JOURNAL OF BACTERIOLOGY, July 1972, p. 112-118

Vol. 111, No. 1
Printed in U.S.A.

Documentation of Auxotrophic Mutation in Blue-Green Bacteria: Characterization of a Tryptophan Auxotroph in *Agmenellum quadruplicatum*

LONNIE O. INGRAM, DUANE PIERSON, JAMES F. KANE,[1] C. VAN BAALEN, AND ROY A. JENSEN

Marine Science Institute, University of Texas, Port Aransas, Texas 78373; The University of Tennessee-Oak Ridge Graduate School of Biomedical Sciences, and Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830; and Department of Microbiology, Baylor College of Medicine, Houston, Texas 77025

Received for publication 14 April 1972

A tryptophan-requiring auxotroph of *Agmenellum quadruplicatum* strain BG1, a species of blue-green bacteria, was isolated by means of a nitrosoguanidine-penicillin procedure. Its growth characteristics were determined, and the enzymological block was identified in the A activity of tryptophan synthetase. Starvation of the auxotroph for tryptophan resulted in the derepression of the synthesis of all five enzymes. The first four enzymes derepressed 2- to 3-fold, and tryptophan synthetase B derepressed 20-fold. In the parental prototroph, BG1, anthranilate synthetase was active in crude extracts with ammonia as the amino donor reactant, but not with glutamine.

A tremendous fraction of genetic and biochemical research has been dependent upon the use of auxotrophic mutants derived from heterotrophic bacteria. In contrast, the obligately phototrophic and lithotrophic bacteria have been neglected. Indeed, no auxotrophic derivative of these microbial groups has been rigorously documented. Rittenberg and Grady (20) reported the isolation of a strain of *Thiobacillus thiooxidans*, after ultraviolet light mutagenesis, which required yeast extract. However, this organism reverted before the specific nature of the nutritional block could be determined or verified. Stevens and Van Baalen (26) reported a similar example with a Casamino Acids-requiring strain of the blue-green bacterium *Agmenellum*. Kumar (12), Singh (21, 22), and Srivastava (24) have reported the isolation of nutritionally deficient mutants of various blue-green bacteria. Few of these claims were substantiated by the isolation of pure clones or by identification of precise nutritional requirements. Li et al. (13) suggested that, in general, auxotrophic mutations occur in autotrophs at a much lower frequency than in heterotrophs. Asato and Folsome (1) suggested that their inability to recover auxotrophic mutants of *Anacystis nidulans* after nitrosoguanidine or ultraviolet light mutagenesis was due to some intrinsic properties of the organism.

In this paper, we describe the isolation in *Agmenellum quadruplicatum* of an auxotroph with an obligate and stable requirement for tryptophan.

MATERIALS AND METHODS

Parental organism and cultivation. *A. quadruplicatum* strain BG1 is an axenic isolate described by C. Van Baalen (27). *A. quadruplicatum* was included in the provisional taxa identified recently by Stanier et al. (25). Cells were routinely grown batchwise at 39 C with continuous gassing (air enriched with 1% CO_2) by the method of Myers (15). Illumination was provided by four 20-w deluxe cool white fluorescent lamps (two on either side). The parent organism, denoted BG1, was grown in medium ASP_2 of Provasoli et al. (19, 27). The tryptophan auxotroph, denoted strain ATR1, was grown in the same manner with the addition of filter-sterilized tryptophan (final concentration, 0.2 mM). Under these growth conditions, the generation times of both strains BG1 and ATR1 were 3.5 hr.

Growth was routinely measured turbidimetrically by use of a Lumetron colorimeter. Dry weights were determined after filtration on tared membrane filter

[1] Present address: Department of Microbiology, University of Tennessee Medical Units, Memphis, Tenn. 38103.

discs (0.45 μm pore size; Millipore Corp.). The disc and cells were dried to a constant weight at 55 C. A culture of strain BG1 at a cell density corresponding to a turbidity of 1.0 contains 0.26 mg of cells (dry weight) per ml.

