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Sustained Production of Amino Acids by Immobilized Analogue-resistant Mutants of a Cyanobacterium *Anacystis nidulans* BD-1

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Batch cultures of *Anacystis nidulans* BD-1 resistant to azaleucine and fluorotyrosine produced and liberated a wide range of amino acids, notably glutamic acid, alanine, phenylalanine, leucine, isoleucine, cysteine and methionine. Sustained liberation for prolonged periods was achieved after immobilization on calcium alginate and the net concentration in the medium was 0.18~0.2 g l^{-1}. While acetohydroxy acid synthase in azaleucine-resistant mutant lost leucine- and isoleucine-sensitivity, fluorotyrosine-resistant strain turned phenylalanine activating. The activities of nitrate assimilating enzymes were also higher in the mutants and were relaxed from ammonium-repression. The metabolic adjustments involved in amino acid overproduction are discussed.

Resistance to amino acid analogues in heterotrophic microorganisms (17) and cyanobacteria (10) results in overproduction and release of amino acids as a consequence of *a)* desensitization of biosynthetic enzymes against feedback responses (11, 16) and *b)* altered amino acid transport (reutilization) properties (12).

Cyanobacteria are phtotrophs, can use inexpensive resources and are easy to manipulate. This makes them useful for production of several metabolites including amino acis (12), which can be recovered from culture filtrates. However, a major obstacle is the permeability barrier which restricts outward transport of amino acids. For example, fluorotryptophan-resistant *Anabaena variabilis* mutants (6) accumulated but not excreted tryptophan, and *Phormidium uncinatum* mutants (10) excreted none of the overproduced amino acids. Prolonged retention of amino acis within the cells can lead to their rapid degradation. To some extent these problems have been overcome by trapping the amino acids as single cell protein (10) and by permeabilizing the cells, thus facilitating the release (9). Finally, immobilization of cells on alginate or other suitable matrices renders the liberation process sustainable (6).

We have investigated the feasibility of amino acid production by non-nitrogen fixing cyanobacteria (10) using nitrate as N source. This was done on the assumption that net nitrogen incorporation by the cells, particularly when immobilized, would be greater than that of N_2-fixing diazotrophic forms (6), in which some heterocysts (comprising a fraction of the cells) are involved.

We report here selection of analogue-resistant mutants of *Anacystis nidulans* which are deregulated with amino acid biosynthetic enzymes, and demonstrate sustained amino acid liberation by immobilized cultures.

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MATERIALS AND METHODS

Organism and Growth

Axenic cultures of *A. nidulans* BD-1 (Collection, Agriculture and Molecular Biology Division, Bhabha Atomic Research Centre, Bombay) were grown phototrophically as described befor (3) in a $NaNO_3$ (5 mM) based BG-11 medium (13).

Analogue-resistant Mutants

The method of Kerby *et al.* (6) was used with some modification. A thick culture suspension was mutagenized in 100 mM citrate buffer (pH 6.5) with 50 μg ml^{-1} of N-methyl-N'-nitro-N-nitrosoguanidine for 45 min, washed with BG-11 medium and after allowing growth in this medium for 36 h, the cells were plated onto 1% (w/v) agar-solidified medium supplemented with 200 μg ml^{-1} each of 4-aza-DL-leucine and m-fluoro-DL-tyrosine. Pin-head colonies of resistant mutants appeared after 20~30 days of incubation at a frequency of 0.08~0.1%. These were restreaked on selective medium two times, before growth in 20 μg ml^{-1} analogue-supplemented liquid medium was attempted. Representative mutants designated AL^R and FT^R were used for further studies.

Immobilization

The procedure of Kerby *et al.* (6) was used with some

modification. Equal volumes of cyanobacterial suspension in K_2HPO_4-free BG-11 medium at chlorophyll *a* (Chl *a*) density of 40 μg ml^{-1} and molten 3.5% (w/v) solution of sodium alginate were mixed and added drop by drop to 0.1 M $CaCl_2$ solution under stirring. The beads were allowed to stant for 2 h, washed thrice with above medium and transferred to phosphate-containing BG-11 medium.

Enzyme Activity Measurements

Enzyme activities of acetohydroxy acid synthase (11), 3-deoxy-D-arabinoheptulosonate-7-phosphate (DAHP) synthase and prephanate dehydratase (5) and of nitrate and nitrite reductases and glutamine synthetase (transferase) (2) were measured in the cell-free extracts as reported elsewhere.

Amino Acids and Other Estimations

Total amino acid concentration in medium was determined after Rosen (15) using L-leucine as a standard. The procedure of Kerby *et al.* (6) was used to determine both cellular and extracellular pools of amino acids. Culture density for this experiment was set approximately at 6~6.5 μg ml^{-1} chlorophyll *a*. Analysis was done using a Pharmacia LKB alpha plus amino acid analyser and lithium citrate buffer system.

The concentrations of chlorophyll *a* and phycocyanin were measured after Mackinney (8) and Allen and Smith (1) respectively. Protein content was estimated by Lowry's method (7).

