The Isolation and Characterization of Mutant Strains of the Blue-green Alga *Anacystis nidulans*

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(Accepted for publication 13 October 1971)

SUMMARY

Procedures for mutagenesis and conditions for selection by penicillin enrichment in the blue-green alga *Anacystis nidulans* have been developed. The characterization of 19 mutant strains involving single or serially induced multiple markers was achieved. In addition to drug-resistant and morphological mutants auxotrophic strains requiring phenylalanine, methionine, biotin or acetate together with strains deficient in sulphate and nitrate reduction have been isolated.

INTRODUCTION

Previous attempts to isolate mutants of blue-green algae have had only limited success, and in particular auxotrophic mutants have proved difficult to obtain. Although several species of blue-green algae are significantly more resistant to γ -radiation than are bacteria (Kraus, 1966) other mutagens, ultraviolet light (u.v.) and *N*-methyl-*N'*-nitro-*N*-nitroso-guanidine (NTG) are comparably lethal to species from both groups of procaryotic organisms. Mutations affecting u.v. and drug resistance have proved the most readily recovered, and several such mutants of the unicellular blue-green alga *Anacystis nidulans* have been reported although not always as clonally pure strains (Kumar, 1962, 1963, 1965; Singh, Singh & Sinha, 1966; Pikalek, 1967; Bazin, 1968; Asato & Folsome, 1969; Shestakov & Khyen, 1970). Morphological and pigment alterations have been shown in mutants of *Anabaena cycadeae* (Singh & Singh, 1964*a*), *Anabaena doliolum* (Singh & Singh, 1964*b*), *Nostoc linchia* (Singh & Tiwari, 1969), *Plectonema boryanum* (Padan & Shilo, 1969), *Agmenellum quadruplicatum* (Ingram & Van Baalen, 1970) as well as in the more studied *Anacystis nidulans* (Van Baalen, 1965; Kunisawa & Cohen-Bazire, 1970). The best characterized auxotrophic mutants of blue-green algae are the nitrate-reductase and nitrite-reductase mutants of *Agmenellum quadruplicatum* described by Stevens & Van Baalen (1970); strains of *Anacystis nidulans* lacking nitrate reductase have also been isolated (Van Baalen, 1965). The requirement for a non-specific reduced carbon source has been reported in a mutant of *Phormidium mucicola* (Srivastava, 1969) and in *Anacystis doliolum* (Singh & Singh, 1964*b*). This communication describes the development of conditions for mutation and penicillin enrichment which have led to the isolation of 19 mutant strains of *Anacystis nidulans* which carry single or multiple mutations affecting morphological, drug resistance and nutritional characters.

METHODS

Organism. The parent strain for all the mutants isolated was *Anacystis nidulans*, no. 625 of the Culture Collection of Algae at Indiana University, Department of Botany, Bloomington, Indiana, U.S.A.

Culture media and conditions. *Anacystis nidulans* was grown in liquid culture and on agar plates as previously described (Herdman, Faulkner & Carr, 1970).

Supplemented media. For the isolation and growth of mutants Allen's media was supplemented with the necessary growth factors at the concentrations listed below: inorganic salts, NH_4Cl , 3 mM; $\text{Na}_2\text{S}_2\text{O}_3$ and NaNO_2 , 1 mM; carbon sources, Na acetate, 2 mM; malic acid and formic acid, 0.5 mM; shikimate, 0.1 mM; vitamins, biotin, thiamine, B_{12} , nicotinic acid, 5 μM ; nucleotides, thymine, thymidine, uracil, 0.5 mM; amino acids, arginine, aspartate, cysteine, glutamate, isoleucine, leucine, methionine, proline, serine, threonine, valine, 1.0 mM; alanine, phenylalanine, 0.1 mM. Tyrosine at 2 mM was used for development of resistant strains; antibiotics, streptomycin 25 $\mu\text{g}/\mu\text{l}$; polymixin-B 100 $\mu\text{g}/\text{ml}$.

Incorporation of [^{14}C]-labelled materials. [$2\text{-}^{14}\text{C}$]acetate (25 μCi , 55 $\mu\text{Ci}/\text{mmol}$) was added to an exponentially growing culture (100 ml) of the acetate requiring mutant 1163 growing in acetate supplemented medium and samples (5 ml) were removed at intervals up to 8 h. Organisms were immediately collected on a membrane filter and washed ($8\times$) with small volumes of medium. After drying, the filters were glued to an aluminium planchette and isotope incorporation estimated in a Nuclear Chicago low background gas-flow counter at an efficiency of 30% and to an accuracy of $\pm 3\%$. The same procedure was used for measurement of [methyl- ^{14}C]methionine incorporation into nitrate-reductase deficient, methionine-requiring mutant 2112.

