Conditions for Mutagenesis of the Nitrogen-fixing Cyanobacterium *Anabaena variabilis*

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Chemically induced mutation in the cyanobacterium *Anabaena variabilis* was studied using resistance to the pyrimidine analogue 5'-fluorocytosine as a genetic marker which can be selected positively. Cytosine is metabolized through uracil and the UMP pyrophosphorylase 'salvage' pathway in this photoautotroph, as it is in enteric bacteria. Treatment with various concentrations of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) gave the highest frequencies of 5FC-resistant mutants when lethality approximated 99%, irrespective of the exposure time or mutagen concentration. The pH of the incubation medium strongly influenced mutation; exposure to MNNG at pH 6.0 yielded 13-fold higher frequencies of mutants than at pH 7.5. The greatest frequency of resistant cells was found after cultures had undergone six or more doublings following mutagenesis. The mutation frequencies obtained by treatment with MNNG were approximately 4- and 25-fold higher than those after exposure under empirically defined conditions to diethyl sulphate or nitrous acid, respectively, and 1.4×10^3 -fold higher than the frequency of spontaneous mutation. Neither chloramphenicol-inhibited nor caffeine-sensitive systems capable of repairing MNNG-induced DNA damage were observed.

INTRODUCTION

Progress in the area of physiological genetics of the cyanobacteria has been lacking, in part due to difficulties in isolating specific mutants (Herdman, 1982) for subsequent genetic analysis by transformation (Buzby *et al.*, 1983) or conjugation (Wolk *et al.*, 1984). Studies on the optimal conditions for the generation of mutants have been undertaken for the unicellular cyanobacteria *Anacystis nidulans* (syn. *Synechococcus leopoliensis*, Rippka *et al.*, 1979) (Herdman & Carr, 1972; Herdman *et al.*, 1980) and *Aphanocapsa* sp. strain 6714 (Astier *et al.*, 1979), as well as the filamentous cyanobacterium *Plectonema boryanum* (Singh & Kashyap, 1977). Although auxotrophic (Currier *et al.*, 1977), photosynthetic (Shaffer *et al.*, 1978) and analogue-resistant (Chapman & Meeks, 1983) mutants of a heterocyst-forming cyanobacterium have been reported, there have been no systematic studies of mutagenesis in these organisms.

The heterocyst-forming cyanobacteria present an experimental system useful for studying the processes of cellular differentiation, N₂ fixation, and, in some strains, symbiotic association. We chose to examine parameters of mutagenesis using *Anabaena variabilis* because it has a high plating efficiency (Wolk & Wojciuch, 1973) and a limited range of mutants have been isolated (Currier *et al.*, 1977). Herein we define optimal conditions for mutagenesis of *A. variabilis* using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) as the mutagen and a positive selection for resistance to the analogue 5'-fluorocytosine (5FC) as the indicator of mutagenic effectiveness. The mutagenic activities of diethyl sulphate (DES) and nitrous acid were also compared under empirically defined conditions.

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Abbreviations: DES, diethyl sulphate; 5FC, 5'-fluorocytosine; 5FU, 5'-fluorouracil; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

METHODS

Strains and culture conditions. *Anabaena variabilis* Kütz. (ATCC 29413) and its uracil-requiring mutant U2 were gifts from C. P. Wolk. The strains were cultured in the basal medium of Allen & Arnon (1955). The basal medium was used at full strength when solidified with 1% (w/v) purified agar or diluted eightfold for growth in liquid culture. Agar was purified by the method of Braun & Wood (1962). The medium for mutant U2 was supplemented with 0.04 mM-uracil. The cultures were incubated at 30 °C under light (8.4 W m⁻²) from cool-white fluorescent lamps; liquid cultures were placed on an orbital shaker at 100 r.p.m. The cultures were routinely checked for contamination microscopically and by plating onto nutrient agar. Cell numbers were determined microscopically using a haemocytometer, and viable c.f.u. were determined by serial dilution and plating.

