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I. An attempt was made to isolate auxotrophic mutants of a blue-green bacterium, Anacystis nidulans requiring the amino acids serine, threonine, glutamate and/or aspartate. The mutagen employed was N-methyl-N'-nitro-N-nitrosoguanidine (NTG). Multiple cycles of penicillin enrichment were performed following mutagenesis. Discussion includes speculation concerning reasons for failure to isolate these auxotrophs.

II. Temperature-sensitive mutants of cyanophage AS-1, which infects the blue-green bacteria Anacystis nidulans and Synechococcus cedrorum, were induced using NTG. Six complementation groups were identified. These were used in two-factor crosses, and the resulting recombination frequencies were used to construct a preliminary genetic map of the cyanophage.

I. ISOLATION OF AUXOTROPHIC MUTANTS OF THE BLUE-GREEN BACTERIUM, *ANACYSTIS NIDULANS*, AND II. ISOLATION AND GENETIC MAPPING OF TEMPERATURE-SENSITIVE MUTANTS OF THE CYANOPHAGE AS-1

by

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APPROVAL PAGE

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INTRODUCTION

I. Little information is currently available about the genetics of the blue-green bacteria (cyanobacteria). The need for research on the genetics of these organisms becomes apparent when one considers the recent advances in recombinant DNA research. Amid the controversy surrounding this technology of "genetic engineering", the possibility of transferring a gene for nitrogen fixation from blue-green bacteria to crop plants is one of the stronger arguments for allowing recombinant DNA research to continue. This possibility may not be too far in the future, in light of the recent successful transplantation of a rat gene for insulin into a bacterium, Escherichia coli (Ullrich et al. 1977). However, before this type of research may be done with the cyanobacteria, the genomes of these microorganisms must be mapped extensively.

Mapping the genomes of the cyanobacteria by recombination frequencies will require two and three factor crosses. Before these may be attempted, however, there must be phenotypic characters, or markers which will signal the recombinational event. Investigators into the genetics of the cyanobacteria therefore need a variety of mutants, including auxotrophs requiring nucleic acid precursors, vitamins and amino acids. Using these markers and employing techniques of transformation and of transduction developed while mapping the genomes of E. coli and other heterotrophic bacteria, it should be possible to map the cyanobacterial genome as well. Orkwiszewski and Kaney (1973) have already shown that transformation can occur in the cyanobacterium, Anacystis nidulans.

Attempts to isolate auxotrophic mutants have met with limited success. Some possible reasons for this difficulty include: 1) extensive duplication of relevant gene loci; 2) some metabolic characteristic which causes auxotrophy to be lethal in these organisms; 3) the relative impermeability of these organisms to amino acids, vitamins, purines and pyrimidines which would render auxotrophs non-supplementable (Kaney et al. 1972); and 4) because cyanobacteria are obligate photoautotrophs, any mutagenic agent that impairs the photosynthetic apparatus will limit survival and will thus reduce the chances of recovering a large number of induced mutants (van Baalen, 1971).

Most of the success to date in this search for mutants has been achieved using a unicellular species of cyanobacterium, Anacystis nidulans. Kaney (1973) has isolated a phenylalanine auxotroph derived from wild-type A. nidulans. Herdman and Carr (1971) have isolated mutants with nutritional requirements including acetate, cysteine, biotin and phenylalanine. Singer and Doolittle (1975) have isolated a leucine-requiring auxotroph.

All of the auxotrophs isolated thus far have been induced using N-methyl-N'-nitro-N-nitrosoguanidine (NTG) as the mutagenic agent. NTG induces in bacteria a high frequency of mutation at doses which result in little killing. NTG induces primarily base substitutions (transitions and transversions), but small deletions have been reported also. At least part of its mutagenic action stems from the generation of diazomethane in solution, an alkylating agent. Mutations are induced primarily at the replication fork, and multiple mutations are common. Used in the mutagenesis of heterotrophic bacteria like E. coli, a kill of 50%

is sufficient to induce high enough mutation rates to insure recovery of auxotrophs (Freese, 1971; Miller, 1972). When used in the mutagenesis of the cyanobacteria, however, investigators have found that it is necessary to reach killing levels of 99.9% to recover any induced mutants (Herdman et al. 1971).

This thesis reports efforts to isolate additional auxotrophic mutants of Anacystis nidulans requiring aspartate, glutamate, threonine and serine.

