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Production of Amino Acids by Analog-Resistant Mutants of the Cyanobacterium *Spirulina platensis*

GIOVANNA RICCARDI,[1]* SILVIO SORA,[2] AND ORIO CIFERRI[1]

Istituto di Microbiologia e Fisiologia Vegetale, University of Pavia, Pavia,[1] and Istituto di Genetica, University of Milan, Milan,[2] Italy

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Mutants of *Spirulina platensis* resistant to 5-fluorotryptophan, β-2-thienylalanine, ethionine, *p*-fluorophenylalanine, or azetidine-2-carboxylic acid were isolated. Some of these mutants appeared to be resistant to more than one analog and to overproduce the corresponding amino acids. A second group was composed of mutants that were resistant to one analog only. Of the latter mutants, one resistant to azetidine-2-carboxylic acid was found to overproduce proline only, whereas one resistant to fluorotryptophan and one resistant to ethionine did not overproduce any of the tested amino acids.

Overproduction of amino acids is a common feature of microbial resistance to analogs of amino acids (3, 6, 14, 16). In general, the overproducing organisms are heterotrophic, and production of metabolites is supported by the organic sources supplied with the medium. To our knowledge, such a phenomenon has been reported rarely for obligate photoautotrophs, a category which includes most cyanobacteria.

Mutants resistant to amino acid analogs have been isolated in *Nostoc muscorum* (15) and *Anabaena* (4), but the mutants were characterized only as far as nitrogen fixation or heterocyst formation was concerned. In the case of *Anacystis nidulans,* mutants resistant to fluorophenylalanine and 2-amino-3-phenylbutanoic acid have been isolated (11). In some of these strains, 3-deoxy-D-arabino-heptonate-7-phosphate synthetase, the first enzyme in the pathway for the biosynthesis of aromatic amino acids, showed a decreased sensitivity in vitro to inhibition by L-tyrosine. Since in *A. nidulans* the enzyme appears to be a single species under feedback control of tyrosine only (7), it is likely that such mutants may overproduce this and, possibly, the other aromatic amino acids. Recently, strains of *Anabaena, Synechococcus,* and *Synechocystis* resistant to different analogs of phenylalanine and to 6-fluorotryptophan, ethionine, and canavanine have been isolated (5). Some of these analog-resistant strains were found to excrete levels of the parental amino acid significantly higher than those excreted by wild-type, sensitive strains. These results demonstrate the existence in cyanobacteria of mutations affecting the mechanisms regulating amino acid biosynthesis. Thus, the strains carrying such mutations overproduce the parental amino acid and consequently become resistant to the toxic effects of the analog.

In the present study, mutants of *Spirulina platensis* resistant to analogs of five different amino acids were isolated and partially characterized. A number of such mutants appeared to be overproducers of amino acids, whereas others did not show enhanced production of these metabolites.

MATERIALS AND METHODS

Strains. *S. platensis* strain C_1 was kindly supplied by G. Florenzano, Istituto di Microbiologia Agraria e Tecnica, University of Florence, Florence, Italy, and was rendered axenic essentially by the procedure of Ogawa and Terui (10), slightly modified (13). The following bacterial strains were used for amino acid bioassays: *Bacillus subtilis* PB1708 (*hisB2 argA3 pheA1*) for phenylalanine (lacking prephenate dehydratase); *B. subtilis* PB168 (*trpC2*) for tryptophan (lacking indole-3-glycerol-phosphate synthase); *B. subtilis* PB1752 (*metC*) for methionine (block unknown); and *Escherichia coli* KX478G3 (*leu ara lac proC purE tsx trp supEG2*) for proline (lacking Δ^1-pyrroline-5-carboxylic acid reductase). *B. subtilis* strains were from the culture collection of the Istituto di Genetica, University of Pavia, Pavia, Italy, whereas *E. coli* was obtained from the culture collection of the Istituto di Genetica, University of Milan, Milan, Italy. Preliminary assays revealed that the nutritional requirements were absolute and could not be satisfied by amino acids different from the one for which each strain was auxotrophic.

