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Arginine Catabolism in the Cyanobacterium *Synechocystis* sp. Strain PCC 6803 Involves the Urea Cycle and Arginase Pathway

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Cells of the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803 supplemented with micromolar concentrations of L-[^{14}C]arginine took up, concentrated, and catabolized this amino acid. Metabolism of L-[^{14}C]arginine generated a set of labeled amino acids that included argininosuccinate, citrulline, glutamate, glutamine, ornithine, and proline. Production of [^{14}C]ornithine preceded that of [^{14}C]citrulline, and the patterns of labeled amino acids were similar in cells incubated with L-[^{14}C]ornithine, suggesting that the reaction of arginase, rendering ornithine and urea, is the main initial step in arginine catabolism. Ornithine followed two metabolic pathways: (i) conversion into citrulline, catalyzed by ornithine carbamoyltransferase, and then, with incorporation of aspartate, conversion into argininosuccinate, in a sort of urea cycle, and (ii) a sort of arginase pathway rendering glutamate (and glutamine) via Δ^1pyrroline-5-carboxylate and proline. Consistently with the proposed metabolic scheme (i) an *argF* (ornithine carbamoyltransferase) insertional mutant was impaired in the production of [^{14}C]citrulline from [^{14}C]arginine; (ii) a *proC* (Δ^1pyrroline-5-carboxylate reductase) insertional mutant was impaired in the production of [^{14}C]proline, [^{14}C]glutamate, and [^{14}C]glutamine from [^{14}C]arginine or [^{14}C]ornithine; and (iii) a *putA* (proline oxidase) insertional mutant did not produce [^{14}C]glutamate from L-[^{14}C]arginine, L-[^{14}C]ornithine, or L-[^{14}C]proline. Mutation of two open reading frames (*sll0228* and *sll1077*) putatively encoding proteins homologous to arginase indicated, however, that none of these proteins was responsible for the arginase activity detected in this cyanobacterium, and mutation of *argD* (*N*-acetylornithine aminotransferase) suggested that this transaminase is not important in the production of Δ^1pyrroline-5-carboxylate from ornithine. The metabolic pathways proposed to explain [^{14}C]arginine catabolism also provide a rationale for understanding how nitrogen is made available to the cell after mobilization of cyanophycin [multi-L-arginyl-poly(L-aspartic acid)], a reserve material unique to cyanobacteria.

Cyanobacteria are prokaryotic organisms that belong to the *Bacteria* domain and are able to carry out oxygenic photosynthesis. Nitrate, ammonium, urea, and atmospheric nitrogen (dinitrogen) are commonly used as nitrogen sources by these organisms (14). Under aerobic culture conditions and combined nitrogen deprivation, some filamentous cyanobacteria fix dinitrogen in specialized cells called heterocysts (49), which transfer fixed nitrogen, in the form of amino acids, to the neighboring vegetative cells (45, 50). Vegetative cells of filamentous cyanobacteria should therefore have the capacity to metabolize an amino acid(s). Additionally, some amino acids, and among them arginine, can be used by some cyanobacteria as a source of nitrogen for growth (33; for a review, see reference 14). Cyanobacteria bear broad-specificity amino acid transport systems (32), and a number of strains, including the unicellular, non-nitrogen-fixer *Synechocystis* sp. strain PCC 6803, have been shown to bear a high-affinity permease for arginine and other basic amino acids (16, 20, 25).

Most cyanobacteria, including *Synechocystis* sp. strain PCC 6803, accumulate as a reserve material multi-L-arginyl-poly(L-aspartic acid), a polymer of aspartate and arginine, also called cyanophycin (41, 43), that is found only in cyanobacteria. Cyanophycin is the product of nonribosomal peptide synthesis catalyzed by cyanophycin synthetase, the product of the *cphA* gene (51), and can represent a cellular nitrogen reserve (1, 2, 28, 42) that in heterocyst-forming cyanobacteria is found in both vegetative cells and heterocysts, where it may serve as a reservoir of newly fixed nitrogen (9). The mobilization of cyanophycin appears to involve a cyanophycinase, the product of the *cphB* gene, which releases an aspartate-arginine dipeptide as an intermediate in the degradation to aspartate and arginine (18, 37). A gene from *Synechocystis* sp. strain PCC 6803 encoding a glycoprotease homologue has also been implicated in cyanophycin degradation (52). Arginine and aspartate must be catabolized to have their nitrogen atoms made available for cellular metabolism.

Two arginine degradation systems commonly found in bacteria are the arginase and the arginine deiminase pathways (11). Arginase produces ornithine and urea from arginine, whereas arginine deiminase produces citrulline and ammonium. In the arginine deiminase pathway, citrulline is catabolized to ornithine and carbamoyl phosphate by ornithine carbamoyltransferase, with the produced carbamoyl phosphate being further metabolized by carbamate kinase, rendering ATP (from ADP), bicarbonate, and ammonium. Arginine utilization by the arginine deiminase pathway is characterized in many bacteria by an abundant ornithine excretion (11). Indeed, some of these bacteria incorporate arginine into the cell by means of an arginine-ornithine antiporter (12), the product of the *arcD* gene in *Pseudomonas aeruginosa* (27). In the arginase pathway, on the other hand, ornithine can be further metabolized to glutamate by the sequential actions of (i) ornithine transaminase (which renders glutamate semialdehyde, which spontaneously dehydrates to Δ^1pyrroline-5-carboxylate) and Δ^1pyrroline-5-carboxylate dehydrogenase or (ii) ornithine

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TABLE 1. Mutants of *Synechocystis* sp. strain PCC 6803 generated in this work[a]

Mutant strain	Gene	ORF	ORF's coordinates	Oligonucleotides' coordinates	Inserted cassette	Insertion site (coordinate[s])
CSMJ1	*argF*	*sll0902*	3227266–3226340	3227260–3227239, 3226350–3226372	C.K2	*Eco*RV (3226968)
CSMJ3	*speB1*	*sll0228*	148942–148022	148912–148892, 148030–148049	C.C1	2 *Nco*I sites (148657, 148503)
CSMJ4	*speB2*	*sll1077*	801206–800034	801207–801189, 800037–800054	C.K3	*Kpn*I (800900)
CSMJ15	*putA*	*sll1561*	1969118–1966146	1967183–1967163, 1966227–1966246	C.C1	*Sma*I (1966756)
CSMJ16	*argD*	*slr1022*	640737–642026	641214–641233, 642276–642257	C.K3	*Sma*I (641750)
CSMJ39	*proC*	*slr0661*	3338682–3339485	3338010–3338029, 3339666–3339647	C.K3	*Eco*RI (3338989)

[a] The names of the generated *Synechocystis* mutants and of the identified genes are presented in the first two columns. The names and chromosome coordinates (24) of the corresponding ORFs are indicated in the third and fourth columns, respectively. The coordinates of the oligonucleotides used for PCR amplification of those ORFs, and the names and insertion sites (in terms of restriction endonuclease name and coordinate) of the inserted antibiotic resistance-encoding gene cassettes are indicated in the last three columns.

cyclodeaminase (which renders proline) and proline oxidase (11). Another catabolic route found in several bacteria is the arginine succinyltransferase pathway, in which arginine is first activated to N^2-succinylarginine, which is transformed to N^2-succinyglutamate through a pathway similar to the arginase pathway, to finally release glutamate (11).

