Mutation Induction in the Cyanobacterium *Gloeocapsa alpicola*

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The lethal and mutagenic effects of ultraviolet light (u.v.), ethyl methanesulphonate (EMS), methyl methanesulphonate (MMS), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and mitomycin C on the unicellular cyanobacterium *Gloeocapsa alpicola* have been investigated. Mitomycin C and EMS did not significantly increase the frequency of streptomycin-resistant mutants above the spontaneous level. MNNG and MMS produced increases of 10 to 30 times the spontaneous frequency. Contrary to previous reports on mutation induction in cyanobacteria, u.v. was the most effective mutagen, increasing the spontaneous mutation frequency 4000-fold. However, since photoreactivating light eliminated most of this mutagenic response it was necessary to rigorously exclude light of wavelengths < 550 nm during post-irradiation growth. Caffeine failed to increase the mutation frequency of u.v.-irradiated cultures, but cells were more sensitive to u.v. killing in the presence of caffeine.

INTRODUCTION

Genetic studies on cyanobacteria have been restricted to a few species, most of which are difficult to manipulate, either because they are filamentous or because they lack reproducible growth on agar-containing media (Ladha & Kumar, 1978). The most widely used unicellular species is *Anacystis nidulans* which, although showing high plating efficiency, has the disadvantage of not being a nitrogen-fixing species (Stanier & Cohen-Bazire, 1977). *Gloeocapsa alpicola* would appear to provide a suitable alternative for genetic studies since it is unicellular, has a high plating efficiency and some strains are capable of fixing atmospheric nitrogen. It also has a high frequency of genetic transformation (Deville, 1975; Devilly & Houghton, 1977). However, one of the major problems that has restricted the development of genetic studies in the cyanobacteria has been the difficulties encountered in the isolation of suitable mutant strains. Mutation induction using antibiotic resistance, auxotrophy, pigmentation changes, temperature sensitivity, filamentation or cyanophage resistance as markers, has been demonstrated in *Anacystis nidulans* (Herdman *et al.*, 1973), *Anabaena doliolum* (Singh & Singh, 1964), *Phormidium mucicola* (Srivastava, 1969), *Agmenellum quadruplicatum* (Ingram *et al.*, 1972), *Synechocystis aquatilis* (Zhevner & Shestakov, 1972), *Cylindrospermum majus* (Singh & Sinha, 1965), *Nostoc linckia* (Singh & Tiwari, 1969), *Plectonema boryanum* (Padan & Shilo, 1969) and *Synechococcus cedrorum* (Kaney, 1973). A wide range of mutagens has been used to induce mutations in cyanobacteria (Delaney *et al.*, 1976). However, many authors have failed to comment on the efficiency of mutagenesis and comparisons are complicated by the lack of standardization of techniques. In general, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, nitrosoamines and nitrosomethyl urea are considered to be efficient mutagens; whereas methyl methanesulphonate, ethyl methanesulphonate and diethyl sulphate are considered to be less effective (Shestakov & Jevner,

1968). X-rays, mitomycin C, hydroxylamine, acridine orange and ultraviolet light are considered to be poor mutagens for cyanobacteria (Shestakov, 1972).

In the present study, the effect of a variety of mutagens on survival and on mutation to streptomycin resistance in *Gloeocapsa alpicola* has been examined. Efficiencies of mutagenesis and optimal conditions for mutation have been determined.

METHODS

Organisms. *Gloeocapsa alpicola* (Lyngbye) Bornet, a unicellular cyanobacterium which produces nitrogenase, was obtained from the Culture Collection of Algae and Protozoa, Cambridge (collection no. 1430/1). *Escherichia coli* strain AB1157 was obtained from the *E. coli* Genetic Stock Center, Yale University School of Medicine, New Haven, Conn., U.S.A.