Media and growth conditions. The minimal medium used for *S. platensis* was that described by Ogawa and Terui (10) and contained the following components (in grams per liter): Na_2CO_3 (4.03), $NaHCO_3$ (16.8), K_2HPO_4 (0.5), $NaNO_3$ (2.5), K_2SO_4 (1.0), NaCl (1.0), $MgSO_4 \cdot 7H_2O$ (0.2), $CaCl_2 \cdot 2H_2O$ (0.04), $FeSO_4 \cdot 7H_2O$ (0.01), and EDTA (0.08). The final pH of the medium was 8.5. Solutions of oligo-elements

(containing [in grams per liter] H_3BO_3 [2.85], $MnCl_2 \cdot 4H_2O$ [1.81], $ZnSO_4 \cdot 7H_2O$ [0.22], $CuSO_4 \cdot 5H_2O$ [0.08], MoO_2 [0.015], NH_4VO_3 [0.023], $K_2Cr_2(SO_4)_4 \cdot 24H_2O$ [0.096], $NiSO_4 \cdot 7H_2O$ [0.048], $Na_2WO_4 \cdot 2H_2O$ [0.018], $Ti_2(SO_4)_3$ [0.04], and $Co(NO_3)_2 \cdot 6H_2O$ [0.044]) were added after autoclaving. Solid media were prepared by adding 1.5% (wt/vol) agar (Biolife). For the isolation of resistant mutants, the same medium was supplemented with 1 g of bacteriological peptone (Oxoid) per liter. Cultures of *S. platensis* were grown at 28°C in minimal medium, on a rotary shaker, and in the light (ca. 4,000 lx). Cell growth was measured routinely either by turbidity (spectrophotometrically at 560 nm) or by cell count in a hemacytometer.

The basal medium for *B. subtilis* and *E. coli* was that described by Davis and Mingioli (2). When necessary, amino acids were added to give a final concentration of 25 μg/ml. Bacterial strains were grown at 37°C in a rotary shaker, and growth was determined spectrophotometrically at 560 nm.

Mutagenesis and selection of mutants. Cultures of *S. platensis* were grown to early stationary phase (2.5×10^5 filaments per ml) and then treated with 100 μg of nitrosoguanidine per ml for 30 or 60 min, to 0.1 to 1% survival. The cells were washed free of mutagen by filtration, plated on minimal medium containing peptone, and incubated for 10 days in the light (confluent growth). This allowed cell division and segregation of mutant genomes. The plates were then replicated with velvet onto minimal medium supplemented with one of the following amino acid analogs: β-2-thienylalanine (10 μg/ml), *p*-fluorophenylalanine (10 μg/ml), 5-fluorotryptophan (50 μg/ml), ethionine (5 μg/ml), and azetidine-2-carboxylic acid (2 μg/ml). Single resistant colonies were picked from such plates and purified by five successive streakings on analog-supplemented media. The mutant strains were kept on solid minimal medium containing the respective analog. The level of resistance was determined on solid minimal medium containing different concentrations of the antimetabolite.

Determination of amino acid production. Amino acid production by mutants of *S. platensis* was determined by microbiological assays, using auxotrophic mutants of *B. subtilis* or *E. coli*. At different times, samples (10 ml) of cultures grown in minimal medium were withdrawn from 1-liter cultures, and the cells were removed by filtration under sterile conditions. Samples (0.5 ml) of the filtrates as such or after dilution were then added to tubes containing 4.5 ml of minimal medium (2), and the tubes were inoculated with an overnight-grown culture of either bacterium to give an optical density of 0.01 at 560 nm. The bacterial cultures were then incubated overnight with shaking at 37°C, and growth was determined spectrophotometrically. The amino acid content of each sample was determined by interpolation on standard curves obtained with known amounts of each amino acid. The dose-response curve was linear within the following concentration ranges: proline, 0.1 to 3 μg/ml; phenylalanine, 0.5 to 2 μg/ml; methionine, 0.5 to 4 μg/ml; and tryptophan, 0.1 to 1 μg/ml.

Chemicals. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine, DL-5-fluorotryptophan, β-2-thienyl-DL-alanine, DL-ethionine, DL-*p*-fluorophenylalanine, L-azetidine-2-carboxylic acid, L-proline, L-tryptophan, L-methionine, and L-phenylalanine were purchased from Sigma Chemical Co.