Arginase activity has been reported for a number of different cyanobacteria, including strains of *Anabaena* sp., *Aphanocapsa* (*Synechocystis*) sp., *Nostoc* sp., and *Oscillatoria* sp. (4, 22, 31, 44, 48). Arginine deiminase activity has also been reported for some strains of *Anabaena* sp., *Aphanocapsa* (*Synechocystis*) sp., and *Nostoc* sp. (22, 31, 48). On the other hand, *Synechococcus* sp. strains PCC 6301 and PCC 7942, which do not synthesize cyanophycin and are unable to transport arginine with high affinity, are known to express an L-amino acid oxidase, the product of the *aoxA* gene, which releases 2-ketoarginine and ammonia from arginine (references 5 and 6 and references therein). This amino acid oxidase is located mainly in the periplasmic space (6) and shows a low affinity for its substrates (15).

In this work, we have investigated arginine catabolism in a cyanobacterium, *Synechocystis* sp. strain PCC 6803, by using an in vivo approach. We have analyzed the fate of exogenously supplied [^{14}C]arginine, [^{14}C]ornithine, and [^{14}C]proline in the wild-type strain and in a series of amino acid metabolism mutants that included strains with mutations in genes encoding ornithine carbamoyltransferase (*argF*), *N*-acetylornithine aminotransferase (*argD*), proline oxidase (*putA*), Δ^1pyrroline-5-carboxylate reductase (*proC*), and two putative homologues of arginase.

MATERIALS AND METHODS

Growth conditions. *Synechocystis* sp. strain PCC 6803 was grown axenically in BG11 (nitrate-containing) medium (38). 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. Some cultures were supplemented with 1 mM (or, where indicated in the text or table footnotes, 5 mM) filter-sterilized L-arginine, L-citrulline, L-ornithine, or L-proline. The *Synechocystis* mutants carrying gene cassette C.K2 or C.K3 (13) were routinely grown with 25 μg of kanamycin · ml^{-1}, and the mutants carrying C.C1 (13) were grown with 10 μg of chloramphenicol · ml^{-1}. *Escherichia coli* strain DH5α was grown in Luria-Bertani medium with, when necessary, 50 μg of ampicillin · ml^{-1}, 50 μg of kanamycin · ml^{-1}, and 25 μg of chloramphenicol · ml^{-1}.

Uptake assays. Cells grown in BG11 medium (supplemented, where indicated in the table footnotes, with an amino acid and/or an antibiotic) were harvested by low-speed centrifugation at room temperature, washed twice with 25 mM *N*-tris(hydroxymethyl)-methylglycine (Tricine)–NaOH buffer (pH 8.1), and resuspended in the same buffer. The concentration of chlorophyll *a* (Chl) in methanolic extracts of the cell suspension was determined (29). The uptake assays were carried out for the times indicated in each experiment at 30°C in the light (white light from fluorescent lamps) and were started by mixing a suspension (1.1 ml) of cells containing 5.5 to 16 μg of Chl with a solution (0.1 ml) of L-[U-^{14}C]arginine (5 to 342 μCi · μmol^{-1}), L-[U-^{14}C]ornithine (256 μCi · μmol^{-1}), or L-[U-^{14}C]proline (257 μCi · μmol^{-1}) (radioactive amino acids were from Amersham or New England Nuclear). The final concentration of amino acid is indicated for each experiment. At the end of the incubation, a 1-ml sample was filtered (0.45-μm-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 used to analyze intracellular labeled metabolites as described below. The rates of amino acid uptake in the 15-min assays presented in Fig. 2 and Tables 2 and 3 were estimated by taking a 0.1-ml sample of the cell suspension 10 min into the incubation period. The sample was filtered, and the cells on the filters were washed as described above. The filters carrying the cells were then immersed in a scintillation cocktail, and their radioactivity was measured. Retention of radioactivity by boiled cells was used as a blank.

In the experiment shown in Fig. 1, to determine the radioactivity incorporated into macromolecules, a sample of the cell suspension was added to ice-cold trichloroacetic acid (TCA; final concentration, 10%), incubated at 4°C for 30 to 60 min, and filtered; the filters were then washed with 5 to 10 ml of ice-cold 10% TCA and immersed in a scintillation cocktail, and their radioactivity was measured. To determine the total acid-stable radioactivity of the cell suspension, samples of the cell suspension were mixed with HCl (final concentration, 0.25 N), vigorously shaken, and combined with a scintillation cocktail, and their radioactivity was then measured.

To determine metabolites produced from the labeled substrate in short-term experiments (see Fig. 3 and 4), a sample of 0.25 to 1 ml of the cell suspension was mixed, without filtering the cells, with 1 to 2 ml of water at 100°C and further incubated for 5 min in a bath of boiling water.

Analysis of labeled metabolites. After uptake assays had been completed, washed filters containing the cells used in the assays were immediately (<30 s) immersed in 2 ml of boiling water and incubated at 100°C for 5 min. Each filter was then withdrawn, and the resulting suspension was centrifuged. Boiled cell suspensions from short-term experiments were also centrifuged at this stage. Samples (1 to 2 ml) from the supernatant solutions were lyophilized and dissolved in 20 to 25 μl of water. Samples of the resulting solutions, corresponding to the extract of an amount of cells equivalent to 0.4 to 2.7 μg of Chl, were applied to 0.1-mm-thick cellulose thin-layer chromatography (TLC) plates (20 by 20 cm; Merck). Two-dimensional separation of amino acids was effected by using the following solvents. In the first system of solvents, the first-dimension solvent consisted of *n*-butanol–acetone–ammonium hydroxide–water (20:20:10:4, vol/vol/vol/vol), and the second-dimension solvent consisted of isopropanol-formic acid-water (20:1:5, vol/vol/vol). In the second system of solvents, the first-dimension solvent consisted of phenol-water (100:28, vol/vol) and the second-dimension solvent consisted of *n*-butanol–acetic acid–water (12:3:5, vol/vol/vol). The TLC plates were analyzed by conventional autoradiography or by electronic autoradiography using a two-dimensional scanner for β particles (InstantImager; Packard), which allows a quantitative analysis of the radioactive spots. Identification of the metabolite originating a radioactive spot was made by cochromatography by supplementing the samples with stable amino acids as markers and visualizing the amino acids after chromatography with a solution of ninhydrin in acetone in the presence of cadmium acetate (3).