Media and growth conditions. The medium for *G. alpicola* was a modification of that described by Allen (1968). Liquid growth medium contained (g l⁻¹ in deionized distilled water): NaNO₃, 1.5; K₂HPO₄, 3.9 × 10⁻²; MgSO₄·7H₂O, 7.5 × 10⁻²; CaCl₂, 3.6 × 10⁻²; citric acid, 1.2 × 10⁻²; ferric citrate, 6.0 × 10⁻³; H₃PO₄, 2.9 × 10⁻³; MnCl₂, 1.8 × 10⁻³; ZnSO₄·7H₂O, 2.22 × 10⁻⁴; Na₂MoO₄·2H₂O, 3.9 × 10⁻⁴; CuSO₄·5H₂O, 7.8 × 10⁻⁵; Co(NO₃)₂·6H₂O, 5.0 × 10⁻⁶. Sodium silicate (0.58 g l⁻¹) and EDTA (10⁻³ g l⁻¹) were added and the mixture was heated to boiling. After cooling, the pH was adjusted to 5.5 and the medium was sterilized at 121 °C for 15 min. After cooling, filter-sterilized Na₂CO₃ was added to a final concentration of 2.0 × 10⁻² g l⁻¹ giving a final pH of 8.0 to 8.5. Flasks (500 ml) containing 100 ml of medium were inoculated with single colonies of *G. alpicola* or a 1 % (v/v) inoculum of an exponentially growing culture and incubated in a Gallenkamp illuminated orbital incubator at 30 ± 1 °C, 120 oscillations min⁻¹ and under constant illumination with white fluorescent light at 2000 lx. Solid medium was prepared by mixing sterile, double-strength salts medium with an equal volume of sterile Difco Bacto-agar (3.6 %, w/v, in distilled water) at 45 °C. After rapid mixing the medium was poured into plastic Petri dishes and the plates were dried at 60 °C for 10 min.

Viable counts were determined by diluting liquid cultures of *G. alpicola* in single salts medium and spreading 0.1 ml appropriate dilutions on dried plates. The plates were incubated at 30 ± 1 °C, 50 % relative humidity and illuminated with gold-yellow light (Thorn International; λ 550 to 700 nm) at an intensity of 2000 lx. Colonies were counted after incubation for 10 to 14 d.

The selective media for *G. alpicola* contained 2 µg streptomycin sulphate ml⁻¹; the minimum concentration of streptomycin to which wild-type *G. alpicola* is sensitive is 0.5 µg ml⁻¹. Mutant counts were made after incubation for 21 d.

Escherichia coli was grown in Oxoid nutrient broth. Non-selective agar for the estimation of viable counts was made by adding 15 g Oxoid no. 1 agar l⁻¹ to broth. Plates were incubated at 37 °C and colonies were counted after 24 h.

Mutagenic treatments. (i) Ultraviolet light (u.v.). *Escherichia coli* was washed and resuspended in phosphate buffer, pH 7.0, prior to irradiation. *Gloeocapsa alpicola* was irradiated in single salts medium. Cultures at a density of 10⁸ cells ml⁻¹ were exposed to 254 nm u.v. from a mercury discharge lamp (Griffin & George) at room temperature; the incident dose rate was 0.19 J m⁻² s⁻¹. The suspensions were magnetically stirred throughout the irradiation period. Irradiations and all subsequent manipulations were carried out under yellow light to prevent photoreactivation. Where indicated, u.v.-irradiated cultures were held in buffer containing 0.2 % (w/v) caffeine for 24 h before washing and plating or diluting in broth. Irradiated *G. alpicola* was plated and incubated with either gold-yellow or white light at 2000 lx. Where indicated, u.v.-irradiated cultures were exposed to blue light (Thorn International; λ 350 to 550 nm) at an intensity of 2000 lx. To ensure maximum photoreactivation, cultures were exposed to blue light for 90 min (Williams *et al.*, 1979). (ii) Ethyl methanesulphonate (EMS) and methyl methanesulphonate (MMS). EMS and MMS were added to exponentially growing cultures incubated under normal growth conditions. Where necessary, the mutagens were diluted either in 0.1 M-phosphate buffer, pH 7.0 (for *E. coli*) or single salts medium (for *G. alpicola*) prior to addition to cultures. (iii) *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). A freshly prepared, filter-sterilized solution was added to exponentially growing cultures incubated under normal growth conditions. (iv) Mitomycin C. Mitomycin C (Sigma) was dissolved in 0.1 M-phosphate buffer, pH 7.0 (for *E. coli*) or single salts medium (for *G. alpicola*) and added to exponentially growing cultures incubated under normal growth conditions.

