Structure and Expression of a Cyanobacterial \textit{ilvC} Gene Encoding Acetoxyhydroxyacid Isomeroeductase

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Acetoxyhydroxyacid isomeroeductase (AHAIR) is the shared second enzyme in the biosynthetic pathways leading to isoleucine and valine. AHAIR is encoded by the \textit{ilvC} gene in bacteria. A 1,544-bp fragment of genomic DNA containing the \textit{ilvC} gene was cloned from the cyanobacterium \textit{Synechocystis} sp. strain PCC 6803, and the complete nucleotide sequence was determined. The identity of the gene was established by comparison of the nucleotide and derived peptide sequences with those of other \textit{ilvC} genes. The highest degree of sequence similarity was found with the \textit{ilvC} gene from \textit{Rhizobium meliloti}. The isolated \textit{Synechocystis ilvC} gene complemented an \textit{Escherichia coli ilvC} mutant lacking AHAIR activity. The expressed \textit{Synechocystis} gene encodes a protein that has a molecular mass of 35.7 kDa and that has AHAIR activity in an in vitro assay. Polyclonal antibodies raised against purified \textit{Synechocystis} AHAIR produced a single band on a Western blot (immunoblot) of a \textit{Synechocystis} cell extract and detected the protein in an extract of an \textit{E. coli ilvC} mutant strain that was transformed with a plasmid containing the \textit{Synechocystis ilvC} gene. The antibody did not react with an extract of an \textit{E. coli ilvC} mutant strain that was transformed with a control plasmid lacking the \textit{Synechocystis ilvC} gene or with an extract of an \textit{E. coli IlvC} control strain.

The biosynthetic pathways leading to isoleucine and valine in microorganisms and plants have three enzymes in common: acetoxyhydroxyacid synthase (EC 4.1.3.18), AHAIR (acetoxyhydroxyacid isomeroeductase) (EC 1.1.1.86), and dihydroyxacyl dehydratase (EC 4.2.1.9) (6). AHAIR, the shared second enzyme of the biosynthetic pathways, catalyzes an unusual two-step reaction involving migration of the α-alkyl group of the acetoxyhydroxyacid to the β-carbon to form an α-keto-β-hydroxy acid, which is then reduced to an α,β-dihydroxy acid with NADPH as a cosubstrate (Fig. 1). Unlike other enzymes that catalyze alkyl group migration, which require vitamin B₁₂ as a cofactor, AHAIR from \textit{Escherichia coli} and \textit{Salmonella typhimurium} does not require a cobalamin or other Co-containing cofactor (13) and requires only Mg²⁺ as a cofactor (2, 9). The enzyme utilizes both 2-acetolactate and 2-aceto-2-oxobutyrate as alternative substrates in the biosynthesis of valine and isoleucine, respectively. In a third reaction, \textit{S. typhimurium} AHAIR is able to catalyze the reduction of α-ketopantoate to pantoate, an intermediate in the biosynthesis of coenzyme A (17). Finally, the \textit{ilvC} gene, which encodes AHAIR, is required for nodulation in \textit{Rhizobium meliloti}, for unknown reasons not involving isoleucine and valine biosynthesis (1).

The biosynthetic pathway leading to the branched-chain amino acids is of interest for herbicide development. Acetoxyhydroxyacid synthase is the target for a variety of herbicides (21). Inhibition of AHAIR also results in some herbicidal effects (3), although much less work has been done in this regard.

In higher plants, the enzymes for isoleucine and valine biosynthesis are encoded in the nuclear genome but are active in the chloroplasts (11, 12). Cyanobacteria are considered to be closely related to chloroplasts of higher plants but, because they are prokaryotes, they offer a less complicated experimental system for the study of this process.