Generation of mutants. The open reading frames (ORFs) of the *Synechocystis* sp. strain PCC 6803 chromosome (24) inactivated in this work are summarized in Table 1. DNA fragments corresponding to those ORFs were amplified by PCR using primers whose coordinates in the strain PCC 6803 chromosome are indicated in Table 1. PCR amplification was carried out in a 50-μl 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, and buffer. The program used for amplification was denaturation for 1 min at 95°C, annealing for 1 min at 55 to 60°C, and polymerization for 1 min at 72°C (30 cycles).

The PCR products were cloned in the vector pGEM-T (Promega). The identity of the cloned fragment was verified by restriction endonuclease analysis or by sequencing with a T7Sequencing Kit (Pharmacia) and [α-^{35}S]thio-dATP. Gene cassette C.K2, C.K3, or C.C1 (13) was inserted by standard procedures into the endonuclease restriction site(s) indicated in Table 1 for each ORF. These restriction sites were unique in the corresponding DNA fragment, except for the two *Nco*I sites in *sll0228*; in this case, a deletion of 155 bp accompanied the insertion of the C.C1 cassette [the *sll0228* insert cloned in pGEM-T was transferred to pBluescript SK(+) before the cassette was inserted].

Transformation of *Synechocystis* sp. strain PCC 6803 with plasmids carrying the disrupted DNA fragments was carried out as described previously (10), except that the cells were spread onto nitrocellulose filters (Nucleopore REC-85). Transformants were selected in BG11 solid medium supplemented with antibiotics (see above) and citrulline in the cases of those with inactivated *slr1022* and *sll0902* or proline in the case of that with inactivated *slr0661*. To facilitate segregation of the mutant chromosomes, kanamycin-resistant (Km^r) or chloramphenicol-resistant (Cm^r) transformants were then grown in liquid medium supplemented with up to 300 μg of kanamycin · ml^{-1} or 20 μg of chloramphenicol · ml^{-1}, respectively.

To test whether the resulting mutant strains were homozygous for the mutant chromosomes, PCR amplification using genomic DNA from each mutant as the template and the corresponding primers was carried out. Segregation was also verified by Southern blot analysis for all the ORFs except *slr1022*, using the corresponding PCR-amplified DNA fragments as probes. Hybridizations were carried out at 65°C according to the recommendations of the manufacturers of membranes. Strains homozygous for the mutated chromosome were obtained for all the disrupted ORFs.

Isolation of genomic DNA from cyanobacteria was carried out as described previously (8). Plasmid DNA from *E. coli* DH5α was isolated by standard methods (39).

Enzyme activities. For determination of ornithine carbamoyltransferase activity in permeabilized cells, BG11-grown cells were harvested by centrifugation at room temperature, washed with 200 mM Tricine-NaOH buffer (pH 8.1), and resuspended in the same buffer at a Chl concentration of 80 to 200 μg · ml^{-1}. The reaction mixture (total volume, 0.46 ml) consisted of 200 mM Tricine-NaOH buffer (pH 8.1), 10 mM carbamoyl phosphate, 10 mM L-ornithine, an amount of cells corresponding to 20 to 50 μg of Chl, and 10 μg of mixed alkyltrimethylammonium bromide (Sigma) per μg of Chl. The reaction was carried out at 30°C for up to 40 min. Reactions run without added ornithine or carbamoyl phosphate were used as blanks. Samples of 100 μl were withdrawn after different incubation times, mixed with 50 μl of 21% ice-cold TCA, incubated at 4°C for 15 min, and centrifuged at 13,000 × *g* for 5 min at 4°C. The citrulline produced in the reaction was colorimetrically determined in a 100-μl sample of the supernatant (7).

For determination of arginase in permeabilized cells, BG11-grown cells were harvested by centrifugation at room temperature, washed with 50 mM Tricine-NaOH buffer (pH 8.5), and resuspended in the same buffer at a Chl concentration of ca. 200 μg · ml^{-1}. These cells were mixed with toluene (0.5 ml · mg of Chl^{-1}), vigorously shaken for 1 min, and used in the following reaction mixture (total volume, 3 ml): 200 mM Tricine-NaOH buffer (pH 8.5), 1 mM $MnCl_2$, 20 mM L-arginine, and an amount of cells corresponding to 70 to 75 μg of Chl. (We later observed that addition of $MnCl_2$ was not necessary to determine this arginase activity.) The reaction was carried out at 30°C for up to 40 min. Reactions run without added arginine were used as blanks. Samples of 0.55 ml were withdrawn after different incubation times, mixed with 20 μl of concentrated sulfuric acid, and centrifuged at 13,000 × *g* for 5 min at 4°C. The ornithine produced in the reaction was colorimetrically determined for a 0.5-ml sample of the resulting supernatant (36).

Levels of activity of arginase and agmatinase (agmatine ureohydrolase) were also determined for cell extracts of cells grown in BG11 medium lacking $NaNO_3$, supplemented with 5 mM L-arginine and 10 mM $NaHCO_3$, and bubbled with a stream of air-CO_2 (99:1, vol/vol). A total of four determinations for two independently prepared cell extracts were performed. The cells were harvested by centrifugation, washed with 50 mM Tricine-NaOH buffer (pH 8.5), and resuspended in the same buffer supplemented with 1 mM dithiothreitol, DNase (ca. 50 μg · ml^{-1}), and protease inhibitors (1 mM [each] phenylmethylsulfonyl fluoride, benzamidine, and aminocaproic acid). This cell suspension was passed through a French press at 20,000 lb/in^2 and centrifuged (8,000 × *g*, 20 min, 4°C), and the supernatant was used for arginase or agmatinase activity determination, as follows. The level of arginase activity was determined as described above for a reaction volume of 1.5 ml without the addition of $MnCl_2$ and with an amount of cell extract containing ca. 7.5 mg of protein. For agmatinase assays, the cell extract was supplemented with 150 μM acetohydroxamic acid (a urease inhibitor) and incubated for 30 min at 30°C before being used in the following reaction mixture (total volume, 0.95 ml): 100 mM Tricine-NaOH buffer (pH 8.5), 1 mM $MnCl_2$, 8 mM agmatine sulfate, and an amount of cell extract containing ca. 6 mg of protein. The reaction was carried out at 30°C for up to 100 min. Reactions run without added agmatine were used as blanks. Samples of 0.1 ml were withdrawn after different incubation times, mixed with 25 μl of 25% perchloric acid, incubated at 4°C for 15 min, and centrifuged at 13,000 × *g* for 5 min at 4°C. The urea produced in the reaction was colorimetrically determined in 100 μl of the resulting supernatant (7). The cell extract protein concentration was determined by a modified Lowry procedure (30) using bovine serum albumin as a standard.

