0273-2289/92/3702—0111$02.20

Amino Acid Overproduction by Analog Resistant Mutants of the Nitrogen Fixing Cyanobacterium *Anabaena* sp 287

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Received March 9, 1992; Accepted August 14, 1992

ABSTRACT

Two amino acid analog resistant mutants of the cyanobacterium *Anabaena* sp 287 were isolated after MNNG mutagenesis. *Anabaena* ST 16, a mutant resistant to the alanine analog D-α-aminobutyric acid and *Anabaena* ST 25, another mutant resistant to the histidine analog 1,2,4-triazole-3-alanine, released alanine and histidine, respectively, into the medium upon immobilization in alginic acid during diazotrophic growth in fluidized bed reactors. The rates of amino acid production by the mutants were 4.3 μmol mg chl^{-1} h^{-1} of D-alanine by *Anabaena* ST 16 and 16.6 μmol mg chl^{-1} h^{-1} of L-histidine by *Anabaena* ST 25. Nitrogen fixation by the mutants was not affected by the extracellular amino acid concentration. While the radioactive carbon flow was followed, the parent strain retained 93% of fixed ^{14}C and released only 7% into the medium. On the other hand, *Anabaena* ST 16 released 13% and *Anabaena* ST 25 released 29% into the medium. These mutants are beneficial in the production of radioactive amino acids using diazotrophic photobiotechnology.

Index Entries: Cyanobacteria; nitrogen fixation; analog resistance; immobilization; amino acid secretion; radioactive carbon flow; photobiotechnology.

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INTRODUCTION

Photosynthetic cyanobacteria, which fix atmospheric nitrogen, produce many nitrogenous exometabolites from air, water, and sunlight (*1*). Among them, ammonium and amino acids have considerable agronomical and industrial significance. The photoproduction of ammonium and amino acids without induction has been shown in many nitrogen fixing cyanobacteria (*2–4*) and the continuous and discontinuous secretion of these products is monitored by immobilization of cells or induced by inhibitors of the nitrogen metabolic pathway (*5*). This has led to the development of a technology involving different strains of cyanobacteria improved for the overproduction and excretion of ammonium (*6,7*) and amino acids (*8,9*) using diazotrophic growth. Overproduction of metabolites can be induced by the selection of mutants that are resistant to analogs of that metabolite (*10*). Deregulation of amino acid biosynthetic pathway owing to analog resistance led to the release of amino acids such as tryptophan, phenyl alanine, methionine, and arginine by strains of *Anabaena* sp 29151, *Synechocystis* sp 29108, *Synechococcus* sp AN TX20 and *Synechococcus* sp 602 (*11*). Similar amino acid analog resistant mutants of *Spirulina platensis* (*12*), *Anabaena variabilis* (*9*), and *Synechococcus* PCC 7942 (*13*) overproduced proline, aromatic amino acids, and arginine and citruline, respectively. We report here the amino acid overproduction by two analog resistant mutants of the cyanobacterium *Anabaena* sp 287 and the flow of carbon and nitrogen fixation under amino excreting conditions.

MATERIALS AND METHODS

Cyanobacterium and Culture Conditions

Anabaena sp 287 was obtained from the Division of Microbiology, IARI, New Delhi, India. Cultures were grown and maintained in BG11 medium at 25°C with or without combined nitrogen (*14*). Other growth conditions were as reported previously (*15*).

Immobilization of *Anabaena* Filaments

One liter of cyanobacterial culture was centrifuged at 6000g for 10 min and washed with BG11 medium devoid of phosphate. The pellet was suspended in the same medium and the suspension was mixed with equal volume of 3% sodium alginate solution and added dropwise into 100 m*M* calcium chloride using a sterile capillary tube. The biocatalyst containing beads were then loaded in fluidized bed 500 mL-continuous culture reactors to collect the amino acids released into the medium with a dilution rate of 0.2 h^{-1}.

Isolation of Amino Acid Analog Resistant Mutants

Short filaments (5–10 cells/filament) of *Anabaena* sp 287 were treated with 250 μg mL^{-1} of MNNG in citrate buffer pH 5.5 for 30 min. After the mutagenic treatment they were washed with phosphate buffer pH 7.0 and suspended in BG11 medium with 2 m*M* NH_4Cl and allowed to grow for 10 h in a rotary shaker (100 rpm). The mutated filaments were spread on wet cellulose nitrate filters and placed on amino acid analog containing BG11 medium devoid of combined nitrogen. The alanine analog D-α aminobuytric acid and the histidine analog 1,2,4-triazole-3-alanine were used at the concentration of 250 μM mL^{-1}. Green colonies appearing after 10 d were transferred to liquid and solid media containing the respective amino acid analogs. Several resistant clones were selected and the total amino acid levels were determined in the culture filtrates as described below. Mutant strains *Anabaena* ST 16 and *Anabaena* ST 25, which released alanine and histidine in higher quantities, respectively, were selected based on the HPLC quantification of amino acids for further studies.