Preparation of extracts. Cell pellets obtained by centrifugation of cultures were stored at −20 C prior to extract preparations. The cell pellets were resuspended in 50 mM potassium phosphate buffer, *p*H 7.0, containing 0.1 M KCl. Phenylmethylsulfonylfluoride, 0.01 mM, created a surface effect that decreased frothing in subsequent disruption procedures. Clumps of cells were dispersed with a short treatment (30 to 45 sec) with a Bronwill Sonifier. Cells were lysed with a high concentration of lysozyme (5 mg per ml) at 40 C for 15 min. This preparation was chilled to ice temperature and exposed briefly (30 to 45 sec) to ultrasonic treatment to complete cell lysis and to disrupt deoxyribonucleate. This procedure gave extracts ranging in protein concentration from 5 to 25 mg per ml. Extracts were not dialyzed or desalted with Sephadex (except those used for the assay of anthranilate synthetase, which were passed through a Sephadex G-25 column).

Enzymological assays. Most of the assay techniques were identical or similar to those described in *Methods in Enzymology* (23). No attempt was made to develop optimal conditions of assay for the activities of the tryptophan pathway enzymes. The assay procedure for activity of anthranilate synthetase, with either glutamine or ammonia as the amino donor, was that described by Kane and Jensen (10).

The assay for phosphoribosylanthranilate transferase activity was performed in a reaction mixture containing 0.6 ml of 0.1 mM anthranilate, 0.3 ml of 100 mM phosphoribosylpyrophosphate, and 0.2 ml of crude extract. The reaction was started with the addition of extract and was incubated for 45 min at 37 C. Substrate disappearance was followed by extracting anthranilate into 2 ml of ethyl acetate and measuring fluorescence in an Aminco Bowman spectrophotofluorometer as previously described (10). The assay for transferase activity was the least accurate owing to the quenching effect caused by the pigments present in the crude extract.

Phosphoribosylanthranilate isomerase was assayed in reaction mixtures containing 0.4 ml of phosphoribosylanthranilate (prepared as described below) and 0.4 ml of crude extract. The reaction was begun by adding substrate and incubating at 37 C. After 45 min, 0.1 ml of extract from *Pseudomonas aeruginosa* P-135, a mutant lacking phosphoribosylanthranilate isomerase and derepressed for the remaining enzymes, was added. Incubation at 37 C was continued for 10 min. Samples of 0.5-ml volume were removed for the determination of indoleglycerol 3-phosphate concentration as described for the assay of indoleglycerol 3-phosphate synthetase. This reaction procedure resembles that described by Crawford and Gunsalus (2).

The reaction mixtures used in the assay of indoleglycerol 3-phosphate synthetase contained 0.2 ml of 6 μM 1-(*o*-carboxyphenylamino)-1-deoxy-D-ribulose 5-phosphate and 0.3 ml of crude extract. The reaction was started at 37 C with the addition of substrate and was incubated for 45 min. The reaction was terminated with the addition of 0.2 ml of 1 M acetate buffer, *p*H 5.0, and 0.2 ml of 1 M $NaIO_4$. After 20 min at room temperature, 0.4 ml of 1 N NaOH was added. Indole 3-aldehyde was extracted with 2.0 ml of ethyl acetate by vortex mixing for 45 sec. After a brief, low-speed centrifugation, the absorbance of indole 3-aldehyde was read in a Gilford spectrophotometer at 290 nm.

The reaction mixture for the assay of the A activity of tryptophan synthetase contained 0.1 ml of indoleglycerol 3-phosphate, 0.06 ml of 1 M serine, 0.04 ml of 10 mM pyridoxal-5-phosphate, 0.01 ml of 100 mM ethylenediaminetetraacetate, and 0.4 ml of crude extract. The reaction was started at 37 C with the addition of indoleglycerol 3-phosphate, and incubation was continued for 45 min. The disappearance of indoleglycerol 3-phosphate was followed as described in the section on the assay of indoleglycerol 3-phosphate synthetase.

