

# Amino Acid Transport in Taxonomically Diverse Cyanobacteria and Identification of Two Genes Encoding Elements of a Neutral Amino Acid Permease Putatively Involved in Recapture of Leaked Hydrophobic Amino Acids

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The activities of uptake of thirteen <sup>14</sup>C-labeled amino acids were determined in nine cyanobacteria, including the unicellular strains *Synechococcus* sp. strain PCC 7942 and *Synechocystis* sp. strain PCC 6803; the filamentous strain *Pseudanabaena* sp. strain PCC 6903, and the filamentous, heterocyst-forming strains *Anabaena* sp. strains PCC 7120 and PCC 7937; *Nostoc* sp. strains PCC 7413 and PCC 7107; *Calothrix* sp. strain PCC 7601 (which is a mutant unable to develop heterocysts); and *Fischerella muscicola* UTEX 1829. Amino acid transport mutants, selected as mutants resistant to some amino acid analogs, were isolated from the *Anabaena*, *Nostoc*, *Calothrix*, and *Pseudanabaena* strains. All of the tested cyanobacteria bear at least a neutral amino acid transport system, and some strains also bear transport systems specific for basic or acidic amino acids. Two genes, *natA* and *natB*, encoding elements (conserved component, NatA, and periplasmic binding protein, NatB) of an ABC-type permease for neutral amino acids were identified by insertional mutagenesis of strain PCC 6803 open reading frames from the recently published genomic DNA sequence of this cyanobacterium. DNA sequences homologous to *natA* and *natB* from strain PCC 6803 were detected by hybridization in eight cyanobacterial strains tested. Mutants unable to transport neutral amino acids, including *natA* and *natB* insertional mutants, accumulated in the extracellular medium a set of amino acids that always included Ala, Val, Phe, Ile, and Leu. A general role for a cyanobacterial neutral amino acid permease in recapture of hydrophobic amino acids leaked from the cells is suggested.

The cyanobacteria are a phylogenetically coherent group of prokaryotic organisms within the eubacteria (12). The cyanobacteria represent, however, a very diverse biological group, as evidenced by their wide span of mean DNA base composition (13), that show a diverse morphology and occur in many different natural habitats (23). Nonetheless, metabolically, the cyanobacteria are a rather homogeneous group of organisms that are characterized by their ability to perform oxygenic photosynthesis and to fix carbon dioxide through the Calvin reductive pentose phosphate pathway. Some cyanobacteria are additionally capable of photoheterotrophic or chemoheterotrophic growth. With regard to the acquisition of nitrogen, most cyanobacteria assimilate nitrate and ammonium, and many strains are also able to assimilate urea or dinitrogen (7). Fixation of dinitrogen in some filamentous cyanobacteria takes place in differentiated cells called heterocysts (30). Some cyanobacteria have been shown to be able to take up some amino acids (7). Amino acids like Arg, Asn, and Gln can be used as sources of nitrogen by a number of cyanobacteria (7), while Arg has been described to serve as a source of carbon for *Synechocystis* (*Aphanocapsa*) sp. strain PCC 6308 (29).

The array of amino acid transport systems present in cyanobacteria has been characterized at a detailed level only for the unicellular *Synechocystis* sp. strain PCC 6803 (8, 19) and the filamentous, heterocyst-forming *Anabaena* sp. strain PCC 7120 (9, 14, 22, 31). Three amino acid transport systems in

strain PCC 6803, one specific for basic amino acids and Gln, one specific for neutral amino acids (excluding Gln), and another one specific for Glu and Gln, have been described (19); however, reported data (19) do not demonstrate that Glu and Gln share a transport system in strain PCC 6803. Three high-affinity amino acid transport systems in strain PCC 7120 have been defined: one for basic amino acids (14) and two with overlapping, though not identical, specificities for neutral amino acids (22). Strain PCC 7120 appears to bear also a low-affinity, passive transport system for basic amino acids (14) and a low-affinity system for the uptake of acidic amino acids (22). Besides having a role in the uptake of extracellular amino acids, some of these transport systems might have a role in the recapture of amino acids that have leaked from the cells as well as in the intercellular transfer of amino acids that takes place in the *Anabaena* filament during diazotrophic growth (22).

In this work, we attempt to define the array of amino acid transport systems present in some diverse cyanobacteria, including strains that are currently being widely investigated but for which data on amino acid uptake are either scarce (e.g., *Anabaena* sp. strain PCC 7937 [see references 3 and 27]) or not available (e.g., *Synechococcus* sp. strain PCC 7942, *Calothrix* sp. strain PCC 7601, and *Pseudanabaena* sp. strain PCC 6903). We have determined the activity of uptake of thirteen <sup>14</sup>C-labeled amino acids in nine cyanobacterial wild-type strains, isolated from some of them some spontaneous amino acid transport mutants, and determined some physiological properties of these mutants. Two genes encoding elements of the neutral amino acid transport system of *Synechocystis* sp. strain PCC 6803 have also been identified.

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TABLE 1. Cyanobacterial strains used in this work

Strain	Description	Reference or source
<i>Anabaena</i> sp. strain PCC 7120 (ATCC 27893)	Wild-type strain belonging to Section IV	23
<i>Anabaena</i> sp. strain PCC 7937 (ATCC 29413)	Wild-type strain belonging to Section IV	23
<i>Nostoc</i> sp. strain PCC 7413 (ATCC 29106)	Wild-type strain belonging to Section IV	23
<i>Nostoc</i> sp. strain PCC 7107 (ATCC 29150)	Wild-type strain belonging to Section IV	23
<i>Calothrix</i> sp. strain PCC 7601	Wild-type strain belonging to Section IV	23
<i>Fischerella muscicola</i> UTEX 1829	Wild-type strain belonging to Section V	Culture Collection of Algae, University of Texas
<i>Pseudanabaena</i> sp. strain PCC 6903 (ATCC 27190)	Wild-type strain belonging to Section III	23
<i>Synechococcus</i> sp. strain PCC 7942	Wild-type strain belonging to Section I	23
<i>Synechococcus</i> sp. strain PCC 6803 (ATCC 27184)	Wild-type strain belonging to Section I	23
CS440	Mutant of strain PCC 7937 isolated as resistant to 100 $\mu$ M azaleucine	This work
CS454	Mutant of strain CS440 isolated as resistant to 150 $\mu$ M hydroxylysine	This work
CS447	Mutant of strain CS440 isolated as resistant to 100 $\mu$ M methionine sulfoximine	This work
CS453	Mutant of strain CS447 isolated as resistant to 100 $\mu$ M hydroxylysine	This work
CS441	Mutant of strain PCC 7413 isolated as resistant to 100 $\mu$ M azaleucine	This work
CS456	Mutant of strain CS441 isolated as resistant to 10 $\mu$ M methionine sulfoximine	This work
CS442	Mutant of strain PCC 7107 isolated as resistant to 100 $\mu$ M azaleucine	This work
CS457	Mutant of strain CS442 isolated as resistant to 25 $\mu$ M hydroxylysine	This work
CS451	Mutant of strain PCC 7601 isolated as resistant to 50 $\mu$ M azaleucine	This work
CS448	Mutant of strain PCC 6903 isolated as resistant to 100 $\mu$ M azaleucine	This work
CS446	Mutant of strain PCC 6903 isolated as resistant to 20 $\mu$ M methionine sulfoximine	This work
CSX28a	Mutant of strain PCC 6803 bearing gene cassette C.K3 inserted into <i>str0467</i> , in the same orientation as the ORF	This work
CSX28b	Mutant of strain PCC 6803 bearing gene cassette C.K3 inserted into <i>str0467</i> , in the orientation opposite to that of the ORF	This work
CSX18a	Mutant of strain PCC 6803 bearing gene cassette C.K3 inserted into <i>str0559</i> , in the same orientation as the ORF	This work
CSX18b	Mutant of strain PCC 6803 bearing gene cassette C.K3 inserted into <i>str0559</i> , in the orientation opposite to that of the ORF	This work

## MATERIALS AND METHODS

**Strains and growth conditions.** The wild-type and amino acid transport mutant strains used in this work are indicated in Table 1. All of these strains were grown axenically in BG11 (nitrate-containing) medium (23). Culture medium for amino acid analog-resistant mutants was supplemented with the corresponding amino acid analog. For plates, the medium was solidified with 1%, separately autoclaved agar (Difco). Cultures were grown at 30°C in the light, with shaking (80 to 90 rpm) for liquid cultures. The amino acid analogs (Sigma) used were  $\delta$ -hydroxylysine (antagonist of Lys), 4-aza-DL-leucine (antagonist of Leu), and L-methionine sulfoximine (antagonist of Met and Glu). (See Table 1 for the concentration of amino acid analog used with each strain.) The amino acid analogs were filter sterilized. *Synechocystis* sp. strain PCC 6803 mutants carrying gene cassette C.K3 (5) were routinely grown with 100 to 300  $\mu$ g of kanamycin ml<sup>-1</sup>.

