Nature of UV resistance in the cyanobacterium *Nostoc linckia*

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After several exposures of *Nostoc linckia* to ultraviolet radiation, an UV-resistant strain was isolated. When this strain was subcultured for 5 to 6 generations in medium supplemented with 1 μg ml^{-1} of acridine orange and then exposed to UV irradiation, the resistant character was lost and it behaved like a wild strain. Agarose gel electrophoretic analysis of crude lysate DNA revealed the presence of three classes of covalently closed circular DNA bands in the UV-resistant strain, but their absence in the strain which had been cultured in acridine orange. This finding suggests the involvement of extra chromosomal genetic elements in UV resistance in *N. linckia*.

RADIOSENSITIVITY responses of micro-organisms are known to vary according to pre- and post-environmental conditions. The use of ultraviolet radiation to induce or isolate mutant forms of animals, plants and micro-organisms has been reported by several workers[1-3]. Some of the chemicals that modify UV survival include the acridines and other related dyes and caffeine. Kumar[4] studied the effects of proflavine on *Anacystis nidulans*. Metallic ions are also known to modify the UV sensitivity in *A. nidulans*[5]. More recently, the effect of ultraviolet-B (280–320 nm) irradiation on physiological activities of several species of cyanobacteria has been studied[6,7]. Nothing is known about the nature of UV resistance in cyanobacteria; it may be under the control of chromosomal genes, cytoplasmic elements, or plasmids. Acridine orange eliminates plasmids from prokaryotic cells[8]. Plasmid-borne genetic determinants are thus identified by comparing the phenotypes of 'cured' derivatives with those of their plasmid-containing parents. Acridine orange pre-treatment would, therefore, be expected to be of some help in testing these alternative hypotheses. Any information on this aspect will be of obvious utility in the case of economically and agriculturally important microbes such as nitrogen-fixing cyanobacteria, because this aspect does not seem to have been studied previously in nitrogen-fixing cyanobacteria.

Methods

The organism used was *Nostoc linckia* (Roth) Born. et Flah., a filamentous, heterocystous and nitrogen-fixing cyanobacterium. Cultures were grown axenically in Allen and Arnon's[9] medium, with no combined nitrogen and adjusted to pH 7.5 after autoclaving. Cultures were grown in air at 26 ± 2°C and illuminated with four cool-white fluorescent lamps at an average intensity of 250 μE m^{-2} s^{-1}. They were shaken by hand thrice daily. Growth of the parent and resistant strains was followed by measuring their absorbance at 660 nm in a Bausch and Lomb Spectronic-20 colorimeter. Heterocyst frequency was determined by counting the number of heterocysts as a function of filament length. Each time about 1000–1100 cells were counted.

For acetylene-reducing activity, a suspension of 2 ml of whole filaments was incubated in a 7-ml serum stoppered tube containing 10% C_2H_2 and placed in a shaker bath. The bath was illuminated from the bottom by six cool-white fluorescent lamps at an average intensity of 300 μE m^{-2} s^{-1}. The ethylene formed was measured by injecting 0.2 ml of the gas phase into a CIC (Chromatography and Inst. Co, Baroda) gas chromatograph equipped with Porapak R column at 50°C with nitrogen as the carrier gas.

The source of UV radiation was a germicidal lamp (American Ultraviolet Co, USA) emitting its main output at 2537 Å with radiation dose of about 85 ergs mm^{-1} s^{-1} at a distance of 22.5 cm. For the isolation of the resistant strain, exponentially grown culture of *N. linckia* was centrifuged, washed thrice with the same medium and homogenized with Pyrex glass beads. Five ml of the suspension was placed in a petri dish and stirred with a sterilized magnetic stirring bar. The inoculum (10 μl) containing 4×10^3 colony-forming units per plate was seeded on agar plates containing Allen and Arnon's medium without combined nitrogen sources. The dose of UV irradiation was varied by varying the time of exposure. After irradiation, cultures were kept in the dark for 24 h to avoid photoreactivation and then incubated in the light. Colonies were scored. Resistant colonies were subcultured individually in test tubes. Finally, a strain, resistant for 10 min UV exposure, was isolated after repeated testing for its resistant character.

The DNAs of the resistant (Nl_{10}) strain and that pretreated and subcultured with acridine orange for about six generations were prepared as described earlier[10]. Standard techniques were used for preparation of DNA and gel electrophoresis[11].