Optimal conditions for mutagenesis. Optimal exposure times to ultraviolet light (u.v.) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) were found by exposing two mutants, 1111 (deficient in nitrate reductase) and 1121 (deficient in sulphate reduction), to the mutagens and observing the dose which gave maximum reversion to wild-type.

Ultraviolet light. Cultures of the mutants were concentrated to 3×10^9 cells/ml by centrifugation and resuspended in fresh growth medium. The suspensions were irradiated at 254 nm under a Camag Universal u.v. lamp (Camlab, Cambridge). Samples (1 ml) were removed at intervals and plated directly on minimal medium to score revertants, and also diluted and plated on supplemented medium to estimate viable count.

Nitrosoguanidine. A 4 ml suspension containing 3×10^9 cells/ml was added to 16 ml growth medium containing NTG (Aldrich Chemical Co. Inc., Milwaukee, Wisconsin, U.S.A.) to a final concentration of 25 $\mu\text{g}/\text{ml}$ at pH 8.0. Samples (1 ml) were removed at intervals, filtered through a sterile Millipore filter, washed, resuspended in sterile-distilled water, and plated as described above.

Optimal conditions for penicillin enrichment. The penicillin enrichment technique was applied to the isolation of mutants of *Anacystis nidulans*. A mixture of wild-type and strain 1111 or wild-type and strain 1121 cells in the ratio wild-type: mutant (3:1) was grown from 5×10^7 to 6×10^8 cells/ml in medium containing NH_4Cl or $\text{Na}_2\text{S}_2\text{O}_3$ respectively. The culture was centrifuged, washed and resuspended in minimal medium at 1×10^7 cells/ml and then allowed to grow for 4 h to permit cessation of growth of mutant cells. Under these conditions wild-type cells divided twice in this period, and the final cell count was 3×10^7 cells/ml. After 4 h growth penicillin (60 $\mu\text{g}/\text{ml}$) was added to the culture. Samples (1 ml) were removed at intervals, filtered, washed to remove traces of penicillin, resuspended in sterile-distilled water and plated on supplemented medium to score the total viable counts. Colonies were then replica plated on to minimal and supplemented medium to score the numbers of wild-type and mutant cells in the sample.

Isolation of auxotrophic mutants. A 2 ml sample of an exponentially growing culture (5×10^8 cells/ml) was added to 8 ml of growth medium containing NTG at a final concentration of 25 $\mu\text{g}/\text{ml}$, and incubated for 60 min. The sample was filtered through a Millipore

filter, washed, and resuspended in 100 ml of supplemented medium. This culture was grown to 5×10^8 cells/ml and a 10 ml sample was filtered, resuspended in minimal medium (100 ml) and exposed to 60 $\mu\text{g/ml}$ penicillin for 2 to 2½ h as described above. Penicillin was removed by filtration and washing with 30 ml water for a 10 ml sample and the cells resuspended in 100 ml supplemented medium and allowed to grow to a density of 3×10^8 to 8×10^8 cells/ml. The culture was diluted and plated on supplemented agar plates as previously described to give 200 to 350 colonies/plate. The colonies were replica plated on to minimal and supplemented agar using filter paper. Colonies which were identified as mutants were picked off, streaked on to supplemented agar, and their growth requirements identified on agar and in liquid medium. This procedure permitted 10,000 to 20,000 colonies to be screened in each experiment.

Isolation of resistance mutants. Cultures were exposed to NTG as described above, and subsequently grown to approximately 8×10^8 cells/ml in the absence of the compound to which resistance was being sought. The culture was then plated directly on to agar containing the compound, and resistant colonies isolated.

Nomenclature of mutants. The proposals of Demerec, Adelberg, Clark & Hartman (1968) have been followed.

RESULTS

Penicillin enrichment. Wild-type and mutant (1111 and 1121) cells exhibited differential survival during exposure to penicillin in minimal medium (Fig. 1). The viable count of strains 1111 and 1121 decreased to 60 and 80 % respectively of the initial values after 2 h exposure to penicillin. The viable count of wild-type cells decreased more rapidly, and after 2 h exposure only 0.5 % of wild-type cells were viable. The viable count of wild-type cells decreased further to 0.15 % after 3 h exposure to penicillin; after this time no further reduction in viability was observed.

Thus exposure to penicillin equivalent to one doubling time (2 h) killed 99.5 % of wild-type cells but only 20 to 40 % of mutant cells. This differential survival represents a 100-fold enrichment of mutant cells.