Mutagenesis. Since a c.f.u. of a filamentous organism is equivalent to the total number of cells in a filament rather than a single cell, cultures of *A. variabilis* in the exponential phase of growth (10⁶–10⁷ cells ml⁻¹ in 50 ml volumes) were fragmented by cavitation in a sonic cleaning bath to reduce the filaments to an average length of two cells (Wolk & Wojciuch, 1973). This procedure provides a more accurate determination of the viable cell number. However, the plating efficiency of fragmented cultures decreased from 99.8% on medium supplemented with ammonium to 72% in the absence of combined nitrogen, apparently because some single-celled filaments exhausted their internal supply of nitrogen before they reached the critical length required for heterocyst differentiation (Wilcox *et al.*, 1975). For reasons of quantification, the basal medium was always supplemented with 2.5 mM-NH₄Cl and buffered with 5 mM-TES or 5 mM-MOPS (Sigma), both adjusted to pH 7.5 with NaOH.

The fragmented cultures were washed twice with basal medium and suspended at a concentration of 10⁶ to 2 × 10⁷ cells ml⁻¹ in a 20 ml volume for exposure to the mutagen. The mutagen was removed by three centrifugal washes (1000 g, 5 min) with basal medium and the treated cultures were suspended in buffered ammonium basal medium.

Stock solutions of MNNG at 10 mg ml⁻¹ were made in glass-distilled water by heating to 45 °C followed by mild cavitation in a sonic cleaning bath to complete solubilization. The stock solution was filter sterilized and 2.0 ml samples were frozen until use and then never refrozen. Various exposure times (5 min to 6 h) and MNNG concentrations (0.025–1.0 mg per ml of cell suspension) were examined. In experiments to determine the pH optimum for MNNG mutagenesis, the fragmented cultures were suspended in either 10 mM-citrate buffer pH 5.0, 5.5 or 6.0, or 10 mM-TES buffer pH 6.5, 7.0 or 7.5.

DES (Sigma) was used as supplied, generally at 5 µl per ml of cell suspension in 20 ml buffered ammonium basal medium, pH 7.5. Various exposure times up to 30 min and concentrations from 2.5 to 30 µl per ml of cell suspension were also examined.

For nitrous acid treatment, fragmented cultures were suspended in 13 ml 0.6 M-acetate buffer, pH 4.5, to which an equal volume of 0.05 M-sodium nitrite was added. At various times, treatment was terminated by adding 5.0 ml samples to 37 ml of 0.067 M-phosphate buffer pH 8.0.

The survivors were screened for resistance to 0.3 mM-5FC or 5'-fluorouracil (5FU; US Biochemical Corp.) on solid media (4–10 plates with 200 µl of suspension per plate), generally after 6 d culture in buffered liquid ammonium basal medium following mutagenesis. In two experiments to determine mutation frequency 1–3 d after exposure to MNNG, approximately 10⁻⁷ cells ml⁻¹ in 200 ml were treated and correspondingly higher numbers of cells plated on 5FC medium.

Enzyme assay. Cytosine deaminase activity was determined with cells made permeable with toluene using a protocol adapted from Stacey *et al.* (1979). A 2.0 ml suspension of *A. variabilis* containing approximately 10¹⁰ cells ml⁻¹ was mixed with 0.5 ml toluene, incubated at room temperature for 5 min in a 15 ml conical centrifuge tube, centrifuged (1000 g, 5 min) and suspended in 0.8 ml basal medium. The cytosine deaminase assay was adapted from Neuhaud & Ingraham (1968) and contained in a final volume of 1.0 ml: 50 mM-Tris/HCl buffer, pH 7.0, 1 mM-cytosine, and 0.8 ml of cells. The reaction was initiated by the addition of toluene-treated cells and terminated at 30 min intervals by transferring 0.1 ml samples to 0.9 ml cold 0.5 M-perchloric acid. The acid-treated samples were centrifuged (1000 g, 5 min) and the absorbance of the supernatant solutions was measured at 282 nm. An absorbance change of 0.208 units was equivalent to a change in cytosine concentration of 250 nmol. Activity was normalized to cellular chlorophyll *a* content, determined as before (Chapman & Meeks, 1983).