*Escherichia coli* DH5 $\alpha$  was grown in Luria-Bertani medium with, when necessary, 50  $\mu$ g of ampicillin ml<sup>-1</sup> and 50  $\mu$ g of kanamycin ml<sup>-1</sup>.

**Isolation of spontaneous mutants.** Spontaneously occurring mutants were isolated in BG11 plates supplemented with inhibitory concentrations (Table 1) of hydroxylysine, azaleucine, or methionine sulfoximine. The plates were inoculated with cells (ca. 10  $\mu$ g of chlorophyll *a* [Chl]) suspended in soft (0.5%) top agar, and individual colonies that appeared about 1 week after inoculation were restreaked on selective medium. (Chl was determined in methanolic extracts [21].)

**Uptake assays.** Cells grown in BG11 medium (supplemented with the appropriate amino acid analog or antibiotic in the case of the mutants) were harvested by low-speed centrifugation at room temperature, washed with 25 mM *N*-tris(hydroxymethyl)-methylglycine (Tricine)-NaOH buffer (pH 8.1), and resuspended in the same buffer. The assays were carried out at 30°C in the light (100 W · m<sup>-2</sup>, white light from incandescent lamps) and were started by mixing a suspension (1 ml) of cells (3 to 15  $\mu$ g of Chl) with a solution (0.1 ml) in Tricine buffer of the amino acid to be tested as a substrate. The amino acids used as substrates were L-[U-<sup>14</sup>C]amino acids (4.5 to 15.1  $\mu$ Ci ·  $\mu$ mol<sup>-1</sup>; Amersham or New England

Nuclear) at a final concentration of 10  $\mu$ M. The total reaction mixture volume was 1.1 ml. After an incubation of 10 min (or, when indicated, 1 min), a 1-ml sample was filtered (0.45- $\mu$ m-pore-size Millipore HA filters were used) and the cells on the filters were washed with 5 to 10 ml of Tricine buffer. The filters carrying the cells were then immersed in scintillation cocktail, and their radioactivity was measured. Retention of radioactivity by boiled cells was used as a blank. The uptake of each amino acid was determined, in the different wild-type strains, from two to six times (up to 17 determinations in the case of strain PCC 7120) and, in the mutants, from two to four times. For the sake of simplicity, the data are presented as the mean of the values obtained in those determinations, which always gave similar results (within 20% of the mean for the wild-type strains and within 30% of the mean for the mutants).

Uptake assays for the determination of  $K_s$  (solute concentration in the extracellular medium that gives one half of the maximum rate of uptake) and  $V_{max}$  values were carried out with the following substrate concentrations: 0.5 to 400  $\mu$ M for Asp in strains PCC 6903, CS446, and CS448; 0.05 to 400  $\mu$ M for Arg in strain PCC 7413; and 0.25 to 400  $\mu$ M for Glu in strains PCC 6803 and CSX28a. The assays were started by the addition of 0.25 ml of a cell suspension (1.4 to 15.7  $\mu$ g of Chl) in Tricine buffer to a solution of substrate in the same buffer. The radioactivity used was 1.4 to 9.3  $\mu$ Ci ·  $\mu$ mol<sup>-1</sup>. The total reaction mixture volume was 1.05 ml, and the incubation time was 1 min.  $K_s$  and  $V_{max}$  values were estimated from Eadie-Hofstee plots of the data obtained in uptake assays with different initial concentrations of amino acid.

**Identification of amino acid transport genes.** The sequences of primers Tr20 and Tr21, used to amplify open reading frame (ORF) *str0467* from *Synechocystis* sp. strain PCC 6803 (17) by PCR, were 5'-GTAATTGACACAGTTTCCATGACGCG-3' and 5'-CTTCTCTTTATTTGCACCACGAGGG-3', respectively. Primers Tr8 and Tr9, used to amplify ORF *str0559*, were 5'-CAGGATGCGTC TATTTTGGCCCC-3' and 5'-CAACGAAATCGGGGAATAAACGCC-3', respectively. PCR amplification was carried out in a 50- $\mu$ l reaction mixture volume containing 2 ng of genomic DNA from strain PCC 6803; 0.2 mM each deoxynucleoside triphosphate; 50 pmol of each primer; 2.5 U of *Taq* polymerase

TABLE 2. Amino acid uptake activities in different strains of cyanobacteria<sup>a</sup>

Amino acid	Uptake activity in strain:									
	<i>Anabaena</i> sp. strain PCC 7120	<i>Anabaena</i> sp. strain PCC 7937	<i>Nostoc</i> sp. strain PCC 7413	<i>Nostoc</i> sp. strain PCC 7107	<i>Calothrix</i> sp. strain PCC 7601	<i>F. muscicola</i> strain UTEX 1829	<i>Pseudanabaena</i> sp. strain PCC 6903	<i>Synechococcus</i> sp. strain PCC 7942	<i>Synechocystis</i> sp. strain PCC 6803	
<b>Basic</b>										
Arg	103.0	59.0	535.7	58.4	79.2	6.3	28.6	0.3	3126	
His	74.1	90.8	147.5	65.9	110.1	20.7	90.2	24.3	63.9	
Lys	115.0	69.6	73.4	59.6	119.7	14.8	53.5	0.8	457.3	
<b>Neutral</b>										
Ala	119.3	119.0	280.4	86.6	191.9	44.3	196.6	29.9	101.0	
Asn	86.6	45.1	122.1	31.8	80.8	12.9	62.6	4.3	10.5	
Gln	73.1	52.0	137.5	34.3	76.7	18.4	106.6	13.4	51.6	
Gly	175.7	113.5	208.0	73.4	182.1	48.7	220.7	6.7	102.8	
Leu	71.0	60.3	155.4	60.0	79.3	26.7	135.7	41.7	79.4	
Phe	72.6	73.0	176.1	53.8	100.5	33.7	137.7	41.8	54.4	
Pro	94.3	89.5	282.7	69.3	117.4	23.0	192.7	0.7	70.2	
Ser	129.4	84.1	258.4	79.3	203.6	34.9	186.8	8.9	69.3	
<b>Acidic</b>										
Asp	18.3	21.9	25.6	4.3	11.2	8.4	286.0	7.1	2.6	
Glu	6.9	11.4	12.2	6.1	5.8	3.2	136.2	11.2	568.3	

<sup>a</sup> The uptake of 10  $\mu\text{M}$  <sup>14</sup>C-labeled amino acid was measured in cells that had been grown in BG11 (nitrate-containing) medium as described in Materials and Methods. Uptake activities, measured in 10-min assays (1-min assay in the case of Arg and Glu in strain PCC 6803), are expressed in nanomoles per milligram of Chl per 10 min.

(Boehringer); and buffer (Boehringer). The program used for amplification was denaturation, 1 min at 95°C; annealing, 1 min at 55°C; and polymerization, 1 min at 72°C (30 cycles).

PCR products were cloned in pGEM-T vector (Promega); plasmids containing PCR products generated with primers Tr8-Tr9 and Tr20-Tr21 were named pCSX10 and pCSX24, respectively. The 1.1-kb kanamycin resistance ( $\text{Km}^r$ ) gene cassette C.K3 (5) was inserted into a unique *Sma*I site of pCSX10 to generate pCSX18a and pCSX18b (both orientations) and into a unique *Sma*I site of pCSX24 to generate pCSX28a and pCSX28b (both orientations). Sequences of plasmids pCSX18b and pCSX28a were verified by using a <sup>17</sup>S sequencing kit (Pharmacia) and [ $\alpha$ -<sup>35</sup>S]-thio-dATP. Transformation of *Synechocystis* sp. strain PCC 6803 with pCSX18a, pCSX18b, pCSX28a, and pCSX28b was carried out as previously described (4), except that the cells were spread onto nitrocellulose filters (REC-85; Nuclepore). Transformants were selected in BG11 solid medium supplemented with 30  $\mu\text{g}$  of kanamycin  $\text{ml}^{-1}$ .  $\text{Km}^r$  transformants were then grown in medium supplemented with 100 to 300  $\mu\text{g}$  of kanamycin  $\text{ml}^{-1}$ . To test whether the resulting mutant strains were homogeneous for the mutant chromosomes, PCR amplification with genomic DNA from the mutants as templates and the corresponding primers was carried out. Amino acid transport activities of these mutants were determined as described above.