Reliability

Experiments were repeated at least three times. Data from typical experiments are given in which deviation from mean did not exceed ±10%.

Chemicals

All chemicals were purchased at their analytical grades from Sigma Chemical Co., St. Louis, U.S.A. and HiMedia, Bombay, India.

RESULTS AND DISCUSSION

In liquid cultures, as low as 20 μg ml^{-1} of the two analogues was found inhibitory to growth of wild-type strain. The growth rates of wild-type strain without, and of resistant mutants without or with, 80 μg ml^{-1} analogue concentrations were similar (generation time; 32~36 h). Concentrations beyond 100 μg ml^{-1} were lethal for the mutants. The mutants with/without analogues appeared more bluish than wild type. The phycocyanin vs. chlorophyll *a* ratios for wild type, AL^R and FT^R strains were respectively 10.4, 16.1 and 13.7.

Table 1 shows the spectrum of free amino acids produced within the cells and released into the medium. Overall, the mutants accumulated a wide range of amino acids, notably glutamic acid which accounted for 49~56% of the total pool. AL^R strain, in addition, produced alanine, isoleucine, leucine, cysteine and methionine and FT^R strain produced phenylalanine and aspartic acid. Comparatively much smaller concentrations of amino acids were detected in the wild-type strain.

Table 1. Internal and extracellular free pools of amino acids in wild type *A. nidulans* and its analogue-resistant mutants.

Amino acid	Amount (μ mol mg^{-1} chlorophyll *a*)				
	Internal			Extracellular	
	Wild type	AL^R	FT^R	AL^R	FT^R
Ala	0.38	2.1	0.4	1.9	0.63
Arg	0.03	0.38	nd	0.44	0.36
Asp	0.02	0.1	1.1	0.04	0.62
Cys	nd	0.54	0.24	0.1	0.04
Glu	0.04	6.9	4.4	2.1	1.5
Gly	0.045	0.3	0.28	0.2	0.37
His	nd	0.36	0.11	nd	0.08
Ile	nd	0.4	nd	0.4	0.06
Leu	nd	0.92	0.07	1.1	0.1
Lys	0.035	0.36	0.26	0.19	0.32
Met	nd	0.46	0.16	nd	0.04
Phe	nd	0.08	0.39	0.04	0.42
Ser	0.03	0.38	nd	0.44	0.03
Thr	0.01	0.3	0.31	0.23	0.3
Tyr	nd	0.12	0.07	0.08	0.11
Val	nd	0.46	0.11	0.62	0.16

nd=not detectable. Amino acid concentrations were determined in 8-d old dense cultures. Within this period no extracellular amino acid was detected in wild-type strain.

Mutants, but not the wild type, excreted most of overproduced amino acids since their individual proportions in intra- and extracellular pools were nearly similar. Permeability barrier does not seem to be a major obstacle in *Anacystis* mutants. However, some amino acids e.g. arginine and isoleucine, were absent in cell extracts but were detected in the medium (Table 1) and this could be due to their rapid degradation.

Extracellular accumulation of amino acids was monitored in free-living and immobilized cells of the mutants (Fig. 1). The total inoculum size for both sets was kept at approximately 10 μg ml^{-1} chlorophyll *a*, which accounted for a concentration of 4.8~5 μg chlorophyll *a* per bead. Under free-living state the mutants liberated amino acids for upto 4 days. The concentration remained stable for another 12 days and then decreased. The growth, however, ceased after 8 days of inoculation. On the other hand, the immobilized cells continued to liberate amino acids for a period of nearly one month, after which time the beads disintegrated. The concentration built up was in the range of 0.18~0.2 g l^{-1} (20~24 mg mg^{-1} chlorophyll *a*). Chlorophyll concentration in the beads did not increase any further, probably due to saturation of colonization. However, if chlorophyll *a* density was kept low (0.4~0.45 μg per bead), the cells grew

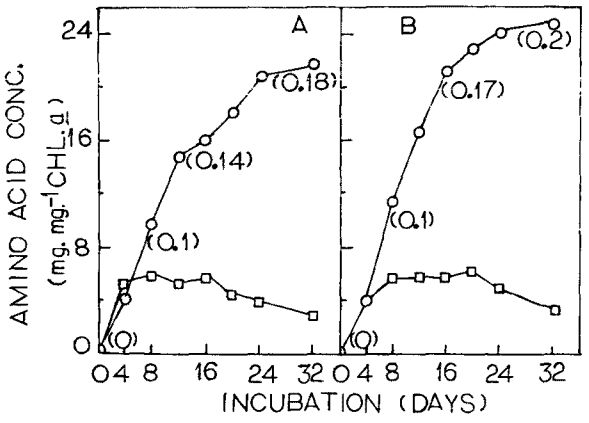

Fig. 1. Extracellular liberation of amino acids in (A) AL^R and (B) FT^R mutants of *A. nidulans* under free-living (—□—) and immobilized (—○—) conditions.
Values in parentheses are of corresponding concentrations in g l^{-1}.

on the beads with a generation time of 42~45 h. Since amino acid liberation was very poor and inconsistent compared to the above beads, a correlation with growth could not be established.