RESULTS

Fate of [^{14}C]arginine. Nitrate-grown cells of *Synechocystis* sp. strain PCC 6803 efficiently take up arginine from the extracellular medium (16, 25). After a few minutes of incubation in the presence of 50 μM [^{14}C]arginine, some of the radioactivity taken up by the cells was found in cold-TCA-precipitable material (Fig. 1), which includes protein and cyanophycin polypeptide. This accounted for about 20% of the radioactivity taken up by the cells (Fig. 1), indicating that a significant amount of radioactivity remained in soluble metabolites. On the other hand, the total radioactivity in the cell suspension decreased during the experiment. This decrease was best determined after acidification with 0.25 N HCl (Fig. 1), suggesting production of [^{14}C]CO_2 (which would be lost from the cell suspension as a gas) and, therefore, metabolism of arginine by the cells. In experiments like that shown in Fig. 1, once the cells had exhausted [^{14}C]arginine from the medium, no other ^{14}C-labeled substance was observed in samples from the extracellular medium subjected to TLC and autoradiography (not shown). This indicates that arginine uptake is not accompanied by release into the extracellular medium of any of its metabolic products other than CO_2.

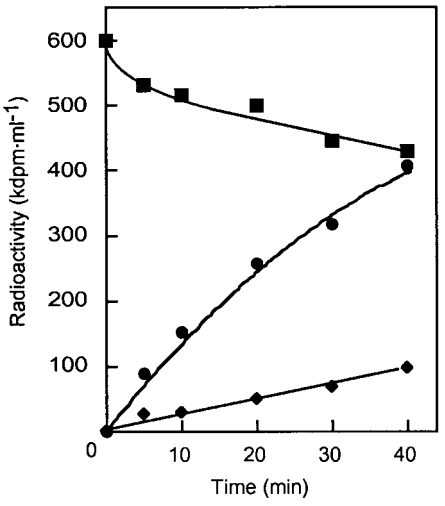

FIG. 1. Utilization of arginine by *Synechocystis* sp. strain PCC 6803. A suspension of nitrate-grown cells containing 10 μg of Chl · ml^{-1} was incubated in Tricine buffer supplemented with 50 μM [^{14}C]arginine (see Materials and Methods for details). ■, Total acid-stable radioactivity in the cell suspension (determined after treatment with 0.25 N HCl); ●, radioactivity in the cells; ◆, radioactivity incorporated into cold-TCA-precipitable material.

The distribution of radioactivity among metabolites present in the soluble fractions of the cells was analyzed by TLC and autoradiography as described in Materials and Methods. Radioactivity from [^{14}C]arginine was distributed, after 15 min of incubation, among a few metabolites (Fig. 2). Cochromatography with stable amino acids identified the main spots as arginine, citrulline, proline, glutamate, glutamine, ornithine, and argininosuccinate. (Glutamine and citrulline spots overlap in the first TLC system of solvents, but the two amino acids could be separated from each other using the second TLC system of solvents [not shown].) In some experiments, a light spot identified as agmatine was also observed. Quantification of the radioactivity in each spot in 18 independent experiments carried out with arginine concentrations of 1 to 30 μM indicated that, in general, apart from arginine itself, more label accumulated in citrulline, proline, or glutamate than in ornithine.

Because in some bacteria arginine catabolism enzymes are induced upon growth in the presence of arginine, we studied [^{14}C]arginine metabolism in cells grown in the presence of arginine. Consistent with a previous report (16), cells that had been grown in culture medium supplemented with arginine showed a lower rate of uptake of [^{14}C]arginine than non-

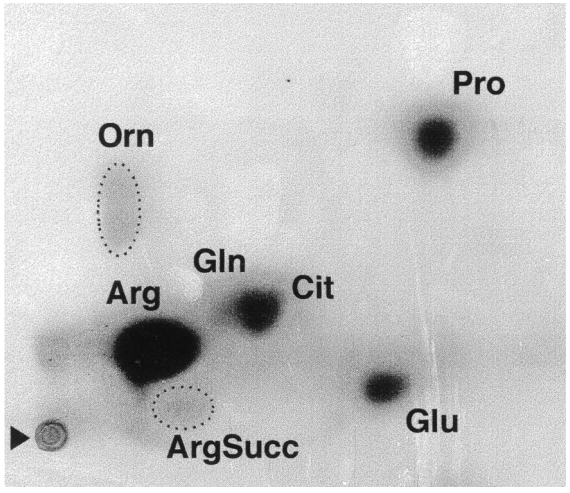

FIG. 2. Production of ^{14}C-labeled metabolites from [^{14}C]arginine in *Synechocystis* sp. strain PCC 6803. A suspension of nitrate-grown cells containing 5 μg of Chl · ml^{-1} was incubated for 15 min in Tricine buffer supplemented with 30 μM [^{14}C]arginine. The rate of arginine uptake was 205 nmol · mg of Chl^{-1} · min^{-1}. Cell metabolites were extracted and analyzed by TLC and autoradiography as described in Materials and Methods. The figure shown corresponds to a TLC developed with the first system of solvents. The amino acids identified were: arginine (Arg), citrulline (Cit), proline (Pro), glutamate (Glu), glutamine (Gln), ornithine (Orn), and argininosuccinate (ArgSucc). Note that the glutamine and citrulline spots overlap. The triangle points to the origin of the chromatography.

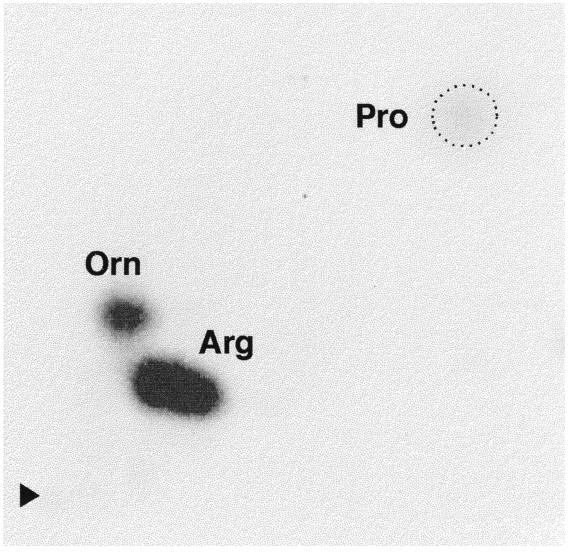

FIG. 3. Short-term metabolism of [^{14}C]arginine by *Synechocystis* sp. strain PCC 6803. A suspension of nitrate-grown cells containing 5.5 μg of Chl · ml^{-1} was incubated for 15 s in Tricine buffer supplemented with 1.9 μM [^{14}C]arginine. A 1-ml sample of the cell suspension was mixed with 2 ml of boiling water, and radioactive metabolites were analyzed as described in Materials and Methods. The figure shown corresponds to a TLC developed with the first system of solvents. The amino acids identified were arginine (which corresponds to intracellular plus extracellular arginine), ornithine, and proline. Abbreviations are as indicated in the legend to Fig. 2. The triangle points to the origin of the chromatography. An experiment run in parallel using cells that had been incubated in boiling water for 5 min before the addition of 1.9 μM [^{14}C]arginine showed only the arginine spot (not shown).

