Approach to Recognition of Regulatory Mutants of Cyanobacteria

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Antimetabolite analogs of essential amino acids are useful as selective agents for isolation of regulatory mutants of cyanobacteria, although we observed striking microbiological differences from other widely used eubacterial systems. Regulatory mutants shown to overproduce and excrete tryptophan, phenylalanine, tyrosine, methionine, or arginine were isolated from four cyanobacteria: Anabaena sp. 29151, Synechococcus sp. 602, Synechococcus sp. AN Tx20, and Synechocystis sp. 29108. Surprisingly, regulatory-mutant colonies did not support a halo of cross-fed wild-type growth on selective medium. Since regulatory mutants were shown to excrete substantial levels of amino acids, it was deduced that poor cross-feeding must reflect a generally low nutritional responsiveness of the cyanobacterial background. This conclusion was confirmed by results which showed that regulatory-mutant cells of cyanobacteria dispersed among wild-type populations of Bacillus subtilis did produce halo colonies on solid analog-containing medium. Cross-feeding between one cyanobacterial pair (a phenylalanine excretor and a phenylalanine auxotroph) was successfully demonstrated in the absence of the analog under conditions in which relatively large masses of each cell population type were spread near one another on agar plates. These results suggest that amino acid excreted by regulatory mutants of cyanobacteria on analog-containing selective medium is transported into nearby wild-type cells too inefficiently to overcome the antimetabolite effects of the analog, thereby failing to generate halos of physiologically resistant background cells. Consistent with this interpretation was the finding that the pheA1 auxotroph from Synechococcus sp. 602 exhibited a linearly proportional dependence of growth rate upon exogenous concentration of L-phenylalanine (below 20 μM). Wild-type B. subtilis serves as a convenient and sensitive test lawn for screening obvious regulatory mutants from among collections of analog-resistant cyanobacterial mutants. Appropriate B. subtilis auxotrophs can be used as convenient indicator strains for the identification of regulatory mutants in cyanobacteria through the observation of synthetic growth responses.

Biochemical perturbations produced by regulatory mutations in bacteria have offered a highly successful approach to the understanding of regulatory mechanisms, enzyme structure, and biosynthetic pathways (6, 8, 16) and to the elucidation of interactions between biosynthetic pathways (10). The ability of analogs to mimic natural end products has been a familiar basis for the selection of regulatory mutants recognized as a fraction of analog-resistant individuals in a sensitive population. Analog-resistant mutants which are defective in the regulation of amino acid biosynthesis have been used to identify those enzyme activities subject to feedback inhibition and enzyme repression. Regulatory mutants are commonly sorted from other classes of resistant mutants by selection of halo colonies. The latter colonial phenotype results from overproduction and excretion of a natural end product which then physiologically relieves the antimetabolite effect, stimulating growth of a background halo of wild-type cells around mutant colonies on the original selection plates (1, 5). The identity of an excreted end product can quickly be established by the ability to cross-feed a particular auxotrophic strain (1). Published descriptions of regulatory mutants in cyanobacteria are virtually nil. An isolated example was with Anacystis nidulans mutants in which relatively modest quantitative differences in the sensitivity of 3-deoxy-D-arabinohexulose-7-phosphate synthase to feedback inhibition by L-tyrosine were observed (14). These mutants were not characterized as excretors of aromatic end products.

Our initial experiments, in which sensitive wild-type cyanobacteria were plated on analog-containing medium, produced surprising results.
Although analog-resistant colonies were readily obtained as spontaneous mutants, colonies surrounded by halos of cross-fed background cells were never seen. In organisms such as Bacillus subtilis or Escherichia coli, excreting regulatory mutants are readily discerned from other classes of resistant mutants, (e.g., transport-deficient mutants) on the original selection plate by observing the cross-feeding of sensitive background cells, manifested as a gradient of growth around the resistant colony (halo).

A priori, this could mean that regulation of biosynthetic pathways in cyanobacteria is so unspectacular that loss of regulation in mutants generates only minor quantitative increases in pathway flow, that analog selection for some unknown reason is unsuitable for recognition of regulatory mutants, or that regulatory mutants are in fact selected by analog resistance but do not cross-feed background cells because of feeble transport of excreted molecules into analog-sensitive background cells. The last explanation is supported by our results.