Electrophoresis was performed in a horizontal slab chamber containing 0.8% agarose (Sigma type II) slab gel in tris-borate buffer (90 mM) at pH 8.0. After adding 1/9 volume of a dye solution containing 50% glycerol,

Table 1. Comparison of growth, heterocyst frequency, acetylene-reducing activity and UV survival of wild type and UV-resistant (Nl_{10}) strains of *Nostoc linckia*. Growth and other conditions were the same as described in the text. Heterocyst frequency was calculated after 72 h of transfer from nitrate-supplemented to nitrogen-fixing conditions. Lethal dose of UV radiation for wild type was 2.0 min.

Organism	Heterocyst frequency (%)	Acetylene-reducing activity (nmol C_2H_2 formed mg^{-1} dry weight h^{-1})	Growth (generation time, h)	UV survival (10 min UV exposure)	
				No treatment	Pretreatment with acridine orange (1 $\mu g\ ml^{-1}$)
Nostoc linckia	5.6	40	48	Lethal	Lethal
Nostoc linckia (Nl_{10}, resistant for 10 min UV exposure strain)	5.6	36	54	20×10^3 colonies (100%)	500 colonies (2.5%)

1% sodium dodecyl sulphate and 0.2% bromophenol blue, the samples (20 μl well^{-1}) were loaded on to the gel. Electrophoresis was carried out for 150 min at 120 V and 40 mA. The gels were stained in ethidium bromide (5 $\mu g\ ml^{-1}$) for 30 min and then seen under long wavelength UV illumination. Photographs of the gel bands were taken using 2B clear and 23 A orange filter. Lambda DNA digested with *Hin*dIII was used as reference for estimating the molecular weight of the unknown plasmid DNAs.

Acridine orange, ethylene diamine tetraacetic acid, disodium salt (Na_2EDTA), ethidium bromide, bromophenol blue, and sodium dodecyl sulphate (SDS) were obtained from Sigma Chemical Co, USA. All other biochemicals were of Analar grade of British Drug Houses (Glaxo), India. The gases used were from Indian Oxygen Ltd, Bombay.

Results

Table 1 presents the data of growth, heterocyst frequency, acetylene reduction and UV survival of wild type and the resistant strain (designated as Nl_{10}). The resistant strain grew slowly, had similar heterocyst frequency but lower acetylene-reducing activity. The observed effect may be attributed to the partial damage to photosynthetic apparatus of the resistant strain[7].

The lethal UV dose for parent strain was two min. Acridine orange (1 $\mu g\ ml^{-1}$) was not inhibitory to either strain. Therefore, this concentration was used for pre-treatment experiments. For the curing experiment, exponential cultures of UV-resistant strain were subcultured six times in nitrogen-free medium supplemented with 1 $\mu g\ ml^{-1}$ of acridine orange. Then, the homogenized suspension was centrifuged, washed and exposed to UV irradiation for 10 min and finally 10 μl of suspension (4×10^3 colony-forming units per plate) was plated. After 15 days, colonies were scored for per cent curing of UV resistance. It is clear from the curing experiment (Table 1) that the resistant character was lost after acridine orange pretreatment of the resistant strain.

Figure 1 shows the electrophoretogram of treated (with 1 $\mu g\ ml^{-1}$ of acridine orange) and pre-treated strains (Figure 1, lanes 2 and 3) respectively. Lane 1 represents *Hin*dIII digested λ-DNA fragments and