supplemented cells. The patterns of [^{14}C]arginine-derived, labeled amino acids were, however, similar in cells from the two growth conditions, except that a relatively high accumulation of [^{14}C]argininosuccinate was observed in the cells grown in the presence of arginine (Table 2, experiment 1).

Nitrate-grown cells preincubated for 1 h with stable citrulline or ornithine took up [^{14}C]arginine at a rate similar to that of control cells that had not been preincubated with the amino acid. Analysis, by TLC and the ninhydrin reaction, of amino acids in extracts of the cells showed that preincubation with citrulline actually resulted in accumulation within the cells of a noticeable amount of stable citrulline (and arginine) and that preincubation with ornithine resulted in the accumulation of stable ornithine. While only a partial decrease in arginine catabolism was observed in cells preloaded with citrulline, catabolism of [^{14}C]arginine was drastically depressed in cells that had been preincubated with ornithine (Table 2, experiment 2). This result indicates a strict control by ornithine of the first step(s) of [^{14}C]arginine catabolism in strain PCC 6803.

Time-course experiments showed that the patterns of labeled metabolites produced from [^{14}C]arginine were similar for incubation periods of 3 to 30 min. Short-term experiments indicated, however, that production of labeled ornithine preceded that of citrulline, proline, or glutamate. Thus, after 15 s of incubation in the presence of 1.9 μM [^{14}C]arginine, radioactivity in metabolites other than arginine was mainly concentrated in ornithine (Fig. 3).

Fate of [^{14}C]ornithine. The fate of [^{14}C]ornithine in *Synechocystis* sp. strain PCC 6803 was investigated. After 2 min of incubation of the cells in the presence of 2.7 μM [^{14}C]ornithine, the main radioactive products of ornithine metabolism

TABLE 2. Fate of [^{14}C]arginine in *Synechocystis* sp. strain PCC 6803

Expt	Growth medium	Preincubation (added amino acid)	% of the sum of radioactivity in ^{14}C-labeled metabolite(s)[a]:					
			Arg	Cit + Gln	Pro	Glu	Orn	ArgSucc
1[b]	BG11		84.7	5.5	4.5	3.4	1.2	0.7
	BG11 + Arg		90.7	2.9	1.6	1.2	1.0	2.5
2[c]	BG11	None	81.4	12.4	0.8	3.8	1.6	ND
	BG11	Citrulline	85.4	12.6	0.4	1.2	0.4	ND
	BG11	Ornithine	99.5	0.2	<0.1	<0.1	0.2	ND

[a] Cell metabolites were analyzed as described in Materials and Methods by using the first TLC system of solvents. ND, not determined. Other abbreviations are as indicated in the legend to Fig. 2. The citrulline plus glutamine spot was usually more than 90% citrulline (Table 3).

[b] Cells grown in BG11 medium or BG11 supplemented with 5 mM arginine were washed and used in arginine uptake assays as described in the legend to Fig. 2. The rate of arginine uptake was 205 (cells grown in BG11) and 47 (cells grown in BG11 plus arginine) nmol · mg of Chl^{-1} · min^{-1}.

[c] Cells grown in BG11 medium were incubated for 1 h at 30°C in the light in Tricine buffer supplemented with 5 mM citrulline, 5 mM ornithine, or no added amino acid. After being washed with Tricine buffer, the cells were incubated for 15 min in Tricine buffer supplemented with 24 μM [^{14}C]arginine. The rates of arginine uptake were similar in the three cell suspensions, about 103 nmol · mg of Chl^{-1} · min^{-1}.

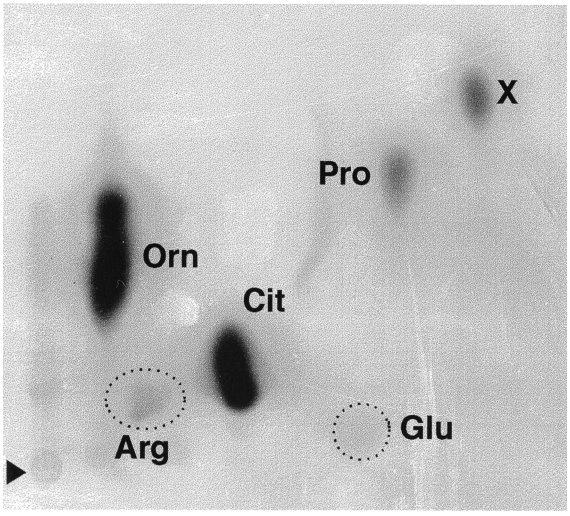

FIG. 4. Production of ^{14}C-labeled metabolites from [^{14}C]ornithine in *Synechocystis* sp. strain PCC 6803. A suspension of nitrate-grown cells containing 10 μg of Chl · ml^{-1} was incubated for 2 min in Tricine buffer supplemented with 2.7 μM [^{14}C]ornithine. A 0.25-ml sample of the cell suspension was mixed with 1 ml of boiling water, and radioactive metabolites were analyzed by TLC and autoradiography as described in Materials and Methods. The figure shown corresponds to a TLC developed with the first system of solvents. The amino acids identified were ornithine (which corresponds to intracellular plus extracellular ornithine), citrulline, arginine, glutamate, and proline. Abbreviations are as indicated in the legend to Fig. 2. X might correspond to Δ^1pyrroline-5-carboxylate, but a definitive identification of this compound was not obtained. The triangle points to the origin of the chromatography.

were citrulline, proline, and an unidentified metabolite (Fig. 4). Some radioactivity was also recovered in arginine, glutamate, and, although it is not seen in Fig. 4, argininosuccinate.