MATERIALS AND METHODS

Microorganisms. All cyanobacterial strains used except Anabaena sp. are unicellular organisms which readily plate out as colony-forming units on solid medium. Synechocystis sp. ATCC 29108 and Anabaena sp. ATCC 29151 were obtained from the American Type Culture Collection, Rockville, Md. Synechococcus sp. 602 and Synechococcus sp. 602 pheA1, a phenylalanine auxotroph, were generous gifts of S. V. Shestakov. Synechococcus sp. AN Tx20 (commonly denoted as Anacystis nidulans in the literature) is ATCC 27144. All wild-type cyanobacterial strains were routinely maintained on Cg10 medium (20) solidified with 1.4% (wt/vol) agar (Difco) sterilized separately. Analog-resistant mutants were maintained on Cg10 medium supplemented with 100 μg of the appropriate analog per ml.

The prototrophic B. subtilis strain NP1 is a derivative of strain 168 (15). The auxotrophic B. subtilis strains used, all derived from strain 168, and their amino acid requirements were E-7, tryptophan; E-34, tyrosine; E-62, phenylalanine; A-140, histidine; A-87, methionine; and A-54, arginine. B. subtilis F-2 is a constitutive regulatory mutant which is resistant to 5-methyltryptophan and is a heavy excretor of tryptophan (11).

Selection of mutants resistant to amino acid analogs. Spontaneous analog-resistant mutants were selected by either (i) placing a few crystals of analog in the middle of Cg10 agar medium spread with a lawn of 10^6 to 10^8 cells or (ii) spreading 10^6 to 10^8 cells on a plate of Cg10 medium containing 100 μg of analog per ml. Plates were incubated at 34°C under constant illumination. Resistant colonies appeared within 7 to 10 days and were then purified by single-colony isolation on analog-containing medium. Isolated single colonies were checked for phenotypic stability and excretion.

Determination of auxotrophic growth response to L-phenylalanine. Cells of Synechococcus sp. 602 pheA1 were grown in Dm medium (19) supplemented with 0.5 mM L-phenylalanine. A 10-ml sample of actively dividing cells (optical density at 500 nm, 0.5) was washed twice with 10-ml portions of sterile unsupplemented Dm medium via cell capture on a 0.45-μm membrane filter (Millipore Corp.). After being washed, the cells were suspended in 10 ml of sterile medium. A 1.0-ml sample of washed cells was added to each of seven 75-ml test tubes containing 50 ml of Dm medium supplemented with various amounts of filter-sterilized L-phenylalanine. The growth vessels were incubated in a 34°C water bath illuminated with fluorescent lights. Care was taken to ensure that all tubes received equal light exposure. Growth vessels were aerated with a 2% (vol/vol) mixture of CO2-air. Samples were aseptically removed at periodic intervals, and the percent transmittance values were determined with a Bausch & Lomb Spectronic 20 colorimeter at both 500 and 600 nm. The values recorded were converted to optical density units. Growth curves obtained by using values at 500 and 600 nm were comparable. At these wavelengths, the contribution of the pigments is less than 10% of total absorbancy. Samples having optical densities greater than 0.4 were appropriately diluted to maintain the range in which optical density is proportional to turbidity.

The wild type grows exponentially, and accurate doubling times are conveniently obtained in the optical density range of 0.1 to 1.0. At turbidity values above 1.0, shading effects complicate growth rate determinations. Growth rates of phenylalanine auxotrophic Synechococcus sp. 602 pheA1 were determined through the 40- to 88-h growth period after the initial lag phase and were calculated as an increment in optical density per hour rather than a doubling time because of the linear nature of the growth curve (see Fig. 3).

Routine identification of regulatory mutants. A biologically sensitive test to identify amino acid excretors in cyanobacterial strains is a heterologous system employing a confluent lawn of B. subtilis. B. subtilis grows readily on Cg10 medium in the light when 0.5% glucose is present as a carbon source. Mixtures of prototrophic populations of cyanobacteria and B. subtilis were compatible during growth. In some cases, auxotrophic lawns of B. subtilis were used for detection of halo growth responses. In other cases, wild-type (prototrophic) lawns sensitive to the analog present in the medium were used to detect potential halo responses.