**Genomic DNA and plasmid isolation.** Isolation of genomic DNA from cyanobacteria was carried out as previously described (2), except for strain UTEX 1829 cells that were frozen with liquid air and broken by grinding, with glass beads, in a mortar. Plasmid DNA from *E. coli* DH5 $\alpha$  was isolated as previously described (24).

**Southern blotting and hybridization.** Blotting of DNA onto GeneScreen Plus membranes (DuPont) was carried out according to the manufacturer's recommendations. Prehybridization and hybridization were carried out in 5 $\times$  SSPE (1 $\times$  SSPE is 0.18 M NaCl, 10 mM sodium phosphate, and 1 mM EDTA [pH 7.4])–5 $\times$  Denhardt's solution (24)–0.5% (wt/vol) sodium dodecyl sulfate (SDS)–50  $\mu\text{g}$  of herring sperm DNA  $\text{ml}^{-1}$ . Hybridization was carried out at 55°C for 15 h. Filters were then washed twice, at the same temperature, with 2 $\times$  SSPE–0.1% SDS for 15 min and thereafter once or twice with 1 $\times$  SSPE–0.1% SDS for 15 min. DNA probes were labeled with a DNA labeling kit (Ready to Go kit; Pharmacia) and [ $\alpha$ -<sup>32</sup>P]dCTP. The *nata*A probe used was a 609-bp *Hpa*I fragment that contained part of *nata*A (591 bp) and 18 bp upstream of it, isolated from a DNA fragment generated by PCR with primers Tr20 and Tr21 and pCSX24 as the template. The *nata*B probe used was a DNA fragment internal to *nata*B generated by PCR with primers Tr8 and Tr9 and pCSX10 as the template.

**Determination of amino acids in the culture medium.** Cells that had been grown in liquid BG11 medium (with the corresponding analog or antibiotic added in the case of mutant strains) for approximately 1 week were harvested, washed, and resuspended in the same medium without analogs, at 1  $\mu\text{g}$  of Chl  $\text{ml}^{-1}$ . After 46 to 51 h of incubation under culture conditions, samples were withdrawn and filtered. A portion of the filtered solution (culture medium without cells) was frozen and lyophilized, and the dry material obtained was dissolved in a small volume of water. Amino acids present in the concentrated

samples were then separated by high-performance liquid chromatography (HPLC).

The method used for reverse-phase HPLC of amino acids involves derivatization of amino acids with orthophthalaldehyde and  $\beta$ -mercaptoethanol and detection by fluorescence emission at 425 nm. A gradient of two solvent mixtures was used (6). Solvent A was methanol-tetrahydrofuran-buffer (2:2:96, vol/vol). Solvent B was methanol-water (65:35, vol/vol). Buffer was 0.05 M  $\text{Na}_2\text{HPO}_4$ –0.05 M sodium acetate, adjusted to pH 7.5 with glacial acetic acid. The gradient program used was as follows: time zero, 0% solvent B; 11.5 min, 50% B; 20.5 min, 100% B; 25.5 min, 0% B; 30 min, 0% B. The Waters chromatograph utilized was equipped with a Waters Resolve C18, 5- $\mu\text{m}$  particle size column (3.9 by 150 mm). An amino acid standard solution (Sigma) was used for calibrating the apparatus. Neither Pro nor Cys could be detected by this method.

## RESULTS

**Amino acid uptake.** In order to investigate the ability of different cyanobacterial strains to take up some amino acids, we checked the incorporation of radioactivity into the cells from an extracellular solution of 10  $\mu\text{M}$  <sup>14</sup>C-labeled amino acid, in 10-min or 1-min assays. Table 2 shows the results obtained with 13 amino acids and nine different cyanobacterial strains. Detailed studies with *Anabaena* sp. strain PCC 7120 (14, 22) and *Synechocystis* sp. strain PCC 6803 (8, 19) have been described previously, these strains being included again in this work for the sake of comparison.

Among taxonomic Section IV (23) strains, *Anabaena* sp. strain PCC 7937 and *Calothrix* sp. strain PCC 7601 exhibited amino acid uptake activities very similar (within a range of 0.52- to 1.6-fold) to those presented by strain PCC 7120. *Nostoc* sp. strain PCC 7413, compared to strain PCC 7120, exhibited uptake activities somewhat higher (from 1.2- to 3-fold) for most amino acids, and 5.2-fold for Arg. We determined the kinetic parameters of Arg uptake in strain PCC 7413 and found the following values:  $K_m$ , 3.3  $\mu\text{M}$ ;  $V_{max}$ , 124  $\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$  of Chl. *Nostoc* sp. strain PCC 7107 exhibited uptake activities somewhat lower (from 0.23- to 0.89-fold) than those exhibited by strain PCC 7120.

*Fischerella muscicola* UTEX 1829, a heterocyst-forming cyanobacterium that belongs to taxonomic Section V (23), showed

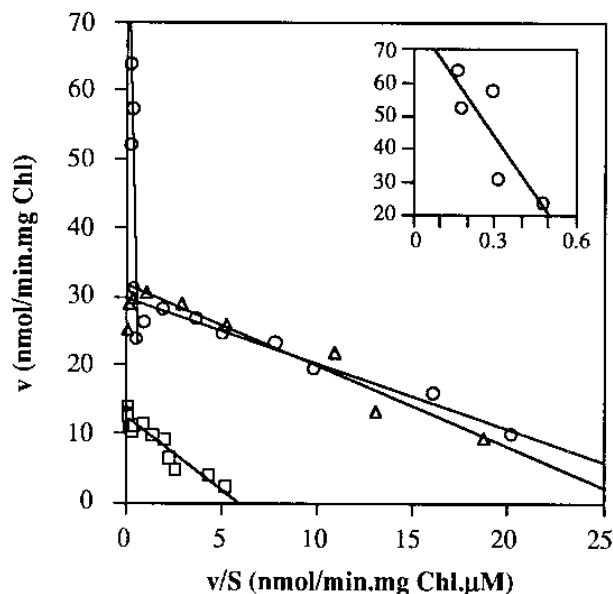


FIG. 1. Eadie-Hofstee plot of the effect of the concentration of Asp on the rate of Asp uptake by *Pseudanabaena* sp. strain PCC 6903 (○), CS446 (□), and CS448 (△). Cells grown in BG11 medium, with added amino acid analogs for the mutants (100  $\mu$ M azaleucine, strain CS448, and 20  $\mu$ M methionine sulfoximine, strain CS446) were washed with Tricine buffer and used in 1-min uptake assays as described in Materials and Methods.  $v$ , rate of uptake of Asp;  $S$ , concentration of Asp.

neutral and acidic amino acid uptake activities which were ca. 30% and ca. 50%, respectively, of those exhibited by strain PCC 7120 (Table 2). On the other hand, the uptake activities of the basic amino acids Arg and Lys in strain UTEX 1829 were rather low, at 6 and 13%, respectively, of the values observed with strain PCC 7120.

*Pseudanabaena* sp. strain PCC 6903 belongs to taxonomic Section III of cyanobacteria (23) and is phylogenetically very distant from the other strains included in this study (12). Strain PCC 6903 showed uptake activities of neutral amino acids that were somewhat higher (1.5-fold as a mean) and uptake activities of Arg and Lys that were lower (0.28- and 0.47-fold, respectively) than those exhibited by strain PCC 7120. However, strain PCC 6903 exhibited a very high activity of uptake of acidic amino acids (Table 2). Determination of the kinetic parameters of uptake of Asp showed two kinetic components with apparent  $K_s$  values of 1.1 and 116  $\mu$ M, respectively (Fig. 1).  $V_{max}$  of the high-affinity component was 32.4  $\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$  of Chl, and  $V_{max}$  of the low-affinity component was, after subtracting  $V_{max}$  for the high-affinity system, 46.2  $\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$  of Chl.

*Synechococcus* sp. strain PCC 7942 and *Synechocystis* sp. strain PCC 6803 are unicellular cyanobacteria that belong to taxonomic Section I (23). Strain PCC 7942 exhibited extremely low activities of uptake of Arg and Lys, suggesting the absence of a basic amino acid transport system in this cyanobacterium, since uptake of His, for which this strain exhibited a moderate activity of transport (Table 2), might take place through a transport system for neutral amino acids. Among the neutral amino acids, strain PCC 7942 took up the nonpolar amino acids Ala, Leu, and Phe at higher rates than the polar amino acids tested (Table 2). With regard to acidic amino acids, strain PCC 7942 showed uptake activities similar to those observed with some other cyanobacteria like the *Anabaena* and *Nostoc* strains included in this study (Table 2). Results obtained in this

work on amino acid uptake in strain PCC 6803 are consistent with previously reported data (8, 19). This strain showed uptake activities of neutral amino acids and His similar to those exhibited by the majority of the cyanobacteria studied, but uptake activities for Arg, Lys, and Glu were highest in strain PCC 6803 than in any other tested cyanobacterium (Table 2). Kinetic parameters of basic amino acid uptake in strain PCC 6803 have been previously investigated (8, 19); we have now determined kinetic parameters of Glu uptake and found the following values:  $K_s$ , 49  $\mu$ M;  $V_{max}$ , 529  $\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$  of Chl.