Analytical Procedures

Chlorophyll *a* was determined in methanol extracts (*16*). Protein was determined using Coomassie blue dye binding method (*17*). Shimadzu Graphicard UV-Visible Spectrophotometer was used for optical density measurements. Total nitrogen of the filaments and culture filtrates was determined as described earlier (*14*).

Determination of Nitrogenase Activity

Nitrogenase activities were estimated by acetylene reduction assay (*18*). Immobilized cyanobacteria were washed with BG11 medium devoid of combined nitrogen and were added to 35 mL of medium in 130 mL-Wheaton bottles. The bottles were sealed with a flanged rubber septum and were placed on a rotary shaker (100 rpm) and illuminated with a quantum flux 75 μeinsteins m^{-1} s^{-1}. Ethylene was resolved in a Hewlett Packard 5830A gas chromatograph using a stainless steel Porapack N column at 110°C as FID temperature. Nitrogenase activities are expressed as nanomoles of C_2H_4 produced/mg protein/h.

Determination of Oxygen Evolution and Consumption

The oxygen evolution and consumption by cyanobacterial filaments was followed polarographically under saturating light using a YSI 4004 Clark Oxygen Electrode. The signal from the electrode was recorded on a

Hitachi Model 200 strip chart recorder. The results are the average of triplicate experiments and expressed in μmol oxygen evolved/consumed/mg chlorophyll/h.

Amino Acid Quantification

Total amino acids were quantified in spent cultures of all analog resistant mutants with ninhydrin reagent (*19*). Among them one mutant resistant to each analog that produced the highest amount was chosen for further study. They were designated as *Anabaena* ST 16 for D-α aminobutyric acid resistance and *Anabaena* ST 25 for 1,2,4-triazole-3-alanine resistance. Comparative quantification of D-alanine and L-histidine was carried out by using Hitachi 655 HPLC components. Standard amino acid separations were made using Sigma amino acids. *O*-Pthaldialdehyde derived amino acids were separated by isocratic elution at the flow of 1 mL min^{-1} using 20 m*M* phosphate buffer pH 6.8 and methanol in the ratio of 64:36 at room temperature.

Estimation of ^{14}C— Carbon Flow and Protein Synthesis

^{14}C-Sodium bicarbonate was used for the determination of the flow of carbon through *Anabaena* sp 287 and its two amino acid overproducing mutants as described previously (*14*). Cultures of the three stains were allowed to grow for ten days (early log phase) and 300 KBq of radioactive sodium bicarbonate was added and the strains were allowed to grow for another 5 d to attain mid-log phase. The culture filtrates were collected after 5 d and dried in a water bath at 60°C. The pellets were also dried at 60°C and then hydrolyzed with 10% tri-chloro acetic acid and this fraction was saved. The pellet obtained after acid hydrolysis was then treated with 1*M* NaOH for hydrolysis at 80°C for 30 min in a water bath. Similar acid and alkali hydrolysis were done for the dried culture filtrates. Among the fractions the acid soluble fraction of the culture filtrates which constitutes the amino acids was passed through Dowex-50 in a column of 30×1.5 cm. The absorbed amino acids from this column were eluted with 1*M* ammonia solution and finally concentrated to 1 mL. The total amino acids were separated by two-dimensional paper chromatography (*20*). The corresponding alanine and histidine spots were identified by a ninhydrin spray. $^{14}CO_2$ fixation was determined in terms of $H^{14}CO_3^-$ incorporation (*14*). After 1 h incorporation the cyanobacterial filaments were washed with BG11 medium containing nonradioactive sodium bicarbonate to remove the unincorporated $H^{14}CO_3^-$. Protein synthesis was determined in terms of ^{14}C-chlorella hydrolysate incorporation (*21*). After 6 h incorporation, the cyanobacterial filaments were washed with BG11 medium containing nonradioactaive chlorella hydrolysate to remove the unincorporated ^{14}C-chlorella hydrolysate. Radioactive counts were monitored in a Minaxi B-TriCarb 4000 series Liquid Scintillation Counter

using 5 mL of scintillation cocktail provided by the counter manufacturers. Values presented in Table 2 are the mean value of six experiments or cultures, whichever applicable to the context.