Analytical techniques. The assay of culture filtrates for protein was done by the method of Bradford (3). Quantitative assays for excreted aromatic amino acids were carried out as follows. Actively growing cultures (optical density at 500 nm, 1.0 to 4.5), incubated in liquid Cg10 medium at 34°C and aerated with a 2% (vol/vol) CO2-air mixture, were centrifuged at 10,000 × g for 5 min in a Sorvall model RC 2-B centrifuge. Portions of the resulting supernatants were filtered through a 0.45-μm Millipore filter, and the filtrate was immediately frozen at -15°C until assayed. Phenylalanine was measured by the fluorescence assay of McCaman and Robins as modified by Wong et al.
(21), except that 50 μl of 25% trichloroacetic acid was added to a 200-μl sample to reduce excessive sample dilution, 1.0 ml of copper reagent was added, and relative fluorescence was measured at an emission wavelength of 470 nm. Tyrosine was determined by the fluorescent assay of Wong et al. (21), except that 50 μl of 20% trichloroacetic acid was added to a 200-μl sample and 200 μl of α-nitroso-β-naphthol reagent was added to 50 μl of trichloroacetic acid supernatant. Tryptophan was measured by the fluorescent decay assay of Guilbault and Froelich (9). All fluorescence measurements were performed with an Aminco-Bowman spectrophotofluorometer.

Biochemicals. All compounds used were reagent grade or of the highest purity available. All analogs were obtained from Sigma Chemical Co., except 3-methylhistidine (Calbiochem) and N-acetylleucine (Schwartz/Mann).

RESULTS

Isolation of regulatory mutants. Four cyanobacterial species were screened for sensitivity to a number of amino acid analogs. Analogs of arginine, methionine, phenylalanine, tryptophan, and tyrosine inhibited growth of one or more strains sufficiently to allow the selection of potential regulatory mutants, expected to possess analog resistance phenotypes. The most useful selective agents found are listed in Table 1. Other analogs tested but found to have little or no inhibitory effect on any of the species screened include 2-methylleucine, N-methylleucine, α-methylmethionine, norleucine, norvaline, α-methyl-m-tyrosine, 5-methyltryptophan, and p-tyrosine. 3-Methylhistidine and 3-aminotyrosine were found to inhibit growth of some of the strains tested, but spontaneous resistant mutants were not recovered.

Recognition of regulatory mutants. It was immediately apparent that although various analog-resistant colonies were easily obtained, none of them supported a halo of cross-fed background growth. This is illustrated by the photograph in Fig. 1A showing resistant colonies of Synechocystis sp. 29108 arising on 4-fluorophenylalanine-containing medium. Even when resistant mutants were purified and spread at greater cell mass onto wild-type lawns on 4-fluorophenylalanine agar, cross-fed halos did not arise (Fig. 1B). On the other hand, many of the resistant mutants initially selected (circled in Fig. 1A) proved to be phenylalanine excretors since each cross-fed a lawn of the auxotrophic Synechococcus sp. 602 pheA1 on minimal medium (Fig. 1C). Control platings established the inability of wild-type Synechococcus sp. 29108 to cross-feed Synechococcus sp. 602 pheA1 even after prolonged incubation.

Similar experiments to recognize the possible excretion of amino acids by other analog-resistant mutants were not initially feasible due to the lack of appropriate cyanobacterial auxotrophs (12). However, B. subtilis NP1 was found to be capable of growth on Cg10 medium supplemented with glucose, and auxotrophic strains derived from B. subtilis NP1 were suitable for rapid identification of cyanobacterial regulatory mutants (i.e., wild-type cyanobacteria did not feed B. subtilis auxotrophs, whereas analog-resistant cyanobacterial excretors produced typical halos within background lawns of the appropriate auxotrophs). For example, the heterologous combination of cyanobacterial tryptophan excretor and B. subtilis tryptophan auxotroph exhibited cross-feeding (Fig. 2A). The same cyanobacterial tryptophan excretors successfully cross-fed in heterologous combination with the B. subtilis wild type on 6-fluorotryptophan (Fig. 2B). Similarly, the heterologous combination of cyanobacterial phenylalanine excretor and wild-type B. subtilis on 4-fluorophenylalanine exhibited cross-feeding (Fig. 2C) in contrast to the negative results obtained in Fig. 1B with the homologous combination of cyanobacteria. When cyanobacterial excretors of tryptophan (Fig. 2D) or of phenylalanine (Fig. 2E and F) were spread onto analog-containing agar spread with heavy cyanobacterial lawns, no cross-feed-