RESULTS AND DISCUSSION

Characteristics of the resistance marker

The growth of wild-type *A. variabilis* was inhibited by 0.3 mM concentrations of either 5FC or 5FU. In enteric bacteria, resistance to 5FC can occur through the inactivation of either cytosine deaminase (EC 3.5.4.1) or UMP pyrophosphorylase (EC 2.4.2.9) (Beck *et al.*, 1972). Cells lacking UMP pyrophosphorylase are resistant to both 5FC and 5FU, while those lacking cytosine deaminase are resistant to 5FC only. Of 100 randomly chosen mutants resistant to 5FC,

Fig. 1. Appearance of 5FC-resistant cells in populations of *A. variabilis*, and changes in viability, after treatment of cavitated suspensions with $0.025 \text{ mg MNNG ml}^{-1}$ at pH 7.0 for 6 h. Samples were removed daily and the number of cells per c.f.u. (Δ), c.f.u. ml^{-1} ($\circ$), and 5FC-resistant mutants ml^{-1} (∇) were determined. From these values the mutation frequencies per c.f.u. ($\bullet$) and per cell ($\blacktriangle$) were calculated. The values plotted represent means of three to five separate experiments.

Table 1. Cytosine deaminase activity of wild-type and mutant strains of *A. variabilis*

Strain	5FC-resistance	5FU-resistance	Specific activity*
Wild-type	—	—	74.9 ± 7.8
5FC-4	+	—	8.8 ± 0.24
5FC-6	+	+	73.8 ± 2.5

* Expressed as $\text{nmol min}^{-1} (\text{mg chlorophyll } a)^{-1}$; values represent the means $\pm$ SE of four experiments.

isolated and selected as described below, 54 were also resistant to 5FU. A representative mutant resistant only to 5FC had 12% of the cytosine deaminase activity of the wild-type (Table 1). Conversely, a mutant resistant to both 5FC and 5FU had a cytosine deaminase activity identical to the wild-type (Table 1).

Currier & Wolk (1978) showed that the enzymes for *de novo* synthesis of uridylic acid, as described for enteric bacteria (O'Donovan & Neuhard, 1970), are present in *A. variabilis*. They assumed that the uracil-dependent growth of mutants of *A. variabilis* that lack the first enzyme of the pathway, aspartate transcarbamylase, resulted from the activity of UMP pyrophosphorylase. The sensitivity of wild-type *A. variabilis* to 5FU and subsequent isolation of 5FU-resistant mutants are consistent with, but do not prove, the presence of such a 'salvage' enzyme. The measured activity of cytosine deaminase, sensitivity to 5FC, and isolation of mutants resistant to 5FC but sensitive to 5FU, all imply that cytosine is metabolized through uracil as in *Salmonella typhimurium* (Beck *et al.*, 1972). This conclusion is supported by the observation that the *A. variabilis* uracil-requiring mutant U2 (Currier *et al.*, 1977) grew equally well when uracil was replaced by cytosine (data not shown).

Segregation of mutant genomes

To establish an optimal time for genomic segregation after MNNG mutagenesis, the numbers of 5FC-resistant mutants ml^{-1} , cells per filament and c.f.u. ml^{-1} were determined daily (Fig. 1).