In the work presented here, the primary sequence of the AHAIR-encoding \textit{ilvC} gene from a unicellular cyanobacterium, \textit{Synechocystis} sp. strain PCC 6803, was determined. The identity of the gene was verified by the successful complementation of an \textit{E. coli ilvC} mutant as well as by the in vitro detection of AHAIR activity in extracts of transformed mutant cells. A polyclonal antibody raised against the purified cyanobacterial enzyme recognized the protein in \textit{Synechocystis} and transformed \textit{E. coli} cell extracts but not those of \textit{ilvC} or wild-type \textit{E. coli}.

**MATERIALS AND METHODS**

**Strains, media, and growth conditions.** \textit{Synechocystis} sp. strain PCC 6803 was a gift from J. G. K. Williams (Du Pont Central Research, Wilmington, Del.). For DNA isolation and protein extraction for the enzyme assay, axenic cultures were grown as described previously (18). \textit{E. coli DH5αMCR} (F⁻ mcrA mcrB mrr ϕ80lacZΔM15 ΔlacZYA-argF)U169 recA1 endA1 hsdR hsdM supE44 λ⁻ thi⁻1 gyrA relA1) cells were obtained from Bethesda Research Laboratories (Bethesda, Md.). \textit{E. coli CU424} (\textit{ilvC}Δ and \textit{galT} λ⁺) was a gift from H. E. Umbarger (Perdue University, West Lafayette, Ind.). LB broth (20) was used as the rich medium. The selective medium used was that of Davis and Mingioë (10) but modified by omitting sodium citrate and increasing the glucose concentration to 0.5% (22). All amino acids except for isoleucine and valine were present at a concentration of 0.5 mM. Solid media were prepared by the addition of 1.5% (wt/vol) Bacto-Agar (Difco, Detroit, Mich.). Ampicillin was used at a concentration of 100 μg/ml. For the selection of colonies containing plasmids with an insertion into the multiple cloning site, each plate was supplemented with 1 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and 4 μg of isopropyl-β-D-thiogalactopyranoside (IPTG).

A previous study identified the NH₂-terminal amino acid...
sequence of a purified Synechocystis protein that was not known at the time to have AHAIR activity (19). A synthetic 81-base oligonucleotide was generated from this sequence, on the basis of E. coli preferred codon usages (26), and the probe was used for hybridization experiments after it was end labeled with $^{32}$P by use of T4 DNA kinase (20). The sequence of the oligonucleotide probe was 5’GGCGGTAT GTACTAGATCAGGATGCAACCTGGATCTGCTG GCGGGCAAACCGTGGCGATCATCGGCTACGGCTACAG3’.

**DNA isolation and library construction.** Except when indicated otherwise, standard procedures were used (20). Synechocystis genomic DNA was isolated from a culture harvested in the exponential phase of growth. Three grams (wt weight) of cells was collected by centrifugation, washed once in ice-cold water, and then resuspended in 8 ml of buffer A (20 mM Tris [pH 7.6], 5 mM EDTA). After the addition of 150 μl of 3.0 M Na-acetate and 250 μl of 10% (wt/vol) sodium dodecyl sulfate (SDS), the cell suspension was dripped into liquid nitrogen and ground into a fine powder with a pestle and a mortar. The cell powder was transferred into 15 ml of phenol that had been equilibrated with buffer A. As soon as the cells thawed, 1 ml of chloroform was added and the DNA was extracted by gentle agitation. Following phase separation, the aqueous phase was reextracted twice with phenol-chloroform-isoamyl alcohol (25:24:1 [vol/vol/vol]) and then twice with chloroform-isoamyl alcohol (24:1 [vol/vol]). The DNA was precipitated with ethanol, resuspended in 2 ml of TE buffer (10 mM Tris-HCl [pH 7.6], 1 mM EDTA), digested with RNase A (final concentration, 120 μg/ml) and proteinase K (final concentration, 50 μg/ml) overnight at room temperature, and extracted with phenol-chloroform-isoamyl alcohol (25:24:1 [vol/vol/vol]) and then with chloroform-isoamyl alcohol (24:1 [vol/vol]). The DNA was again precipitated with ethanol, resuspended in 1.5 ml of TE buffer, and used in the following procedures.

