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Synechocystis PCC 6803 Contains a Single Gene for the β Subunit of Tryptophan Synthase with Strong Homology to the *trpB* Genes of *Arabidopsis* and Maize (*Zea mays* L.)[1]

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We report the sequence of the *trpB* gene of the cyanobacterium *Synechocystis* sp. PCC 6803. This gene was cloned from a plasmid library by functional complementation of a *trpB* mutant of *Escherichia coli* K-12. Among the known *trpB* sequences, the *Synechocystis* gene bears the greatest homology to the duplicated *trpB* genes of *Arabidopsis thaliana* and *Zea mays*. Southern and northern blotting analyses suggest that *Synechocystis* contains only a single *trpB* gene. In contrast to all other prokaryotes, *Synechocystis* has a *trpB* gene that is monocistronic. Attempts to construct a *trpB* null mutant of *Synechocystis* by standard techniques were unsuccessful, suggesting that this organism is unable to concentrate tryptophan from the external medium.

Trp metabolism has been extensively studied in a wide variety of organisms (Yanofsky, 1984; Hütter et al., 1986; Crawford, 1989). In every case, the individual steps in Trp biosynthesis involve seven catalytically identical steps. The final two reactions, catalyzed by Trp synthase, involve the conversion of indole-3-glycerol-P and Ser to Trp and glyceraldehyde-3-P. Indole is an enzyme-bound intermediate in this process (Miles, 1991). In eubacteria and archaebacteria, Trp synthase contains two different subunits organized in the form of an $\alpha_2\beta_2$ tetramer. Each subunit catalyzes one of two partial reactions. The α subunit is responsible for forming indole from indoleglycerol-P, whereas the β subunits catalyze the formation of Trp from indole and Ser. The genes encoding the two subunits of Trp synthase, *trpA* and *trpB*, are generally found within polycistronic operons, an arrangement that enables the two proteins to be expressed coordinately. In fungi, Trp synthase is a homodimer, each subunit of which has both α and β catalytic centers. In green plants such as *Arabidopsis thaliana* and *Zea mays*, the situation is quite different. Each of these organisms has duplicate unlinked genes for the Trp synthase β subunit (Berlyn et al., 1989; Last et al., 1991; Wright et al., 1992). These observations suggest a general pattern of redundancy for aromatic amino acid biosynthetic enzymes in higher plants, although an exception to this notion has recently been described (Rose et al., 1992).

A number of intriguing questions have been raised by these recent advances. Although the Trp biosynthetic enzymes of green plants are encoded by unlinked nuclear genes, the proteins are targeted to the chloroplast. In the case of Trp synthase, there presumably exists a mechanism to ensure that separately expressed α and β subunits reach the chloroplast in stoichiometrically equivalent amounts. Because the photosynthetic cyanobacteria are considered to be ancestral to the chloroplasts of eukaryotic green plants, it is of considerable interest to understand the structural organization of aromatic amino acid biosynthetic genes in these organisms.

In the present work, we cloned a monocistronic, intronless *trpB* gene from the cyanobacterium *Synechocystis* sp. PCC 6803 by using selection for functional complementation of an *Escherichia coli trpB* missense mutant. The complete nucleotide sequence of this gene and its flanking regions are reported. By computer analysis, the *Synechocystis trpB* gene was found to bear a strong resemblance to the corresponding genes of *Arabidopsis* and maize.

MATERIALS AND METHODS

Construction of a Genomic Library of *Synechocystis* sp. PCC 6803

Genomic DNA from *Synechocystis* sp. PCC 6803, isolated following cesium chloride density gradient centrifugation, was partially digested at room temperature with restriction endonuclease *Sau*3A to generate fragments with an average length of 3 kb. The resulting fragments were inserted into pBluescript SK(+) (Stratagene) that had been linearized with *Bam*HI and dephosphorylated with calf intestinal alkaline phosphatase. Strain DH5α (Sambrook et al., 1989) was transformed with the ligation mixture. The resulting library contained approximately 10^7 independent clones.

