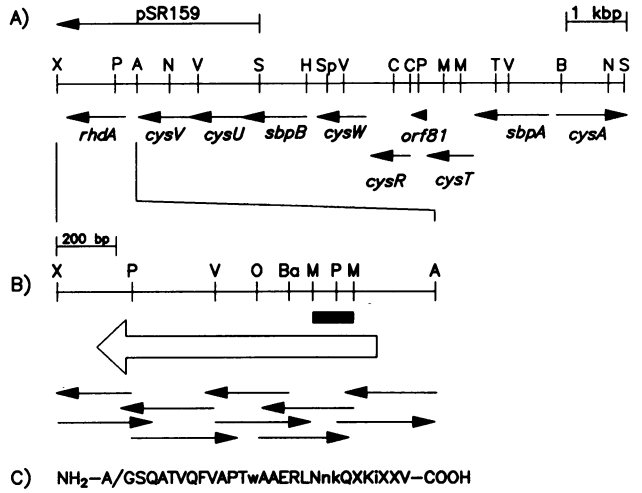

FIG. 4. *rhdA* gene in a cluster of sulfur-regulated genes. (A) Restriction map of a region of the *Synechococcus* sp. strain PCC 7942 genome that is regulated by sulfur availability. The restriction sites shown on the map are abbreviated as follows: X, *Xba*I; P, *Pst*I; A, *Sca*I; N, *Nae*I; V, *Eco*RV; S, *Sal*I; H, *Hin*dIII; Sp, *Sph*I; C, *Cla*I; M, *Sma*I; T, *Stu*I; B, *Bgl*II; O, *Xho*I; Ba, *Bam*HI. The extent of each gene and the gene designation are given below the map. See Laudenbach and Grossman for a detailed discussion of this region (24). (B) Restriction map of the *rhdA* gene. Sequencing was done in both directions from each of the sites on the map as represented by the dark arrows. The extent of the transcript is represented by the large open arrow. The *Sma*I fragment that was deleted in the mutant strain is shown as a solid rectangle under the map. (C) Amino acid sequence from the amino terminus of the isolated 33-kDa polypeptide. Capital letters indicate unambiguous residues; lowercase letters indicate residues with weaker signals. The first residue was determined to be either A or G.

onies to ensure that the mutants were homozygous (44). Confirmation of the gene disruption was done by Southern hybridizations. As shown in Fig. 8B, the *rhdA* gene hybridized to a 3.5-kb *Eco*RV genomic DNA fragment from the mutant strain and a 1.7-kb *Eco*RV fragment from the wild type. This 1.8-kb change in fragment size reflects both the integration of the 2.0-kb spectinomycin resistance gene and the loss of the 0.16-kb *Sma*I fragment. An additional hybridization signal of approximately 9.0 kb was detected in both strains and resulted from hybridization to DNA downstream of the *Eco*RV site located in the 3′ end of the *rhdA* gene. Wild-type copies of the *rhdA* gene were not detected in the mutant strain.

Total soluble proteins were isolated from the *rhdA* mutant grown in the presence and absence of sulfate and analyzed by polyacrylamide gel electrophoresis (Fig. 8A). As expected, the 33-kDa polypeptide was not present in the *rhdA* mutant. However, the mutant strain grew at approximately the same rate as wild-type cells on sulfate (300 or 40 μM initial concentration), thiosulfate (300 or 20 μM), and tetrathionate (300 or 10 μM).

It has been reported previously that there is a small increase in rhodanese activity in cyanobacteria deprived of sulfate (31). When *Synechococcus* sp. strain PCC 7942 was deprived of sulfur, it exhibited a small increase in both rhodanese and thiosulfate reductase activities (results not shown). While the *rhdA* mutant did not show an increase in the activities of these enzymes during growth in sulfur-limited medium, the levels of the enzymes were not significantly different between the mutant and wild-type strains. Additionally, the purified 33-kDa protein had low levels of rhodanese activity but failed to show any thiosulfate reductase activity. These results suggest that the *rhdA* gene product has only low levels of rhodanese activity and that this catalytic activity may not represent its primary function in the acclimation process. The rhodaneselike protein encoded by *orf2* in *S. erythraea* also exhibited essentially no rhodanese activity (12).