Amino acid catabolism in *argF*, *argD*, *proC*, and *putA* mutants. Making use of the available complete sequence of the chromosome of *Synechocystis* sp. strain PCC 6803 (24), we sought the isolation and analysis of *Synechocystis* mutants lacking some enzyme activities that, as discussed below, might be involved in arginine catabolism. ORF *sll0902* of the *Synechocystis* genome would encode a protein homologous to ornithine carbamoyltransferases (*argF* gene product) from various biological sources. A *Synechocystis sll0902* mutant was isolated as described in Materials and Methods and named CSMJ1. This strain strictly required citrulline (or arginine) for growth, being unable to grow when ornithine replaced citrulline, and did not show any detectable ornithine carbamoyltransferase activity. (Ornithine carbamoyltransferase activity detected in the wild-type strain was 1.92 μmol · mg of Chl^{-1} · min^{-1}.) No in vivo production of [^{14}C]citrulline from [^{14}C]ornithine was observed in strain CSMJ1, whereas the extent of labeling in proline, glutamate, and glutamine was higher than in the wild-type strain (Table 3, experiment 1). These observations indicate that *sll0902* is indeed the *argF* gene of strain PCC 6803 and that this is the only gene encoding an ornithine carbamoyltransferase in this cyanobacterium. Metabolism of [^{14}C]arginine in strain CSMJ1 was altered with respect to that in the wild-type strain. In four independent experiments, the amount of label that accumulated as [^{14}C]citrulline was reduced to 14 to 20% of the values found in the wild-type strain, whereas the amount of label that accumulated as [^{14}C]proline, [^{14}C]glutamate, and [^{14}C]glutamine increased about twofold (see data from a representative experiment in Table 3, experiment 2).

TABLE 3. [^{14}C]arginine, [^{14}C]ornithine, and [^{14}C]proline catabolism in *Synechocystis* sp. mutants[a]

Expt	Strain	Genotype	Growth medium	Substrate	% of the sum of radioactivity in ^{14}C-labeled metabolites:							
					Arg	Cit	Gln	Cit + Gln	Pro	Glu	Orn	ArgSucc
1	PCC 6803	Wild type	BG11 + Cit	[^{14}C]Orn	1.0	61.7	2.4		31.2	2.6	1.2	0.0
	CSMJ1	*argF* mutant	BG11 + Cit	[^{14}C]Orn	0.0	0.0	5.1		85.8	5.7	3.5	0.0
2	PCC 6803	Wild type	BG11 + Cit	[^{14}C]Arg	36.5	36.3	0.1		17.6	5.7	2.6	1.3
	CSMJ1	*argF* mutant	BG11 + Cit	[^{14}C]Arg	39.2	5.9	4.6		36.7	10.6	0.9	2.1
3[b]	PCC 6803	Wild type	BG11 + Cit	[^{14}C]Arg	15.5			14.5	9.3	59.7	0.3	0.7
	CSMJ16	*argD* mutant	BG11 + Cit	[^{14}C]Arg	19.7			12.6	4.7	62.4	0.2	0.5
4	PCC 6803	Wild type	BG11 + Cit	[^{14}C]Orn	1.2			52.7	40.2	3.2	1.6	1.1
	CSMJ16	*argD* mutant	BG11 + Cit	[^{14}C]Orn	1.6			52.0	40.4	3.3	1.5	1.2
5	PCC 6803	Wild type	BG11 + Pro	[^{14}C]Arg	14.1	19.9	5.5		11.8	47.7	0.3	0.7
	CSMJ39	*proC* mutant	BG11 + Pro	[^{14}C]Arg	21.7	66.2	1.4		5.8	3.7	0.2	1.0
6	PCC 6803	Wild type	BG11 + Pro	[^{14}C]Orn	11.6	61.4	2.3		17.5	4.7	0.7	1.7
	CSMJ39	*proC* mutant	BG11 + Pro	[^{14}C]Orn	18.5	73.0	1.5		4.1	0.4	0.1	2.4
7[c]	PCC 6803	Wild type	BG11	[^{14}C]Pro				20.1	27.2	52.7		
	CSMJ15	*putA* mutant	BG11	[^{14}C]Pro				0.0	100	0.0		
8	PCC 6803	Wild type	BG11	[^{14}C]Orn	47.1			24.0	10.7	15.4	2.8	0.0
	CSMJ15[d]	*putA* mutant	BG11	[^{14}C]Orn	56.9			29.8	7.0	0.0	4.4	0.7
9	PCC 6803	Wild type	BG11	[^{14}C]Arg	40.3	29.6	2.9		7.9	17.1	2.1	0.0
	CSMJ15[d]	*putA* mutant	BG11	[^{14}C]Arg	28.4	40.0	0.0		24.5	0.0	3.3	0.7

[a] Cells grown in BG11 medium or BG11 supplemented with the indicated amino acid (and the appropriate antibiotics for the mutants) were used in 15-min uptake assays, with the indicated substrates added at 1 to 2 μM. Arginine and ornithine, but not proline, were exhausted from the medium in the course of the assays. Each experiment was performed two to four times (except experiment 6, which was done only once), and the results of a representative experiment are presented. When only the first TLC system of solvents was used, the radioactivity of the citrulline plus glutamine spot is presented. Abbreviations are as indicated in the legend to Fig. 2.

[b] The cells used in this experiment, grown in BG11 plus citrulline, were incubated for 18 h in BG11 medium before being used in the uptake assays.

[c] The rate of proline uptake was 1.4 nmol · mg of Chl^{-1} · min^{-1} in both strains PCC 6803 and CSMJ15.

[d] Agmatine was detected in strain CSMJ15 incubated with either [^{14}C]ornithine (1.2%) or [^{14}C]arginine (3.1%).

ORF *slr1022* of the *Synechocystis* genome is the putative *argD* gene encoding *N*-acetylornithine aminotransferase. An *slr1022* mutant, strain CSMJ16, that behaved as an arginine auxotroph was generated as described in Materials and Methods. As expected from an *argD* mutant, strain CSMJ16 could also be grown in citrulline- or ornithine-supplemented media. This mutant was not impaired or only moderately impaired in the production of [^{14}C]proline and [^{14}C]glutamate from [^{14}C]arginine or [^{14}C]ornithine (Table 3, experiments 3 and 4).

ORF *slr0661* of the *Synechocystis* genome is the putative *proC* gene encoding Δ^1pyrroline-5-carboxylate reductase, a proline biosynthesis enzyme. An *slr0661* mutant, strain CSMJ39, that behaved as a proline auxotroph was generated as described in Materials and Methods. Production of [^{14}C]glutamate plus [^{14}C]glutamine from [^{14}C]arginine (Table 3, experiment 5) or [^{14}C]ornithine (Table 3, experiment 6) was reduced in strain CSMJ39 to 10 and 27%, respectively, of that found with the wild type, implicating the *proC* gene product in arginine and ornithine catabolism. On the other hand, although its production was impaired in the mutant, the presence of [^{14}C]proline among the products of [^{14}C]arginine and [^{14}C]ornithine catabolism in strain CSMJ39, a proline auxotroph, was unexpected (see Discussion).