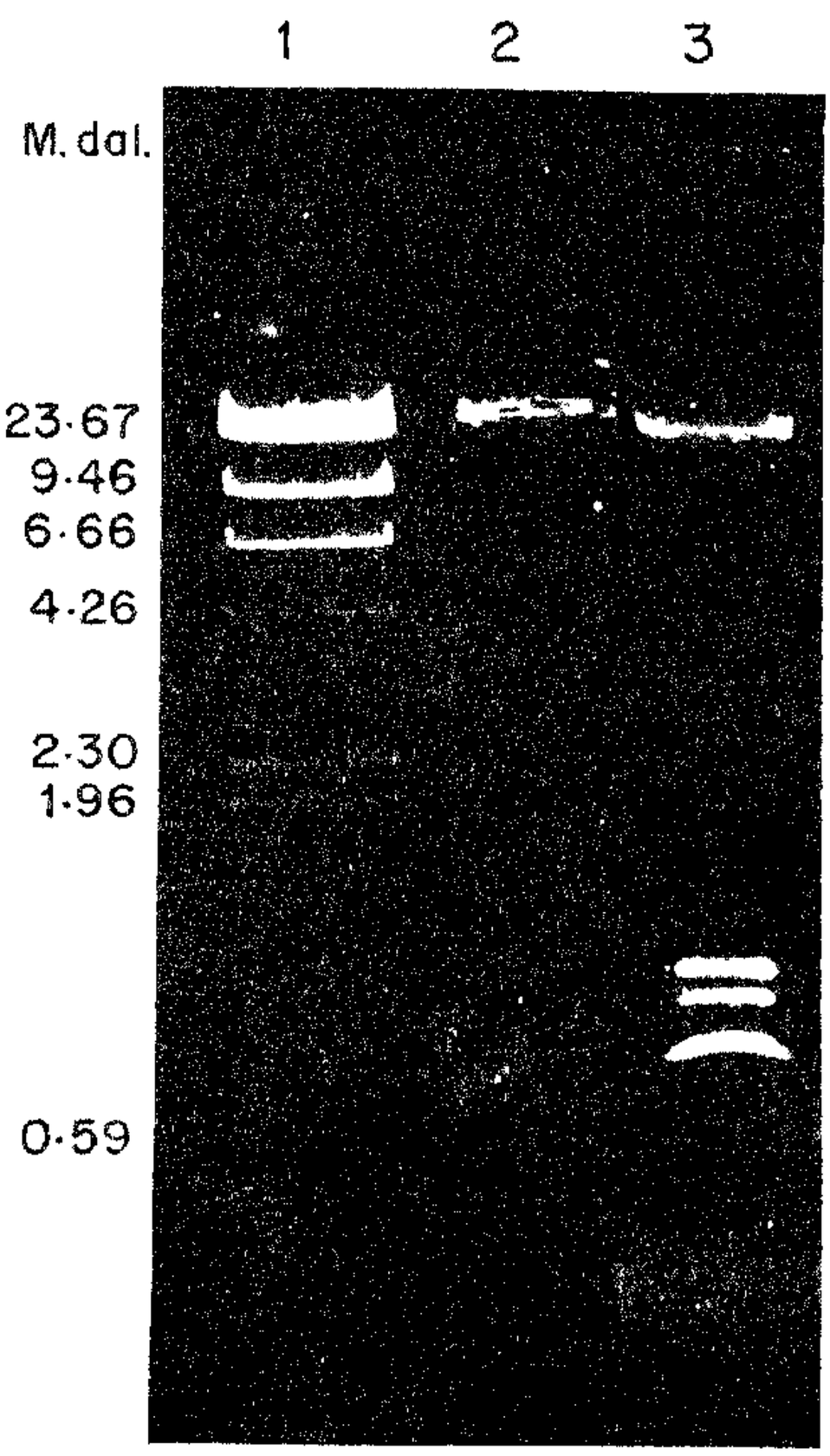

Figure 1. Agarose gel electrophoretic separation of crude lysate DNA from treated (lane 2) and pre-treated (lane 3) strains of *Nostoc linckia*. Lambda *Hin*dIII DNA digest used as reference standard P, lane 1.

shows seven bands corresponding to linear fragments of different molecular weight classes. The seventh band was seen under UV illumination and it was very faint and 0.8 cm downward of the third plasmid band of pre-treated resistant (Nl_{10}) strain. This Nl_{10} strain shows three plasmid bands. With reference to the λ-DNA fragments, all the three plasmid bands were found to lie between 1.96 and 0.59 Md. This is only a rough

approximation, and exact determination will require further analysis. Interestingly, when treated with acridine orange, the Nl_{10} strain lost all the three covalently closed circular DNA bands. Acridine orange-mediated curing of plasmids is evident from the loss of plasmid bands of treated strain (Figure 1, lane 2) but the loss of UV resistance character raises the question whether this trait is plasmid-borne. Although extant information on cyanobacterial plasmids indicates that they are cryptic, the change observed by us seems to indicate that plasmids may possibly have a role to play in UV resistance in *N. linckia*.

Acridines have been employed to cure or disinfect bacterial cells carrying sex factor or other episomes in an autonomous state in the cytoplasm and also to distinguish between their autonomous state, in which they are susceptible to curing by acridines, and the integrated state, in which they are not affected by acridines[12]. The loss of UV resistance seems to be due to loss of a particular class of plasmid, harbouring genes for UV resistance.

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