DISCUSSION

We have defined the localization of a prominent protein with an apparent molecular mass of 33 kDa that is only present in sulfur-limited cells and have isolated and characterized the gene encoding this protein. This protein can constitute between 1 and 3% of the total soluble protein in sulfur-starved cells. It is located at the periphery of the cell, as determined by immunocytochemical localization, and can be released from the cell by osmotic shock treatment (usually indicative of periplasmic proteins). Both the subcellular location and the prominence of the protein suggested that it might be a substrate-binding protein, specific for a sulfur-containing compound. Periplasmic substrate-binding proteins in the enteric bacteria can constitute as much as 1% of total cellular protein.

In the process of attempting to isolate the gene encoding this protein, we discovered that it was contiguous to a cluster of sulfur-regulated genes important for sulfate uptake. Analysis of the *rhdA* gene and transcript provided some insights into both the biosynthesis and function of the 33-kDa protein. The protein is probably synthesized as a precursor, with a presequence of 37 amino acids and a cleavage site that conforms to the rules of von Heijne (40). This is rather long for a signal peptide, although long

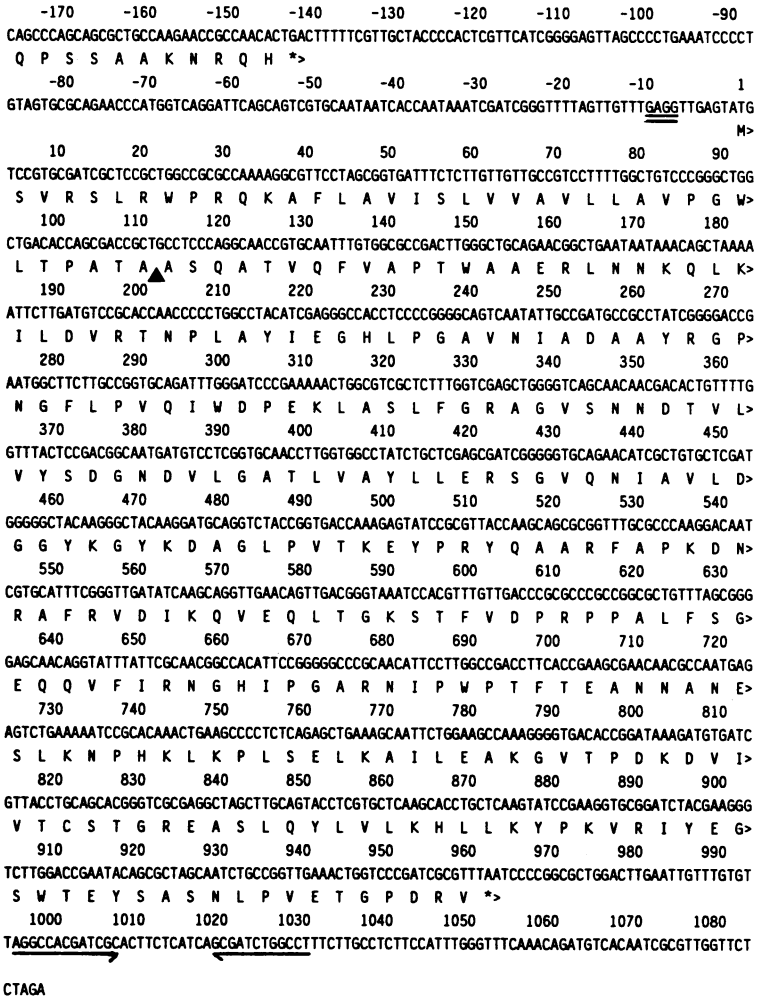

FIG. 5. Nucleotide sequence of the *rhdA* gene. The protein probably begins at the methionine codon located at nucleotide 1 (discussed in text). A putative ribosome-binding site (double underlined) precedes the initiator methionine by seven nucleotides. A solid arrowhead indicates the signal peptide cleavage site. Nucleotides with the potential to form a hairpin-loop structure at the 3′ end of the gene, beginning 31 nucleotides past the termination codon, are underlined with arrows.

presequences have been found for other cyanobacterial proteins targeted to the lumen of the thylakoid membranes (39). The initiator Met codon is preceded by a cyanobacterial ribosome-binding site (GAGG) by the optimal distance of seven nucleotides. Although this Met codon is likely to be the site of translation initiation, translation in cyanobacteria can also initiate at Val codons. From the deduced protein sequence, codons for Val are located at positions −36 and −22 relative to the first amino acid of the mature polypeptide. A possible ribosome-binding site was observed for Val-36 (GAGT positioned six nucleotides from the CGT valine codon); however, no likely ribosome-binding site was observed for Val-22.

