SPONTANEOUS AND INDUCED MUTATION FREQUENCIES IN THE BLUE-GREEN ALGA *ANACYSTIS NIDULANS*

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The relative frequency of spontaneous and nitrosoguanidine-induced mutation to streptomycin resistance in the blue-green alga *Anacystis nidulans* has been estimated both in the forward and reverse directions. Nitrosoguanidine (NTG) proved very effective at acidic *p*H for both inducing and reverting the mutants. The frequency of NTG-induced mutation to streptomycin resistance was about one hundred times higher than the spontaneous one. NTG-induced revertible mutants which, besides reverting spontaneously, were reverted by NTG itself, possibly by transitional changes. The frequency of reverse mutation was significantly higher than the induced forward frequency and may account for the remarkably high genetic stability of the alga.

INTRODUCTION

The procaryotic organization, coupled with aerobic photosynthesis and extreme degree of ecological stability mark the blue-green algae as a most fascinating group of micro-organisms in which the genetic basis of environmental stability could profitably be studied. In comparison to other microbes, higher plants and animals, the mechanisms of mutagenesis in Cyanophyceae seem apparently inefficient although spontaneous and induced mutation to drug resistance, especially to streptomycin, is well-established for certain unicellular blue-green algae (Singh *et al.* 1966; Kumar 1968; Padan and Shilo 1969).

Ultraviolet radiation and N-methyl N' nitro N-nitrosoguanidine (NTG) have been extensively used in the study of mutagenesis in blue-green algae and NTG has proved comparatively more productive than UV in this respect (Kumar 1968; Van Baalen 1965; Stevens and Van Baalen 1969). However, neither of these two agents has proved as effective in Cyanophyceae as in other photosynthetic or non-photosynthetic microbes.

Most previous studies of mutagenesis in blue-green algae have been qualitative, dealing exclusively with forward mutations involving antibiotic-resistance, non-sporulation and filamentation and virtually no work has been done on the quantitative characterization of forward and reverse mutation for any single character in any blue-green alga.

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In the present paper we report and quantitatively compare the results of NTG-induced forward and reverse mutations to streptomycin-resistance in the blue-green alga *Anacystis nidulans*.

Materials and Methods

Organism

Anacystis nidulans Drouet (Myers' strain), a fast-growing unicellular blue-green alga, was used for the mutation studies. Its ability to grow in suspension in liquid cultures and to form clones on agar plates makes it a suitable organism amenable to quantitative studies.

Culture medium

The Hughes *et al.* medium as modified by Allen (1968) was routinely used for culturing the alga. The cells to be treated with NTG were grown in liquid medium and after treatment with NTG they were inoculated on agarized medium, solidified with 1–1.5 per cent agar, in petri dishes of 100 mm diameter. Aliquots containing 6×10^4 cells each were spread on each plate. Cultures were grown and maintained at $38 \pm 2°C$ and about 500–600 lux.

Chemicals and mutagen

Dihydrostreptomycin sulphate (potency 745 units/mg, Glaxo) was used for screening streptomycin-resistant mutants occurring spontaneously or induced (or reversed) by mutagenic treatment with N-methyl N'-nitro N-nitrosoguanidine (NTG; Aldrich Chemical Co., Milwaukee, Wisconsin). A fresh solution of the mutagen was prepared for each experiment. A schedule similar to that devised for bacteria (Adelberg *et al.* 1965) for determining the lethal and mutagenic action of NTG was first followed in this study. In one set of experiments, exponentially growing cells were treated at different concentrations of NTG (0, 1, 10, 30, 40, 50 and 100 μg/ml) for 30 min in phosphate-citrate buffer of *p*H 5.5. The other set involved treatment of the cells with a constant dose of NTG (*i.e.*, 30 μg/ml) for varying time periods. The cell-NTG mixture was then appropriately diluted and plated on culture medium at an inoculum size of 6×10^4 cells/plate. Plates inoculated with treated cells and the control plates were incubated for nearly two weeks in the light and the per cent survival was estimated by colony counts.

Estimation of forward mutations

Exponentially growing cells were harvested by centrifugation and divided into two sets, one set serving as the control and the other being treated with 30 μg NTG/ml for 30 min. Treated and untreated samples were seeded on 2% agar plates each and after proper settling of the inocula on each plate, a second layer of molten agar at 45–50°C containing 10 μg streptomycin/ml was overlayered on each of the 15 plates from each set. The remaining 6 plates served as controls for determination of per cent viability of NTG-treated and untreated samples.