II. AS-1 is a cyanophage that infects two species of cyanobacteria, Anacystis nidulans and Synechococcus cedrorum. It was originally isolated and characterized by Safferman et al. in 1971. To date, there are no known lysogenic strains. The largest cyanophage so far described, AS-1 has a polyhedral head with a diameter of 90 nm and a tail:head ratio of about 3 to 1 (Safferman, 1972). It exhibits first-order adsorption kinetics, with about 85% adsorbed to the cyanobacterial host in one hour (Safferman, 1972). There is a latent period of 8½ hours, followed by a rise period of 7½ hours with a yield of about 50 cyanophage per infected cell. Photosynthesis must be maintained by the host for viral replication (Allen et al. 1976).

One of the host species, Anacystis nidulans, is one of the better characterized cyanobacteria in terms of both physiology and genetics, although even for this species information is by no means abundant. Thus, it would be logical to concentrate initial cyanophage genetic research on AS-1, since knowledge obtained through this endeavor may complement information about the host, and vice-versa. This thesis also reports the isolation of temperature-sensitive mutants of cyanophage

AS-1, and their use in construction of a preliminary genetic map of the cyanophage.

MATERIALS AND METHODS

I. The parent strain used in the search for auxotrophic mutants was Anacystis nidulans, IU 625, kindly provided by Dr. Robert Safferman of the Environmental Protection Agency, Cincinnati, Ohio.

Growth conditions: Cultures were grown at room temperature (25-30 C), in modified Chu No. 10 medium (Cannon et al. 1971), on a shaker (New Brunswick Scientific Company). Illumination was provided by cool-white fluorescent lamps at an average intensity of 150 foot-candles.

Mutagenesis: Mutagenesis was carried out using N-methyl-N'-nitro-N-nitrosoguanidine (NTG) (Sigma). A kill curve was determined using a constant concentration of NTG and varying time (Miller, 1972) to indicate when a 99.9% kill of wild-type cells was achieved, since this amount of kill has proven most successful for mutation of the cyanobacteria (Herdman et al. 1971). Table 1 (see Appendix) shows that 99.9% kill occurred at 15 minutes using a final concentration of 100µg/ml NTG. Stock NTG, prepared just before use, was added at a final concentration of 100µg/ml to a 100ml exponentially growing culture of A. nidulans at a cell density of approximately 10^8 cells/ml (cell density was estimated by use of a Klett-Summerson photoelectric colorimeter). The culture was then incubated for 15 minutes as previously described. Cells were then centrifuged (Sorvall RC2-B, 5900 X G) and washed three times with Chu broth with final suspension in Chu broth to which the amino acids aspartate, glutamate, threonine and serine (Sigma) had been added to a final concentration of 100µM. These amino acids were chosen because

they have been shown to be assimilated by A. nidulans (Smith, 1971). The culture was then grown to 10^8 cells/ml, during which time it was centrifuged, washed and finally suspended in amino acid supplemented Chu medium every three days.

Penicillin enrichment: When a cell density of 10^8 cells/ml was again reached the culture was centrifuged and washed with Chu minimal (Chu without the amino acids) to remove amino acids and resuspended in unsupplemented Chu medium. Eight hours of incubation allowed for a reduction in the growth rate (and thus cell wall synthesis) of any induced auxotrophs. Penicillin G (Sigma), prepared just before use, was then added at a final concentration of 500 units/ml. Cultures were then incubated as previously described for 24 hours, which had been shown to cause a 99% kill of wild-type cells (Table 2, see Appendix). Following this penicillin treatment cells were centrifuged and washed to remove penicillin with final resuspension in amino acid supplemented Chu. Three cycles of penicillin enrichment were done, since Delaney et al. (1974) have reported increased recovery of auxotrophs using this technique.

Recovery of mutants: Possible mutants were recovered by diluting aliquots of the treated culture in supplemented Chu (pH 7.0), plating onto amino acid supplemented 1.5% Chu agar plates, transferring by sterile toothpick the resulting isolated colonies to a second set of supplemented Chu plates in an orderly arrangement, and replica-plating these colonies to Chu minimal. Absence of growth on the minimal plate by a transferred colony tentatively indicated auxotrophy. Suspected colonies were further tested in liquid and on solid media to validate the auxotrophic nature of the isolate. The pH of the plates used was

adjusted so that the initial pH was 8.0 and the final pH was 7.0 (Herdman et al. 1973).