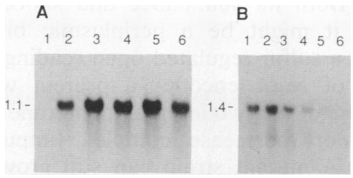

FIG. 6. Northern analysis of RNA from cells after various periods of sulfur stress. Approximately 5 μg of total RNA was applied to each lane. (A) RNA from cells grown in complete medium (lane 1) and cells grown in medium devoid of sulfur for 1 (lane 2), 2 (lane 3), 4 (lane 4), 6 (lane 5), and 12 (lane 6) h were hybridized to an *rhdA* gene-specific probe. (B) The RNA in lanes 1 to 6 was the same as that used in lanes 1 to 6 of panel A, but it was hybridized to a probe specific for the phycocyanin gene (*cpcBA*) (23). The sizes of the transcripts (in kilobases) are shown to the left of each panel.

The gene sequence also establishes the presence of a hairpin-loop structure that begins 36 nucleotides after the TAA termination codon. Hairpin-loop structures in the RNA have been implicated both in transcription termination and in altering transcript stability. In *Rhodobacter capsulatus*, such a structure might be important for limiting the action of a 3′ exonuclease, thereby extending the half-life of the segment of the transcript encoding the bacterial light-harvesting proteins (5, 9). Hairpin-loop structures have also been implicated in the maintenance of stability of chloroplast transcripts (36). In this case, these structures are often associated with proteins that have been hypothesized to

```
Orf2(15)   nlntdgvvfA evdedttayd gghipgaikl dwknelqdHv rrd------- ---------- -fvnrEgfek LlsakGigNd
RhdA       ASQATVQFVA PTWAAERLNN KQLKILDVRT NPLAYIEGHL PGAVNIADAA YRGPNGFLPV QIWDPEKLAS LFGRAGVSNN
Rhd (12)   kwlAesvrag kvgpglrvld aswyspgtRe arkeYlErHv PGAsffdiee cRdkaspyeV mlpseagfAd yvGslGiSNd

Orf2       DTVilYggnN --nwfAayay wyfklyGhsd vklLDGGrKk weldGrelTK EePnraAtay kaqepdAsir afrdeVvdai
RhdA       DTVLVYSDGN D-VLGATLVA YLLERSGVQN IAVLDGGYKG YKDAGLPVTK EYPRYQAARF APKDNRAFRV DI-KQV-EQL
Rhd        thVvVYngdd lgsfyAprVw wmfrvfGhrt vsVLnGGfrn wlkeGhPVTs EpsRpepAiF katlNRsllk ty-eQVlEnL

Orf2       gnKnlvdVrs pdefagklla pahlpQesaq RaGHIPsAiN vPWskaa-ne dgtf------ --KsdeELKq vygeaGldtD
RhdA       TGK-STFVDP RPP--AlFSG ----EQQVFI RNGHIPGARN IPWPTFTEAN NANESLKNPH KLKPLSELKA ILEAKGVTPD
Rhd        esKrfqlVDs Raq--grylG tqpepdaVgl dsGHIrGsvN mPfmdFl-te NgfE------ --KspeELrA mfEAKkVdlt

Orf2       KDtIayCriG ersShtwfVL reLLghtnVk nYdGSWTEYg slvgvpienP qeqga
RhdA       KDVIVTCSTG REASLQYLVL KHLLKYPKVR IYEGSWTEYS ASNLPVETGP DRV
Rhd        KplIaTCrkG vtAchiaLaa -yLcgkpdVa IYdGSWfEwf hrapPetwvs qgkg
```

FIG. 7. Comparison of the *rhdA* gene product with bovine liver rhodanese (Rhd) and a rhodaneselike protein (Orf2) from *S. erythraea*. Regions of identity or conservation are indicated by asterisks and capital letters, respectively. Dashes denote gaps introduced to maximize the sequence alignment.

change the susceptibility of the mRNAs to nucleolytic attack.