<table>
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<th>Analog</th>
<th>Anabaena sp. 29151</th>
<th>Synechocystis sp. 29108</th>
<th>Synechococcus sp. 602</th>
<th>Synechococcus sp. AN Tx20</th>
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* Defined as the ability to carry out syntrophic cross-feeding of appropriate auxotrophs of B. subtilis.

* NI, no inhibition.

TABLE 1. Selection of analog-resistant mutants of cyanobacteria
Fig. 1. Examination of phenylalanine cross-feeding in homologous combinations of cyanobacteria. (A) Photograph of resistant colonies selected from a confluent lawn of Synechocystis sp. 29108 cultured on 4-fluorophenylalanine-containing medium. Circled colonies were isolated, purified, and tested for ability to cross-feed a wild-type lawn of Synechocystis sp. on analog-containing medium (B) a lawn of phenylalanine auxotroph pheA1 on Cg10 medium (C).

Fig. 2. Comparison of cross-feeding results in homologous and heterologous combinations of cyanobacteria and B. subtilis. Populations of the following were spread with an inoculating loop onto an agar surface previously spread with the indicated confluent lawn: (a) B. subtilis tryptophan excretor F-2, (b) wild-type Anabaena sp. 29151, (c) Anabaena sp. tryptophan excretor 6FT2, (d) Anabaena sp. tryptophan excretor 6FT1, (e) Synechocystis sp. phenylalanine excretor 4FP30, (f) Anabaena sp. phenylalanine excretor 4FP1, (g) Anabaena sp. phenylalanine excretor 4FP2, and (h) Synechocystis sp. phenylalanine excretor 4FP31. Confluent lawns corresponding with each panel shown are: (A) B. subtilis tryptophan auxotroph E-7 on Cg10 minimal medium, (B) B. subtilis wild-type NP1 on 6-fluorotryptophan medium, (C) B. subtilis wild-type NP1 on 4-fluorophenylalanine medium, (D) Anabaena sp. 29151 wild type on 6-fluorotryptophan medium, (E) Anabaena sp. 29151 wild type on 4-fluorophenylalanine medium, and (F) Synechocystis sp. 29108 on 4-fluorophenylalanine medium.
ing between these homologous-combination pairs was observed.

The approach of using heterologous combinations of *B. subtilis* auxotrophic lawns and potential cyanobacterial amino acid excretors was exploited to screen for various classes of regulatory mutants. A very large fraction of the analog-resistant mutants isolated in four cyanobacterial strains were regulatory mutants (Table 1). Only obvious excretors were scored as regulatory mutants, and it is possible that some of the nonexcreting analog-resistant mutants were also regulatory mutants.

**Amino acid excretion by regulatory mutants.** Quantitative measurements of amino acid excretion were made in seven mutants representing four organisms (Table 2). Strains were grown in liquid culture, and the extracellular growth fluid was assayed as described above. Growth supernatants did not contain detectable levels of protein. The wild-type organisms tested accumulated little or no phenylalanine in the growth medium, compared with the elevated levels found in regulatory mutants resistant to the indicated fluoro derivatives of phenylalanine. The most dramatic instance was with 4-fluorophenylalanine-resistant mutants of *Synechocystis* sp. 29108. The two mutants, 4FP30 and 4FP31, also overproduce tryptophan and tyrosine and have been shown to possess a mutant 3-deoxy-D-arabinohexulosonate-7-phosphate synthase that is desensitized to allosteric control (Hall and Jensen, unpublished data). Mutant 6FT2, a tryptophan excretor from *Anabaena* sp. 29151, illustrates another obvious example of a regulatory mutation.