**Amino acid transport spontaneous mutants.** The isolation of mutants resistant to toxic amino acids (8) or to toxic amino acid analogs (14, 19, 22) has permitted the identification of some amino acid transport systems in *Anabaena* sp. strain PCC 7120 and *Synechocystis* sp. strain PCC 6803. We have been able to isolate mutants resistant to some toxic amino acid analogs from five of the cyanobacterial strains described above and have determined their activity of uptake of 13 amino acids.

Strain CS440, a mutant of *Anabaena* sp. strain PCC 7937 isolated as a clone resistant to azaleucine, was severely impaired in the uptake of neutral amino acids but showed relatively high levels of uptake of acidic amino acids and of Arg and Lys (Table 3). Strain CS440 would be impaired in a transport system for neutral amino acids which would also be responsible for a significant fraction of the uptake of His. The uptake of basic amino acids still observed in mutant CS440 was inactivated in a hydroxylysine-resistant derivative of that strain (strain CS454) (Table 3), and the uptake of acidic amino acids still observed in mutant CS440 was inactivated in a methionine sulfoximine-resistant derivative (strain CS447) (Table 3). To obtain a mutant derived from strain PCC 7937 that missed all the amino acid uptake activities that we have determined, we isolated a hydroxylysine-resistant derivative of strain CS447, mutant CS453, which indeed showed very low levels of uptake of all of the tested amino acids (Table 3).

Azaleucine-resistant derivatives of *Nostoc* sp. strains PCC 7413 and PCC 7107, mutants CS441 and CS442, respectively, were mainly impaired in the uptake of neutral amino acids (Table 3). However, whereas strain CS441 showed levels of uptake of basic and acidic amino acids which were lower than those of its parental strain PCC 7413, strain CS442 actually showed uptake activities of basic amino acids and Asp which were higher than those of its parental strain PCC 7107 (Table 3). The uptake of acidic amino acids, as well as that of His and Lys, was further impaired in a methionine sulfoximine-resistant mutant of strain CS441, strain CS456 (Table 3). On the other hand, the uptake of basic amino acids, but not that of acidic amino acids, was severely impaired in a hydroxylysine-resistant mutant of strain CS442, strain CS457 (Table 3). An azaleucine-resistant derivative of *Calothrix* sp. strain PCC 7601, mutant CS451, exhibited a phenotype very similar to that of mutant strain CS441 (Table 3).

An azaleucine-resistant mutant of *Pseudanabaena* sp. strain PCC 6903, mutant CS448, was severely impaired not only in the uptake of neutral amino acids but also in that of basic amino acids, and a similar phenotype was exhibited by a methionine sulfoximine-resistant mutant of strain PCC 6903, mutant CS446 (Table 3). Additionally, the two mutants showed uptake activities of acidic amino acids which were lower than those exhibited by the wild-type strain PCC 6903. As described above, strain PCC 6903 showed two kinetic components for the uptake of Asp. Mutants CS446 and CS448 showed only the high-affinity component (Fig. 1), suggesting that the low-affinity uptake of Asp was mediated by the transport system that also mediates the uptake of neutral and basic amino acids.

TABLE 3. Amino acid uptake activities of amino acid transport mutants derived from various strains of cyanobacteria<sup>a</sup>

Amino acid	Uptake activity (%) in mutant strains <sup>b</sup> derived from the indicated PCC wild-type strain										
	PCC 7937				PCC 7413		PCC 7107		PCC 7601	PCC 6903	
	CS440	CS454	CS447	CS453	CS441	CS456	CS442	CS457	CS451	CS448	CS446
<b>Basic</b>											
Arg	81	8	81	5	65	58	128	2	40	1	2
His	50	11	38	1	28	15	89	4	21	11	3
Lys	75	<1	70	<1	59	31	136	4	26	<1	<1
<b>Neutral</b>											
Ala	19	4	3	2	4	1	11	2	2	<1	<1
Asn	8	7	5	3	3	<1	5	5	2	16	7
Gln	15	15	1	2	5	1	27	33	6	9	3
Gly	9	3	11	5	1	2	7	9	<1	<1	<1
Leu	2	<1	<1	<1	<1	<1	10	9	<1	3	2
Phe	<1	2	<1	2	1	<1	6	6	<1	<1	2
Pro	<1	<1	<1	<1	<1	<1	2	2	<1	<1	<1
Ser	7	6	11	1	2	<1	6	7	1	1	<1
<b>Acidic</b>											
Asp	67	67	4	3	36	8	175	202	34	47	26
Glu	71	79	3	2	29	15	92	77	40	47	51

<sup>a</sup> The uptake of 10  $\mu$ M <sup>14</sup>C-labeled amino acid was measured in 10-min assays. Data of uptake activities in the mutants are presented as a percentage of the value exhibited by the corresponding wild-type strain.

<sup>b</sup> Strain CS440 is an azaleucine-resistant mutant of strain PCC 7937; strain CS454 is a hydroxylysine-resistant mutant of strain CS440; strain CS447 is a methionine sulfoximine-resistant mutant of strain CS440; strain CS453 is a hydroxylysine-resistant mutant of strain CS447; strain CS441 is an azaleucine-resistant mutant of strain PCC 7413; strain CS456 is a methionine sulfoximine-resistant mutant of strain CS441; strain CS442 is an azaleucine-resistant mutant of strain PCC 7107; strain CS457 is a hydroxylysine-resistant mutant of strain CS442; strain CS451 is an azaleucine-resistant mutant of strain PCC 7601; strain CS448 is an azaleucine-resistant mutant of strain PCC 6903; strain CS446 is a methionine sulfoximine-resistant mutant of strain PCC 6903.

**Genes encoding amino acid transport elements.** The availability of the DNA sequence of about 1 Mb of the genome of *Synechocystis* sp. strain PCC 6803 (17) allowed us to investigate the possible involvement in amino acid transport of some ORFs whose putative protein products show homology to permease elements. The experimental approach used involved amplification by PCR and cloning of ORFs of interest, interruption of the cloned ORF with gene cassette C.K3 (that encodes resistance to kanamycin and lacks transcriptional terminators), transformation of strain PCC 6803 with the mutated ORF, and determination of amino acid transport activities of the generated *Synechocystis* mutants. (Details of cloning and inactivation of two ORFs are described in Materials and Methods, and a similar methodology was used for all other ORFs investigated.) The ORFs investigated, and the protein(s) to which the putative products of the ORFs would be homologous, were the following: *slr0075*, *sll0484*, *slr0467*, and *sll0374*, products homologous to the conserved component of ABC-type transport systems; *sll0064*, a product homologous to periplasmic binding proteins for glutamine; *slr0700*, a product homologous to a 4-amino butyrate permease (GABA) and to cationic amino acid permeases; *sll0606* and *slr0559*, products homologous to periplasmic binding proteins for leucine, isoleucine, and valine (no homology for the putative products of *sll0606* and *slr0559* was reported by Kaneko et al. [17]), but the indicated homology was found in an analysis performed by us of the reported 1 Mb of *Synechocystis* DNA sequence); and *slr0468*, a product with no homology to any known protein (this ORF was studied because it is adjacent to *slr0467*).

A DNA fragment of 1,048 bp comprising the whole *slr0467* ORF (801 bp) was amplified by PCR and cloned (see Materials and Methods for details). Gene cassette C.K3 was inserted into *slr0467*, generating plasmids pCSX28a and pCSX28b (Fig. 2) that were transferred to strain PCC 6803 by transformation

and selection for Km<sup>r</sup>. Chromosomally homogeneous clones bearing inactivated *slr0467* were named strains CSX28a and CSX28b, respectively. Strains CSX28a and CSX28b were severely impaired in uptake of the neutral amino acids Ala, Gly, Leu, Phe, Pro, and Ser (Table 4).