RESULTS

Isolation of analog-resistant mutants. Over 200 resistant mutants were isolated for the five tested analogs (Table 1): two analogs of phenylalanine and one analog each of proline, tryptophan, and methionine. Except in the case of 5-fluorotryptophan, for which only one resistant mutant was isolated (frequency of 1.1×10^{-7} per plated filament, each filament containing about 100 cells), mutants resistant to the other analogs appeared with frequencies varying from 1.2×10^{-6} to 7.1×10^{-6} per plated filament. Variation was observed in the degree of resistance. Certain mutants—for example, some of those resistant to ethionine and the majority of those selected on azetidine-2-carboxylic acid—were resistant to analog concentrations more than 100-fold higher than the minimal inhibitory concentration of the sensitive strain.

Mutants selected for resistance to analogs of one amino acid were often resistant also to analogs of other amino acids. The phenomenon was especially striking when the different amino acids apparently did not share the same biosynthetic pathway. The sensitivities to the five tested amino acid analogs of 290 mutants of *S. platensis*, each selected as resistant to one analog only, were assayed on solid media. All 65 mutants resistant to the analogs of phenylalanine were resistant also to ethionine and azetidine-2-carboxylic acid, but not to 5-fluorotryptophan. Among the ethionine-resistant mutants, 74 (e.g., ET7) were resistant also to *p*-fluorophenylalanine, β-2-thienylalanine, and azetidine-2-carboxylic acid, whereas 6 (e.g., ET17) were resistant only to ethionine. Similarly, 3 azetidine-2-carboxylic acid-resistant mutants (e.g., AZ1) were inhibited by other analogs, whereas the remaining 142 (e.g., AZ8) were re-

TABLE 1. *Number of resistant mutants isolated and level of resistance*

Amino acid analog	Parental strain's minimal inhibitory concn (μg/ml)	Resistant mutants	
		No. isolated	Resistance range (μg/ml)
5-Fluorotryptophan	5	1	300
β-2-Thienylalanine	2.5	38	100–200
Ethionine	2	80	300–400
p-Fluorophenylalanine	2.5	27	20–80
Azetidine-2-carboxylic acid	1	145	10–150

sistant to *p*-fluorophenylalanine, β-2-thienylalanine, and ethionine (representative mutants are shown in Table 2). It is noteworthy that the only mutant resistant to 5-fluorotryptophan was sensitive to all other tested analogs, and none of the mutants resistant to the other four analogs was found to be resistant to 5-fluorotryptophan. Thus, there appeared to exist among the mutants isolated some that were resistant to one analog only (e.g., FT1, AZ1, and ET17) and others that were resistant to more than one analog (e.g., TA35, PF27, ET7, and AZ8). The latter mutants were resistant even to analogs of amino acids that, apparently, did not share a common biosynthetic pathway (e.g., proline and methionine or phenylalanine and proline).

Amino acid production. An organism resistant to an amino acid analog may overcome the toxic effect of the antimetabolite by developing a discriminatory mechanism that either prevents the entry into the cell of the analog or that selects, at the level of protein synthesis, the amino acid against the analog. Alternatively, the resistant cell may degrade the analog or it may alter the regulation of the pathway leading to the biosynthesis of the parental amino acid. The latter case results in the overproduction of the amino acid in the cell and, often, its excretion in the medium. To ascertain if the latter mechanism was operating in the mutants of *S. platensis*, the production of amino acids was determined during growth of the cross-resistant (Fig. 1) and non-cross-resistant (Fig. 2) mutants. The growth curves of AZ8, PF27, and TA35 (Fig. 1) were essentially the same as that of the wild type (Fig. 2), whereas that of mutant ET7 was greatly reduced, reaching a plateau at a cell concentration approximately 50% lower than that of the other strains (Fig. 1). The parental strain did not secrete significant quantities of the four tested amino acids into the medium. Small amounts of phenylalanine, methionine, and proline were released into the medium only as cell lysis occurred, after about 30 days of incubation. Tryptophan was practically undetectable, even after cell lysis. Four mutants tested (AZ8, PF27, TA35, and ET7) overproduced large amounts of phenylalanine, and part (about 50%) was secreted by the cells during growth, before lysis. Proline and methionine were also secreted in concentrations significantly higher than those produced by the parental strain. Similarly, during lysis, levels of amino acids higher than those secreted by the parental strain were released into the medium. Thus, it appeared that the four tested mutants cross-resistant to azetidine-2-carboxylic acid, *p*-fluorophenylalanine, β-2-thienylalanine, and ethionine all produced the amino acids at concentrations greater than those produced by the wild type. Although they were selected independently, as mutants resistant to azetidine-2-carboxylic acid, *p*-fluorophenylalanine, or β-2-thienylalanine, it is possible that AZ8, PF27, and TA35 carry mutations in the same gene. Mutant ET7 appeared to be similar to the above-mentioned mutants, although its growth characteristics and the level of amino acid production differentiated it from AZ8, PF27, and TA35.