The purified DNA was digested to completion with various restriction enzymes. After size fractionation on an agarose gel, the DNA fragments were transferred to a nitrocellulose membrane and screened by Southern hybridization to the end-labeled oligonucleotide probe. The DNA fragments corresponding in size to the location of the positive signal on the Southern blot were purified from a second agarose gel and ligated to a pBluescript cloning vector that had been digested with the corresponding restriction enzyme and dephosphorylated with calf intestinal alkaline phosphatase. E. coli DH5αMCR was transformed to ampicillin resistance with the ligation mixture. The resulting partial library was grown on LB plates containing ampicillin, transferred to nitrocellulose, and rescreened. Plasmid DNA was isolated from positive colonies and screened by Southern blot analysis.

Plasmid DNA was isolated by the boiling-lysis method. Cells from an overnight culture were harvested by centrifugation and resuspended in a buffer containing 50 mM Tris-HCl [pH 8.1], 50 mM EDTA, 8% sucrose, and 5% Triton X-100. After the addition of lysozyme (final concentration, 750 μg/ml), the cell suspension was incubated on ice for 10 min and then placed in a boiling water bath for 40 s. Cell
solution were stored buffered saline. Sodium azide (0.05% phosphate-buffered) were centrifugation, resulting plasmid DNA was used for cloning and sequencing experiments.

DNA sequence analysis was carried out by the dyeoxy-nucleotide chain termination method as described previously (20). Intact plasmid DNA was used as a template. Several restriction fragments were subcloned into the pBluescript vector and sequenced by use of the flanking primers. All sequences were confirmed on the basis of the complementarity of the sequences obtained from both strands.

Protein extraction. For the AHAIR assay and Western blotting (immunoblotting), cells were grown to the mid-exponential growth phase, collected by centrifugation, resuspended in buffer B [150 mM N-tris(hydroxymethyl)methylglycine (pH 7.9), 300 mM glycerol, 40 mM MgCl2, 1 mM dithiothreitol], and disrupted by two passages through a French pressure cell. The homogenate was cleared by centrifugation at 17,000 x g for 1 h. The resulting supernatant fluid was used for the enzyme assay.

A glycerol gradient-purified *Synechocystis* protein fraction containing AHAIR and glutamyl-tRNA reductase was prepared as previously described (19).

Enzyme assays. AHAIR activity was assayed by measuring the disappearance of the NADPH *A*260 in a reaction mixture (1 ml) that contained buffer B plus enzyme extract, 0.2 mM NADPH, and 10 mM acetolactate.racemic acetolactate, prepared as previously described (19). The homogenate was cleared by centrifugation at 17,000 x g for 1 h. The resulting supernatant fluid was used for the enzyme assay.

A glycerol gradient-purified *Synechocystis* protein fraction containing AHAIR and glutamyl-tRNA reductase was prepared as previously described (19).

Preparation of antibodies. Antigen was purified as described previously for glutamyl-tRNA reductase (19). The protein was isolated from an SDS-polyacrylamide gel by electroelution, lyophilized, and resuspended in water. The antigen solution, containing approximately 250 µg of protein, was emulsified with complete Freund's adjuvant at a ratio of 1:1 (vol/vol). A rabbit (5-kg female New Zealand White) was immunized by subcutaneous injection. The antigen preparation for booster immunizations was made as described above but with incomplete Freund's adjuvant. The booster shots, containing approximately 150 µg of protein, were given at 6, 8, and 10 weeks after the initial immunization. The rabbit was bled before each booster shot and thereafter on a weekly schedule. The blood was allowed to clot at room temperature for 3 h. The serum was separated by centrifugation and incubated at 55°C for 30 min to inactivate the complement system. Serum immunoglobulins were precipitated with (NH4)2SO4 at 50% of the saturating concentration, pelleted by centrifugation, redissolved in phosphate-buffered saline, and dialyzed against phosphate-buffered saline. Sodium azide (0.05% [wt/vol]) was added to the dialyzed antibody fraction. Aliquots of the antibody solution were stored at -20°C until further use.