The activity of tryptophan synthetase B was assayed by the procedure of Meduski and Zamenhof (14). The reaction was started at 37 C by adding 0.2 ml of substrate mixture (14) to 0.3 ml of crude extract. Incubation was continued at 37 C for 45 min. The reaction was terminated with 0.05 ml of 5,5-dimethyl-1,3-cyclohexanedione followed by the addition of trichloroacetic acid to a final concentration of exactly 5%. Unreacted indole was extracted into 3 ml of toluene by mixing with vortex action for 30 sec, and phase separation was achieved by low-speed centrifugation. A 2-ml amount of the toluene layer was mixed with 0.5 ml of 0.5% 4-dimethylaminocinnamaldehyde in propanol, and the mixture was incubated for 10 min at 37 C; 0.25 ml of H_2SO_4 (8% in 95% ethanol) was then added and mixed. After incubation at 37 C for 10 min, 2.0 ml of 50% acetic acid was added and mixed. The samples were held on ice for 10 min, mixed for 30 sec by vortex action, and centrifuged for 5 min at low speed. Indole concentrations in the bottom layers were estimated by measuring absorbance at 635 nm.

Biochemicals. Anthranilic acid was purchased from Calbiochem. The disodium salt of ribose-5-phosphate, serine, pyridoxal-5'-phosphate, phenylmethylsulfonylfluoride, and phosphoribosylpyrophosphate were obtained from Sigma Chemical Co. 1-Methyl-3-nitro-1-nitrosoguanidine, 4-dimethylaminocinnamaldehyde, and 5,5-dimethyl-1,3-cyclohexanedione were purchased from Aldrich Chemical Co. (Ethylenedinitrilo)-tetraacetic acid, disodium salt, was from Matheson Co., Inc. Lysozyme, three times crystallized, was from Worthington Biochemical Corp.

Chorismate was prepared as the barium salt (6) and was estimated to have a purity of 96% (uncorrected for solvation). It was stored under vacuum at −20 C in a desiccator. Phosphoribosylanthranilate was prepared just prior to assay by mixing 0.25 ml of 1 M anthranilate in 95% ethanol with 0.25 ml of 1 M ribose-5-phosphate. The nonenzymatic reaction was allowed to continue for 5 min at room temperature, and then the reaction was terminated by dilution,

200-fold, with cold 0.1 M triethanolamine hydrochloride buffer, pH 8.6. 1-(o-Carboxyphenylamino)-1-deoxy-D-ribulose 5-phosphate and indoleglycerol 3-phosphate were prepared as described previously (3). Other chemicals were of the highest quality commercially available.

RESULTS

Mutagenesis and isolation of a tryptophan auxotroph. A solution of 1-methyl-3-nitro-1-nitrosoguanidine was prepared just before use by dissolving 2 mg in 20 ml of ASP_2 medium at 45 C. The solution was immediately cooled to 22 C and sterilized by filtration. A 5-ml amount of the nitrosoguanidine solution was mixed with 5 ml of BG1 culture in the logarithmic phase of growth (6×10^7 cells/ml) and incubated at 22 C for 5 min. The mutagenized cells were washed twice by centrifugation and resuspension in sterile medium. The final suspension was adjusted to a volume of 4 ml, and 2 ml was used to inoculate a sterile growth tube containing 20 ml of medium. The mutagenized culture was returned to suitable growth conditions (light, 1% CO_2, 39 C) and incubated for 16 hr. At this time, filter-sterilized penicillin G was added to a final concentration of 0.05 μg/ml. After an additional 24 hr of incubation under growth conditions, serial dilutions were spread on solidified ASP_2 (1% Difco agar) containing L-phenylalanine, L-tyrosine, or L-tryptophan (each at 0.2 mM). The plates were sealed with Scotch tape (no. 810), inverted, and incubated beneath banks of 20-w tungsten lamps. After penicillin treatment, lysis was visually apparent as loss of turbidity and was confirmed by microscopic examination.

After 4 days of incubation, 312 potential auxotrophs (104 from each of the aromatic supplements) were picked with sterile toothpicks and streaked on plates containing the respective amino acid supplements. These were replica-plated after incubation on unsupplemented and aromatic-supplemented medium. Plates were inspected for the presence of auxotrophs. Several clones showed enhanced growth on each of the respective supplements. However, in only one clone was growth completely blocked in the absence of supplement. This isolate grew well on the tryptophan-supplemented plate. It was grown in liquid culture in the presence of tryptophan and cloned by two successive spread platings of appropriate dilutions. The final clonal isolate was designated strain ATR1. The growth rate, pigmentation, cellular morphology, and ultrastructure of strain ATR1 were identical to that of the parent, strain BG1. Other mutants having leaky requirements were not characterized further.