A DNA fragment of 893 bp internal to ORF *slr0559* was amplified by PCR and cloned (see Materials and Methods for details). Gene cassette C.K3 was inserted into the amplified region of *slr0559*, rendering plasmids pCSX18a and pCSX18b (Fig. 2) that were used to generate, by transformation of strain PCC 6803 strains CSX18a and CSX18b, respectively. These strains were chromosomally homogeneous for inactivated *slr0559* and exhibited very low rates of uptake of the neutral amino acids Ala, Gly, Leu, Phe, Pro, and Ser (Table 4).

Inactivation of neither *slr0467* nor *slr0559* substantially affected the activity of uptake of Gln and of basic amino acids (Table 4) (His can behave to some extent as a neutral amino acid). Insertional mutants of *slr0467* and *slr0559* were impaired in the uptake of Asp (Table 4). Because strain PCC 6803 was able to take up Asp only at a very low rate (Table 2), it is possible that a significant fraction of this uptake took place through a neutral amino acid transport system. Inactivation of *slr0467* resulted in a 33 to 53% inhibition of the uptake of Glu, an effect that was not reproduced by inactivation of *slr0559* (Table 4). Because the mutants of *slr0467* were essentially devoid of activity of transport of neutral amino acids (except Gln), we used one of these mutants (strain CSX28a) to analyze Glu transport in the absence of any contribution of a neutral amino acid transport system. Kinetic parameters of Glu uptake in strain CSX28a were determined, and the following values were found:  $K_s$ , 44  $\mu$ M;  $V_{max}$ , 389 nmol  $\cdot$  min<sup>-1</sup>  $\cdot$  mg<sup>-1</sup> of Chl. Kinetic parameters of Glu uptake were also determined in the presence of Gln at 0.01, 0.1, or 1 mM, and  $K_s$  and  $V_{max}$  values similar to those obtained in the absence of Gln were found.

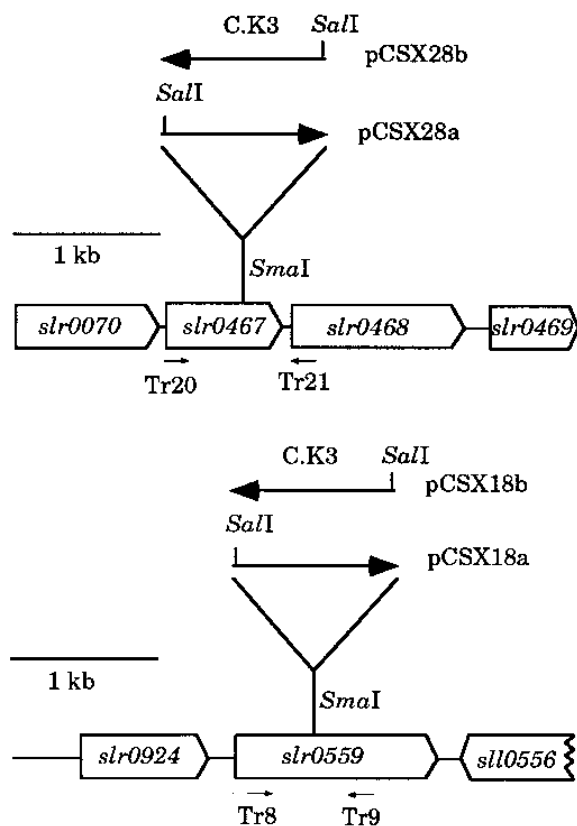


FIG. 2. Schematic representation of the genomic regions around ORFs *slr0467* (map position, 334 kb) and *slr0559* (map position, 532 kb) in the genome of *Synechocystis* sp. strain PCC 6803 (17). None of the ORFs neighboring *slr0467* or *slr0559* would encode transport-related proteins. The locations of the primers used for PCR amplification (Tr20, Tr21, Tr8, and Tr9) and of the *Sma*I restriction sites where gene cassette C.K3 was inserted in *slr0467* and *slr0559* are shown. The orientation of C.K3 in the plasmids bearing inactivated *slr0467* or *slr0559* is also indicated.

The amino acid transport phenotype of the insertional mutants of ORFs *slr0467* and *slr0559* suggests that these are genes encoding elements of a neutral amino acid permease. We shall refer to these genes as *nata* (*slr0467*) and *natB* (*slr0559*) (*nat* derives from neutral amino acid transport). Chromosomically homogeneous mutants were obtained for all of the other investigated ORFs except *slr0075*. None of those mutants was impaired in the transport of any of the 12 amino acids tested (those amino acids that are included in Table 4).

Since all of the cyanobacterial strains that were studied in this work showed the ability to take up neutral amino acids (Table 2), we investigated, by means of Southern hybridization analysis, the presence in those cyanobacteria of DNA sequences homologous to *nata* and *natB* from strain PCC 6803 (Fig. 3). Under the conditions of the experiment whose results are shown in Fig. 3, hybridization signals were observed, with the two probes, with all of the tested strains except PCC 7937. However, with strain PCC 7937 DNA, hybridizing bands of 3.2 kb with the *nata* probe and of 8 kb with the *natB* probe were observed with a larger amount of loaded *Hind*III-restricted DNA (not shown).

**Amino acids in the extracellular medium.** Because some amino acid transport mutants of both *Anabaena* sp. strain PCC 7120 (22) and *Synechocystis* sp. strain PCC 6803 (19) have been shown to release some amino acids to the extracellular me-

TABLE 4. Amino acid uptake activities of *Synechocystis* sp. strain PCC 6803 insertional mutants of ORFs *slr0467* and *slr0559*<sup>a</sup>

Amino acid	Uptake activity in mutants of ORF:			
	<i>slr0467</i>		<i>slr0559</i>	
	CSX28a	CSX28b	CSX18a	CSX18b
<b>Basic</b>				
Arg	92	71	100	98
His	70	70	73	68
Lys	80	88	94	84
<b>Neutral</b>				
Ala	<1	<1	<1	1
Gln	86	86	100	94
Gly	<1	<1	<1	1
Leu	<1	<1	<1	1
Phe	<1	<1	<1	1
Pro	<1	<1	<1	<1
Ser	5	3	4	5
<b>Acidic</b>				
Asp	27	19	27	23
Glu	67	47	88	84

<sup>a</sup> The uptake of 10  $\mu$ M <sup>14</sup>C-labeled amino acid was measured in 10-min assays, except in the case of Arg and Glu (1-min assays). Data of uptake activities in the mutants are presented as a percentage of the value exhibited by the wild-type strain PCC 6803.

dium, we tested accumulation of amino acids in media where some of the mutants described above had been incubated for approximately 48 h. The spontaneous mutants investigated were those isolated as azaleucine-resistant derivatives of Section IV cyanobacteria and the azaleucine- and methionine sulfoximine-resistant derivatives of *Pseudanabaena* sp. strain PCC 6903. The HPLC chromatograms shown in Fig. 4 indicate that all of the tested mutants accumulated in the outer medium, at higher concentrations than their parental wild-type strains, a set of amino acids that included Ala, Val, Phe, Ile, and Leu. The amino acid analog-resistant mutants of *Pseudanabaena* sp. strain PCC 6903, strains CS446 and CS448, also accumulated in the outer medium some other amino acids, including Asp, Glu, Ser, Gly, Thr, and Tyr, that were also released to some extent by wild-type strain PCC 6903 (Fig. 4).

An azaleucine-resistant mutant of *Synechocystis* sp. strain PCC 6803 has previously been shown to release some amino acids to the extracellular medium (19). We have now determined the concentration of amino acids in the medium in cell suspensions of the *nata* and *natB* insertional mutants, strains CSX28a and CSX18b, respectively. After 46 h of incubation in BG11 medium, extracellular accumulation of Ala, Val, Phe, Ile, and Leu was observed with both mutants (Fig. 5).