Bacteria Strains and Culture Media

Escherichia coli K-12 strains SP948 [*trpB* (missense, derived from YS111), Δ*recA*, *srl*::Tn10], and SP974 [Δ(*trpBA-tonB*), *cysB*, *tna2*, Δ*recA*, *srl*::Tn10 (Zhao and Somerville, 1992)] were

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Abbreviations: ACH, acid-hydrolyzed casein; KAPA, DNA fragment bearing the kanamycin-resistance determinant of Tn 903.

used as tester strains. The nutritional phenotypes of these strains are given in Table I. Minimal medium was salt mix E of Vogel and Bonner (1956) supplemented with Glc (0.2%), thiamine (1.0 $\mu g\ mL^{-1}$), biotin (0.1 $\mu g\ mL^{-1}$), and 1.5% Bacto-agar (Difco). The following nutritional supplements were used: L-Trp, 50 $\mu g\ mL^{-1}$; L-Cys, 50 $\mu g\ mL^{-1}$; indole, 10 $\mu g\ mL^{-1}$; and ACH, 0.2%. Nutrient agar was used as a complete medium. When necessary, 50 mg L^{-1} of ampicillin was present in media; 25 mg L^{-1} of kanamycin was present.

DNA Sequencing and Computer Analysis

DNA sequencing was performed on single-stranded DNA templates by the dideoxy chain termination procedure (Sanger et al., 1977) using Sequenase (United States Biochemical). Single-stranded phagemid DNA was prepared by superinfection of *E. coli* strain JM101 or JM109 with the helper phage M13K07 (Sambrook et al., 1989). In addition to the original *trpB* clone (pGPZ4000; Fig. 1), selected via functional complementation, several subclones with inserts of different sizes, orientations, or DNA contents were generated, using pBluescript SK(+) (Stratagene) pBGS18+ and pBGS19+ (Spratt et al., 1986) or M13 mp19 (Sambrook et al., 1989). Nucleotide and amino acid sequence analysis was carried out using software from the Genetics Computer Group, made available via the Purdue University Agricultural Campus Laboratory for Computational Biochemistry.

Southern and Northern Analysis

Southern blotting was performed using 2 μg of *Synechocystis* genomic DNA for each restriction endonuclease digestion. A DNA fragment of 1250 bp containing the *trpB* gene, generated by PCR, was labeled with [^{32}P]dCTP by random primer labeling (Promega). This fragment was used as a probe in both Southern (genomic) blotting experiments and for northern analysis. Total RNA was isolated from cells of *Synechocystis* sp. PCC 6803 (Golden et al., 1987). RNA (5 μg) was subjected to electrophoresis on a denaturing 1.2% agarose gel containing formaldehyde. For analysis, the separated RNA molecules were transferred to reinforced nitrocellulose membranes (Stratagene).

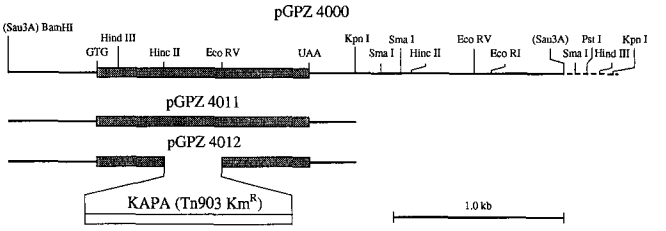

Figure 1. Restriction endonuclease cleavage map of the 3.2-kb segment of DNA that contains the *Synechocystis trpB* gene. The closed bar represents the *trpB*-coding region. GTG and UAA are the initiation codon and stop codon, respectively. The solid line represents the insert, and the dashed line represents vector DNA (pBluescript SK+). The lower map is that of the null mutant clone pGPZ4012. The KAPA (Tn903Km^R) fragment (Barany, 1985) is represented as an open bar. For further details, see text.

Disruption of *trpB*

Plasmid pGPZ4000 was digested with restriction endonuclease *Kpn*I, diluted and treated with T4 DNA ligase. The resulting subclone, named pGPZ4011 (Fig. 1), was fully able to complement the lesion in *trpB* tester strains of *E. coli*. Plasmid pUC-4-KAPA (Pharmacia) was digested with *Hinc*II, and the KAPA (Tn903Km^R) fragment was purified electrophoretically. Plasmid pGPZ4011 was digested with *Eco*RV and *Hinc*II and then treated with T4 DNA ligase in the presence of the KAPA fragment. The resulting plasmid, pGPZ4012 (Fig. 1), conferred kanamycin resistance and was unable to reverse the phenotype of the *E. coli trpB* tester strains SP948 and SP974.