The growth curves of three non-cross-resistant mutants were similar to that of the parental strain (Fig. 2). With the exception of mutant AZ1, which seemed to release proline at concentrations similar to those excreted by the cross-resistant mutants (Fig. 1), none of these mutants appeared to excrete into the medium and to contain intracellularly phenylanine, methionine, proline, and tryptophan at concentrations higher than those of the wild-type strain. Mutants FT1 and ET17 may have become resistant to the analogs through a mechanism that did not involve an alteration in the regulation of amino acid biosynthesis.

TABLE 2. *Single and multiple resistance of different mutants*

Mutant	Growth on medium containing:				
	5-Fluorotryptophan, 200 μg/ml	Ethionine, 200 μg/ml	*p*-Fluorophenylalanine, 40 μg/ml	β-2-Thienylalanine, 200 μg/ml	Azetidine-2-carboxylic acid, 150 μg/ml
TA35	−	+	+	+	+
PF27	−	+	+	+	+
ET7	−	+	+	+	+
ET17	−	+	−	−	−
AZ1	−	−	−	−	+
AZ8	−	+	+	+	+
FT1	+	−	−	−	−

DISCUSSION

Mutants resistant to analogs of amino acids appear to be very common in *S. platensis*. In this investigation almost 300 mutants were isolated by using five different amino acid analogs. Some of the mutations seem to be very frequent, since, for instance, more than 100 mutants resistant to azetidine-2-carboxylic acid and 80 resistant to ethionine were isolated. In contrast, only one mutant was found to be resistant to the tested tryptophan analog, 5-fluorotryptophan. As already reported by other investigators (4, 5), there seems to exist in cyanobacteria a wide variation in the frequency of appearance of mutants resistant to the analogs of this amino acid. It is possible that such differences are due to