## DISCUSSION

**Cyanobacterial amino acid transport systems.** A summary of the amino acid transport systems that, as discussed below, can be defined in those cyanobacteria that we have studied is presented in Table 5. Amino acid transport activities exhibited by some cyanobacteria but not yet inactivated by mutation are also depicted in Table 5. The four cyanobacterial strains belonging to Section IV as described by Rippka et al. (23) that, in addition to *Anabaena* sp. strain PCC 7120, have been included in this study, namely, *Anabaena* sp. strain PCC 7937, *Nostoc* sp. strains PCC 7413 and PCC 7107, and *Calothrix* sp. strain PCC 7601, showed a pattern of amino acid uptake activities (Table 2) and allowed the isolation of a set of mutants (Table 3) that

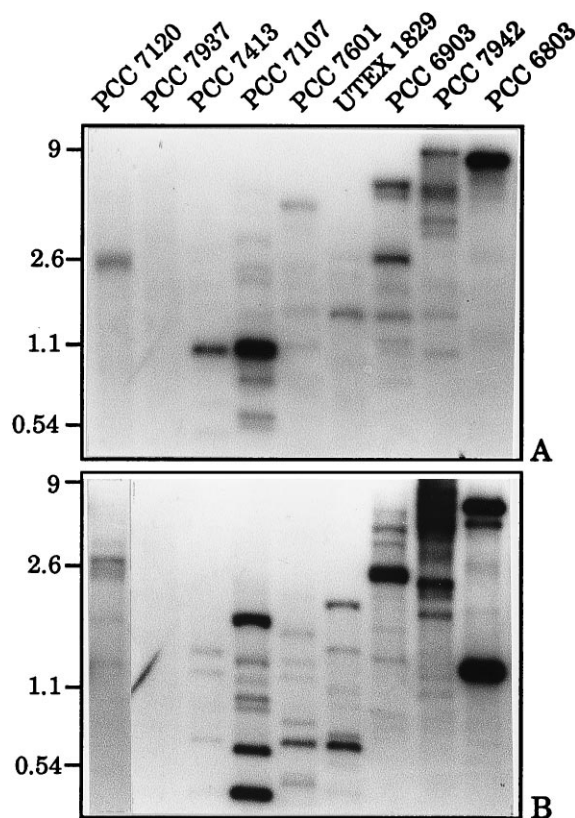


FIG. 3. Hybridization of *natA* (A) or *natB* (B) to genomic DNA from several cyanobacteria. Genomic DNA was restricted with *Hind*III (strain PCC 7120 in panel A and strain PCC 7942), *EcoRV* (strain PCC 7120 in panel B and strain PCC 7937), *Mva*I (strains PCC 7413, PCC 7107, PCC 7601, and UTEX 1829), or *Dra*I (strains PCC 6903 and PCC 6803). Southern blots were probed with a 609-bp fragment of *natA* (A) or an 893-bp fragment of *natB* (B) (see Materials and Methods). Sizes in kilobases are indicated on the left.

indicate the presence in these four strains of a transport system for neutral amino acids. In contrast to strain PCC 7120, where two different systems for the uptake of neutral amino acids have been detected (22), strains PCC 7937, PCC 7413, PCC 7107, and PCC 7601 would bear a single transport system for neutral amino acids, as suggested by the low activity of uptake of neutral amino acids remaining in the azaleucine-resistant mutants that were isolated (Table 3). In strains PCC 7937, PCC 7413, and PCC 7601, this transport system seems to transport also, to some extent, basic and acidic amino acids, since azaleucine-resistant mutants derived from these strains were somewhat impaired in the uptake of basic and acidic amino acids as well (Table 3). The transport system for neutral amino acids in strain PCC 7937 probably corresponds to that previously described for the same organism as a Leu transporter (27).

The activities of transport of basic and acidic amino acids observed with the Section IV cyanobacteria studied in this work (Table 2) were similar to those described for strain PCC 7120 (22). The presence in *Anabaena* sp. strain PCC 7937 of transport systems specific for basic and acidic amino acids is suggested by the behavior of strains isolated as mutants resistant to hydroxylysine (that showed inactivation of transport of Arg, His, and Lys) and methionine sulfoximine (that showed inactivation of transport of Asp and Glu), respectively (Table 3). The Gln uptake activity remaining in the azaleucine-resistant mutant of strain PCC 7937, strain CS440, was lost in the

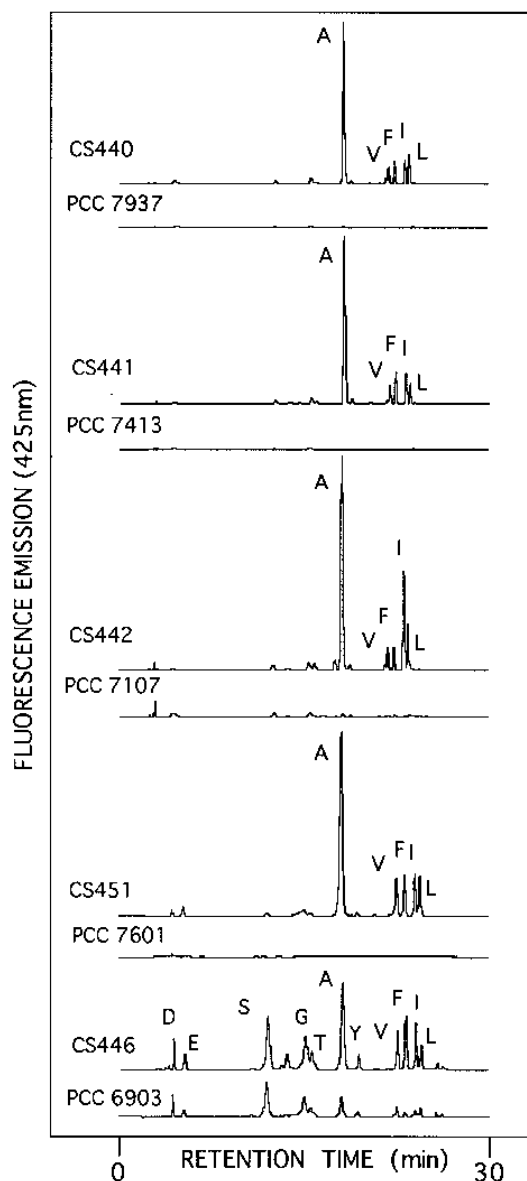


FIG. 4. HPLC chromatograms of samples from the extracellular medium of cultures of *Anabaena* sp. strain PCC 7937 and its azaleucine-resistant derivative strain CS440, *Nostoc* sp. strain PCC 7413 and its azaleucine-resistant derivative strain CS441, *Nostoc* sp. strain PCC 7107 and its azaleucine-resistant derivative strain CS442, *Calothrix* sp. strain PCC 7601 and its azaleucine-resistant derivative strain CS451, and *Pseudanabaena* sp. strain PCC 6903 and its methionine sulfoximine-resistant derivative strain CS446. Cells grown on BG11 medium (supplemented with amino acid analogs in the case of the mutants) were washed, resuspended in BG11 medium, and incubated for approximately 48 h under culture conditions. Samples of the cell suspensions were filtered, and the filtrates were concentrated and subjected to HPLC analysis as described in Materials and Methods. The amino acids indicated are as follows: A, Ala; V, Val; F, Phe; I, Ile; L, Leu; D, Asp; E, Glu; S, Ser; G, Gly; T, Thr; Y, Tyr. We do not know the identity of the substance originating the peak between S and G in the sample from strain CS446. Results similar to those obtained with strain CS446 were obtained with strain CS448. The concentrations of Ala in the extracellular medium of the cell suspensions were 6.4 to 12.1  $\mu$ M in the case of the mutants, 0.04 to 0.36  $\mu$ M for the wild-type heterocyst formers, and 1.4  $\mu$ M for strain PCC 6903.

methionine sulfoximine-resistant derivative of strain CS440, strain CS447. This suggests that Gln can also be transported by the acidic amino acid transport system of strain PCC 7937, which is consistent with the proposal of a common element for

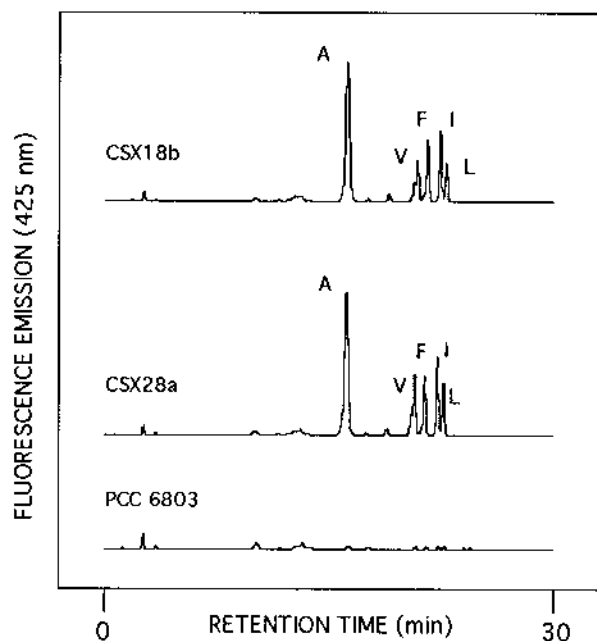


FIG. 5. HPLC chromatograms of samples from the extracellular medium of cultures of *Synechocystis* sp. strain PCC 6803 and of insertional mutants CSX18b (*natB*) and CSX28a (*natA*). Cells grown in BG11 medium (supplemented with kanamycin in the case of the mutants) were washed, resuspended in BG11 medium, and incubated for 46 h under culture conditions. Samples of the cell suspensions were filtered, and the filtrates were concentrated and subjected to HPLC analysis as described in Materials and Methods. The amino acids indicated are as follows: A, Ala; V, Val; F, Phe; I, Ile; L, Leu. The concentrations of Ala were 0.15  $\mu$ M (strain PCC 6803), 7.4  $\mu$ M (strain CSX28a), and 7.5  $\mu$ M (strain CSX18b).