**Nature of analog inhibition of growth.** Since homologous combinations of cyanobacterial pairs cross-fed in the absence of an analog (excretors and auxotrophic lawns) but not in the presence of an analog exposure (excretors and prototrophic lawns on analog-containing medium), the possibility that analog exposure was bactericidal was tested. Lawns of *Synechocystis* sp. 29108 and *Anabaena* sp. 29151 were spread to confluence on Cg10 medium containing 100 μg of 4-fluorophenylalanine per ml, perhaps the most inhibitory analog tested. The agar plates were incubated at 34°C under constant illumination. Each day over a period of 1 week, 0.1 ml of a 20 mM mixture of the three aromatic amino acids was added to a well cut in the agar medium with a cork borer. In both species, continued viability after 4 days of analog inhibition was demonstrated by observation of good growth around each amino acid-containing well. Hence, the primary analog effect appears to be bacteriostatic, as in other bacteria. Generally, recovery of viable cyanobacteria was minimal after 7 days of analog exposure.

**Relationship of amino acid transport.** When single cells of the phenylalanine-excreting mutants were plated on lawns of phenylalanine auxotroph *Synechococcus* sp. 602 pheA1, cross-feeding was not apparent until 4 to 6 days after appearance of the excreting mutant colonies. This delayed response suggested feeble transport. Accordingly, it seemed likely that a lack of active transport, or a very low level of transport, might explain the relatively poor cross-feeding results with homologous combinations of cyanobacteria on analog-containing medium. This would be consistent with the finding of background halos of *B. subtilis*, which has an excellent transport system (2, 7), around cyanobacterial regulatory mutants on analog containing medium, in contrast to the failure of homologous cyanobacterial combinations to cross-feed.

When the transport of L-[14C]phenylalanine into cells of *Agmenellum quadruplicatum* or *Syne-
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**chococcus** sp. AN Tx20 was compared with *B. subtilis* under the conditions specified by D'Ambrosio et al (7), transport rates in cyanobacteria were indeed found to be roughly two orders of magnitude less than those of *B. subtilis*. At 50 μM L-[¹⁴C]phenylalanine, 0.2 nmol of amino acid was transported in 5 min per mg of cell mass in *Synechococcus* sp. AN Tx20 compared with 29 nmol/mg per 5 min in *B. subtilis*. At 5 μM L-[¹⁴C]phenylalanine the transport rate in *B. subtilis* was 8 nmol/mg per 5 min compared with a rate of <0.05 nmol/mg per 5 min in *Synechococcus* sp. AN Tx20.

If amino acid transport were sufficiently deficient, one might predict a direct dependence of growth rate of cyanobacterial auxotroph upon exogenous amino acid present at low concentrations. In organisms with an active transport mechanism, the uptake of low concentrations of amino acids required by auxotrophs is adequate to maintain the wild-type growth rate, even in a range that distinctly limits the ultimate mass yield. The family of growth curves shown in Fig. 3 reveals that differing rates of growth were indeed obtained at different exogenous concentrations of L-phenylalanine supplied in the growth medium to a cyanobacterial phenylalanine auxotroph. It was convenient to use linear coordinates to represent growth rates at initial L-phenylalanine concentrations lower than 25 μM since the kinetics of growth in this concentration range approximate linearity. At concentrations of L-phenylalanine greater than 100 mM, growth was exponential and equaled the 9-h doubling time of the wild type. Figure 4 is a plot of the data points obtained when growth rate (determined from the slope of each curve in Fig. 3) is plotted as a function of the concentration of exogenous L-phenylalanine initially supplied. A linear proportionality of growth rate and L-phenylalanine concentration was observed below 15 μM. In *E. coli* or *B. subtilis*, a comparable manipulation of growth rate through adjustment of exogenous L-phenylalanine can only be achieved in the chemostat.

**DISCUSSION**

Analogs of metabolites such as amino acids inhibit microbial growth by acting as end product mimics. Antimetabolite effects are readily reversed in the presence of the natural end product (1). Thus, regulatory mutants which overproduce end product are selected as resistant colonies on analog-containing medium. One expects that wild-type cells contiguous to a regulatory mutant colony will be phenotypically resistant because of the presence of excreted end product. Halo formation around colonies of analog-resistant mutants has apparently never been described in cyanobacteria. This could mean that regulatory mechanisms (and therefore the possibility of genetic deregulation) are rather undeveloped or even nonexistent for many biosynthetic pathways in cyanobacteria. Repression control of biosynthetic pathways in cyanobacteria does indeed appear to be minimal (4) as in fact is the case with many other nonenteric bacteria (17). However, allosteric control of