Stability and growth requirements of the auxotroph, ATR1. A suspension of ATR1 was centrifuged, washed, and added to ASP_2 medium containing agar (1%) at 45 C to yield approximately 10^6 cells/ml. Samples (15 ml) were immediately poured into sterile plastic petri plates (10 cm) and allowed to solidify. Various compounds were tested for their ability to support the growth of strain ATR1 by overlaying the solidified suspension with sterile filter paper discs (12.7 mm in diameter) containing 2 μmoles of the test compounds. Tyrosine, phenylalanine, tyramine, phenylpyruvate, p-hydroxyphenylpyruvate, erythrose, pyruvate, shikimate, chorismate, anthranilic acid, and indole were all unable to support visible growth of strain ATR1 after 1 week of incubation (as described earlier). Only tryptophan produced a dense halo of growth (Fig. 1) in the vicinity of the disc. This growth response is quite comparable to that found with tryptophan auxotrophs of heterotrophic microorganisms such as *Escherichia coli*, *Bacillus subtilis*, or *Pseudomonas aeruginosa*. The testing of indole and anthranilic acid as growth factors was hindered by their toxicity, which is perhaps the consequence of photoproduct formation in the presence of light. Mutant ATR1 required frequent transfer on

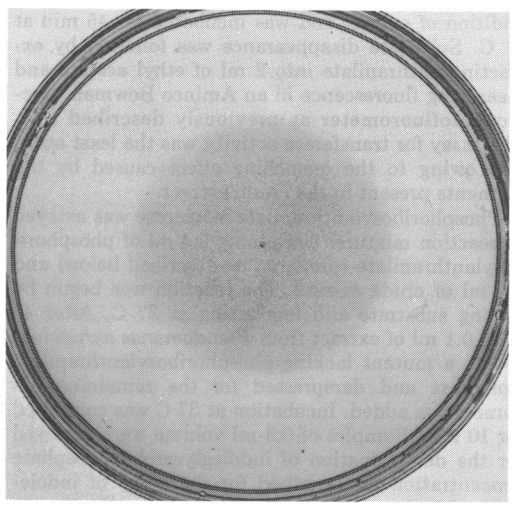

FIG. 1. *Photograph of agar plate showing growth response of mutant ATR1 to* L-*tryptophan. A pour plate was seeded with* 10^6 *cells per ml of basal medium. Several crystals of* L-*tryptophan were placed on the agar in the center of the plate. Growth occurred after incubation at 39 C.*

stock slants to prevent autoinhibition and subsequent loss of viability.

From the strong growth response on agar plates (Fig. 1), the level of the tryptophan requirement was judged to be quite low. No inhibitory response to the higher concentrations of tryptophan was detected. With the use of liquid culture, a more quantitative assessment of the tryptophan requirement was made. Various amounts of an exponentially growing suspension of strain ATR1 containing 0.2 mM L-tryptophan were added to sterile 20-ml growth tubes containing unsupplemented medium. These were incubated under growth conditions and monitored turbidimetrically. As demonstrated in Fig. 2, mutant ATR1 has an absolute requirement for tryptophan. Maximal growth yields comparable to those of the parental strain BG1 were obtained at tryptophan concentrations above 0.01 mM. Below 0.01 mM tryptophan, not only growth yield, but also growth rate, was proportional to added tryptophan. Continued incubation of starved cultures caused progressive pigment destruction. Cells changed from a blue-green to green, to yellow, and finally to white. Incubation of these cultures for 2 weeks resulted in no reversion to wild type or greening. As a check for stability of strain ATR1, 2×10^7 cells in 0.2 ml of medium containing 0.2 mM tryptophan were inoculated into each of 10 sterile culture tubes containing 20 ml of unsupplemented medium and were incubated for 4 days. Growth was monitored turbidimetrically. All cultures turned white after exhaustion of tryptophan. The culture in one tube became green after 7 days. From this, a maximal reversion frequency can be estimated to be 2×10^{-8}.