Finally, the RhdA polypeptide shows some similarity to bovine liver rhodanese and a rhodaneselike protein of *S. erythraea*. The identity to rhodanese is approximately 26% along the entire sequence. Although a specific biological function for rhodanese is not known, it is a ubiquitous enzyme that catalyzes the cleavage of a sulfane bond and the transfer of a thiol group to any of a number of thiophilic acceptor molecules. It has been suggested that the enzyme plays a role in the detoxification of certain molecules, such as cyanide and sulfide (43), and that it may have the capacity to serve as a thiosulfate reductase (43). It may also perform the oxidation of thiosulfate to sulfate in such organisms as *Chromatium* spp. and *Thiobacillus novellus* (34). It has even been suggested to play a role in the insertion of sulfur into proteins containing labile sulfur molecules (8). While the deduced amino acid sequence of the 33-kDa protein presented here does have some similarity to bovine liver rhodanese, it probably does not serve the same biological function. The 33-kDa polypeptides displays very low rhodanese activity, and inactivation of the *rhdA* gene does not significantly lower either the rhodanese or thiosulfate reductase activity in the cell. In addition, the mutant strain grew well on sulfate, thiosulfate, and tetrathionate even though the RhdA polypeptide was absent. While a rhodaneselike protein from *S. erythraea* also did not exhibit any classical rhodanese activity, elimination of the protein limited the utilization of a number of inorganic sulfur sources (12).

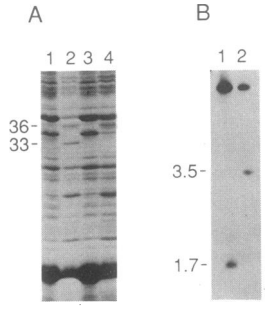

FIG. 8. Analysis of wild-type and *rhdA* mutant strains for the presence of the 33-kDa polypeptide. (A) Total soluble proteins from sulfur-sufficient (lanes 1 and 3) and sulfur-deprived (lanes 2 and 4) cells from the wild type (lanes 1 and 2) and the *rhdA* mutant (lanes 3 and 4) were resolved on a 12 to 18% polyacrylamide gradient gel. Markers used to determine the apparent molecular masses (in kilodaltons) of the protein bands are the same as in Fig. 1. (B) DNA gel blot hybridizations confirming that the recombinant clone containing the inactivated *rhdA* gene had integrated into *Synechococcus* sp. strain PCC 7942 by a double recombination event. Lane 1 contains wild-type genomic DNA, and lane 2 contains *rhdA* mutant DNA, both of which were digested with *Eco*RV. The probe was a 2.0-kb restriction fragment which contained the entire *rhdA* gene as well as 5'- and 3'-flanking sequences. Size markers are in kilobase pairs.

At this point in our understanding of sulfur acquisition in *Synechococcus* sp. strain PCC 7942, it is not possible to assign a definitive role to the 33-kDa protein in the acclimation process. However, given its proximity to genes encoding the sulfate transport system, its specific and high-level accumulation under sulfur deprivation conditions, its localization to the periplasmic space, and its similarity to rhodanese, the protein is probably involved in the acquisition of sulfur compounds. Both its abundance and subcellular location suggest that it might be a periplasmic binding protein. Additionally, a sulfur-regulated open reading frame located downstream of *rhdA* encodes a protein with properties similar to those of the two integral membrane proteins of the sulfate transport permease complex (unpublished data). Since the *rhdA* mutant strain can still grow on the same inorganic sulfur compounds as wild-type cells, the 33-kDa protein may be involved in the transport of a sulfur compound not tested or may not be absolutely required for uptake of a specific sulfur compound but may enhance transport. This appears to be the case for the sulfate- and thiosulfate-binding proteins in *E. coli* (16, 33) and *Synechococcus* sp. strain PCC 7442 (24; unpublished data). It is also possible that a second gene encoding a protein that functions

in a capacity similar to the 33-kDa polypeptide is present on the cyanobacterial genome.

ACKNOWLEDGMENTS

We thank Glenn Ford for his help with the computer analysis of the data and Jane Edwards for both typing the manuscript and preparing some of the figures. We also thank Fran Thomas, who helped us obtain the electron micrographs.

This work was supported by a grant from the North Atlantic Treaty Organization, the Carnegie Institution of Washington, and the National Science Foundation (grant DCB 8916301).