![Fig. 3. Growth response of *Synechococcus* sp. 602 pheAl as a function of increasing concentrations of exogenous L-phenylalanine. Within the range of optical density shown, the parental wild type grows exponentially at a doubling time of 9 h.](http://www.asm.org/jb/)

![Fig. 4. Proportional relationship of growth rate and exogenous L-phenylalanine concentration. The growth rates on the ordinate scale are expressed as increment of optical density at 500 nm per hour × 10³.](http://www.asm.org/jb/)

http://jb.asm.org/ on February 1, 2021 by guest
regulatory enzymes has been found in most, if not all, biosynthetic pathways where it has been sought.

In this study analog-resistant mutants of cyanobacteria were shown to excrete levels of end product that were qualitatively similar to mutants isolated in other procaryotes such as B. subtilis. On original selection medium the former mutants do not cross-feed the sensitive background. However, the ability of cyanobacterial regulatory mutants to cross-feed wild-type lawns of analog-sensitive B. subtilis on analog-containing medium eliminates the possibility that the presence of analog somehow diminishes excretion of end product in cyanobacteria. The unexpected obstacle to rapid identification of excreting mutants, i.e., lack of wild-type growth halos on selection plates, was overcome by the use of B. subtilis tester lawns which proved to be growth compatible with the cyanobacterial strains utilized. It is likely that other species of bacteria capable of growth on minimal salts medium supplemented with glucose, such as E. coli, will also prove to be growth compatible with species of cyanobacteria, thus making available a wide array of auxotrophic indicator strains for the identification of regulatory mutants in cyanobacteria. Many of the regulatory mutants proved to be stable through several transfers on nonselective medium and so should provide useful genetic markers.

The results of cross-feeding experiments with a variety of B. subtilis-cyanobacterial heterologous combinations in comparison with homologous combinations reveal that cyanobacterial lawns respond poorly to excreted end product. Most likely this is the consequence of weak transport capability in cyanobacteria, shown to be qualitatively poor relative to B. subtilis. The linear dependence of the growth rate of Synechococcus sp. 602 pheAl upon the exogenous concentration of L-phenylalanine further suggests the absence of active transport for L-phenylalanine. In various species of autotrophic bacteria (including Synechococcus sp. AN Tx20) the rate of amino acid assimilation was found to vary proportionally with exogenous concentration, a finding interpreted to reflect generally poor transport of amino acids (17). The absence of sugar permeases has been postulated to explain the failure of obligately photoautotrophic cyanobacteria to grow in the dark at the expense of various sugars, including glucose (18). A comparative study of glucose utilization by Aphanoocapsa sp. 6714, a facultative photo- and chemoheterotroph, with glucose utilization by two obligate photoautotrophs, Synechococcus sp. 6301 and Aphanoocapsa sp. 6308, indicated that the failure of the latter two organisms to grow with glucose was due to the absence of an effective glucose permease (13). It would not be surprising, therefore, to find that many, if not all, cyanobacteria lack permeases for amino acids.

In summary, one can envision the following scenario of events during the emergence of a cyanobacterial regulatory-mutant colony on analog-containing selective medium. Initially, very little excreted metabolite is elaborated because of the small cell population within the growing clone. Nearby wild-type cells must be able to transport via a high-affinity system to scavenge the low concentrations of metabolite present. The metabolite analog, present at high concentration, will most likely exacerbate transport problems in a competitive fashion. Relatively slow growth of cyanobacteria will result in greater opportunity for diffusion of excreted metabolite through the agar relative to generation time, thereby effectively diluting the concentration of metabolite encountered by background cells. In contrast, the efficient high-affinity transport of amino acids such as phenylalanine in B. subtilis provides a sensitive response to excreted metabolites even under plating conditions in which a single cell generates a colony supporting a cross-fed halo.

ACKNOWLEDGMENTS

This investigation was supported in part by Public Health Service research grant AM-19447 from the National Institutes of Health and in part by funds from the Center for Somatic cell Genetics and Biochemistry.

We gratefully acknowledge the help of Joan Markiewicz with the illustrative art work.

LITERATURE CITED