II. Organisms and growth conditions: Wild-type AS-1 cyanophage was kindly provided by Dr. Robert Safferman of the Environmental Protection Agency, Cincinnati, Ohio. Host Anacystis nidulans, IU 625, was grown as described in part I of Materials and Methods. AS-1 was assayed using the soft-agar overlay technique (Safferman et al. 1964). Non-permissive growth conditions (40 C) were maintained using a BOD incubator (Precision Scientific, model 805) with illumination provided by cool-white fluorescent lamps at an average intensity of 175 foot-candles.

Mutagenesis: Five ml of a 1 week old culture of A. nidulans in logarithmic growth phase and at a cell density of 10^7 cells/ml (Klett-Summerson photoelectric colorimeter) were infected by wild-type AS-1 at a multiplicity of infection (m.o.i.) of approximately 1. After 30 minutes of adsorption at room temperature (25-30 C), the culture was membrane filtered (0.45μm, Millipore Corp.), washed with Chu broth to remove unadsorbed virus, and diluted into 50 ml of Chu broth to which NTG (Sigma) had been added at a final concentration of 100μg/ml. After 7 and 8 hours of growth in the light, 1 ml aliquots were removed and diluted 100-fold into Chu broth. Lysis was completed by shaking the resulting 100 ml samples for two minutes with 10 ml of chloroform.

Isolation of mutants: The mutagenized stock of AS-1 was plated on a lawn of A. nidulans and incubated for 48 hours to obtain well isolated plaques. Individual plaques were then picked with sterile toothpicks onto two plates of A. nidulans lawns, and incubated at 40 C and room temperature (25-30 C), respectively. Plaques that did not show lytic

activity at 40 C, but did at room temperature were then picked and inoculated into 10 ml cultures of A. nidulans at room temperature. After this culture lysed, stocks were prepared both by shaking with 2 ml chloroform and by centrifugation (Sorvall RC2-B) followed by membrane filtration (0.22μm, Millipore Corp.). The resulting lysates were then assayed at room temperature and at 40 C.

Complementation studies: To determine whether the isolated mutants were indeed defective for different gene products, complementation tests were performed. Drops of two different stock cyanophages at approximately 10^6-10^7 plaque-forming units/milliliter (PFU/ml) were placed on a lawn of A. nidulans and then immediately put into the 40 C BOD incubator. After 48 hours of incubation, lytic activity indicated complementation. Controls included single-phage drops and wild-type AS-1 incubated at room temperature and at 40 C.

Two-factor crosses: Ten ml cultures of A. nidulans were infected with two complementing temperature-sensitive mutants at an m.o.i. of 10 for each cyanophage, and incubated at room temperature for 7½ hours. After shaking with 2 ml of chloroform, the resulting lysate was assayed at room temperature for total cyanophage yield and at 40 C for indication of wild-type recombinants. The resulting recombination frequencies were used to construct a preliminary genetic map of cyanophage AS-1.

RESULTS

I. Eighteen hundred colonies from a culture of Anacystis nidulans treated as described in Materials and Methods I were picked and tested for auxotrophy using the replica plating technique. Of these, 35 exhibited auxotrophic behavior upon initial transfer to minimal medium during replica plating. Each of these was then streaked onto supplemented medium and after sufficient growth, an isolated colony picked and streaked onto minimal medium. Three of these suspects showed no growth on this second minimal plate. An isolated colony was then again picked from the supplemented medium and streaked onto a minimal plate. Some growth was observed on these plates for each of the suspected auxotrophs, indicating prototrophy or a return to prototrophy. An isolated colony of each suspect was then picked and inoculated into 4 ml of both supplemented Chu broth and minimal Chu broth. Growth in both supplemented and minimal was observed for all but one of the suspects. Work is continuing toward positive determination of the auxotrophic nature, (or lack of) for this isolate. In view of the observed growth on solid minimal Chu medium, it is doubtful that this isolate will prove to be auxotrophic.

II. Thirty-two possible temperature-sensitive mutants of cyanophage AS-1, as determined by the plaque-transfer to room temperature and 40 C lawn of A. nidulans, were isolated. Each was subsequently titered at RT and 40 C and 20 were found to be truly temperature-sensitive (Table 3, see Appendix). Complementation studies revealed 6 complementation groups (Table 4, see Appendix). Two-factor crosses were performed, and the

resulting recombination frequencies are seen in Table 5 (see Appendix). These frequencies were used to construct the preliminary genetic map of AS-1 as seen in Figure 1 (see Appendix).