Greater production of amino acids is expectedly the result of overwhelming consumption of the nitrogen source and altered regulation of amino acid biosynthetic enzymes. Several amino acids exert repression on nitrate reductase as also on nitrogenase (14). Ammonia generated by deamination can also promoto repression (2). Thus, greater production of amino acids should restrict nitrate utilization. Hence, it was a matter of interest to examine the regulatory behaviour of nitrate and nitrite reductases in the mutants. The activities of the two enzymes, as also of glutamine synthetase, were found several fold higher in both the mutants than the wild type strain (Table 2). Similar results were found with key nitrogen metabolising enzymes in analogue-resistant mutants of other cyanobacteria (6, 10).

Nitrate-grown cultures were supplemented with 1 mM NH_4Cl or 1 mM glutamine and 5 mM HEPES/NaOH, pH 8.0. The extracts were prepared from so incubated and from nitrate-grown control cultures, and activities of nitrate and nitrite reductases were measured (Table 2). While in the wild type the activities were inhibited owing to repression, the mutant enzymes were only marginally affected. Thus, mutation derepressed these enzymes. The elevated activities in the mutants can be explained from the fact that in wild-type strain some degree of repression is always imposed from nitrate-derived ammonia.

Acetohydroxy acid synthase enzyme was found in extracts of *A. nidulans*, and its activity was inhibited *in vitro* by isoleucine, leucine and valine, being effective in a decreasing order (Table 3). Same reponse was found in FT^R mutant. In cyanobacteria only valine- and leucine-sensitive enzyme has been reported, whereas in bacteria isoleucine inhibition has also been found (10, 11). The enzyme in AL^R mutant was deregulated in a manner that its sensitivity towards leucine and isoleucine inhibition was lost. This property of the enzyme could be responsible for excess production of the two amino acids (Table 1).

One of the significant contributions in cyanobacterial classification since the work of Rippka *et al.* (13) is the use of enzymological patterns. Properties of aromatic amino acid biosynthetic enzymes viz. cofactor specificity, nature and extent of feedback inhibition and activity ratios of enzymes, responsible for common end products have been used as keys (4). Based on these properties, the genus *Synechococcus*, of which *A. nidulans* is a member, has been divided into two distinct subgroups. Wild type BD-1 strain exhibited retro-Tyr type DAHP synthase (inhibited by tyrosine alone), $NADP^+$-specific

Table 2. Comparative activities of enzymes of nitrate metabolism in wild type *A. nidulans* and analogue-resistant mutants.

Enzyme	Incubation* in nitrate cultures	Activity (n mol min^{-1} mg^{-1} protein)		
		Wild type	AL^R	FT^R
Nitrate reductase	None	10.9	35.7	47.6
	NH_4Cl	0.8	32.8	44.3
	Gln	1.2	33.6	45.2
Nitrite reductase	None	18.0	75.0	83.2
	NH_4Cl	2.6	70.0	81.8
	Gln	3.3	69.5	80.2
Glutamine synthetase	None	940	9100	10100

*NH_4Cl or glutamine were added to nitrate-grown cultures and further incubated for 12 h.

Table 3. Comparative activities of enzymes of aromatic and branched-chain amino acid biosynthesis in wild type *A. nidulans* and analogue-resistant mutants.

Enzyme	Addition*	Activity (n mol min^{-1} mg^{-1} protein)		
		Wild type	AL^R	FT^R
Acetohydroxy acid synthase	None	3.21	3.7	2.9
	Leu	1.6	3.5	1.8
	Ile	0.9	3.4	0.8
	Val	2.5	1.7	2.2
DAHP synthase	None	2.9	2.8	3.3
	Tyr	1.6	1.8	2.1
Prephanate dehydratase	None	2.3	1.5	4.0
	Phe	0.2	0.6	5.8
	Tyr	6.4	3.9	8.4

*Amino acids (0.2 mM) were added to the extracts and incubated at 30°C for 4~6 min. Controls without additions were kept in parallel.

shikimate dehydrogenase and high ratios (22-24) between $NADP^+$-arogenate and NAD^+-prephanate dehydrogenases, both leading to tyrosine. These characters point to this strain being akin to *Synechococcus* PCC 6301 (4). Further, prephanate dehydratase, responsible for phenylalanine synthesis, was product-sensitive but was activated by tyrosine (Table 3).

Both AL^R and FT^R mutants shared the same features of the wild type except that in the latter prephanate dehydratase was activated by phenylalanine (Table 3), a characteristic presumably responsible for overproduction of this amino acid.

While considering cyanobacteria for commercial production of amino acids, it would be desirous to select those strains in which per capita nitrogen incorporation is high and amino acid liberation is considerably unrestricted.

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