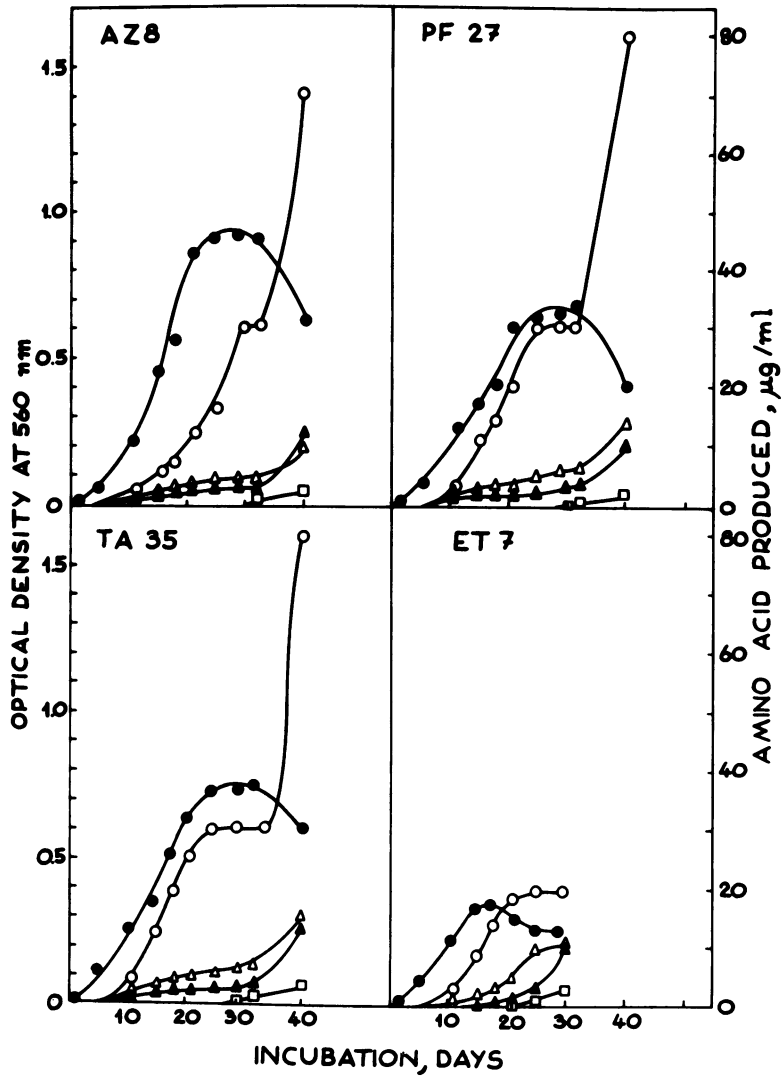

FIG. 1. *Growth of and amino acid production by cross-resistant mutants. Cultures of cross-resistant mutants were grown in minimal medium. Portions of the culture filtrate were removed for amino acid determination. Growth is expressed as the optical density at 560 nm of a 1:10 dilution of the culture. Cell lysis occurred after approximately 32 days of incubation. Symbols: ●, growth; ▲, proline production; ○, phenylalanine production; △, methionine production; □, tryptophan production. Strains: AZ8, a mutant selected as resistant to azetidine-2-carboxylic acid; PF27, a mutant selected as resistant to p-fluorophenylalanine; TA35, a mutant selected as resistant to β-2-thienylalanine; ET7, a mutant selected as resistant to ethionine.*

differences in the permeabilities or sensitivities of the different cyanobacteria to the analogs tested. One of the most common mechanisms of resistance appears to be that due to mutations in the systems regulating amino acid biosynthesis (5, 11). Such regulatory mutants become resistant to analogs by overproducing the parental amino acids. The effect of this type of mutation is twofold. The higher amino acid content of the cell results in a reduction of the uptake of the amino acid and its analog from the medium. In addition, the analog entering the cell is further diluted by the excess of the amino acid present intracellularly. Two mutants resistant to the tested phenylalanine analogs, *p*-fluorophenylalanine and β-2-thienylalanine, were found to excrete into the growth medium concentrations of the amino acid significantly higher than those excreted by the parental strain. Besides phenylalanine, the mutants seemed to overproduce