DISCUSSION

I. This failure to isolate threonine, serine, aspartate, and glutamate-requiring auxotrophs of A. nidulans remains unexplained. Using similar methods, Herdman and Carr (1971) reported a maximum induced mutation frequency of 3.4×10^{-2} for A. nidulans. Isolation of acetate-requiring auxotrophs was reported by these investigators at a frequency of 5×10^{-3}, and biotin, phenylalanine and methionine-requiring auxotrophs were isolated at a frequency of approximately 5×10^{-4} following a single cycle of penicillin enrichment. Delaney et al. (1974) reported that auxotrophs of A. nidulans could be recovered at rates of 10^{-3} to 10^{-4} following several cycles of penicillin enrichment. It follows then that screening of 1000 to 10,000 colonies from a properly treated culture of A. nidulans should yield positive results.

Kaney (1973) has reported a spontaneous reversion rate for a phenylalanine-requiring auxotroph of A. nidulans of 3.0×10^{-8}. This rate is not high enough to explain the apparent return to prototrophy observed in the isolates suspected initially of auxotrophy.

Other investigators have noted the difficulty encountered in recovering induced auxotrophs of A. nidulans and other cyanobacteria (Herdman et al. 1971; Li et al. 1967; Kaney et al. 1972). Whatever the reason for this resistance to mutation, it may perhaps be understood in terms of evolution and the natural selective pressures acting upon these organisms which have existed since the Precambrian (Brock, 1973). It must be remembered that most species of cyanobacteria, including A. nidulans,

are obligate photoautotrophs. Their distribution within the biosphere places them in habitats which contain very few organic nutrients. Many heterotrophs, like Escherichia coli, are found in habitats which abound in these same organic nutrients (amino acids, vitamins, etc.). If a spontaneous mutation occurs in the genome of a heterotroph which causes the organism to become auxotrophic for one of these nutrients, the selective pressure which might eliminate this new strain is not as great, since the nutrients are readily available from the surrounding environment. The selective pressure is not as great then to conserve intact those genes which code for the enzymes necessary for normal metabolism of the nutrient.

In the case of an obligate photoautotroph such as A. nidulans however, whose normal habitat contains scarce quantities of these same nutrients, there is selective pressure toward conservation of these loci, since inability to obtain the nutrients from the environment would render mutations in these genes lethal. Thus evolution toward a genome which somehow conserves these loci is favored, whether the mechanism be more efficient repair mechanisms, multiple copies of the loci, etc..

Thus, it is interesting to note that all success to date in isolating auxotrophs of A. nidulans has been achieved using NTG as the mutagenic agent. It is known that NTG produces multiple mutations which most often occur in clusters (Freese, 1971). If there are in fact multiple copies of pertinent loci in A. nidulans, they would most likely be close to one another, having arisen by duplication. In order to induce an auxotrophic mutant then, the mutagenic agent would have to cause mutation in all copies of the locus. Since it would therefore require a multiple-hit

action by the mutagen, the frequency of recovery of auxotrophs would be correspondingly low. Perhaps this is precisely what NTG has done in the few cases of induced auxotrophs, and it may only require more time before more auxotrophs are isolated.

Colonies from the treated cultures of A. nidulans are being further screened, and it is hoped that as the total number of tested colonies increases, a new auxotroph will yet be found.

II. Given the relative ease with which temperature-sensitive mutants of AS-1 were isolated (as determined by the initial test of isolated plaques at room temperature and 40 C), it came as somewhat of a disappointment when no complementation was detected between this first group of isolates. Assuming that the lack of complementation meant that all the isolates were in fact copies of the same temperature-sensitive mutant, and must therefore be sister progeny, the mutagenesis time was adjusted from 8 hours to 7 hours, which is well within the latent period of the cyanophage (8½ hours). After this adjustment had been made, several complementing temperature-sensitive mutants were isolated. Recombination data has been compiled for only three of these loci. Further mutagenesis has yielded an additional 20 temperature-sensitive phages. Complementation studies are currently being performed with these to determine if they delineate new loci. Additional recombinational data from the three remaining mutants, and those which will hopefully be yielded from the complementation studies with the 20 new isolates, should render more complete the map of AS-1 presented in Figure 1.

It is important to note that these temperature-sensitive mutants are not inactivated by exposure to the restrictive temperature. Lysates

exposed to 40 C in the BOD incubator retain their infectivity at 26 C with no significant drop in cyanophage titer. This means that it is the cyanophage's "functional potential", and not structural integrity which is affected by the mutation.