Table 2. *Effect of pH of the suspension medium on MNNG-induced mutation frequencies in A. variabilis*

Cultures were exposed to 1.0 mg MNNG ml⁻¹ for 15 min and subsequently grown in liquid medium for 6 d before determination of mutation frequencies. The values represent the means $\pm$ SE of four to six experiments.

pH	Frequency of 5FC-resistant mutants
5.0	$(0.446 \pm 0.13) \times 10^{-4}$
5.5	$(1.48 \pm 0.58) \times 10^{-4}$
6.0	$(3.98 \pm 1.00) \times 10^{-4}$
6.5	$(1.18 \pm 0.90) \times 10^{-4}$
7.0	$(0.943 \pm 0.09) \times 10^{-4}$
7.5	$(0.30 \pm 0.02) \times 10^{-4}$

Colonies resistant to 5FC did not appear in numbers greater than predicted from the spontaneous rate of mutation until the liquid suspensions had been cultured for 3 d after mutagenesis. The total numbers of 5FC-resistant mutants increased for at least 8 d, as did the mutation frequency normalized to c.f.u. However, when normalized to total cells, the maximal frequency appeared to be reached by day 6. This discrepancy in normalized values results from the opposing processes of cellular division and lysis discussed below. A segregation period of 6 d was used in subsequent experiments, although for convenience mutation frequency was calculated per c.f.u.

The number of c.f.u. continued to decrease after mutagenesis until after 2 d only 36% of the c.f.u. which were present immediately after mutagenesis remained. Between 2 and 6 d after mutagenesis the number of c.f.u. increased 14-fold. This growth response is unusual considering the filamentous nature of *A. variabilis*; one might predict an increase in cell number but this would not correspond to an increase in c.f.u. It took 4 d from the time of mutagenesis for a filament to double its length and the average filament length began to increase at the same time as the number of c.f.u. began to stabilize. This implies that continued lysis of newly formed daughter cells resulted in filament breakage and the subsequent increase in c.f.u. The competing variables of cellular division and cellular lysis in mutagenized cultures precluded calculations of the actual growth rate of *A. variabilis* under these conditions, although the density of the cultures was seen to increase. Batch cultures of wild-type cells had a doubling time of 20–24 h under identical growth conditions (data not shown). Thus, we assume that the 3 d culture period before increased numbers of 5FC-resistant mutants were detected corresponded to approximately three doublings and would reflect the segregation of five to eight copies of the genome per cell of *A. variabilis*. Genome multiplicity of this order, or greater, is not unusual in cyanobacteria (Doolittle, 1979).

MNNG-induced mutagenesis

The frequency of spontaneous 5FC-resistant mutants, determined by plating fragmented cultures directly onto 5FC plates, was $2.8 \times 10^{-7} \pm 1.7 \times 10^{-7}$ per c.f.u. (mean of three experiments $\pm$ SE). The average size of a c.f.u. in these experiments was 1.6 ± 0.2 cells.

The effects of three parameters on MNNG-induced mutation to 5FC resistance were examined: duration of exposure, pH of the suspension medium, and concentration of the mutagen. The viability and mutation frequency of *A. variabilis* after treatment with MNNG at 1.0 mg ml⁻¹ at pH 6.0 and 7.0 for various periods are shown in Fig. 2(a). A 75% reduction in viable c.f.u. occurred at either pH within 5 min of exposure and reached 99.9% by 30 min. The maximal mutation frequency at pH 7.0 was observed after 20 min of exposure and corresponded to >99% lethality. A similar pattern of lethality and mutation frequency was seen at pH 6.0. The optimal pH for MNNG-induced mutagenesis of *A. variabilis* was 6.0 (Table 2). The mutation frequency at pH 6.0 was more than 4- and 13-fold higher than the frequencies of cultures exposed at pH 7.0 and 7.5 respectively.