The colonies surviving the streptomycin challenge were tentatively scored as streptomycin-resistant clones and repeatedly tested for their stability. One of the streptomycin-resistant colonies arising spontaneously was picked up, grown in basal liquid medium and later used in studies of NTG-induced reverse mutation.

Estimation of reverse mutation

The same procedure as adopted in the study of forward mutation was employed in this study except that NTG-treated streptomycin-resistant samples were allowed to grow to form colonies on the agar medium before being overlayered with a second layer of molten agar supplemented with 10 μg streptomycin/ml.

The frequency of forward or reverse mutations was estimated by scoring the number of colonies surviving or not surviving the challenge of exposure to 10 μg/ml of streptomycin.

RESULTS

A preliminary study of the conditions for effective mutagenicity of NTG in *A. nidulans* revealed that NTG lethality is considerably enhanced when it is mixed with the agar medium held at about 45°C before pouring than when it is mixed with the phosphate-citrate buffer suspension. A similar observation has been made by Zamenhof *et al.* (1966) who reported that freshly prepared NTG plates were invariably toxic.

The results plotted in Fig. 1 indicate that the NTG dose-effect curve obtained by varying the NTG concentration against a fixed time is sigmoidal, having a broad shoulder, and a steep final slope. When the NTG dose was varied by varying the duration of treatment against a constant concentration, the resulting curve was almost exponential (Fig. 2). A comparison of Figs. 1 and 2 will suggest two modes of killing by NTG, viz., one operating at concentrations lower than 30 μg/ml and the other effective at 30 μg/ml or higher concentrations. The NTG-kill is sigmoidal at lower doses and exponential at higher doses.

Characterization of induced streptomycin-resistant mutants

The induced streptomycin-resistant (ISR) mutants were picked up and grown clonally in the absence of streptomycin, 15–20 times extending over a 3-month period. These clones were characterized on the basis of (*a*) form and size of cells and colonies, and (*b*) their ability to back mutate both spontaneously and following NTG treatment.

Cell and colony morphology

The cells of the ISR mutant were longer (25.6–38.4 μ) than the wild strain (1.6–2.2 μ) and appeared as long aseptate filaments. These filaments were longer in streptomycin-supplmented medium than in its absence. The filamentous mutant phenotype was found to be a stable trait. A similar report was made for a marine blue-green alga by Ingram and Van Baalen (1970). The average colony diameters of the ISR mutants obtained on streptomycin-free and streptomycin-supplemented plates measured 110–130 μ and 168–205 μ respectively, after 15-day incubation. The wild type colonies and those of ISR mutant growing on streptomycin-free plates did not differ significantly in their dimensions.

Reverse mutation of ISR mutants

Table I shows that NTG is effective in increasing the frequency of forward mutation to streptomycin-resistance. A hundred-fold increase over the spontaneous

yield was noted. The data summarised in Table II show that NTG was able to revert the mutants it had induced and the frequency of reversion was 3.3×10^5 times higher than the induced forward frequency. The spontaneous reversion frequency of ISR mutants was also higher than in the forward direction but comparatively lower than the induced reversion frequency. Both spontaneous and induced revertants were found to resemble the non-mutant parent as regards their streptomycin sensitivity.

TABLE I

Frequency of induced vs *spontaneous mutation to streptomycin resistance (Forward mutation)*

Mutagen	Resistant mutants/2×10^8 colony forming units (averages)	Frequency/2×10^8 cells plated	Viability
None	3	1×10^{-8}	68%
30 µg NTG/ml	200	100×10^{-8}	42%

TABLE II

Frequency of induced vs *spontaneous mutation to streptomycin sensitivity (Reverse mutation)*

Mutagen	Total no. of colonies surviving on 15		Frequency of reverse mutation (as % of revertants/survivors)
	SM-free plates	SM-supplemented plates	
None	99500	97675	1.8
30 µg NTG/ml	60000	40200	33
Net NTG-induced revertants		= 33-1.8 = 31.2%	

DISCUSSION

Nitrosoguanidine acts on replicating DNA and causes killing or mutation depending upon the viability of NTG-damaged DNA (Kimball and Setlow 1972). The sigmoidal killing of *A. nidulans* by lower doses of NTG implicates some process counteracting NTG lethality. Stevens and Van Baalen (1969) reported that a marine blue-green alga *Agmenellum quadruplicatum* possesses a repair system analogous to dark repair system which operates against NTG lethality. The synergistic effect of solid medium on NTG lethality is found to be so severe that any size of inoculum fails to overcome this toxicity. Nothing is known about the biochemical basis of the increased NTG lethality in view of lack of information on conditions affecting DNA replication in blue-green algae. However, it seems certain that the efficiency of a mutagen depends greatly on the procedure used.