Post-treatment of cultures. After incubation with chemical mutagens for the appropriate time, cultures were immediately diluted 10-fold in warm growth medium, centrifuged in a Microfuge (Beckman), washed and resuspended in growth medium. The time taken to remove the mutagens by centrifugation was less than 2 min.

Estimation of induced mutation. Mutagen-treated cultures were diluted and spread on non-selective plates

Fig. 1. Effect of EMS (a), mitomycin C (b), MNNG (c) and MMS (d) on survival (●) and mutation (■) in *G. alpicola*.

Fig. 2. Effect of u.v. on survival (a) and mutation (b) in *G. alpicola* showing the influence of illumination and caffeine. Post-irradiation treatment of the cells: □, white light illumination, no caffeine; ●, gold light illumination, no caffeine; ○, gold light illumination, 0.015 % caffeine; ■, gold light illumination, 0.20 % caffeine.

to estimate survival. Cultures were also diluted 100-fold into the appropriate broth and incubated under normal growth conditions until fully grown. These cultures were concentrated 10-fold, concentrated 30-fold, plated directly or diluted and plated on selective agar medium to estimate the number of mutants and were also diluted and spread on non-selective agar to estimate the number of cells plated. Untreated cultures were used as controls. The mutation frequency was expressed as the number of mutants per 10^9 viable cells plated.

Effect of caffeine on viability of G. alpicola. Exponentially growing cultures were diluted and plated on agar medium containing a range of concentrations of caffeine. Plates were incubated as previously described.

RESULTS AND DISCUSSION

The isolation of mutants resistant to antibiotics, amino acids and amino acid analogues is a reliable indication of mutation in cyanobacteria (Delaney *et al.*, 1976). In the present study, resistance to $2 \mu\text{g}$ streptomycin ml^{-1} was used as a marker. A streptomycin concentration of $100 \mu\text{g}$ ml^{-1} was required to inhibit these mutants compared with $0.5 \mu\text{g}$ ml^{-1} for the wild-type strain. The effects of EMS, mitomycin C, MNNG, MMS and u.v. on survival and mutation in *G. alpicola* are illustrated in Figs 1 and 2. The results presented are an average of at least three independent experiments and each point represents at least 150 mutant colonies. At frequencies close to the spontaneous level, cells were concentrated 10- or 30-fold. However, at these low frequencies mutant counts varied between experiments. The spontaneous frequency shown is an average value obtained over all experiments. All other frequencies have been corrected for variation in the spontaneous frequency between experiments. The development of resistance to streptomycin was a stable character, resistance being maintained for more than 10 subcultures in the absence of challenge.

EMS and mitomycin C failed to increase significantly the frequency of mutants above the spontaneous frequency, although at these low frequencies mutant counts were variable and small increases in frequency were probably undetectable (Fig. 1a, b). Although EMS is considered to induce mutations independently of the DNA repair mechanisms (Ishii & Kondo, 1975), it is non-mutagenic for the *rad-6* mutant of *Saccharomyces cerevisiae* (Bridges, 1976) and for obligate methane-oxidizing bacteria (Williams & Shimmin, 1978) and is only marginally mutagenic for *Micrococcus radiodurans* (Sweet & Moseley, 1974, 1976). The repair-dependent mutagen, mitomycin C, upon reduction in the cell, produces a mixture of mono-adducts and thermostable cross-links between complementary DNA