REFERENCES

1. **Allen, M. M.** 1984. Cyanobacterial cell inclusions. Annu. Rev. Microbiol. **38**:1–25.
2. **Allen, M. M.** 1969. Simple conditions for the growth of unicellular blue-green algae on plates. J. Phycol. **4**:1–3.
3. **Ames, G. F.-L.** 1986. Bacterial periplasmic transport systems: structure, mechanism and evolution. Annu. Rev. Biochem. **55**:397–425.
4. **Ames, G. F.-L., and A. K. Joshi.** 1990. Energy coupling in bacterial periplasmic permeases. J. Bacteriol. **172**:4133–4137.
5. **Belasco, J. G., T. Beatty, C. W. Adams, A. von Gabain, and S. N. Cohen.** 1988. Differential expression of photosynthesis genes in *Rhodopseudomonas capsulata* results from segmental differences in stability within the polycistronic *rxc* transcript. Cell **40**:171–181.
6. **Bishop, L., R. Agbayani, Jr., S. V. Ambudkar, P. C. Maloney, and G. F.-L. Ames.** 1989. Reconstitution of a bacterial periplasmic permease in proteoliposomes and demonstration of ATP hydrolysis concomitant with transport. Proc. Natl. Acad. Sci. USA **86**:6953–6957.
7. **Block, M. A., and A. R. Grossman.** 1988. Identification and purification of a derepressible alkaline phosphatase from *Anacystis nidulans* R2. Plant Physiol. **86**:1179–1184.
8. **Cerletti, P.** 1986. Seeking a better job for an underemployed enzyme. Trends Biochem. Sci. **129**:369–372.
9. **Cheng, C. Y.-A., and J. Belasco.** 1990. Degradation of *puf* LMX mRNA in *Rhodobacter capsulatus* is initiated by nonrandom endonucleolytic cleavage. J. Bacteriol. **172**:4578–4586.
10. **Chua, N.-H., and F. Blomberg.** 1979. Immunochemical studies of thylakoid membrane polypeptides from spinach and *Chlamydomonas reinhardtii*. J. Biol. Chem. **254**:215–233.

10a. **Collier, J. L., and A. Grossman.** Unpublished data.