ORF *sll1561* is the putative *Synechocystis putA* gene encoding proline oxidase. An insertional mutant of this ORF, CSMJ15, that showed no production of [^{14}C]glutamate and [^{14}C]glutamine from [^{14}C]proline (Table 3, experiment 7) was generated as described in Materials and Methods. (Note that the spot of citrulline plus glutamine analyzed in Table 3, experiment 7, would correspond only to [^{14}C]glutamine.) Strain CSMJ15 was also unable to produce [^{14}C]glutamate (and [^{14}C]glutamine) from [^{14}C]ornithine or [^{14}C]arginine (Table 3, experiments 8 and 9). Lack of production of glutamate was accompanied by accumulation of other labeled metabolites like citrulline, argininosuccinate, and, with [^{14}C]arginine as the substrate, proline.

Arginase activity. Arginine-dependent production of ornithine was detected in cells of strain PCC 6803 made permeable with toluene. The activity found, 20 to 50 nmol of ornithine · mg of Chl^{-1} · min^{-1}, accounts for the observed rate of in vivo arginine catabolism, 10 to 20 nmol of arginine metabolized · mg of Chl^{-1} · min^{-1}. In an attempt to identify the arginase-encoding gene, two *Synechocystis* ORFs, *sll0228* and *sll1077*, whose putative protein products show homology to arginases (and agmatinases) from several biological sources, were inactivated as described in Materials and Methods. The corresponding *Synechocystis* mutants, named CSMJ3 and CSMJ4, respectively, exhibited arginase activities, as determined in cell extracts, identical to that found in the wild-type strain, about 0.43 nmol · min^{-1} · mg of protein^{-1}. Strain CSMJ3, however, lacked any agmatinase activity, while strain CSMJ4 showed about 69% of the agmatinase activity found in the wild type. The agmatinase activity detected in cell extracts of strain PCC 6803 was about 0.28 nmol · min^{-1} · mg of protein^{-1}.

DISCUSSION

Synechocystis sp. strain PCC 6803 is able to accumulate large amounts of [^{14}C]arginine incorporated from the extracellular medium. Assuming an intracellular volume of 125 μl · mg of Chl^{-1} (5 μl · mg of protein^{-1}) (23, 35), the intracellular concentration of [^{14}C]arginine reached in the experiments summarized in Table 2 was about 10 mM, representing intracellular/extracellular concentration gradients of about 1,000 (see also reference 25). Even larger concentration gradients should be reached in the cells in experiments like those described in Table 3 where arginine was exhausted from the medium. These arginine concentration gradients would be built up by the basic amino acid permease known to operate in this cyanobacterium (16, 25).

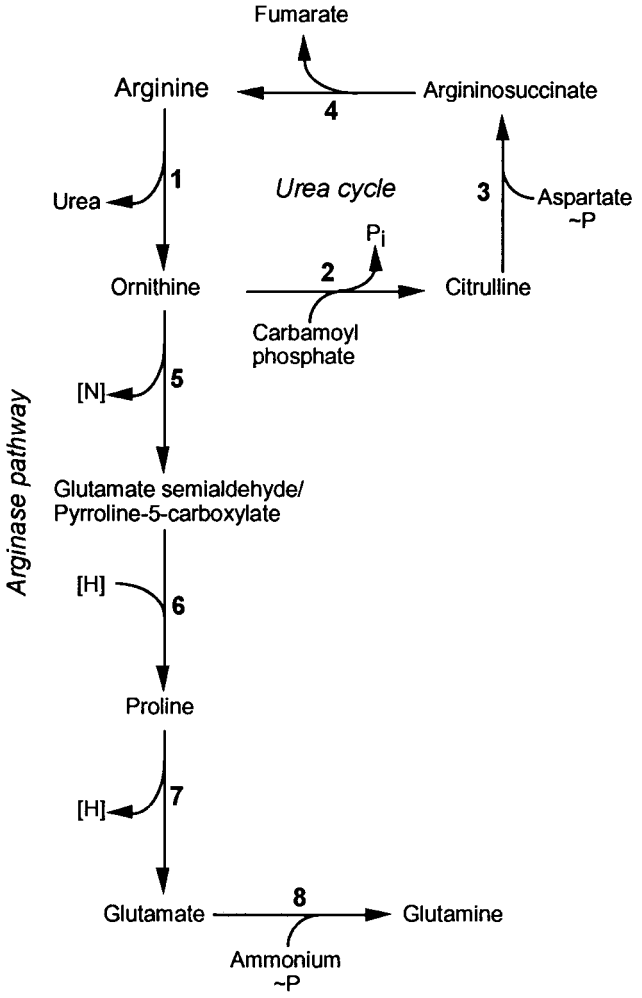

FIG. 5. Schematic representation of arginine catabolism in *Synechocystis* sp. strain PCC 6803. [H], reducing power (normally in the form of NADH, reaction 6, or of NADH and $FADH_2$, reaction 7); [N], nitrogen recovered through a transamination reaction (normally with 2-oxoglutarate as the acceptor); ~P, energy-requiring reaction (energy provided by the hydrolysis of ATP). Enzymes: 1, arginase; 2, ornithine carbamoyltransferase (*argF* gene product, mutated in strain CSMJ1); 3, argininosuccinate synthetase; 4, argininosuccinate lyase; 5, ornithine transaminase; 6, Δ^1pyrroline-5-carboxylate reductase (*proC* gene product, mutated in strain CSMJ39); 7, proline oxidase (*putA* gene product, mutated in strain CSMJ15); 8, glutamine synthetase.

Arginine is subjected to catabolism in *Synechocystis* sp. strain PCC 6803. A scheme summarizing our proposal for the implicated metabolic pathways is presented in Fig. 5. Although [^{14}C]citrulline is a conspicuous product of [^{14}C]arginine, generation of [^{14}C]ornithine, the reaction catalyzed by arginase, appears to represent the initial step in arginine degradation, with [^{14}C]citrulline being synthesized from [^{14}C]ornithine (Fig. 4) by anabolic ornithine carbamoyltransferase. This notion is based in the following observations: (i) the products of extracellularly supplied [^{14}C]ornithine paralleled those of [^{14}C]arginine, (ii) production of [^{14}C]citrulline from [^{14}C]arginine was largely reduced in an *argF* (ornithine carbamoyltransferase) mutant, and (iii) [^{14}C]ornithine was more abundant than [^{14}C]citrulline in short-term experiments of [^{14}C]arginine metabolism. Relatively low levels of label accumulated as [^{14}C]ornithine in cells fed with [^{14}C]arginine. This might be a conse-

quence, at least in part, of inhibition by ornithine of arginine catabolism (Table 2, experiment 2).