Targeted Mutagenesis of *Synechocystis* sp. PCC 6803

Cells of the wild-type strain *Synechocystis* sp. PCC 6803 were transformed according to previously described methods (Williams, 1988; Chitnis et al., 1989) with pGPZ4012 DNA. The *trpB* gene of this plasmid was disrupted by the KAPA (Tn903Km^R) fragment. Kanamycin-resistant cells were allowed to undergo segregation for a few generations by a combination of single-colony selection on solid medium and

Table I. *Growth phenotypes of E. coli trpB mutants and derivatives harboring pGPZ4000*

Only relevant genotypes and nutritional supplements are listed. L-Cys (50 $\mu g\ ml^{-1}$) was present in all media used in the scoring of the nutritional phenotypes of SP974 and its plasmid-bearing derivatives. The complete genotype of strains and media composition are described in "Materials and Methods." Single colonies of each strain were picked from rich media supplemented with the appropriate antibiotics. Each colony was diluted in 0.05 mL of saline and then streaked to the test media. After 3 d incubation at 37°C, the growth properties were scored: +, growth; −, no growth.

Strain	Relevant Genotype	Growth on Minimal Media with Different Nutritional Supplements			
		Tryptophan	ACH	ACH indole	Indole
SP974	Δ(*trpBA*)	+	−	−	−
SP948	*trpB*	+	−	−	−
SP974 (pGPZ4000)	Δ(*trpBA*)/*trpB*$^+$	+	−	+	−
SP948 (pGPZ4000)	*trpB*/*trpB*$^+$	+	+	+	+

growth in liquid BG11 medium (Allen, 1968) containing Trp. During subculture, the kanamycin concentration was gradually increased from 5 to 50 $\mu g\ mL^{-1}$.

RESULTS

Isolation of a *Synechocystis trpB* Clone by Complementation in *E. coli* of a *trpB* Mutation

Plasmid DNA (1.2 μg) of a *Synechocystis* sp. PCC 6803 genomic library was used to transform the *E. coli trpB* tester strain SP948 using electroporation (Bio-Rad). Appropriately diluted samples of the transformation mixture were spread onto both nutrient agar ampicillin plates and minimal-Glc ampicillin plates supplemented with ACH (0.2%) and indole (10 $\mu g\ mL^{-1}$). A number of colonies appeared on the latter plates after several days of incubation (37°C). Among 10^5 ampicillin-resistant transformants (scored on rich medium), eight colonies were found on the selective medium after 3 d. Plasmid DNA extracted from each isolate was introduced into *E. coli* strain SP974. Transformants were selected on nutrient agar supplemented with Cys and ampicillin. Only one of the eight candidates was capable of growth on minimal-casein hydrolysate-indole medium (Table I). It is probable that the other seven colonies were $TrpB^+$ revertants that arose within the tester strain prior to transformation and became ampicillin resistant by acquiring a plasmid from the library that was unrelated to Trp synthase. The plasmid that conferred a $TrpB^+$ phenotype, named pGPZ4000, was also introduced into strain SP948 to confirm the originally observed plasmid-specific phenotypes. The nutritional phenotypes of SP974, SP948, and derivatives of these strains harboring pGPZ4000 are shown in Table I. Because exogenous indole is absolutely required for the growth of the *trpA* strain SP974 harboring pGPZ4000, this plasmid must contain a functional $trpB^+$ gene but not a functional $trpA^+$ gene. Trp biosynthesis supported by pGPZ4000 appeared to be barely adequate, because growth on minimal-salts indole media of cells lacking the Trp synthase α subunit (*trpA* gene product) did not occur unless ACH was present. Presumably, this amino acid-rich supplement (for composition, see Bogosian et al., 1990) minimizes the physiological stress associated with subadequate levels of Trp.

Primary Structure of the *Synechocystis trpB* Gene Product

The deduced amino acid sequence of the *Synechocystis* Trp synthase β protein (412 amino acids) is shown in Figure 2. There was no evidence of a complete or partial open reading frame on either side of the *trpB* gene bearing structural homology to any known Trp synthase α polypeptide. The *Synechocystis trpB* gene product bears significant homology with most of the other known Trp synthase β polypeptides. Among 21 Trp synthase β polypeptide sequences that were analyzed, those from the three photosynthetic organisms *A. thaliana*, Z. *mays*, and *Synechocystis* sp. PCC 6803 showed the highest degree of homology (Fig. 3). There was 73.8% identity with the *A. thaliana* TRP B, as compared to 49.0% identity with the *E. coli* β subunit and 52.9% identity with the β domain of *Saccharomyces cerevisiae* (data not shown). A significant difference between the *trpB* sequences of *Synechocystis* and those of higher green plants is that the transit peptides present in the Trp synthase β subunit sequences of *A. thaliana* and Z. *mays* are missing from the *Synechocystis* enzyme. The initiation codon of the *Synechocystis trpB* open reading frame is GTG rather than ATG. Among the known *trpB* genes, only that of *Caulobacter crescentus* begins with GTG.