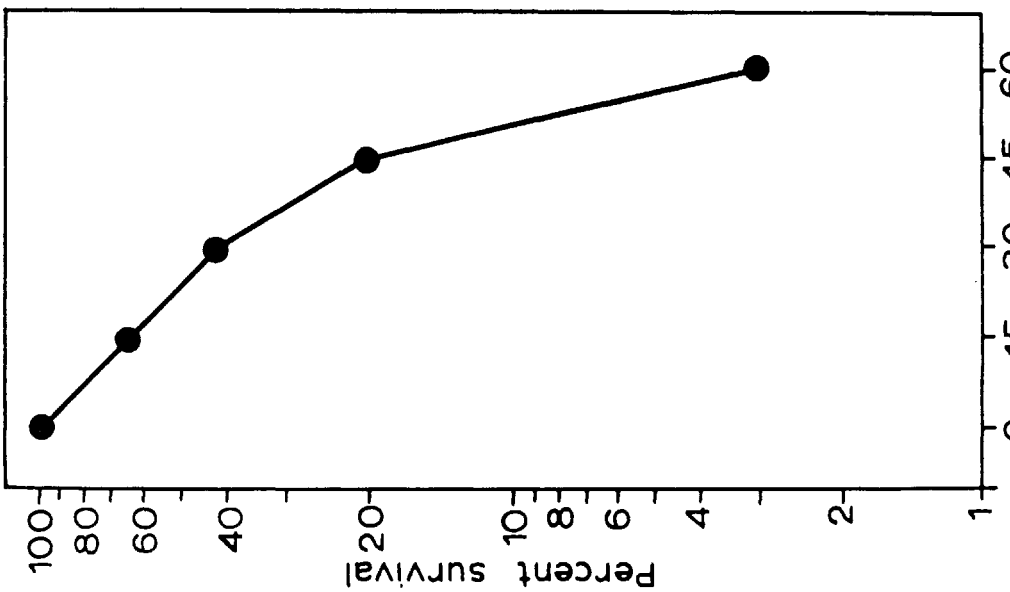

FIG. 2. Survival of *A. nidulans* following treatment with nitrosoguanidine for different durations of time.