Production of [^{14}C]citrulline and [^{14}C]argininosuccinate from [^{14}C]arginine-derived [^{14}C]ornithine might appear to be a futile cycle (note that argininosuccinate should release arginine by action of argininosuccinate lyase). If, however, the proposed pathway is operative in cyanophycin mobilization, it would provide a means for utilization of nitrogen from aspartate, whose amino group would be released as urea, in a sort of urea cycle (Fig. 5). The ability to degrade arginine appears to be constitutively expressed in *Synechocystis* sp. strain PCC 6803, since growth in culture medium supplemented with arginine did not stimulate arginine catabolism (Table 2, experiment 1). This lack of stimulation suggests that the arginine degradation machinery has a role independent of the availability of arginine as a nutrient in the extracellular medium. Such a role could obviously be in the metabolism of arginine (as well as of aspartate) released from cyanophycin. *Synechocystis* sp. strain PCC 6803 also constitutively expresses urease (A. Valladares, A. Herrero, and E. Flores, unpublished data), which would release ammonia (two molecules) and CO_2 from the molecule of urea produced in the arginase reaction. Decomposition of [^{14}C]urea derived from [^{14}C]arginine, as it has been shown for *Synechocystis* sp. strain PCC 6308 (48), can account for a substantial fraction of the [^{14}C]CO_2 release that we have observed (Fig. 1).

Two ORFs, *sll0228* and *sll1077*, whose putative protein products show homology to arginases and agmatinases from different biological sources, are found in the genome of *Synechocystis* sp. strain PCC 6803 (24). Their products have been putatively assigned the roles of arginase and agmatinase, respectively (24). We have shown, however, that neither of them is responsible for the arginase activity detected in strain PCC 6803. ORF *sll0228* clearly encodes an agmatinase and, therefore, represents an *speB* gene, as may also be the case for *sll1077*. Arginase and related enzymes constitute a protein family in which two different groups are discernible (34). Consistent with our results, the deduced polypeptides of *sll0228* and *sll1077* fall within the group of arginase-related enzymes rather than within that of true arginases (34). A small polypeptide showing an arginase-like activity that has been named "L-arginine-metabolizing enzyme" has recently been characterized for *Synechocystis* sp. strain PCC 6803 (A. E. Gau and E. K. Pistorius, Abstr. IX Int. Symp. Phototrophic Prokaryotes, p. 138, 1997) and may be responsible for the arginase activity that we have detected. This polypeptide is the product of ORF *sml0007* and is homologous to the photosystem II PsbY polypeptides of higher plants (17). Apart from arginase, three enzymes are required for the urea cycle, namely, ornithine carbamoyltransferase, argininosuccinate synthetase, and argininosuccinate lyase. These are arginine biosynthesis enzymes that must be normally expressed in cells and have been detected in cyanobacteria (19, 21, 26, 47). On the other hand, no ORF encoding a putative homologue of arginine deiminase is found in the *Synechocystis* genome. Therefore, the route by which [^{14}C]argininosuccinate and a low level of [^{14}C]citrulline are generated from [^{14}C]arginine in the *argF* mutant is currently unknown.

The second part of the arginase pathway, i.e., generation of glutamate from ornithine, appears to be operative in *Synechocystis* sp. strain PCC 6803, since production of [^{14}C]glutamate from both [^{14}C]arginine and [^{14}C]ornithine was evident in our experiments. [^{14}C]Proline was also produced to a large extent from both [^{14}C]arginine and [^{14}C]ornithine. Because no ORF that would determine a protein homologous to known ornithine cyclodeaminases is present in the *Synechocystis* genome (24), conversion of ornithine into glutamate would likely involve as a first step a transamination to render glutamate semialdehyde/Δ^1pyrroline-5-carboxylate. The transamination reaction, using 2-oxoglutarate as an acceptor for the transferred amino group, may be catalyzed by *N*-acetylornithine aminotransferase (the *argD* gene product of the arginine biosynthesis pathway), which, in different bacteria, is also known to be able to use ornithine as a substrate (11). However, our data with the *argD* mutant suggest that ArgD is not important for ornithine degradation in *Synechocystis* sp. strain PCC 6803. The gene encoding the aminotransferase that might participate in ornithine catabolism in this cyanobacterium has not yet been identified.

ORF *sll1561*, the *putA* gene encoding proline oxidase, is required to generate [^{14}C]glutamate from [^{14}C]proline, [^{14}C]ornithine, or [^{14}C]arginine. PutA might act as a Δ^1pyrroline-5-carboxylate dehydrogenase, rendering glutamate. However, because [^{14}C]proline is produced in [^{14}C]arginine and [^{14}C]ornithine catabolism (Fig. 2 and 4), reduction of Δ^1pyrroline-5-carboxylate to proline can represent an intermediate step in ornithine degradation. Our analysis of the effect of a *proC* mutation on arginine and ornithine catabolism supports this view (Table 3, experiments 5 and 6). The involvement of proline as an intermediate in ornithine degradation has also been suggested for *E. coli* and *Pseudomonas putida* (40, 46). Finally, labeled glutamine, which is produced from [^{14}C]glutamate by glutamine synthetase, was observed among the products of [^{14}C]ornithine or [^{14}C]arginine catabolism.

Another arginine-metabolizing enzyme putatively encoded in the *Synechocystis* genome (24) is arginine decarboxylase, which would produce agmatine from arginine. Agmatine was indeed observed in some of our arginine catabolism assays (Table 3, footnote *d*), but this alternative catabolism pathway was apparently less important in *Synechocystis* sp. strain PCC 6803 under our experimental conditions.

Some production of [^{14}C]proline from [^{14}C]arginine or [^{14}C]ornithine was observed in the *proC* mutant, strain CSMJ39. Therefore, although the pathway shown in Fig. 5 appears to represent the main route for arginine and ornithine catabolism in *Synechocystis* sp. strain PCC 6803, an additional pathway producing proline from arginine and ornithine seems to be operative in this cyanobacterium, and some growth of strain CSMJ39 in arginine-supplemented BG11 plates was indeed observed (not shown). However, because strain CSMJ39 behaves like an auxotroph, such a pathway appears not to produce a substantial amount of proline in cells not supplemented with arginine or ornithine.

The proposed arginine catabolism scheme, combining the arginase pathway and the urea cycle (Fig. 5), represents a rather unique mode of arginine catabolism and provides a rationale for understanding how nitrogen is made available to the cell during assimilation of arginine taken up from the extracellular medium as well as during cyanophycin granule mobilization. Interestingly, *Synechococcus* sp. strain PCC 7942, a strain that does not synthesize cyanophycin, exhibits a mode of arginine catabolism (6) that is in sharp contrast to that described in this work for *Synechocystis* sp. strain PCC 6803.

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