11. **Conley, P. B., P. G. Lemaux, and A. R. Grossman.** 1985. Cyanobacterial light-harvesting complex subunits encoded in two red-light-induced transcripts. Science **230**:550–553.
12. **Donadio, S., A. Shafiee, and C. R. Hutchinson.** 1990. Disruption of a rhodaneselike gene results in cysteine auxotrophy in *Saccharopolyspora erythraea*. J. Bacteriol. **172**:350–360.
13. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132**:6–13.
14. **Green, L. S., and A. R. Grossman.** 1988. Changes in sulfate transport characteristics and protein composition of *Anacystis nidulans* R2 during sulfur deprivation. J. Bacteriol. **170**: 583–587.
15. **Green, L. S., D. E. Laudenbach, and A. R. Grossman.** 1989. A region of a cyanobacterial genome required for sulfate transport. Proc. Natl. Acad. Sci. USA **86**:1949–1953.
16. **Hryniewicz, M., A. Sirka, A. Palucha, A. Böck, and D. Hulanicka.** 1990. Sulfate and thiosulfate transport in *Escherichia coli* K-12: identification of a gene encoding a novel protein involved in thiosulfate binding. J. Bacteriol. **172**:3358–3366.
17. **Hunkapillar, M. W., E. Lujan, F. Ostrander, and L. E. Hood.** 1983. Isolation of microgram quantities of proteins from polyacrylamide gels for amino acid sequence analysis. Methods Enzymol. **91**:227–236.
18. **Jeanjean, R., and E. Broda.** 1977. Dependence of sulfate uptake by *Anacystis nidulans* on energy, on osmotic shock and on sulfate starvation. Arch. Microbiol. **114**:19–23.
19. **Jensen, T. E., and J. W. Rachlin.** 1984. Effect of varying sulfur deficiency on structural components of a cyanobacterium *Synechococcus leopoliensis*: a morphometric study. Cytobios **41**: 35–46.
20. **Koj, A.** 1968. Enzymic reduction of thiosulfate in preparations from beef liver. Acta Biochim. Polon. **15**:161–169.
21. **Kredich, N. M.** 1987. Biosynthesis of cysteine, p. 419–429. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
22. **Laemmli, U. K.** 1970. Cleavage of a structural protein during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
23. **Lau, R. H., S. D. Blakely, G. Alvarado-Urbina, J. E. Bailly, J. A. Condie, and P. C. K. Lau.** 1989. Duplication of the phycocyanin operon in the unicellular cyanobacterium *Anacystis nidulans* R2. Gene **80**:375–380.
24. **Laudenbach, D. E., and A. R. Grossman.** 1991. Characterization and mutagenesis of sulfur-regulated genes in a cyanobacterium: evidence for function in sulfate transport. J. Bacteriol. **173**: 2739–2750.
25. **Lawry, N. H., and T. E. Jensen.** 1979. Deposition of condensed phosphate as an effect of varying sulfur deficiency in the cyanobacterium *Synechococcus* sp. (*Anacystis nidulans*). Arch. Microbiol. **120**:1–7.
26. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
27. **Ploegman, J. H., G. Drent, K. H. Kalk, and W. G. J. Hol.** 1978. Structure of bovine liver rhodanese. I. Structure determination of 2.5Å resolution and a comparison of the conformation and sequence of its two domains. J. Mol. Biol. **123**:557–594.
28. **Prentki, P., and H. M. Krisch.** 1984. In vitro insertional mutagenesis with a selectable DNA fragment. Gene **29**:303–313.
29. **Russell, J., L. Weng, P. S. Keim, and R. L. Heinrikson.** 1978. The covalent structure of bovine liver rhodanese. Isolation and partial structural analysis of cyanogen bromide fragments and the complete sequence of the enzyme. J. Biol. Chem. **253**:8102–8108.
30. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-termination inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
31. **Schmidt, A.** 1987. Sulfur metabolism in cyanobacteria. Methods Enzymol. **167**:572–583.
32. **Schmidt, A., I. Erdle, and H.-P. Kost.** 1982. Changes in C-phycocyanin in *Synechococcus* 6301 in relation to growth on various sulfur compounds. Z. Naturforsch. C **37**:870–876.
33. **Sirko, A., M. Hryniewicz, D. Hulanicka, and A. Böck.** 1990. Sulfate and thiosulfate transport in *Escherichia coli* K-12: nucleotide sequence and expression of the *cysTWAM* gene cluster. J. Bacteriol. **172**:3351–3357.
34. **Smith, A. J., and J. Lascelles.** 1966. Thiosulfate metabolism and rhodanese in *Chromatium* sp. strain D. J. Gen. Microbiol. **42**:357–370.
35. **Sorbo, B. H.** 1955. Rhodanese. Methods Enzymol. **2**:334–337.
36. **Stern, D. B., and W. Gruissem.** 1987. Control of plastid gene expression: 3′ inverted repeats act as mRNA processing and stabilizing elements, but do not terminate transcription. Cell **51**:1145–1157.
37. **Tandeau de Marsac, N., W. E. Borrias, C. J. Kuhlemeier, A. M. Castetes, G. A. Van Arkel, and C. A. A. M. J. van den Hondel.** 1982. A new approach for molecular cloning in cyanobacteria: cloning of an *Anacystis nidulans met* gene using a Tn*901*-induced mutant. Gene **20**:111–119.
38. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
39. **Van der Plas, J., A. Bovy, F. Kruyt, G. deVrieze, E. Dassen, B. Klein, and P. Weisbeck.** 1989. The gene for precursor of plastocyanin from the cyanobacterium *Anabaena* sp. PCC 7937: isolation, sequence and regulation. Mol. Microbiol. **3**:275–284.

40. **von Heijne, G.** 1986. A new method for predicting signal sequence cleavage sites. Nucleic Acids Res. **14**:4683–4690.
41. **Wanner, G., G. Henkelman, A. Schmidt, and H.-P. Kost.** 1986. Nitrogen and sulfur starvation of the cyanobacterium *Synechococcus* 6301. An ultrastructural, morphometrical and biochemical comparison. Z. Naturforsch. C **41**:741–750.
42. **Weng, L., R. L. Heinrikson, and J. Wesley.** 1978. Active site cysteinyl and arginyl residues of rhodanese. A novel formation of disulfide bonds in the active site promoted by phenylglyoxal. J. Biol. Chem. **253**:8109–8119.
43. **Westley, J.** 1973. Rhodanese. Adv. Enzymol. **39**:327–368.
44. **Williams, J. G. K.** 1988. Construction of specific mutations in photosystem II photosynthetic reaction center by genetic engineering methods in *Synechocystis* 6803. Methods Enzymol. **167**:766–778.