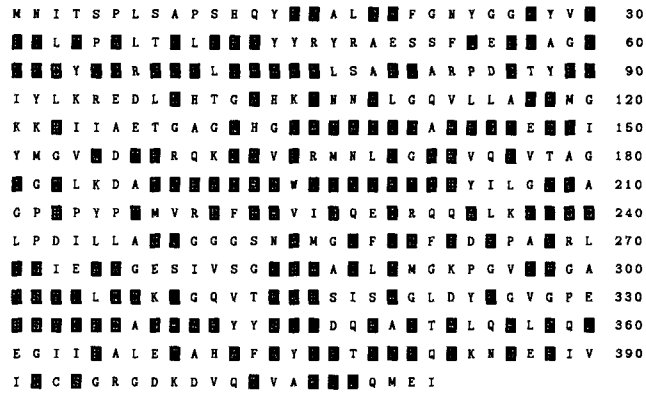

Figure 2. Amino acid sequence of the *trpB* gene of *Synechocystis* sp. PCC 6803, as deduced from the nucleotide sequence. Residues shown in black boxes are conserved between the *Synechocystis* and the land plants Z. *mays* and *A. thaliana*. Residues shown in normal type are either absolutely conserved between all known Trp synthases or display substantial variation from organism to organism.

Southern and Northern Analysis

A 1250-bp DNA fragment containing *trpB*, synthesized by PCR, was labeled with [^{32}P]dCTP by random priming. In Southern blots, this probe hybridized predominantly to the expected bands in each restriction digest (Fig. 4), indicating that *trpB* is present in single copy within the genome of *Synechocystis* sp. PCC 6803. For those restriction endonuclease that do not cleave *Synechocystis* genomic DNA within the *trpB*-coding sequence (*Eco*RI, lane 1; *Bam*HI, lane 2; *Not*I, lane 7), Southern blotting revealed essentially only single hybridizing fragments of mol wts higher than that of the radiolabeled probe. Southern blotting analysis of genomic DNA that had been cleaved with *Hin*dIII alone or in combination with a second enzyme (lanes 3, 5, and 6) showed two strongly hybridizing bands, as expected from the existence of a *Hin*dIII site situated approximately 42 codons from the N terminus of the gene (Fig. 1). Restriction endonuclease *Sty*I is predicted to cleave within the *trpB* gene of *Synechocystis* at two locations, near codons 255 and 290. From the nucleotide sequence on either side of *trpB*, hybridizing *Sty*I fragments of 1442, 1068, and 104 bp were predicted (data not shown). Radiolabeled bands corresponding to the two larger *trpB*-specific *Sty*I fragments were observed (Fig. 4, lane 8). The third predicted fragment of 104 bp was not observed; presumably, it had migrated off the bottom of the gel. The Southern blotting analysis thus tends to rule out the existence of duplicate *trpB* genes in *Synechocystis* PCC 6803.