Western blotting. Proteins were separated by SDS-poly-acrylamide gel electrophoresis. After electrophoresis, the proteins were transferred to a nitrocellulose membrane by a previously described procedure (8, 24). After the transfer, the remaining free binding sites on the membrane were blocked by incubation with TBS (20 mM Tris-HCl [pH 7.4], 0.9% NaCl) containing 5% (wt/vol) bovine serum albumin (BSA) for 1 h at 37°C. The blots were washed in TBS containing 0.1% (wt/vol) BSA for four 5-min periods. Binding of the anti-AHAIR antibodies was accomplished by incubating the blots in antibody binding solution (TBS containing 1% [wt/vol] BSA, 0.05% Tween 20, and a 1:10,000 dilution of the antibody stock solution) at room temperature for 2 h. After the blots were washed as described above, they were incubated with antibody binding solution containing anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (Fisher Scientific, Pittsburgh, Pa.) at room temperature for 1 h and then washed four times. Horseradish peroxidase activity was localized with H2O2 and 3,3'-diaminobenzidine as substrates.

**General methods.** Proteins were assayed by the dye binding method of Bradford (7). Oligonucleotides were synthesized by the phosphoramidite method (5) on an Applied Biosystems, Inc. (Foster City, Calif.), model 394 synthesizer. Restriction endonucleases and other DNA modification enzymes were obtained commercially and used in accordance with manufacturer specifications. The pBluescript II SK phagemid cloning vector was obtained from Stratagene (La Jolla, Calif.). T7 DNA polymerase (Sequenase) and a sequencing kit were from U.S. Biochemical Corp. (Cleveland, Ohio). Nicotelloleose membranes (BT83; 0.2-µm-pore size) were from Schleicher & Schuell (Keene, N.H.). All other reagents were from Sigma (St. Louis, Mo.), Fisher, or Research Organics (Cleveland, Ohio). Nucleotide sequence analysis was performed with The DNA Inspector IIe (Textco, West Lebanon, N.H.). The DNA data base at GenBank was searched with the FASTA program (15).

**Nucleotide sequence accession number.** The sequence reported here has been submitted to GenBank and has the accession number L03713.

**RESULTS**

Characterization of the *Synechocystis* *ih*B gene. *Synechocystis* genomic DNA was digested with three different restriction enzymes, BamHI, EcoRI, and XbaI. Southern blots showed a single band for each restriction digest when screened with an oligonucleotide probe whose sequence was derived from the NH2-terminal peptide sequence of purified *Synechocystis* AHAIR (data not shown). The sizes of the BamHI, EcoRI, and XbaI fragments were 1.5, 8.5, and approximately 20 kb, respectively. The linearized pBluescript cloning vector yielded no bands. The genomic DNA fragments corresponding to the 1.5-kb BamHI band were isolated and ligated into a plasmid vector that had been precut with BamHI. The ligation mixture was used to transform *E. coli* DH5αMCR cells. The transformants were grown on LB medium supplemented with ampicillin. The resulting partial library was screened by colony hybridization with the same oligonucleotide probe as that used for the Southern blot analysis. Positive colonies were isolated and rescreened. Several separate clones were identified, and their plasmids contained identical inserts. One of these insert-containing plasmids was designated pSR1. The 1.5-kb insert was subcloned for sequencing purposes by use of the available restriction sites for EcoRI, Sau3AI, and MspI. The resulting subclones were sequenced by use of primers that
were complementary to flanking vector sequences or synthetic oligonucleotides. The full-length DNA sequence of the 1.5-kb insert is shown in Fig. 2. The insert carries one complete open reading frame with the start codon ATG located at bp 304 to 306. The open reading frame encodes a protein of 331 amino acids with a predicted molecular mass of 35,753 Da. Amino acids 2 to 41 are identical to those determined for the purified protein by NH2-terminal peptide sequence analysis (19). The methionine encoded by the start codon is missing from the protein.