Temperature-sensitive mutations extend to most, if not all genes. The ability to isolate mutations affecting essential functions allows these functions to be analysed, by infecting the host at the restrictive temperature and observing, by EM studies, what synthetic or morphogenetic step is blocked (Hayes, 1968). These mutations will permit investigations into the physiological aspects of the host-parasite relationship between AS-1 and its hosts.

For example, unlike the LPP-1 Plectonema boryanum infection, in which illumination is necessary only during the eclipse period for full maturation and normal yield of cyanophage particles, AS-1 development requires illumination during the full 16 hour lytic cycle (Allen et al. 1976). AS-1 development is thus more dependent upon the photosynthetic apparatus of the host. This has been substantiated by EM studies of the AS-1-Anacystis infection in which it was observed that the photosynthetic lamallae were not altered morphologically until just before lysis, in contrast with the LPP-1-Plectonema infection in which invagination of the lamallae occurs early in the infection (Pearson et al. 1975).

Using these temperature-sensitive mutants then, it might be possible to determine what triggers the abandonment of dependence upon the photosynthetic apparatus, or when this occurs, by infecting the host with a temperature-sensitive mutant for which the affected product has been identified, and observing whether or not invagination of the lamallae occurs at the non-permissive temperature during infection.

Investigations of this design will first require that the products affected by the mutations be identified. It is hoped that work may be soon begun toward this end.

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APPENDIX A: Tables

TABLE 1

Survival of Wild-Type *Anacystis nidulans*

During Exposure to NTG, 100μg/ml

Time (min.)	Viable cells/ml	% Survival
0	4×10^6	100.00
15	6×10^3	0.15
30	1.3×10^3	0.033
60	1.5×10^3	0.037
120	1.1×10^3	0.027

TABLE 2

Survival of Wild-Type Anacystis nidulans

During Exposure to Penicillin G. 500 units/ml

Time (hrs.)	Viable cells/ml	% Survival
0	1.5×10^5	100
1	1.5×10^5	100
2	8.0×10^4	53
3	1.0×10^5	67
4	6.0×10^4	40
5	2.9×10^4	20
6	7.0×10^4	45
12	5.0×10^4	33
24	1.7×10^3	1.1

TABLE 3

Titer of AS-1 Temperature-Sensitive Mutants

Mutant No.	Phage titer (PFU/ml) at: RT*	40 C
AS-1 wt	10^7	10^7
TS-1	10^7	0
TS-2	10^7	0
TS-3	10^7	0
TS-4	10^6	0
TS-5	10^6	10^4
TS-8	10^6	0
TS-9	10^6	0
TS-11	10^7	0
TS-12	10^7	10^5
TS-13	10^6	10^5

TABLE 3 (continued)

Mutant No.	Phage titer (PFU/ml) at: RT*	40 C
TS-15	10^5	0
TS-17	10^6	0
TS-22	10^6	0
TS-25	10^7	0
TS-28	10^8	0
TS-29	10^7	0
TS-30	10^7	10^4
TS-31	10^7	0
TS-32	10^7	0
TS-36	10^7	10^2

*Room Temperature

TABLE 4

Complementation Test Results

TS	1	2	3	4	5	8	9	11	12	13	15	17	22	25	28	29	30	31	32	36
1	-	-	-	-	-	-	-	+	+	-	-	-	+	+	-	-	+	-	-	-
2			-	-	-	-	-	+	+	-	-	-	+	+	-	-	+	-	-	-
3				-	-	-	-	+	+	-	-	-	+	+	-	-	+	-	-	-
4																				
5																				
8																				
9																				
11									+	-	-	-	+	+	-	-	+	-	-	-
12													+	+	-	-	+	-	-	-
13																				
15																				
17																				
22														+	-	-	+	-	-	-
25																-	+	-	-	-
28																				

TABLE 4 (continued)

TS	1	2	3	4	5	8	9	11	12	13	15	17	22	25	28	29	30	31	32	36
29																				
30																				
31																				
32																				
36																				

\+ = Complementation was positive using test described in Materials and Methods II
\- = Complementation was negative using test described in Materials and Methods II
blank = Test was not performed

TABLE 5

Results of Two-Factor Crosses Between TS Mutants

Cross	RT*	40 C	% Recombination
1 + 22	7.8×10^5	1.48×10^4	1.9
1 + 25	1.94×10^5	9.8×10^3	5.0
22 + 25	9.9×10^5	2.45×10^4	2.5

*Room Temperature

APPENDIX B: Figure

FIGURE 1

Preliminary Genetic Map of Cyanophage AS-1