the transport of Glu and Gln in this cyanobacterium (3). The affinity of cells of strains PCC 7120 and PCC 7937 for Glu is relatively low ( $K_s$ , 465  $\mu$ M for strain PCC 7120 [22] and 100  $\mu$ M and 1.4 mM for strain PCC 7937 [3]), but a transport system with a high affinity for acidic amino acids ( $K_s$  for Glu, 0.1 to 0.3  $\mu$ M;  $K_s$  for Asp, 2 to 3  $\mu$ M) has been described for a symbiotic *Nostoc* sp. (26). The transport activities exhibited by strain CS457, a hydroxylysine-resistant derivative of strain CS442, demonstrate that also in *Nostoc* sp. strain PCC 7107 the ability to transport basic and acidic amino acids can be separated by mutation (Table 3). Simultaneous inactivation of uptake of Arg, His, and Lys in mutant CS457 suggests the presence of a single transport system specific for basic amino acids in strain PCC 7107. No such clear separation between the ability to transport acidic amino acids and the ability to transport basic amino acids was obtained with *Nostoc* sp. strain PCC 7413 through the analysis of a methionine sulfoximine-resistant derivative of strain CS441, mutant CS456 (Table 3). However, this mutant still exhibited a considerable activity of uptake of Arg, suggesting the presence in strain PCC 7413 of a transport system rather specific for Arg. Indeed, strain PCC 7413 exhibited a very high activity of transport of Arg, with a  $K_s$  of 3.3  $\mu$ M, a figure similar to that of the high-affinity basic amino acid transport system of strain PCC 7120 ( $K_s$  for Arg, 1.7  $\mu$ M) (14). We were unable to isolate methionine sulfoximine- or hydroxylysine-resistant mutants of *Calothrix* sp. strain PCC 7601 and, therefore, have no direct evidence to separate the basic and acidic amino acid transport activities of this strain.

*F. muscicola* UTEX 1829, also a heterocyst-forming cyanobacterium, showed relatively low uptake activities of all of the tested amino acids, especially of basic amino acids (Table

2). No mutant of strain UTEX 1829 resistant to azaleucine could be isolated, and therefore, we currently do not know the complement of amino acid transport systems of *F. muscicola*.

*Pseudanabaena* sp. strain PCC 6903 appears to bear at least two amino acid transport systems: one general amino acid permease able to transport basic, neutral, and acidic amino acids, and a permease able to transport Asp with high affinity ( $K_s$ , 1.1  $\mu$ M). The mutants (strains CS446 and CS448) missing the general amino acid transport system still exhibited some activity of transport of acidic amino acids (Fig. 1; Table 3). Competition studies (data not shown) have indicated that the transport system taking up Asp with high affinity could also recognize, with lower affinity, Glu.

The unicellular cyanobacterium *Synechococcus* sp. strain PCC 7942 appears to express a transport system for neutral, nonpolar amino acids which might also transport His and, to a lesser extent, Gln and some other neutral amino acids (Table 2). This system could be identical to the branched-chain amino acid transport system previously detected in *Anacystis nidulans* (a cyanobacterium closely related to strain PCC 7942) (20). While strain PCC 7942 does not appear to bear a transport system for basic amino acids, it might bear a system similar to those present in heterocyst-forming cyanobacteria able to take up acidic amino acids.

In the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803, transport systems for neutral amino acids (excluding Gln) and for basic amino acids (and Gln) have been clearly defined previously, and the presence of a high-affinity transport system for Gln that would also transport Glu has been suggested (19). However, lack of inhibition by Gln of Glu uptake, as observed with strain CSX28a, suggests that strain PCC 6803 bears a transport system which is specific for Glu and different from the high-affinity Gln transport system defined by Labarre et al. (19).

Results obtained in this and previous (19, 22) studies indicate the general presence in cyanobacteria of at least one transport system able to mediate the uptake of neutral amino acids. The specificity of this system is not identical in all cyanobacteria. Thus, as discussed above, the permease present in *Synechococcus* sp. strain PCC 7942 is rather specific for nonpolar, neutral amino acids, whereas that present in *Pseudanabaena* sp. strain PCC 6903 is able to transport, in addition to neutral amino acids, basic and acidic amino acids, to the point that it appears to behave as a general amino acid permease.

**Neutral amino acid permease genes and physiology.** A number of ORFs putatively encoding elements of membrane transport systems are found in the ca. 1 Mb of DNA sequence of the genome of *Synechocystis* sp. strain PCC 6803 that has been published (17). We have tested, by means of mutation, whether some of those ORFs might be involved in the uptake of amino acids. Eight ORFs whose putative products are homologous to elements of amino acid permeases from other organisms were chosen for analysis; an ORF adjacent to one of these ORFs was also investigated. We were able to generate chromosomally homogeneous mutants of eight of those ORFs, and two of them were found to be required for transport of neutral amino acids in strain PCC 6803 (Table 4). The *natA* gene (ORF *slr0467* of the genomic sequence of strain PCC 6803) would encode a 267-residue polypeptide homologous to the conserved component of ABC-type permeases, and the *natB* gene (*slr0559*) would encode a 454-residue polypeptide homologous to the periplasmic binding protein of some multicomponent permeases (that are of the ABC type) and to the metabotropic glutamate receptor of *Rattus norvegicus* (rat). *Synechocystis* sp. strain PCC 6803 has been described to bear a neutral amino acid transport system able to transport all of the neutral amino



TABLE 5. Amino acid transport systems or activities in some cyanobacterial strains

Strain	Substrate amino acid(s) for transport system or activity <sup>a</sup>		
	Group 1	Group 2	Group 3
<i>Anabaena</i> sp. strain PCC 7120 <sup>b</sup>	Neutral amino acids, systems I and II	*Acidic amino acids	Basic amino acids, high- and *low-affinity systems
<i>Anabaena</i> sp. strain PCC 7937	Neutral (and acidic and basic) amino acids	Acidic amino acids (and Gln)	Basic amino acids
CS440	—	+	+
CS454	—	+	—
CS447	—	—	+
CS453	—	—	—
<i>Nostoc</i> sp. strain PCC 7413	Neutral (and acidic and basic) amino acids	Acidic and basic amino acids	*Arg
CS441	—	+	+
CS456	—	—	+
<i>Nostoc</i> sp. strain PCC 7107	Neutral amino acids	*Acidic amino acids (and Gln)	Basic amino acids
CS442	—	+	+
CS457	—	+	—
<i>Calothrix</i> sp. strain PCC 7601	Neutral (and acidic and basic) amino acids	*Acidic and basic amino acids	
CS451	—	+	
<i>F. muscicola</i> UTEX 1829	*Neutral, acidic, and basic amino acids		
<i>Pseudanabaena</i> sp. strain PCC 6903	Amino acids in general	*Asp (and Glu)	
CS448	—	+	
CS446	—	+	
<i>Synechococcus</i> sp. strain PCC 7942	*Neutral, nonpolar amino acids (and His, Gln, Gly, Ser, and Asn)	*Acidic amino acids	
<i>Synechocystis</i> sp. strain PCC 6803 <sup>c</sup>	Neutral amino acids (excluding Gln)	*Glu	Basic amino acids (and Gln) and Gln, *high affinity system
CSX28a	—	+	+
CSX28b	—	+	+
CSX18a	—	+	+
CSX18b	—	+	+

<sup>a</sup> Transport systems that have been defined by mutation are presented with indication of their substrate amino acids; amino acids that are transported only partially (or with a lower rate or affinity) by those systems are shown in parentheses. Transport activities or putative transport systems that are exhibited by a cyanobacterial strain but that have not been inactivated by mutation are indicated by an asterisk. +, same as for the parent strain; —, no transport system or activity for amino acids given for parent strain.

<sup>b</sup> For a more detailed description of the amino acid transport systems of this strain, see references 14 and 22.