As a result of many dry weight determinations, the cell yield of 20 ml of a culture of strain BG1 at a density of 1.0 (10% T, Lumetron) is known to be 5.2 mg. On the basis of the cell yield obtained with the various tryptophan concentrations used in Fig. 1, the tryptophan requirement of strain ATR1 represents 0.4% of the dry weight. This value is consistent with the total amino acid composition of other blue-green bacteria (5).

Enzymatic profile of mutant ATR1. Clearly, mutant ATR1 is blocked in the gene specifying the A component of tryptophan synthetase (Table 1). All of the enzyme activities comprising the tryptophan pathway in other microorganisms were detected in the parental BG1. The enzymological proof identifying the lesion in tryptophan synthetase A is consistent with the positive colorimetric test obtained for the accumulation of indoleglycerol

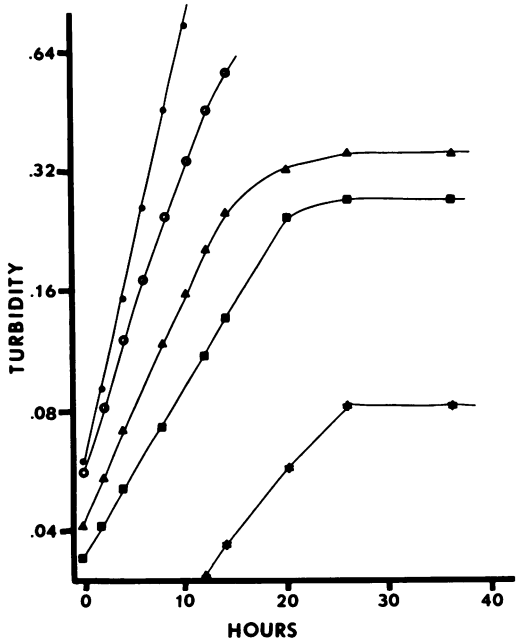

FIG. 2. *Obligate nutritional requirement of mutant ATR1 for L-tryptophan. Growth curves are plotted in which culture turbidity measured in a Lumetron colorimeter is plotted as a function of incubation time. The curve at the far left is the average of values for two cultures, one containing 0.2 mM and the other 0.01 mM L-tryptophan. Tryptophan did not limit growth in these cultures, and the growth rate is identical to that of BG1. The growth-limiting concentrations of tryptophan from left to right of the remaining curves were 5, 2, 1.5, and 0.5 μM, respectively.*

3-phosphate with ferric chloride reagent. The lack of a growth response to indole, expected a priori because of the intact, derepressible tryptophan synthetase B activity, may be due to lack of permeability to the membrane or to toxicity caused by indole, or to both. Indeed, both anthranilate and indole cause some inhibition of growth in the parental strain BG1. The lack of tryptophan synthetase A in mutant ATR1 is all the more apparent since starvation of the auxotroph for tryptophan resulted in the derepression of synthesis of all five of the enzymes. A culture of mutant ATR1 (line 2 of Table 1) was allowed to deplete tryptophan originally supplied at a concentration of about 2 μM (see Fig. 2). Subsequent incubation under conditions of starvation was continued for 4 hr before the cells were harvested for extract preparation. Under these conditions of tryptophan limitation, the first four enzymes derepressed two- to threefold. In contrast, tryptophan synthetase B was derepressed 20-fold.

TABLE 1. *Enzyme profiles of strains BG1 and ATR1*

Strain and growth conditions	Enzyme activity[a]					
	1	2	3	4	5a	5b
Reference specific activities[b]	0.07	0.154	1.23	0.13	0.081	0.081
Relative specific activities[c]						
ATR1 + 0.2 mM tryptophan	1.0	1.0	1.0	1.0	ND	1.0
ATR1 − tryptophan	2.9	2.4	1.9	2.4	ND	20.2
BG1 − tryptophan	1.2	0.6	1.5	0.8		4.1
BG1 + 0.2 mM tryptophan						0.8
BG1 − tryptophan						5.0

[a] Enzyme activities are denoted in order of function in tryptophan biosynthesis. Enzymes 1 to 5b are, respectively, anthranilate synthetase, 5-phosphorylribose 1-pyrophosphate phosphoribosyl transferase, *N*(5′-phosphoribosyl) anthranilic acid isomerase, indoleglycerol 3-phosphate synthetase, tryptophan synthetase A, and tryptophan synthetase B. Activities given for anthranilate synthetase are with ammonia used as the amino donor.