The same radiolabeled *trpB* probe in northern blots re-

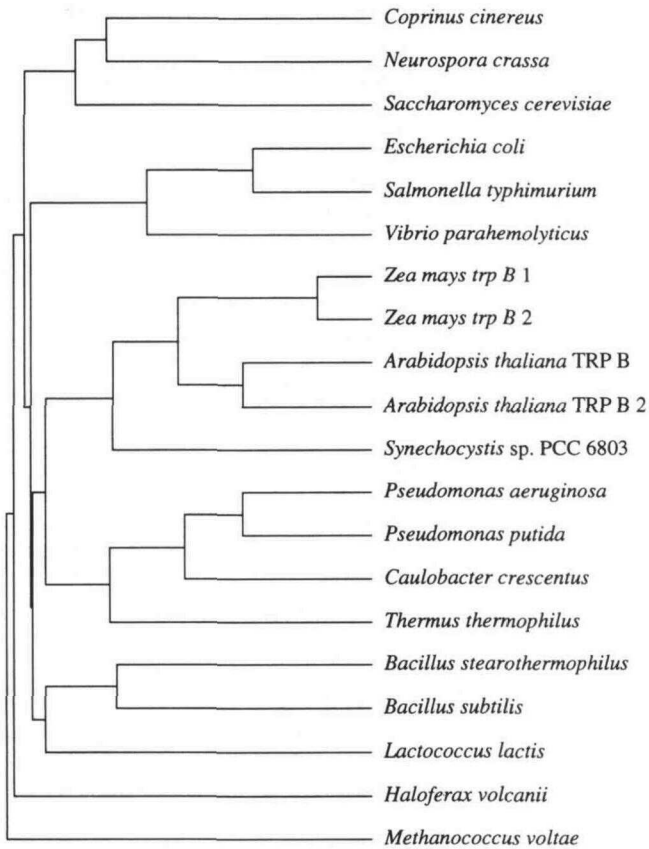

Figure 3. Phylogenetic tree showing possible evolutionary relationship among Trp synthase β polypeptides. The comparison analysis and plot were carried out using the program PILEUP of the Genetics Computer Group, version 7.2. PILEUP executes a series of progressive pairwise alignments between sequences to generate similarity scores. These scores are used to create a clustering order, represented here in the form of a phylogenetic tree. The clustering strategy utilizes unweighted pair-group arithmetic averages (Sneath and Sokal, 1973). The origin of data are: *Coprinus cinereus* TRP1 β peptide, Skrzynia et al. (1989); *Neurospora crassa trp-3* β peptide, Burns and Yanofsky (1989); *S. cerevisiae* TRP5 β peptide, Zalkin and Yanofsky (1982); *E. coli trpB,* Crawford et al. (1980); *S. typhimurium trpB,* Crawford et al. (1980); *Vibrio parahemolyticus trpB,* Crawford et al. (1991); *Z. mays trpB* genes, Wright et al. (1992); *A. thaliana* TRPB, Berlyn et al. (1989); *A. thaliana* second *trpB,* Last et al. (1991); *Synechocystis* sp. PCC 6803 *trpB,* (this work); *Pseudomonas aeruginosa trpB,* Hadero and Crawford (1986); *Pseudomonas putida trpB,* Crawford and Eberly (1986); *C. crescentus trpB,* Ross and Winkler (1988); *Thermus thermophilus* HB27 *trpB,* Koyama and Furukawa (1990); *Bacillus stearothermophilus trpB,* Ishiwata et al. (1989); *Bacillus subtilis trpB,* Henner et al. (1984); *Lactococcus lactis* sp. *lactis trpB,* Bardowski et al. (1992); *Haloferax volcanii trpB,* Lam et al. (1990); *Methanococcus voltae trpB,* Sibold and Henriquet (1988). Polypeptide segments carrying transit peptides or other unrelated structures were excluded during the analysis.

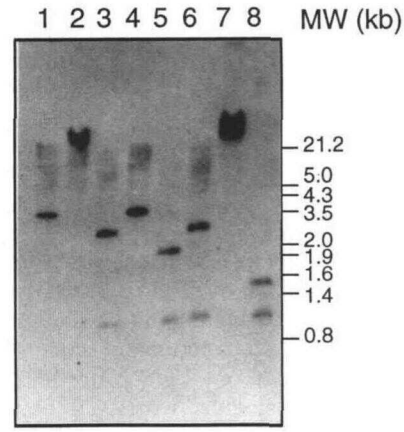

Figure 4. Southern blot analysis of genomic DNA from *Synechocystis* sp. PCC 6803. Genomic DNA was digested with *Eco*RI (lane 1), *Bam*HI (lane 2), *Hin*dIII (lane 3), *Eco*RI plus *Bam*HI (lane 4), *Eco*RI plus *Hin*dIII (lane 5), *Bam*HI plus *Hin*dIII (lane 6), *Not*I (lane 7), and *Sty*I (lane 8). The digested DNA was subjected to electrophoresis on 0.8% agarose, transferred to supported nitrocellulose, and probed with a ^{32}P-labeled 1250-bp *trpB* fragment (see "Materials and Methods"). For a more detailed discussion of these data, see the text.

vealed a single RNA species of approximately 1400 nucleotides (Fig. 5). Given the size of the *trpB* gene (1236 bp) and provided that there is no processing of the primary transcript, its mRNA is most likely monocistronic.