<sup>c</sup> For a more detailed description of the amino acid transport systems of this strain, see references 8 and 19.

acids but Gln (19). Since the insertional mutants of *natA* and *natB* were unable to transport those amino acids that are substrates of the neutral amino acid transport system, we suggest that *natA* and *natB* encode elements of an ABC-type permease for neutral amino acids in *Synechocystis* sp. strain PCC 6803. The proteins showing highest identities to the putative *natA* and *natB* products are BraF (an ATP-binding protein or conserved component) (40% identity) and BraC (periplasmic substrate-binding protein) (26% identity), respectively, of the high-affinity branched-chain amino acid transport system of *Pseudomonas aeruginosa* (15, 16). The *natA* and *natB* genes are not clustered in the genome of strain PCC 6803, and at least another gene, encoding the membrane core of the permease (1), is yet to be identified for this transport system. DNA sequences hybridizing to probes of the *natA* and *natB*

genes were detected in all of the cyanobacteria included in this work. Since, as discussed above, all of these cyanobacteria exhibit the ability to transport at least some neutral amino acids, it is possible that *natA* and *natB* genes are widespread among cyanobacteria.

We have previously raised the possibility that a role for the neutral amino acid transport system I of *Anabaena* sp. strain PCC 7120 (the main way of uptake of Ala, Leu, and Phe into the cell) is recapture of amino acids that have leaked from the cells (22). Results obtained in this work, together with those previously reported for strains PCC 7120 (22) and PCC 6803 (19), suggest that accumulation in the extracellular medium of Ala, Val, Phe, Ile, and Leu is a general feature of cyanobacterial mutants lacking a permease responsible for the uptake of neutral amino acids. Because the spontaneous, amino acid

analog-resistant mutants analyzed in this work appeared at frequencies of  $10^{-6}$  to  $10^{-5}$  per cell, they most likely were the result of single mutations associated with a loss-of-function effect. Moreover, insertional inactivation of either *natA* or *natB* also resulted in accumulation in the outer medium of Ala, Val, Phe, Ile, and Leu. The accumulation of hydrophobic amino acids in the medium of cell suspensions of the mutants can be readily understood if (i) hydrophobic amino acids undergo some leakage from cyanobacterial cells, as might be the general case in bacteria (18), and (ii) the permease operating transport of neutral amino acids in cyanobacteria has a physiological role in recapture of such leaked amino acids. It should be noted that release of amino acids by cyanobacteria is a phenomenon that has been known for a long time (10, 11, 25, 28).

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#### REFERENCES

- Ames, G. F.-L. 1988. Structure and mechanism of bacterial periplasmic transport systems. *J. Bioenerget. Biomembr.* **20**:1–18.
- Cai, Y., and C. P. Wolk. 1990. Use of a conditionally lethal gene in *Anabaena* sp. strain PCC 7120 to select for double recombinants and to entrap insertion sequence. *J. Bacteriol.* **172**:3138–3145.
- Chapman, J. S., and J. C. Meeks. 1983. Glutamine and glutamate transport by *Anabaena variabilis*. *J. Bacteriol.* **156**:122–129.
- Chauvat, F., L. De Vries, A. Van der Ende, and G. A. Van Arkel. 1986. A host-vector system for gene cloning in the cyanobacterium *Synechocystis* PCC 6803. *Mol. Gen. Genet.* **204**:185–191.
- Elhai, J., and C. P. Wolk. 1988. A versatile class of positive-selection vectors based on the nonviability of palindrome-containing plasmids that allows cloning into long polylinkers. *Gene* **68**:119–138.
- Farfán, M. J., E. Martín-Rendón, and I. L. Calderón. 1996. Effect of gene amplification on threonine production by yeast. *Biotechnol. Bioeng.* **49**:667–674.
- Flores, E., and A. Herrero. 1994. Assimilatory nitrogen metabolism and its regulation, p. 487–517. *In* D. A. Bryant (ed.), *The molecular biology of cyanobacteria*. Kluwer Academic Publishers, Dordrecht, The Netherlands.
- Flores, E., and A. M. Muro-Pastor. 1990. Mutational and kinetic analysis of basic amino acid transport in the cyanobacterium *Synechocystis* sp. PCC 6803. *Arch. Microbiol.* **154**:521–527.
- Flores, E., and M. I. Muro-Pastor. 1988. Uptake of glutamine and glutamate by the dinitrogen-fixing cyanobacterium *Anabaena* sp. PCC7120. *FEMS Microbiol. Lett.* **56**:127–130.
- Flynn, K. J., and J. R. Gallon. 1990. Changes in intracellular and extracellular  $\alpha$ -amino acids in *Gloeothece* during  $N_2$ -fixation and following addition of ammonium. *Arch. Microbiol.* **153**:574–579.
- Fogg, G. E. 1952. The production of extracellular nitrogenous substances by a blue-green alga. *Proc. R. Soc. Ser. B* **139**:372–397.
- Giovannoni, S. J., S. Turner, G. J. Olsen, S. Barns, D. J. Lane, and N. R. Pace. 1988. Evolutionary relationships among cyanobacteria and green chloroplasts. *J. Bacteriol.* **170**:3584–3592.
- Herdman, M., M. Janvier, J. B. Waterbury, R. Rippka, and R. Y. Stanier. 1979. Deoxyribonucleic acid base composition of cyanobacteria. *J. Gen. Microbiol.* **111**:63–71.
- Herrero, A., and E. Flores. 1990. Transport of basic amino acids by the dinitrogen-fixing cyanobacterium *Anabaena* PCC 7120. *J. Biol. Chem.* **265**:3931–3935.
- Hoshino, T., and K. Kose. 1989. Cloning and nucleotide sequence of *braC*, the structural gene for the leucine-, isoleucine-, and valine-binding protein of *Pseudomonas aeruginosa* PAO. *J. Bacteriol.* **171**:6300–6306.
- Hoshino, T., and K. Kose. 1990. Cloning, nucleotide sequences, and identification of products of the *Pseudomonas aeruginosa* PAO *bra* genes, which encode the high-affinity branched-chain amino acid transport system. *J. Bacteriol.* **172**:5531–5539.
- Kaneko, T., A. Tanaka, S. Sato, H. Kotani, T. Sazuka, N. Miyajima, M. Sugiura, and S. Tabata. 1995. Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC6803. I. Sequence features in the 1 Mb region from map positions 64% to 92% of the genome. *DNA Res.* **2**:153–166.
- Krämer, R. 1994. Secretion of amino acids by bacteria: physiology and mechanism. *FEMS Microbiol. Rev.* **13**:75–94.
- Labarre, J., P. Thuriaux, and F. Chauvat. 1987. Genetic analysis of amino acid transport in the facultatively heterotrophic cyanobacterium *Synechocystis* sp. strain 6803. *J. Bacteriol.* **169**:4668–4673.
- Lee-Kaden, J., and W. Simonis. 1982. Amino acid uptake and energy coupling dependent on photosynthesis in *Anacystis nidulans*. *J. Bacteriol.* **151**:229–236.
- Mackinney, G. 1941. Absorption of light by chlorophyll solutions. *J. Biol. Chem.* **140**:315–322.
- Montesinos, M. L., A. Herrero, and E. Flores. 1995. Amino acid transport systems required for diazotrophic growth in the cyanobacterium *Anabaena* sp. strain PCC 7120. *J. Bacteriol.* **177**:3150–3157.
- Rippka, R., J. Deruelles, J. B. Waterbury, M. Herdman, and R. Y. Stanier. 1979. Generic assignments, strain histories and properties of pure cultures of cyanobacteria. *J. Gen. Microbiol.* **111**:1–61.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Stewart, W. D. P. 1963. Liberation of extracellular nitrogen by two nitrogen-fixing blue-green algae. *Nature* **200**:1020–1021.
- Strasser, P., and G. Falkner. 1986. Characterization of the glutamate/aspartate-transport system in a symbiotic *Nostoc* sp. *Planta* **168**:381–385.
- Thiel, T. 1988. Transport of leucine in the cyanobacterium *Anabaena variabilis*. *Arch. Microbiol.* **149**:466–470.
- Watanabe, A. 1951. Production in cultural solution of some amino acids by the atmospheric nitrogen-fixing blue-green algae. *Arch. Biochem. Biophys.* **34**:50–55.
- Weathers, P. J., H. L. Chee, and M. M. Allen. 1978. Arginine catabolism in *Aphanocapsa* 6308. *Arch. Microbiol.* **118**:1–6.
- Wolk, C. P., A. Ernst, and J. Elhai. 1994. Heterocyst metabolism and development, p. 769–823. *In* D. A. Bryant (ed.), *The molecular biology of cyanobacteria*. Kluwer Academic Publishers, Dordrecht, The Netherlands.
- Xu, P., and P. J. McAuley. 1990. Uptake of amino acids by the cyanobacterium *Anabaena* ATCC 27893. *New Phytol.* **115**:581–585.