Fig. 2. Effect of MNNG (a), DES (b), and nitrous acid (b) on the viability (c.f.u.) and frequency of 5FC-resistant mutants (per c.f.u.) in cultures of *A. variabilis* as a function of exposure time. For MNNG treatments (1.0 mg ml^{-1}), the cavitated suspensions were suspended in buffered ammonium basal medium at pH 7.0 (Δ , $\blacktriangle$) or 10 mM-citrate buffer at pH 6.0 ($\circ$, $\bullet$). The cavitated samples for treatment with $5 \mu\text{l DES ml}^{-1}$ ($\square$, $\blacksquare$) were suspended in buffered ammonium medium, while those for exposure to nitrous acid (∇ , $\blacktriangledown$) were suspended as described in Methods. At the noted times, samples were removed, washed or inactivated (nitrous acid) and suspended in buffered ammonium basal medium. Samples were immediately plated to determine c.f.u. ml^{-1} (open symbols); mutation frequencies (closed symbols) were determined after 6 d growth in liquid culture. The numbers represent means of four (MNNG at pH 6.0 and 7.0, and nitrous acid) or three (DES) separate experiments; the SEs were less than 10% of the recorded values.

The shapes of the curves depicting viability and mutation frequency at pH 6.0 with 0.025 and 0.250 mg MNNG ml^{-1} (data not shown) were similar to those at 1.0 mg ml^{-1} (Fig. 2a). The maximum mutation frequencies obtained in these experiments were, in order of increasing MNNG concentration, 1.5×10^{-4} , 1.2×10^{-4} and 2.4×10^{-4} . These frequencies resulted only when lethality approached 99% (exposure times of 6, 4 and 0.5 h with respect to increasing MNNG concentration).

The physiological response of *A. variabilis* to MNNG-induced mutation was similar to that of heterotrophic bacteria such as *Escherichia coli* (Adelberg *et al.*, 1965) and *Haemophilus influenzae* (Kimball & Setlow, 1974) only with respect to the optimal pH during incubation. This similarity is probably a consequence of the increased stability of MNNG at acid compared to alkaline pH. In *A. variabilis*, maximal mutation frequencies were obtained when lethality approached 99%, irrespective of the MNNG concentration or exposure time. Comparable results at higher pH were obtained by others for *A. variabilis* (Currier *et al.*, 1977), *Agmenellum quadruplicatum* (Stevens & van Baalen, 1969), and *Anacystis nidulans* (Herdman & Carr, 1972). However, in *E. coli* there is no direct correlation between lethality and mutation; rather, maximal mutation frequencies occur at survival values between 43 and 75% of the initial population (Adelberg *et al.*, 1965). There is no current biochemical information to explain the dramatic difference between cyanobacteria and *E. coli* with respect to the lethal and mutagenic effects of MNNG.

Experiments with additional mutagens

DES is an alkylating agent with a mode of action similar to that of MNNG (Bautz & Freese, 1960). The number of c.f.u. of *A. variabilis* was reduced by 99.8% within 30 min of exposure to

Table 3. *Effects of caffeine and chloramphenicol (CAM) on MNNG-induced mutation frequencies and c.f.u. of A. variabilis*

Mutagenesis was performed on fragmented cultures using 0.25 mg MNNG ml⁻¹ for a 4 h exposure at pH 6.0. Samples were removed, washed twice, and suspended in buffered ammonium medium with or without 0.2 mg caffeine or 0.005 mg CAM ml⁻¹; these compounds were removed by centrifugal washing after 6 d (caffeine) and after 2 or 6 d (CAM) and the cultures were suspended in fresh buffered ammonium medium. The numbers of c.f.u. ml⁻¹ and mutation frequency were determined 6 d after mutagenesis. All values represent means $\pm$ SE of three (CAM) or four (caffeine) separate experiments.