Ultraviolet mutagenesis. The survival of strains 1121 (Fig. 2) and 1111 (Fig. 3) to u.v. differed, in that the culture of strain 1111 appeared to contain a proportion of u.v.-resistant cells which were not observed in cultures of strain 1121. Maximum reversion frequencies, however, occurred at similar doses in both mutants, i.e. at 3 % survival of 1121 and 1 % survival of 1111. The number of mutants increased rapidly with increasing exposure to u.v. up to this point, and then decreased at a rate parallel to the inactivation of the culture. The maximum reversion frequencies were 2.3×10^{-5} for 1121 and 1.8×10^{-5} for 1111.

NTG mutagenesis. The response of strain 1111 to NTG (25 $\mu\text{g/ml}$) is shown in Fig. 4: the viable count decreased with increasing exposure. The number of mutants increased rapidly with time, until a maximum was attained after 60 min exposure. At this time, 0.4 % of the population were viable and 3.52 % of these survivors were revertants to wild-type. Of these revertants 3.41 % (0.12 % of the total survivors) had acquired filamentous form (Fil).

Characterization of mutants. The mutants isolated by the methods described above are shown in Table 1. Nitrate reductase-deficient mutants were isolated at high frequencies (approximately 1×10^{-2}) after penicillin treatment, but were found at lower frequency (1×10^{-4}) without enrichment. Acetate-requiring mutants were observed with frequencies of 1×10^{-5} in the absence of enrichment and 5×10^{-3} following enrichment. Biotin, phenylalanine and methionine-requiring strains were recovered with frequencies of approximately

Fig. 1. The survival of wild-type *Anacystis nidulans* (□—□), and mutants 1121 (○—○) and 1111 (●—●) during exposure to penicillin, 60 μ g/ml.

Fig. 2

Fig. 3

Fig. 2. Survival of strain 1121 (○—○) and reversion to wild type (●—●) during u.v. irradiation.

Fig. 3. Survival of strain 1111 (○—○) and reversion to wild-type (●—●) during u.v. irradiation.

Fig. 4. The survival (O—O) of strain 1111 to NTG and mutation to wild-type (*Nit*⁺) (●—●) and *Nit*⁺ *Fil* mutants (□—□).

5×10^{-4} following enrichment, and were not isolated in experiments where enrichment was not applied. NTG-induced mutants resistant to streptomycin, polymixin-B or tyrosine arose at low frequencies in the range 10^{-5} to 10^{-7} . Filamentous mutants were found at high frequencies (10^{-2} to 10^{-3}) even though penicillin enrichment could not be applied to mutants of this type.

The growth rates of the mutants isolated were similar ($\pm 10\%$) to that observed in wild-type *Anacystis nidulans*, i.e. 0.11 doublings/h. As far as they were tested the auxotrophic strains described had a specific growth requirement; for example 1163 (Ace) did not grow on malate, citrate, fumarate, glutamate, glycine, glucose or glyoxylic acid, and the requirement for methionine of strain 2112 (*Nit* Met) could not be replaced by $\text{Na}_2\text{S}_2\text{O}_3$, cysteine, homocysteine, choline, formate, acetate, pyruvate or vitamin B_{12} . For auxotrophic mutants the accumulation of ^{14}C -labelled nutrient was followed during growth. Under the conditions employed the acetate requiring strain 1163 incorporated $[2\text{-}^{14}\text{C}]\text{acetate}$ at a linear rate twice that of the wild-type; after 7 h growth incorporation ceased. The assimilation of $[\text{methyl-}^{14}\text{C}]\text{methionine}$ by the methionine requiring strain 2112 was faster than the wild-type, but after 2 h entry into both cultures continued at a reduced, similar, rate up to a period of at least 8 h.

DISCUSSION

The results demonstrate that u.v. is a relatively inefficient mutagen for *Anacystis nidulans*, giving a maximum crude mutation frequency of 2.3×10^{-5} for the two genes tested. NTG, however, was 1000-fold more effective in mutagenesis, giving a maximum mutation frequency of 3.4×10^{-2} and producing double mutants with a frequency of up to 1.2×10^{-3} . The maximum mutation frequency resulting from exposure to NTG occurred at a dose where 0.4 % of the culture was viable. This is much less than in bacteria, in which maximum mutation frequencies occur at doses which permit 50 % survival of the treated culture (see Adelberg, Mandel & Chen, 1965).