Three putative cyanobacterial functional sites (23) were found upstream from the start codon (Fig. 2); a ribosome binding site at bp 291 to 296 and a −10 box and a −35 box for transcription at bp 251 to 256 and 229 to 234, respectively.

A search of the GenBank database revealed that the open reading frame of pSR1 has significant homology with other known AHAIR-encoding genes (e.g., those from R. melliloti, E. coli, Neospora crassa, and Saccharomyces cerevisiae). The highest similarity (63.1% identity in a 1,018-bp overlap) was to the R. melliloti ilvC gene (1) (Fig. 3). Similarity to the E. coli ilvC gene was also detected, but compared with the Synechocystis and R. melliloti ilvC genes, the E. coli ilvC gene contains an insert of approximately 400 bp in the third of its length (27). The insert separates two regions of high similarity (52.2% identity in a 646-bp overlap and 60.0% identity in a 145-bp overlap). A short region of high similarity to the S. cerevisiae AHAIR-encoding gene (16) was detected (57.1% identity in a 219-bp overlap). Little if any similarity to the spinach AHAIR-encoding gene (12) was detected.

A comparison of the deduced amino acid sequence of the open reading frame in pSR1 with deduced IlvC amino acid sequences (Fig. 4) indicates significant similarity to the R. melliloti protein (178 of 331 residues identical, for 53.8% similarity) and less similarity to the E. coli protein (114 of 331 residues identical, for 34.4% similarity). Very little similarity of the Synechocystis protein to spinach AHAIR was detected (55 of 331 residues identical, for 16.6% similarity). The best-fit peptide alignment of the spinach protein has a 63-residue (relative to the E. coli protein) or a 109-residue (relative to the Synechocystis and R. melliloti proteins) N-terminal extension that contains the transit peptide sequence for transport into chloroplasts (12). The E. coli and spinach proteins have apparent 136- and 137-residue inserts, respectively, relative to the Synechocystis and R. melliloti proteins. The inserts occur at positions 75% toward the C termini of the latter.

A putative NADPH binding sequence motif, G-X-G-X-X-
A(G)-X-X-X-A, that was previously reported to be conserved in the AHAIR proteins of spinach, E. coli, and S. cerevisiae (12) was also present in the Synechocystis IlvC protein at residues 24 to 33. However, another sequence that was highly conserved in the spinach, E. coli, and S. cerevisiae proteins and that was proposed to be a candidate for the catalytic site (12) was not detected in the Synechocystis protein, nor was it present in the R. melliloti protein (1).

Synechocystis DNA in pSR1 has an incomplete second open reading frame on the strand opposite that of the ilvC gene, beginning at bp 240 to 242 and continuing to the end of the inserted DNA in the direction opposite that of ilvC (Fig. 2). This observation is of possible significance because in E. coli, the ilvY gene is located at a similar location and in a similar reading direction relative to ilvC (27), ilvY encodes a positive activator of ilvC in E. coli (27). R. melliloti DNA also contains a divergent second open reading frame at a similar position relative to ilvC (1). No sequence similarity was detected, at the nucleotide level or the deduced amino acid level, between the divergent second open reading frame of Synechocystis DNA in pSR1 and the E. coli ilvY gene or the R. melliloti divergent second open reading frame.

**Complementation of an E. coli ilvC mutant.** E. coli CU424 has been described as a mutant that is defective in the ilvC gene, which encodes AHAIR in that species (14). This strain requires supplementation of minimal growth medium with isoleucine and valine for growth. Plasmid pSR1 was used to transform competent E. coli CU424 cells. As a control, the pBluescript cloning vector without an insert was used in a parallel transformation. E. coli CU424 cells transformed with the control plasmid were able to grow on rich medium or on selective medium that was supplemented with isoleucine and valine but not on selective medium without supplementation (Table 1). In contrast, IlvC+ control cells transformed with pBluescript and CU424 cells transformed with pSR1 were able to grow on both unsupplemented selective and rich media. This result indicates that plasmid pSR1 can complement E. coli CU424 cells to isoleucine and valine prototrophy.