Chemicals

Standard amino acids, amino acid analogs, and MNNG (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine) were purchased from Sigma Chemical Co., USA, and other chemicals were acquired from E. Merck AG, Germany. Sodium alginate was the product of Aldrich Chemical Co., USA. Radioactive isotopes were purchased from Bhabha Atomic Research Center, Trombay, Bombay, India.

RESULTS

Isolation of Amino Acid Secreting Mutants

Mutant strains of *Anabaena* sp 287 resistant to 250 μM mL^{-1} of D-α aminobutyric acid and 1,2,4-triazole-3-alanine were isolated. In order to obtain mutants having regulatory mutation in the biosynthetic pathway allowing enhanced production of alanine or histidine and to eliminate the mutants arising because of the lack of analog uptake or nontoxic metabolism of the analog, the mutants were screened for the extracellular amino acid levels. Among them *Anabaena* ST 16 and *Anabaena* ST 25 produced higher levels of alanine and histidine, respectively, because of their resistance to the amino acid analogs, caused by deregulation in the amino acid biosynthetic pathway.

Amino Acid Secretion and Nitrogen Fixation

Table 1 shows the time course release of alanine and histidine by the mutants *Anabaena* ST 16 and *Anabaena* ST 25, respectively. The parent strain grown under similar conditions did not liberate amino acids into the medium. However, the mutant strains did not exhibit higher nitrogenase activity when compared to the parent (Table 1). On the other hand the mutants exhibited similar growth rate as the parent (data not shown).

Photosynthesis, Respiration, and Protein Synthesis by *Anabaena* Strains

Table 2 shows the comparison of the photosynthetic and respiratory functions of the parent and amino acid overproducing mutants. Photosynthesis and respiration as determined by oxygen evolution and consumption was neither enhanced nor reduced in the mutant strains. However, the mutants showed enhancement in $^{14}CO_2$ fixation (Table 1).

Table 1
Time Course Release of Alanine and Histidine by Immobilized *Anabaena* ST 16 and *Anabaena* ST 25, Respectively[a]

	Anabaena sp 287			*Anabaena* ST 16			*Anabaena* ST 25		
Time, h	1*	2	3*	1*	2	3*	1*	2	3*
0	—	148.3±7.1	273.50	0.0	153.4±8.8	322.51	0.0	150.5±3.4	315.12
12	—	148.3±6.0	276.15	4.3	154.3±2.6	310.11	16.1	151.1±6.1	311.44
24	—	147.1±5.0	272.11	4.0	153.4±8.5	318.55	16.0	152.3±5.6	315.15
36	—	147.1±5.0	273.46	4.1	150.1±5.5	320.31	16.6	154.3±6.0	311.50
48	—	146.9±8.0	269.43	4.3	151.2±6.0	322.55	16.6	151.2±8.5	314.65
72	—	148.3±6.0	270.17	3.9	154.4±3.6	314.41	16.1	152.1±4.1	315.12
96	—	148.3±7.4	272.08	4.2	153.4±6.1	322.51	16.4	153.9±6.3	313.55
120	—	147.9±6.5	274.73	4.0	152.1±4.1	320.58	16.6	151.1±5.0	315.12

(—) Not detectable.
1. Rate of amino acid (alanine or histidine) production (μmol mg chl^{-1} h^{-1}).
2. Nitrogenase activity (nmol mg protein^{-1} h^{-1}).
3. $^{14}CO_2$ fixation (μmol fixed mg chl^{-1} h^{-1}).
* Values are the average of six determinations.
[a]Nitrogenase activity and $^{14}CO_2$-fixation in comparison with the parent, *Anabaena* sp 287.

The enhancement was significantly higher (12–17%) in *Anabaena* ST 16 and *Anabaena* ST 25 when compared to the parent *Anabaena* sp 287. Protein synthesis by *Anabaena* ST 25 was found to be lower than the other two strains (Table 2).

Flow of Radioactive Carbon Through *Anabaena* Strains

When the radioactive carbon flow through *Anabaena* sp 287 and its mutant strains was determined during growth, the mutant strains exhibited enhanced incorporation of $NaH^{14}CO_3^-$ in the cultures. The levels were 18 and 15% higher in *Anabaena* ST 16 and *Anabaena* ST 25 respectively when compared to *Anabaena* sp 287 (Table 2). Similarly, the ^{14}C-compounds released into the medium were higher in the mutant strains. When compared to the parent (7%), *Anabaena* ST 16 excreted 13% and *Anabaena* ST 25 released 29% of the total ^{14}C-fixed in the cultures. However, *Anabaena* ST 16 retained higher amount of ^{14}C-fixed inside the cells (105%) than the parent (93%) and *Anabaena* ST 25 (86%).