[b] Reference specific activities are representative of strain BG1 extracts from cells grown in minimal salts-glucose medium. Activities are given in nanomoles per minute per milligram of crude extract. These activities can be related directly to the relative activities shown in line 3.

[c] Mutant ATR1 was repressed by growth in the presence of 0.2 mM tryptophan, and was arbitrarily given a relative specific activity of 1.0 for each of the enzyme activities assayed. The notation ND is activity not determined.

Even after derepression, no activity of tryptophan synthetase A was detected, a result consistent with the nonleaky phenotype of mutant ATR1.

Regulation of tryptophan biosynthesis in strain BG1. The levels of the first four enzymes appeared to be about maximally repressed in strain BG1 after growth in a minimal medium. However, tryptophan synthetase B activity was usually elevated fourfold compared to a comparable extract prepared from cells grown in the presence of tryptophan. Confirmation of this result is shown by the data given in the bottom two lines of Table 1. In this experiment, a sixfold difference in tryptophan synthetase B activities was found.

Anthranilate synthetase in every microbial species examined is an allosteric protein inhibited by tryptophan. Figure 3 illustrates that anthranilate synthetase from *A. quadruplicatum* is no exception. The enzyme activity was inhibited 50% at about 2 μM tryptophan, and inhibition approached 100% at 10 μM. No activity was found for anthranilate synthetase when glutamine was used as the amino donor reactant. However, activity was found when ammonia was supplied as the amino donor.

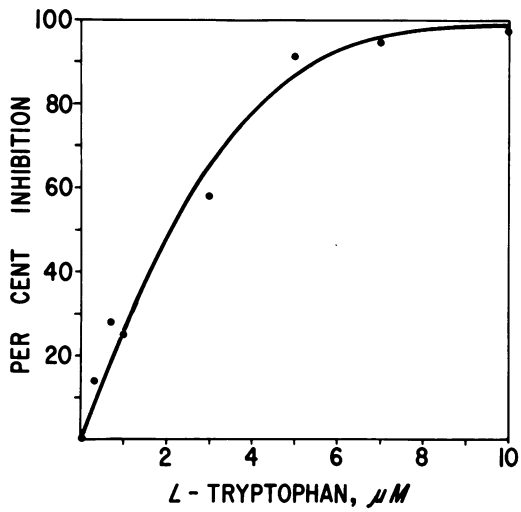

FIG. 3. *Inhibition curve for anthranilate synthetase activity showing per cent inhibition as a function of* L*-tryptophan concentration. Mutant ATR1 was derepressed after 4 hr of starvation for tryptophan. An extract was prepared as described in Materials and Methods, and was passed through a small Sephadex G-25 column; 189 μg of crude protein was used in the assay procedure described (10) for anthranilate synthetase (amn).*

DISCUSSION

It can be concluded that auxotrophic mutants of blue-green bacteria can be obtained. Such mutants should assist the formulation of the details of gene-enzyme relationships, as has been done so successfully in other bacterial groups. The properties of the tryptophan synthetase A auxotroph are similar in many respects to comparable tryptophan auxotrophs in other taxa. The biochemical pathway of tryptophan synthesis appears to be identical in all microorganisms studied. Tryptophan-dependent growth yields, derepression of enzyme

synthesis in response to starvation for tryptophan, and allosteric control of anthranilate synthetase by tryptophan all qualitatively resemble other microbial systems. Since the blue-green bacteria represent a major group of microorganisms which are largely uncharacterized (25), comparative aspects of the regulation of biosynthetic (and other) pathways are of considerable interest.