**In vitro detection of AHAIR activity expressed from pSR1.** E. coli CU424/pSR1, E. coli CU424/pBluescript, and E. coli DH5α MCR/pBluescript were grown in liquid selective medium containing ampicillin and IPTG. The cultures were harvested in the exponential growth phase, and enzyme extracts were prepared. E. coli CU424/pBluescript cells did not grow during the first 35 h (Table 1). However, E. coli CU424/pSR1 cells, which carried the Synechocystis ilvC gene, grew as well as E. coli DH5α MCR/pBluescript cells, used as an AHAIR-positive control. The specific activity of AHAIR from the E. coli CU424/pSR1 cell extract was about 14 times higher than that of AHAIR from the E. coli DH5α MCR/pBluescript cell extract.

**Synechocystis AHAIR.** The purified Synechocystis protein fraction that was previously reported to contain glutamyl-tRNA reductase activity (19) also contained AHAIR activity. Even though the protein was purified with the objective of obtaining homogeneous glutamyl-tRNA reductase and was apparently homogeneous on the basis of SDS-polyacrylamide gel electrophoresis and NH2-terminal peptide sequence data, the specific activity of AHAIR was 100-fold higher than that of glutamyl-tRNA reductase (1.5 μmol versus 15.1 nmol of product formed min⁻¹ mg of protein⁻¹, respectively). The specific activity of Synechocystis AHAIR was comparable to the values of 1.9 and 2.5 μmol min⁻¹ mg of protein⁻¹ reported for the S. typhimurium and E. coli enzymes, respectively (2, 9). Mg²⁺ was absolutely required for activity (data not shown), as has been reported for AHAIR from other sources (2, 9, 11, 13). To determine whether the substrates for AHAIR and glutamyl-tRNA reductase might compete for a single catalytic site, we determined glutamyl-tRNA reductase activity in the presence of the AHAIR substrate, acetolactate, at concentrations ranging up to 10 mM. Acetolactate had no effect on glutamyl-tRNA reductase activity at any tested concentration.

**Immunological detection of AHAIR.** The purified Synechocystis protein was used as an antigen for the immunization of a rabbit. Antibody against the protein was detected in the serum from the first bleeding, which occurred 6 weeks after the initial immunization. For routine immunodetection on Western blots, a 1:10,000 dilution of the antibody stock derived from the third or fourth bleeding was used. The antibody reacted with SDS-denatured purified Synechocystis AHAIR on a Western blot and detected Synechocystis AHAIR with a high specificity on a blot of total cellular
FIG. 2. Nucleotide sequence of the Synechocystis genomic DNA inserted in plasmid pSR1 and the deduced amino acid sequence of the ilvC gene product. Boldfacing indicates residues that were previously identified by NH2-terminal peptide sequencing of the purified protein (19). The underlined amino acid sequence was used to design the oligonucleotide probe. Nucleotide sequences that are recognized by the restricted enzymes mentioned in the text are underlined by a single underline. Restriction sites that were not used in the cloning and sequencing experiments are indicated parenthetically. The putative ribosome binding site and the −10 and −35 transcription signal sites are underlined by a double underline, a dashed underline, and a dotted underline, respectively. A translation start codon for a possible open reading frame on the opposite strand is indicated by underlining with asterisks.
protein (Fig. 5). Preimmune serum did not recognize any protein under the same conditions. The antibody did not neutralize or precipitate native Synechocystis AHAIR or glutamyl-tRNA reductase (data not shown). The antibody reacted with three specific bands on blots of total cellular protein from *E. coli* ilvC cells containing plasmid pSR1. One of the bands corresponds in molecular mass to Synechocystis AHAIR, and the other two bands have slightly higher molecular masses. No bands were detected in a blot of *E. coli* ilvC+ control cells, indicating that *E. coli* AHAIR was not recognized by the antibody.