Extracellular ^{14}C-Amino Acid Levels

Amino acid fractions were obtained through Dowex chromatography from the total ^{14}C-extracellular compounds released by the parent and the mutant strains. When compared to the parent, *Anabaena* ST 16 and *Anabaena* ST 25 released three and eight times higher amount of amino acids, respectively (Table 2). However, in the case of ^{14}C-total extracellular compounds, these strains produced twice and four times higher amounts,

Table 2
Photosynthetic Oxygen Evolution, Respiratory Oxygen Consumption, Protein Synthesis, and the Flow of Radioactive Carbon in *Anabaena* sp 287 and Its Amino Acid Excreting Mutants

Strain	μmol O_2 mg^{-1} chl h^{-1}		^{14}C-Chlorella hydrolysate incorporation	^{14}C-Carbon (fixed)	^{14}C-Carbon (retained)	^{14}C-Carbon (released)	Total extracellular	Extracellular amino acids		^{14}C-alanine	^{14}C-histidine
	Evolved	Consumed	($\times 10^6$ dpm mg^{-1} chl min^{-1})		($\times 10^5$ dpm mg^{-1} chl min^{-1})		^{14}C-amino acids	Percent of total ^{14}C-fixed	Percent of total ^{14}C-released	(dpm mg^{-1} chl min^{-1})	
Anabaena sp 287	375	125	1.15	52.0 (100)	48.3 (93)	3.7 (7)	0.93	1.78	25	0.08×10^4	0.17×10^4
Anabaena ST 16	378	120	1.07	61.8 (118)	54.6 (105)	6.7 (13)	2.55	4.13	38	0.86×10^5	0.17×10^4
Anabaena ST 25	371	123	0.88	60.0 (115)	45.0 (86)	15.0 (29)	7.95	13.25	53	0.38×10^4	2.18×10^5

Values in parentheses indicate the percentage.

Table 3
Fate of Fixed Nitrogen (Total Nitrogen g^{-1} Dry Wt)
During Growth of *Anabaena* sp 287 and Its Mutant Strains

Strain	Cellular nitrogen	Extracellular nitrogen	Culture nitrogen status
Anabaena sp 287	3.83±0.03	0.25±0.04 (6.08)	4.11±0.04
Anabaena ST 16	4.24±0.06	0.32±0.01 (7.10)	4.56±0.07
Anabaena ST 25	4.00±0.01	0.66±0.46 (14.16)	4.66±0.47

Values in parentheses indicate the percentage of extracellular nitrogen in the total culture nitrogen.

respectively, when compared to the parent. *Anabaena* sp 287 released only 1.78% of the total ^{14}C-fixed by the culture. On the other hand *Anabaena* ST 16 and *Anabaena* ST 25 released 4.13 and 13.25% of the total ^{14}C-fixed by the cultures. The ^{14}C-amino acids secreted into the medium were 25% in the case of the parent and 38 and 53% by the mutants *Anabaena* ST 16 and *Anabaena* ST 25, respectively. Though 25% was constituted by amino acids in the total ^{14}C-compounds secreted by *Anabaena* sp 287, none of the amino acid peaks were detectable during HPLC separation of the culture filtrates. Probably, many amino acids were secreted by the parent in insignificant quantities. When the ^{14}C-total amino acids were separated, *Anabaena* ST 16 and *Anabaena* ST 25 recorded higher amounts of ^{14}C-alanine and ^{14}C-histidine respectively when compared to the parent strain (Table 2).

Fate of Fixed Nitrogen

Table 3 shows the culture nitrogen status in *Anabaena* sp 287 and its mutants *Anabaena* ST 16 and *Anabaena* ST 25 during diazotrophic growth. *Anabaena* sp 287 released 6.08% of the nitrogen fixed into the medium. On the other hand *Anabaena* ST 25 released 14.16%, which correlated with its higher release of ^{14}C-total compounds into the medium (Table 2). *Anabaena* ST 16 recorded 7.10% of the total nitrogen fixed, slightly higher than the parent as observed in the ^{14}C-total compounds released into the medium.