Table 1. *Effects of mutagens on the survival of Gloeocapsa alpicola and Escherichia coli AB1157*

Treatment	Units	D ₃₇ survival values*		
		<i>G. alpicola</i>	<i>E. coli</i>	<i>G. alpicola</i> <i>E. coli</i>
U.v. + yellow light	J m ⁻²	99	130	0.76
U.v. + white light	J m ⁻²	480	220	2.18
U.v. + yellow light + 0.2 % caffeine	J m ⁻²	75	124	0.61
MMS	% min	3.5	11.8	0.29
MNNG	µg ml ⁻¹ h	9.5	35.0	0.27
EMS	% min	47.2	134.0	0.35
Mitomycin C	µg ml ⁻¹ h	4.0	2.0	2.00

* D₃₇ is the dose of mutagen required to reduce the viability to 37 % of its original value and represents the average dose required to inactivate a cell.

Fig. 3. Effect of caffeine on the colony-forming ability of unirradiated *G. alpicola*.

strands. Repair of these lesions may give rise to mutational events (Kimball, 1978; Hanawalt *et al.*, 1979). The absence of mutation induction and the shoulderless survival curve (Fig. 1*b*) may indicate that *G. alpicola* is unable to repair DNA cross-links.

MNNG was moderately mutagenic for *G. alpicola* and, as with many heterotrophic bacteria, maximum mutagenesis was found at high levels of survival (Fig. 1*c*). However, the 10-fold increase in mutation frequency does not justify its adoption as an efficient mutagen for cyanobacteria. MMS increased the frequency of streptomycin-resistant mutants 30-fold (Fig. 1*d*). *Gloeocapsa alpicola* was approximately three times more sensitive to the lethal effects of EMS, MMS and MNNG than was *E. coli* (Table 1).

Contrary to previous reports which have indicated that u.v. is a very poor mutagen, only marginally increasing mutation frequencies in cyanobacteria, this study has shown that u.v. is a most efficient mutagen for *G. alpicola*, increasing the frequency of streptomycin-resistant mutants by as much as 4000 times the spontaneous frequency (Fig. 2). However, previous studies have measured u.v.-induced mutation after incubation in the presence of white light illumination, whereas in this study, the mutants were scored after incubation in the presence of non-photoreactivating yellow light. Illumination with white light increased survival and reduced the yield of mutants in u.v.-irradiated *G. alpicola* (Fig. 2). An extremely efficient mechanism of photoreactivation has been demonstrated in *G. alpicola* (Williams *et*

al., 1979) and many other cyanobacteria (Ladha & Kumar, 1978). Photoreactivation in *E. coli* is an error-free mechanism which increases survival and decreases mutagenesis in u.v.-irradiated cultures by monomerizing cyclobutane pyrimidine dimers *in situ* (Witkin, 1976; Hanawalt *et al.*, 1979).

Caffeine is fairly toxic to *G. alpicola* when incorporated into the growth medium (Fig. 3). A concentration of 0.015 %, just below the toxic threshold, had no effect on the survival of u.v.-irradiated *G. alpicola* (Fig. 2), or on the loss of photoreversibility of lethal u.v. damage (E. Williams, unpublished results) and presumably does not inhibit dark repair processes. A higher concentration (0.2 %), although extremely toxic to growing cells of unirradiated *G. alpicola*, had no effect on the viability of non-growing cultures held for periods up to 80 h before washing and plating (Williams *et al.*, 1979). This concentration of caffeine inhibited loss of photoreversibility of lethal u.v. damage (Williams *et al.*, 1979) and reduced the viability of irradiated cultures (Fig. 2), presumably by inhibiting pre-replication dark repair events. However, 0.2 % caffeine failed to increase the yield of u.v.-induced mutants.

In conclusion, provided that light of wavelengths < 550 nm is rigorously excluded during post-irradiation manipulation and growth, u.v. is a very efficient mutagen for *G. alpicola*. MMS and MNNG provide reasonable alternatives.

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