Anthranilate synthetase is one of a number of amidotransferase enzymes which, in most organisms, can use either glutamine or ammonia as an amino donor in vitro. Presumably, glutamine serves as the natural substrate under most physiological conditions. We have speculated that a mutant of *B. subtilis* lacking glutamine reactivity may use ammonia in vivo (11). In this context, it is of considerable interest that the anthranilate synthetase from *A. quadruplicatum* lacks glutamine reactivity, the tentative conclusion drawn from our data. Other genera of the blue-green bacteria, such as *Anacystis nidulans*, have been reported to possess glutamine-reactive anthranilate synthetase activity (28). It is possible that in *A. quadruplicatum* a glutamine-binding protein may be labile. As is the case in other systems, the ammonia-reactive species is inhibitable by tryptophan.

In general, the information existing in the literature suggests that repression of biosynthetic enzymes by end products in blue-green bacteria may not be an important control mechanism. Lack of control by repression has been suggested to be a possible cause for the excretion of large amounts of nitrogenous compounds by some blue-green bacteria (4, 8). Pearce and Carr reported an apparent lack of metabolic control by repression in *Anabaena variabilis* during autotrophic growth in the presence of acetate (16, 18) or glucose (17). An apparent lack of repression control is further supported by examination of the effects of exogenous arginine on seven enzymes involved in its biosynthesis and degradation (8, 9). An alternative interpretation was presented, albeit with less vigor (8). These data are probably too scanty to allow more than a tentative conclusion on this point. Nevertheless, it is intriguing that another major group, the pseudomonads, usually do not possess biosynthetic enzymes subject to regulation by repression (7). A striking exception is represented by the enzymes of tryptophan biosynthesis which vary in response to tryptophan concentration at least 50-fold. It is a curious coincidence that the same exception characterizes the *A. quadruplicatum* system. Even more coincidental is the differential derepression of tryptophan synthetase. In *P. putida* (2) and *P. aeruginosa* (Calhoun and Pierson, *unpublished data*), tryptophan synthetase is induced by indoleglycerol 3-phosphate, whereas the synthesis of the remaining four enzymes is repressed by tryptophan. The data given in Table 1 are suggestive of a similar situation in *A. quadruplicatum*. Starvation of a tryptophan synthetase A mutant (which would result in indoleglycerol 3-phosphate accumulation) results in a 20-fold derepression of tryptophan synthetase B, under conditions in which the other enzymes are derepressed two- to threefold. More detailed studies with other classes of tryptophan auxotrophs will be required to establish the validity of this possibility.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant AM 13105 from the National Institute for Arthritis and Metabolic Diseases. L. O. I. is a post doctoral trainee supported by subcontract 3322 from the Biology Division of Oak Ridge National Laboratory to the Univ. of Tennessee. Oak Ridge National Laboratory is operated by Union Carbide Corp. for the U.S. Atomic Energy Commission.

LITERATURE CITED

1. Asato, Y., and C. E. Folsome. 1969. Mutagenesis of *Anacystis nidulans* by N-methyl-N'-nitro-N-nitrosoguanidine and UV irradiation. Mutat. Res. **8**:531–536.
2. Crawford, I. P., and I. C. Gunsalus. 1966. Inducibility of tryptophan synthetase in *Pseudomonas putida*. Proc. Nat. Acad. Sci. U.S.A. **56**:717–724.
3. Creighton, T. E., and C. Yanofsky. 1970. p. 365–380. *In* H. Tabor and C. W. Tabor (ed.), Methods in enzymology, vol. 17A. Academic Press Inc., New York.
4. Fogg, G. E. 1962. Extracellular products, p. 475–492. *In* R. A. Lewin (ed.), Physiology and biochemistry of algae. Academic Press Inc., New York.
5. Fowden, L. 1954. A comparison of the compositions of some algal proteins. Ann. Bot. (London) **18**:257–266.
6. Gibson, F. 1964. Chorismic acid: purification and some chemical and physical studies. Biochem. J. **90**:256–261.
7. Hegeman, G. D., and S. L. Rosenberg. 1970. The evolution of bacterial enzyme systems. Annu. Rev. Microbiol. **24**:429–462.
8. Hood, W., and N. G. Carr. 1971. Apparent lack of control by repression of arginine metabolism in blue-green algae. J. Bacteriol. **107**:365–367.
9. Hood, W., A. G. Leaver, and N. G. Carr. 1969. Extracellular nitrogen and the control of arginine biosynthesis in *Anabaena variabilis*. Biochem. J. **114**:12P.
10. Kane, J. F., and R. A. Jensen. 1970. The molecular aggregation of anthranilate synthase in *Bacillus subtilis*. Biochem. Biophys. Res. Commun. **41**:328–333.
11. Kane, J. F., and R. A. Jensen. 1972. Metabolic interlock. The dual function of a folate pathway gene as an extra-operonic gene of tryptophan biosynthesis. J. Biol. Chem. **247**:1587–1596.
12. Kumar, H. D. 1966. Some experiments on the induction of nutritionally-deficient mutants in the blue-green alga *Anacystis nidulans*. Advan. Frontiers Plant Sci. **14**:133–142.