DISCUSSION

Excretion of a variety of organic compounds by cyanobacteria during different stages of growth has been reported (*22–28*). However, importance had all along been placed on nitrogenous compounds and attempts had been made to quantify them (*3,4,29*). Since the native strains of cyanobacteria release only small quantities of nitrogenous compounds, mutants overproducing those compounds have been isolated (*6,8,18*).

Such mutants are useful to study the biochemical mechanisms involved in the deregulation of amino acid biosynthetic pathways. In addition, they are of much biotechnological importance in producing nitrogenous substances using solar energy, water, and atmospheric air, and this process is less expensive than the chemical processes that are used to synthesize these nitrogenous compounds.

The amino acid overproducing mutants of *Anabaena* sp 287 isolated in this study were obtained after MNNG mutagenesis. Mutant strains *Anabaena* ST 16 and *Anabaena* ST 25 released alanine and histidine, respectively, into the medium upon immobilization in alginic acid, though they lack higher nitrogenase activities when compared to the parent strain (Table 1). A naturally occurring amino acid-secreting cyanobacterium *A. siamensis* produced a wide range of amino acids (*3*). However, the quantities of individual amino acids released by that strain were lower than the amounts released by the mutants of *Anabaena* sp 287 described here and the mutants of *A. variabilis* (*6*). Though photosynthetic and respiratory activities were similar to the parent, *Anabaena* ST 25 exhibited reduction in the rate of protein synthesis. Such impaired protein synthesis accounted for its more release of amino acids into the medium when compared to the other two strains. The flow of carbon through the three strains traced by labeled carbon demonstrated the higher release of ^{14}C-exometabolites by the two mutant strains (Table 2). Nearly half of the fraction of ^{14}C-exometabolites was constituted by amino acids in the mutant strains when compared to the parent (Table 2). As a photobiotechnological application *Anabaena* ST 16 and *Anabaena* ST 25 released more amino acids from the fixed carbon (4.13 and 13.25%, respectively) and more nitrogenous substances from the fixed nitrogen (7.10 and 14.16%, respectively) when compared to the release by *Anabaena* sp 287 (1.78% of fixed carbon and 6.08% of fixed nitrogen). The release of ^{14}C-labeled individual amino acids, alanine, and histidine by *Anabaena* ST 16 and *Anabaena* ST 25, respectively, in higher quantities also confirmed the results obtained from HPLC quantification of the amino acids (Tables 1 and 2).

Toxic effects of amino acid analogs can be overcome by overproduction of the parental amino acid due to the deregulation in the amino acid biosynthetic pathway (*10*). It is well known that 1,2,4-triazole-3-alanine resistant mutants of bacteria are unable to repress histidine operon functions (*30*). Mutant strains of *Methanococcus voltae* resistant to 1,2,4-triazole-3-alanine were able to excrete histidine, proline, phenyl alanine, and tyrosine in various combinations (*31*). 1,2,4-triazole-3-alanine has been shown to be incorporated into protein and to cause repression of the histidine operon by mimicking the regulatory effect of histidine on enzyme production (*32*). Eventually, the deregulation of histidine biosynthetic pathway caused by altered enzyme(s) led to the release of the parent amino acid in a higher level. Similar amino acid analog resistant mutants of several strains of *Synechococcus* sp (*13,21,33,34,36–38*), *Synechocystis* (*8*),

and *Anabaena* (*9,35*) were found to overproduce parental amino acids due to the deregulation of the enzymes. Sustained photoproduction of a wide spectrum of amino acids by 6-fluorotryptophan resistant mutants of *A. variabilis* was also reported in immobilized systems, alanine being the major component and isoleucine, leucine, glycine, and phenyl alanine in significant quantities (*8*). Based on the ^{14}C-total amino acid release by the two amino acid analog resistant mutants of *Anabaena* sp 287, apart from the secretion of alanine or histidine being the major component, they also secreted other amino acids in significant quantities as reported in other strains of cyanobacteria. This might be owing to the deregulation of the respective amino acid biosynthetic pathway in *Anabaena* ST 16 and *Anabaena* ST 25. However, further studies on the enzymes of the alanine and histidine biosynthetic pathways in the mutant strains will throw light on the type of mutations occurring in *Anabaena* ST 16 and *Anabaena* ST 25. Since significant portions of the total ^{14}C released compounds were constituted by ^{14}C-amino acids, these mutants will be useful for the production of isotopically labeled amino acids using photodiazotrophic-technology.

ACKNOWLEDGMENTS

We thank Council of Scientific and Industrial Research and Indian Council of Agricultural Research, New Delhi, India for financial assistance.

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