Treatment	C.f.u. ml ⁻¹	Frequency of 5FC-resistant mutants
No caffeine	$(4.9 \pm 0.6) \times 10^5$	$(1.36 \pm 0.9) \times 10^{-4}$
+ Caffeine	$(4.0 \pm 1.6) \times 10^5$	$(1.12 \pm 0.8) \times 10^{-4}$
No CAM	$(1.7 \pm 0.6) \times 10^6$	$(2.3 \pm 1.9) \times 10^{-4}$
+ CAM, 2 d	$(1.6 \pm 1.2) \times 10^6$	$(1.1 \pm 0.6) \times 10^{-4}$
+ CAM, 6 d	$(0.8 \pm 0.1) \times 10^6$	$(0.8 \pm 0.2) \times 10^{-4}$

5 μ l DES per ml of cell suspension (Fig. 2b). The frequency of 5FC-resistant mutants increased with time of exposure up to 30 min. Increasing the concentration of DES to 20 μ l ml⁻¹ resulted in a doubling of the mutation frequency relative to treatment with 5 μ l ml⁻¹ but at similar lethalities. The maximal mutation frequency produced by exposure to 20 μ l DES ml⁻¹ was 0.98×10^{-4} . However, at this concentration droplets of the compound were visible in the medium, indicating saturation of the medium and implying that the most effective concentration of DES was between 5 and 20 μ l per ml of cell suspension.

A 5 min treatment of *A. variabilis* with 0.05 M-nitrous acid resulted in a 98% reduction in c.f.u. and a mutation frequency of 1.5×10^{-5} (Fig. 2b). Increasing the time of exposure caused a decrease in viable c.f.u., but had little or no effect on the mutation frequency.

While substantially higher mutation frequencies were obtained with MNNG and DES, these agents tend to produce closely linked multiple mutations (Guerola *et al.*, 1971). The mutagenic activity of nitrous acid in causing AT to GC transitions is more likely to result in single-site lesions (Zimmerman, 1977). This, and the observation that nitrous acid can induce mutations in cyanobacteria at a frequency approximately 54-fold higher than the spontaneous rate, indicates that more useful mutants may be generated by nitrous acid than by MNNG or DES.

Effects of chloramphenicol and caffeine on mutation frequency

The unicellular cyanobacterium *Anacystis nidulans* (Delaney & Carr, 1975), as well as *E. coli* (Kato, 1977), have postreplication repair systems active after UV- or ethylmethane sulphonate-induced chromosomal damage that are inhibited by caffeine. Treatment with caffeine, at 200 μ g ml⁻¹, reduced the viability of *A. variabilis* cells that survived exposure to MNNG by about 20%, but did not increase the mutation frequency (Table 3).

Chloramphenicol, at low concentrations (2.5 μ g ml⁻¹), enhances both UV- (Kato, 1977) and MNNG- (Sklar, 1978) stimulated mutation frequencies in *E. coli* by preventing the induced synthesis of repair enzymes. The presence of up to 5 μ g chloramphenicol ml⁻¹ during the entire 6 d segregation period after exposure to MNNG lowered both viability and mutation frequency of *A. variabilis* (Table 3). Removal of chloramphenicol 2 d after mutagenesis had no detectable effect on either the number of c.f.u. or the mutation frequency.

The apparent lack of a caffeine-sensitive or inducible repair system capable of affecting MNNG-induced lesions in *A. variabilis* does not exclude the presence of other types of repair systems. A variety of repair systems have been reported in cyanobacteria (Stevens & van Baalen, 1969; Tang & Asato, 1978).

The results of this study indicate that mutant phenotypes of a heterocyst-forming cyanobacterium can be recovered in high frequencies from the cells surviving chemical mutagenesis. Irrespective of the chemical mutagen, the incubation time following exposure and prior to selection or counterselection must allow for sufficient cell doublings to segregate

multiple copies of the genome. The major problem confronting physiological genetic studies in cyanobacteria is not mutation, but selection of the desired mutant. For example, we have shown that the structural gene for the enzyme glutamine synthetase can be altered at high frequency (Chapman & Meeks, 1983), but we (and others) have consistently failed to isolate mutants requiring glutamine, in direct selections or after counterselection (Chapman, 1984). This conclusion is supported by the fact that only a limited number of cyanobacterial mutants have been described that are auxotrophic for organic metabolites (Herdman, 1982).

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