13. Li, S. L., G. P. Redei, and C. S. Gowans. 1967. A phylogenetic comparison of mutation spectra. Mol. Gen. Genet. **100**:77–83.
14. Meduski, J. W., and S. Zamenhof. 1968. Quantitative determination of methylindoles. Anal. Biochem. **24**: 202–208.
15. Myers, J. 1950. The culturing of algae for physiological research, p. 45–51. *In* J. Bunel, G. W. Prescott, and L. H. Tifanny (ed.), The culturing of algae. Charles F. Kettering Foundation, Yellow Springs, Ohio.
16. Pearce, J., and N. G. Carr. 1967. The metabolism of acetate by the blue-green algae, *Anabaena variabilis* and *Anacystis nidulans*. J. Gen. Microbiol. **49**:301–313.
17. Pearce, J., and N. G. Carr. 1968. The incorporation and metabolism of glucose by *Anabaena variabilis*. J. Gen. Microbiol. **54**:451–462.
18. Pearce, J., C. K. Leach, and N. G. Carr. 1969. The incomplete tricarboxylic acid cycle in the blue-green alga *Anabaena variabilis*. J. Gen. Microbiol. **55**:371–378.
19. Provasoli. L., J. J. A. McLaughlin, and M. R. Droop. 1957. The development of artificial media for marine algae. Arch. Mikrobiol. **25**:392–428.
20. Rittenberg, S. C., and R. P. Grady. 1950. Induced mutants of Thiobacillus thioxidans requiring organic growth factors. J. Bacteriol. **60**:509–510.
21. Singh, R. N., and H. N. Singh. 1964. Ultraviolet induced mutants of blue-green algae. I. *Anabaena cycadeae* Reinke. Arch. Mikrobiol. **48**:109–117.
22. Singh, R. N., and H. N. Singh. 1964. Ultraviolet induced mutants of blue-green algae. II. *Anabaena doliolum* Bharadwaja. Arch. Mikrobiol. **48**:117–121.
23. Smith, O. H., and C. Yanofsky. 1962. Enzymes involved in the biosynthesis of tryptophan, p. 794–806. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 5. Academic Press Inc., New York.
24. Srivastava, B. S. 1969. Ultra-violet induced mutations to growth factor requirement and penicillin resistance in a blue-green alga. Arch. Mikrobiol. **66**:234–238.
25. Stanier, R. Y., R. Kunisawa, M. Mandel, and G. Cohen-Bazire. 1971. Purification and properties of unicellular blue-green algae (order *Chroococcales*). Bacteriol. Rev. **35**:171–205.
26. Stevens, S. E., Jr., and C. Van Baalen. 1970. Growth characteristics of selected mutants of a coccoid blue-green alga. Arch. Mikrobiol. **72**:1–12.
27. Van Baalen, C. 1962. Studies on marine blue-green algae. Bot. Mar. **4**:129–139.
28. Weber, H. L., and A. Bock. 1969. Regulation of the chorismic acid branch-point in aromatic acid synthesis in blue-green and green algae. Arch. Mikrobiol. **66**:250–258.