All the mutants reported in this paper were isolated after exposure to NTG at pH 8. Preliminary experiments showed no difference in the mutagenic action of NTG in growth medium (pH 8) or in phosphate buffer at pH 5. At pH 8, diazomethane, a decomposition

Table 1. *Mutants isolated in Anacystis nidulans*

Auxotrophic, drug resistance and morphological mutants of *Anacystis nidulans* isolated following treatment with NTG. All mutants isolated were stable, except the three acetate mutants. Strain 1161 had a very high spontaneous reversion frequency; when colonies were replicated from acetate to minimal medium each colony developed between 1–5 revertant sectors. Strains 1162 and 1163 had less extreme, but still high, reversion frequencies, 1 in 10^4 and 2 in 10^6 cells respectively. Mutants requiring the following growth factors were not found, after screening 1×10^4 to 3×10^4 cells for each one: alanine, arginine, aspartate, glutamate, leucine, iso-leucine, proline, serine, shikimate, threonine, valine, thymine, thymidine, uracil, fumarate, malate, thiamine, vitamin B₁₂, nicotinic acid.

Strain	Mutant	Derivation	Growth requirements	Comments
1111	nit-1	Wild-type	NH ₄ Cl or NaNO ₂	Nitrate reductase deficient
1121	cys-1	Wild-type	Cysteine or Na ₂ S ₂ O ₃	Deficient in sulphate reduction
1141	bio-1	Wild-type	Biotin	—
1142	bio-2	Wild-type	Biotin	—
1161	ace-1	Wild-type	Acetate	—
1162	ace-2	Wild-type	Acetate	—
1163	ace-3	Wild-type	Acetate	Growth in acetate only
1151	phe	Wild-type	Phenylalanine	—
2112	nit-1 met-1	1111	NH ₄ Cl or NaNO ₂ + methionine	—
2113	nit-1 met-2	1111	As 2122	—
2111	nit-1 str-1	1111	NH ₄ Cl or NaNO ₂	Resistant to streptomycin 25 µg/ml
2121	cys-1 str-3	1121	Cysteine or Na ₂ S ₂ O ₃	Streptomycin resistant
2141	bio-1 str-2	1141	Biotin	Streptomycin resistant
3141	bio-1 nit-2 str-2	2141	Biotin + NH ₄ Cl or NaNO ₂	—
3113	nit-1 met-2 pmx	2113	NH ₄ Cl or NaNO ₂ + methionine	Resistant to polymyxin-B 100 µg/ml
1141	tyr	Wild-type	—	Resistant to tyrosine
1101	fil-1	Wild-type	—	Filamentous, class II
1102	fil-2	Wild-type	—	Filamentous, class I
3111	fil-4 nit-1 str-1	2111	NH ₄ Cl or NaNO ₂	Filamentous class I; streptomycin resistant

product of NTG, is thought to be the principle agent involved in mutagenesis (Cerdeña-Olmedo & Hanawalt, 1968). The penicillin enrichment technique proved valuable in the isolation of auxotrophic mutants, since the frequency of isolation of such mutants was increased by approximately 20 to 100-fold by this technique. Thus Nit and Ace mutant strains were isolated at frequencies of 1×10^{-2} and 5×10^{-3} respectively following enrichment of the cultures, but at the lower frequencies of 1×10^{-4} and 1×10^{-5} respectively in the absence of enrichment. The degree of enrichment illustrated by these values was in general agreement with the results obtained during the development of the technique. The nitrate reductase deficient mutants appear similar to that described by Van Baalen (1965) and the filamentous mutants (Fil) possessed morphological changes similar to those described by Kunisawa & Cohen-Bazire (1970), in that 1101 was a long single filament (analogous to class II of Kunisawa & Cohen-Bazire) and 1102 possessed cells of variable length up to tenfold that of the wild-type (Kunisawa & Cohen-Bazire, class I).

The relative difficulty in isolation of auxotrophic and other mutant phenotypes of blue-green algae has been noted by other workers but it is evident that *Anacystis nidulans* is sensitive to NTG to an extent comparable to other procaryotes. Although some phenotypes (e.g. Nit, Fil) were isolated at relatively high frequencies, others were not found after extensive screening. The reason for the isolation of only a narrow spectrum of mutants of *A. nidulans* is not known, but may be related to the autotrophic metabolism of the organism since the same difficulties are encountered in the isolation of mutants of autotrophic, eucaryotic algae (Li, Redei & Gowans, 1967).

The use of the techniques described in this paper has led to the isolation of auxotrophic mutants which have not previously been reported in blue-green algae, namely those requiring cysteine (thiosulphate), biotin, methionine, phenylalanine and acetate, and the tyrosine-resistant mutant.

The authors are indebted to Dr B. M. Faulkner for valuable advice and M. H. acknowledges financial support from the Science Research Council.

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