**DISCUSSION**

In a previous report, a protein fraction having glutamyl-tRNA reductase activity was purified to apparent homogeneity from *Synechocystis* sp. strain PCC 6803 (19). NH₂-terminal peptide sequence analysis of the purified protein indicated the presence of only one peptide. A portion of this sequence was used for designing the synthetic oligonucleotide cloning probe for the experiments reported here. Analyses of the nucleotide and inferred amino acid sequences of the cloned *Synechocystis* DNA revealed no similarity to any reported sequence for *hema*, the gene that encodes glutamyl-tRNA reductase. However, the sequences were highly similar to those reported for the *Rhizobium meliloti* ilvC gene, which encodes AHAIR (1). The *Rhizobium meliloti* ilvC gene had not been deposited in the GenBank data base at the time that it was searched for sequences similar to the *Synechocystis* NH₂-terminal peptide sequence.

Reexamination of the purified *Synechocystis* protein indicated that both AHAIR and glutamyl-tRNA reductase activities were present. Attempts to separate AHAIR activity from glutamyl-tRNA reductase activity by isoelectric focusing and additional glycerol gradient centrifugations were unsuccessful. It was only when the extract of the *E. coli* strain that was transformed with a plasmid containing cloned *Synechocystis* DNA was examined that AHAIR activity, but not glutamyl-tRNA reductase activity, was detected.

It is provisionally concluded that the purified *Synechocystis* protein fraction contained both proteins and that the NH₂-terminal peptide sequence previously ascribed to glutamyl-tRNA reductase is instead that of AHAIR. An alternative possibility that could not be excluded is that a homogeneous protein purified from *Synechocystis* catalyzes both reactions but that the recombinant protein lacks some cofactor or modification that is required for glutamyl-tRNA reductase activity. However, the fact that acetolactate did not act as a competitive inhibitor of the glutamyl-tRNA reductase activity of the purified *Synechocystis* protein argues against this possibility.

If the apparently homogeneous purified *Synechocystis* protein did in fact contain two separate proteins, it is possible that the glutamyl-tRNA reductase was blocked at its NH₂ terminus, a situation that would have prevented the detection of a second protein by NH₂-terminal sequence analysis, and/or that the molar proportion of glutamyl-tRNA reductase in the protein fraction was so low that only AHAIR was detected. The latter possibility is supported by the fact that, after silver staining of SDS-polyacrylamide gels, only a single band was detected, even in lanes that were overloaded with the *Synechocystis* protein fraction (19). The apparent molecular mass of the SDS-denatured *Synechocystis* protein was 39 kDa, a value that corresponds well to the peptide molecular mass of 35.8 kDa that was deduced for *Synechocystis* AHAIR from the DNA sequence. In contrast, the recently reported sequence of the *Synechocystis* glutamyl-tRNA reductase-encoding *hema* gene indicates that it encodes a 47.5-kDa peptide whose NH₂-terminal region has no similarity to that of *Synechocystis* AHAIR (25).