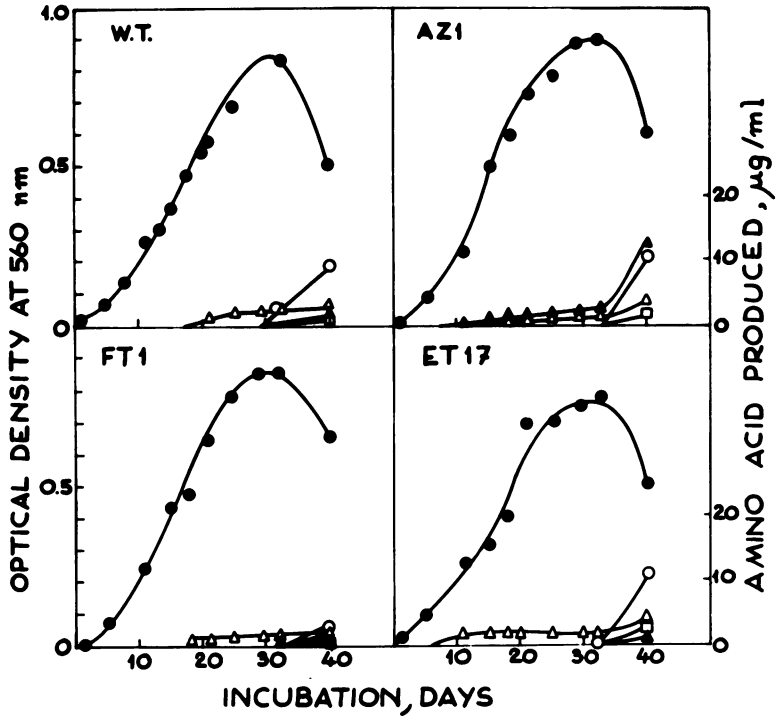

FIG. 2. *Growth and amino production by the parental strain and three non-cross-resistant mutants. Strains: W. T., wild type; AZ1, a mutant resistant to azetidine-2-carboxylic acid; FT1, a mutant resistant to 5-fluorotryptophan; ET17, a mutant resistant to ethionine. Symbols are as defined in the legend of Fig. 1.*

also proline and methionine, but not tryptophan. This type of mutation seems to be present also in mutants selected as resistant to azetidine-2-carboxylic acid or ethionine. Indeed, some of azetidine-2-carboxylic acid- or ethionine-resistant strains overproduced, besides the parental amino acids, phenylalanine. The latter amino acid is the one excreted in larger amounts by the tested regulatory mutants, regardless of the analog used for selection. It seems quite likely that, for certain strains, such as AZ8, PF27, and TA35, the same type of mutation is responsible for resistance to *p*-fluorophenylalanine, β-2-thienylalanine, azetidine-2-carboxylic acid, and ethionine. These mutants appear, then, to carry mutations in the mechanisms regulating amino acid biosynthesis. The result of such a mutation is the overproduction of amino acids. The higher amino acid concentration in the cellular pool may reduce the uptake of the analog into the cell and, in addition, dilute the analog entering the cell. It remains to be established why the concentration of phenylalanine overproduced was always much higher than those of proline and methionine. Similarly, it would be of considerable interest to ascertain why a presumably single mutation results in the overproduction, and hence in resistance to analogs, of amino acids that are synthesized through different and apparently unrelated pathways. However, similar findings already have been reported. For instance, a *B. subtilis* mutant resistant to an analog of histidine has been found to be resistant also to 5-methyltryptophan, possibly as the result of a "metabolic interlock" effect due to regulatory interactions of different, apparently unrelated biosynthetic pathways (8). Similarly, carrot tissue cultures selected for resistance to azetidine-2-carboxylic acid have been found to be resistant also to 5-methyltryptophan (R. Cella unpublished data; C. T. Harmas, unpublished data). A mutant of *Anabaena* strain CA resistant to L-methionine-DL-sulfoximine, an analog of glutamine, was found to be resistant also to 7-azatryptophan (4).

A somewhat different case is that of mutant AZ1, which, in contrast with the wild type, seems to overproduce only proline and to be resistant only to azetidine-2-carboxylic acid. This strain may carry a more typical regulatory mutation, resulting in the derepression of the specific pathway leading to overproduction of the parental amino acid only. Another group of mutants was that comprising such strains as FT1 and ET17,

which, although resistant to one analog, produced little, if any, excess amino acid. It may be surmised that these mutants carry lesions in the permeases for the specific amino acids and, as a consequence, their analogs. Alternatively, the mutations may involve the mechanisms responsible for incorporating the analogs into proteins. Mutations leading to the production of altered aminoacyl-tRNA synthetases, capable of discriminating between amino acids and their analogs, are quite common in bacteria (9).

Finally, although unlikely, a selective degradation of the analogs to nontoxic products cannot be ruled out for the moment.

Both groups of mutants appear to be very stable, having maintained their characteristics for over 1 year of continuous subculturing. Therefore, they may provide useful markers for genetic experiments in *Spirulina*.

Finally, the ease of isolation of mutants with increased intracellular pools of selected amino acids may be of considerable interest in the case of such an organism as *Spirulina*, which is considered a promising source of single-cell protein for animal and, possibly, human nutrition (1, 12). From this point of view, it is important that some of the mutants possessing large intracellular pools of amino acids, such as AZ8, PF27, and TA35, grow at rates and attain final cell concentrations comparable to those of the wild-type strain.

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