Attempts at Targeted Mutagenesis of *trpB*

Extensive efforts were made to generate a *trpB* null mutant of *Synechocystis* ("Materials and Methods"). Although kanamycin-resistant transformants were readily obtained, South-

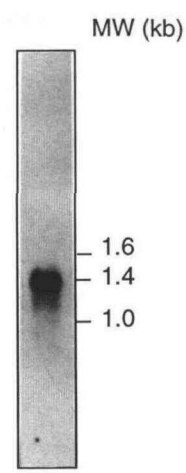

Figure 5. Northern blot analysis of *trpB* of *Synechocystis* sp. PCC 6803. Total RNA (5 μg) was subjected to electrophoresis on 1.2% agarose containing formaldehyde, transferred to a supported nitrocellulose membrane, and hybridized with a *trpB*-specific probe. The size standards were synthesized in vitro using T7 RNA polymerase and cloned, sequenced DNA fragments as templates.

ern blotting analysis of segregated colonies (not shown) revealed the presence of both wild-type and null mutant copies of the gene after several generations of antibiotic selection and segregation. None of the segregated kanamycin-resistant transformants were Trp auxotrophs. In a parallel experiment, null mutants in the *psaL* gene, which encodes a subunit of PSI (Chitnis et al., 1993), were readily obtained.

DISCUSSION

By functional complementation of an *E. coli trpB* point mutant (YS111), we isolated a *trpB*$^+$ clone of *Synechocystis* sp. PCC 6803 DNA from a partial *Sau*3A library. The cloned cDNA was characterized by sequencing, and the inferred polypeptide sequence was analyzed by eye and computationally. As expected, the *Synechocystis trpB* polypeptide lacked the chloroplast transit sequences of the β polypeptides from *A. thaliana* and *Z. mays*. The *Synechocystis trpB* gene uses GTG rather than ATG as the translation initiation codon, a situation that may contribute to the low in vivo complementation activity. Although uncommon, GTG is sometimes used for initiating translation in *Synechocystis* (Carpenter et al., 1990).

Sequence analyses of the clone revealed a long open reading frame of 1236 bp (412 codons) that bore significant homology to each of the known Trp synthase β polypeptides. The greatest homology was with the β polypeptides of *Z. mays* and *A. thaliana*. The sequences of Trp synthase β polypeptides from different organisms are known to be well conserved (Miles, 1991). Several residues within the β subunits of *E. coli* and/or *Salmonella typhimurium* are considered to be either functionally or structurally important (Miles, 1991). However, only three residues, namely, Lys^{87} (Miles et al., 1989; Lu et al., 1993), Glu^{109} (Kayastha and Miles, 1990; Brzovic et al., 1992), and Glu^{350} (Kayastha et al., 1991) have been proven by site-directed mutagenesis to be catalytically essential. The corresponding amino acid residues within the *Synechocystis* β subunit (Lys^{105}, Glu^{127}, and Glu^{368}, Fig. 2) are conserved. A structurally important residue, Gly^{281} of the *E. coli* β subunit, which has been characterized in detail through studies of a conventional missense mutation, *trpB8* (Zhao and Somerville, 1992, 1993), is fully conserved in the *Synechocystis* β subunit.

Unexpectedly, attempts at targeted mutagenesis of *Synechocystis* based on the cloned *trpB* gene were unsuccessful. The inability of the mutant gene to segregate to the homoplastic state could reflect an essential and irreplaceable role of the *trpB* gene in the metabolism of this cyanobacterium. Similar observations have been made with other genes of cyanobacteria (Murphy et al., 1990). Alternatively, and more likely, *Synechocystis* may be unable to carry out Trp uptake because of the absence of an appropriate permease system.

DNA sequence analysis also suggested that there was no *trpA* gene in the vicinity of the cloned *trpB* gene. Results of northern analysis fully support this conclusion. A single mRNA species, approximately 1400 nucleotides in size (Fig. 5), was detected by northern blotting. This result suggests that the *trpB* of *Synechocystis*, like *Z. mays* and *A. thaliana*, is monocistronic. In other prokaryotes, each of the *trpB* genes lie within polycistronic operons containing the *trpA* gene and frequently *trpC*, *trpD*, and *trpE*. In eukaryotes, except for the land plants *Z. mays* and *A. thaliana*, the β polypeptides are fused with the α polypeptide. The monocistronic *trpB* gene characterized in this study strongly supports the argument that cyanobacteria such as *Synechocystis* are derived from common ancestors of green plants such as *Z. mays* and *A. thaliana*.

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