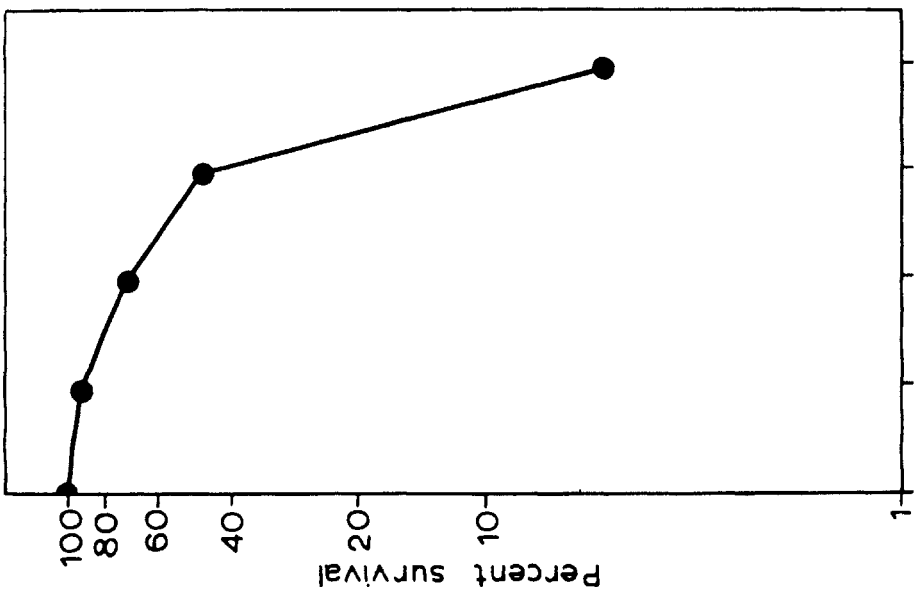

FIG. 1. Nitrosoguanidine dose-effect curve for *Anacystis nidulans*

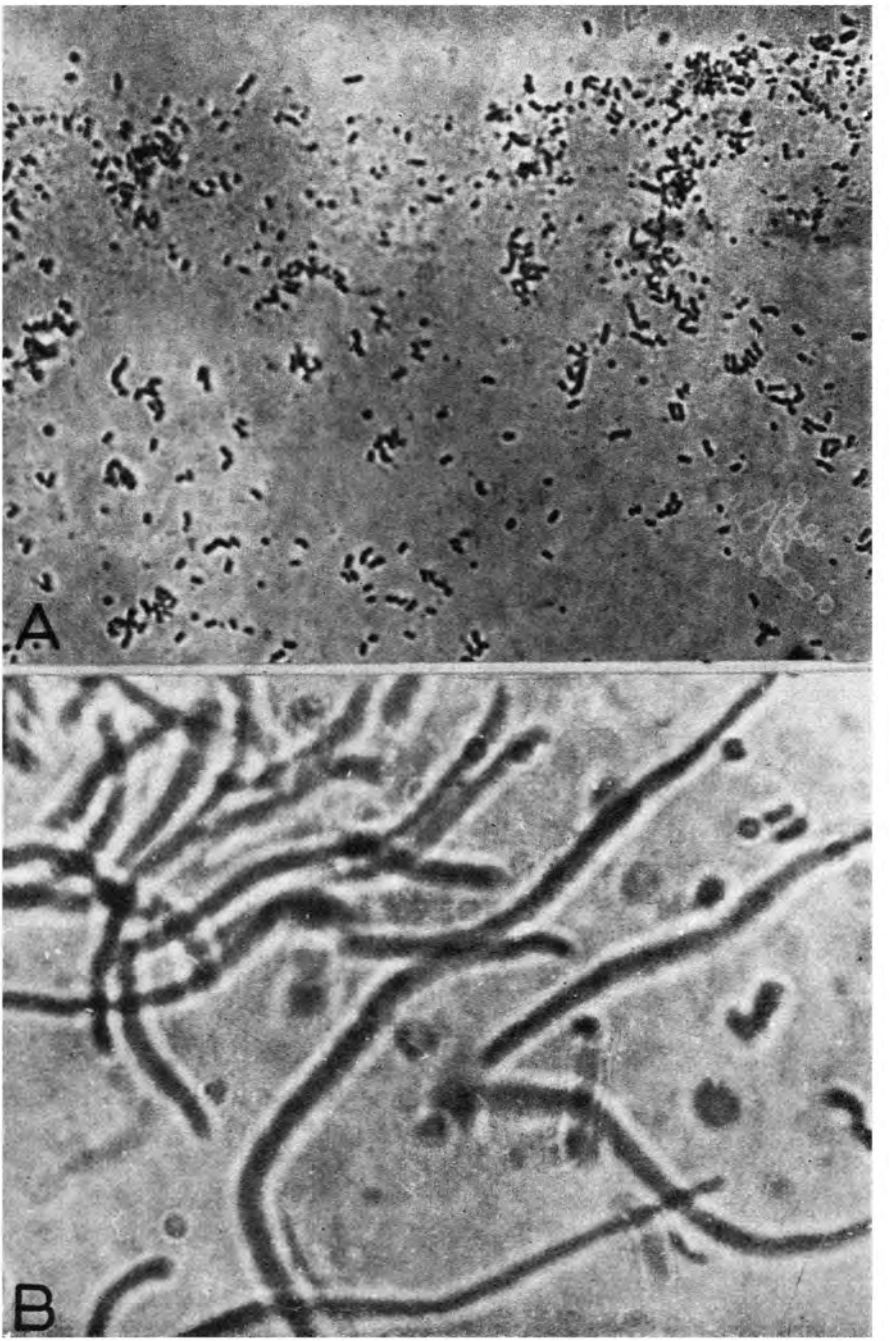

FIG. 3. *A*, photomicrograph of untreated (wild) cells of *A. nidulans*. × 650; *B*, photomicrograph of nitrosoguanidine-induced streptomycin-resistant filamentous mutant of *A. nidulans* grown in streptomycin-containing medium. × 850.

The production of filamentous cells and larger colonies as compared to those of the wild type by NTG treatment suggests that the apparent gene specificity of a mutagen may concern not so much the primary reaction between the gene and the mutagen as its selective influence on the secondary processes by which its effects on the genetic material are translated into the observed mutants (Auerbach 1967).

Both spontaneous and NTG-induced streptomycin-resistant mutants have been found to be revertible suggesting thereby that streptomycin resistance mutation is a point mutation and that NTG is a mutagen causing point mutation in *A. nidulans*.

Our findings on NTG-induced reverse mutation are in agreement with those of Hartley (1970), who observed that one-half of the NTG-induced mutants of *Aspergillus nidulans* at the XDH loci were reverted back by NTG itself by transitional type (GC to AT or AT to GC) base pair changes fairly readily. A very high frequency of reverse mutation in comparison to the forward frequency in the present work appears significant and may be one of the reasons for the apparent genetic stability of the alga. This point may also explain why most blue-green algae are generally resistant to frequent genetic variations.

Acknowledgement

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