The AHAIR proteins described so far appear to fall into two size classes. As determined from enzyme purification and/or translation of the encoding gene, the proteins from *Synechocystis* (this work), *Rhizobium meliloti* (1), and *Saccharomyces cerevisiae* (16) have molecular masses in the range of 35 to 40 kDa, whereas the spinach (11, 12), *E. coli* (27), and *Salmonella typhimurium* (13) proteins have masses of approximately 54 to 57 kDa. The size difference can largely be accounted for by a region of approximately 135 amino acid residues (equivalent to 14.8 kDa) that is present within the central portion of the larger proteins but is absent from the smaller proteins. This region apparently neither is required for nor interferes with catalytic function in heterologous hosts, since the smaller-protein-encoding *Synechocystis* ilvC gene can complement an *E. coli* ilvC mutant strain (this work) and the larger-protein-encoding *E. coli* ilvC gene can complement an *Rhizobium meliloti* ilvC mutant strain (1).
The dissimilarity of the *Synechocystis* and *E. coli* IVC proteins is further indicated by the fact that the antibody raised against the *Synechocystis* protein does not react with any proteins in extracts of *E. coli* IVC+ control cells or IVC mutant cells. The high specificity of the antibody for AHAIR is indicated by the fact that it reacts with a single band in a Western blot of total soluble *Synechocystis* protein. It is not known why the antibody detects two higher-molecular-mass bands, in addition to one having the correct molecular mass, in the extract of *E. coli* cells that were transformed with plasmid pSR1. One possibility is that the *E. coli* translation apparatus fails to reliably terminate translation of the *Synechocystis* gene at the correct position.

In conclusion, the low overall similarity of the *Synechocystis* IVC gene and its translated protein to those of spinach and the high similarity to those of *R. meliloti* are surprising. It is generally presumed that chloroplasts are descended from and therefore have gene homology with the cyanobacteria, even though many genes have been transferred to the nucleus from their presumed original location in the proto-chloroplast genome. As an example of the evolutionary closeness of chloroplasts to cyanobacteria and their
TABLE 1. Complementation of an E. coli ilvC mutant and AH AIR activity in cell extractsa

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<th>Strain/plasmid</th>
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<td>LB + ampicillin</td>
<td>Selective + ampicillin + lie + Val</td>
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<td>DH5aMCR/pBluescript</td>
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<td>CU424/pSR1</td>
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a Cells were transformed as described in Materials and Methods. pSR1 is the plBluescript vector carrying the Synechocystis ilvC gene. pBluescript (without an insert) was used as the control plasmid. Plates were incubated overnight at 37°C. Symbols: +, colonies were formed; -, no colonies were formed. The presence of IPTG did not influence growth under any conditions. For the determination of AH AIR activity, cells were grown on selective medium containing ampicillin and IPTG. Enzyme was extracted and assayed as described in Materials and Methods. One unit of specific activity is defined as the oxidation of 1 nmol of NADPH min⁻¹ mg of protein⁻¹. Less than 1 nmol of NADPH was oxidized per min per mg in the absence of an added substrate. CU424/pBluescript cells did not grow in this medium within 35 h; therefore, AH AIR activity could not be determined.

greater distance from R. meliloti, cyanobacteria and plants synthesize the tetrpyrrole precursor δ-aminolevulinic acid from glutamate via the tRNA-dependent five-carbon pathway, a process that is distinctly different from the δ-aminolevulinic acid synthase-catalyzed condensation of glycine and succinyl-coenzyme A that occurs in R. meliloti and other members of the α-purple group of cubacteria (4). The opposite conclusion that could be derived about the relatedness of plants, cyanobacteria, and α-purple bacteria from comparing their ilvC sequences illustrates the hazard of using a single gene or protein to infer overall evolutionary relatedness.

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ADDENDUM

After this paper was submitted for publication, the DNA sequence for the ilvC gene from B. subtilis was deposited in the GenBank sequence data base under accession number L03181. Sequence comparisons revealed that the B. subtilis ilvC gene and its translated protein are more similar to those of Synechocystis sp. strain PCC 6803 than are those of R. meliloti. The translated B. subtilis and Synechocystis ilvC proteins are 65.0% identical. The B. subtilis ilvC gene and its translated protein, like those of R. meliloti and the Synechocystis sp., lack the extra internal residues found in the E. coli and spinach sequences. Thus, the B. subtilis ilvC sequence supports the conclusions that, with respect to this gene, the cyanobacteria are phylogenetically closer to bacteria such as R. meliloti and B. subtilis than to E. coli and that these bacteria are more distantly related to plants than is E. coli.

REFERENCES


