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Intergeneric transfer of streptomycin-resistance marker between two blue-green algae

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Abstract. Intergeneric transfer of streptomycin-resistance marker from a unicellular blue-green alga *Anacystis nidulans* to a filamentous blue-green alga *Anabaena doliolum* was demonstrated. Mutants of *A. nidulans* resistant to streptomycin, occurring spontaneously or mutagenically-induced could be isolated easily. Naturally occurring streptomycin-resistant mutants of *A. doliolum* could not be detected. Attempts at isolating such mutants either in nitrogen-free medium or in nitrate containing medium were unsuccessful. However, a streptomycin-resistant strain (recombinant) of *A. doliolum* could be isolated in a mixed culture of streptomycin-sensitive *A. doliolum* and streptomycin-resistant *A. nidulans*.

Keywords. Intergeneric-cross; streptomycin-resistance; *Anabaenadoliolum; Anacystis nidulans;* genetic recombination.

1. Introduction

Compared to studies on the genetics of nitrogen-fixing bacteria, very little is known on the genetics of nitrogen fixation in blue-breen algae (Van Baalen 1973; Delaney *et al* 1976). Kumar (1962) for the first time demonstrated parasexuality in a unicellular blue-green alga *Anacystis nidulans*. This phenomenon of recombination was later confirmed in *A. nidulans* by Bazin (1968) and in *Cylindrospermum majus* by Singh and Sinha (1965). Singh (1967), by selecting sporulation as the genetic marker, showed the occurrence of recombination in the filamentous heterocystous nitrogen-fixing blue-green alga *A. doliolum*. Stewart and Singh (1975) have reported the transfer of gene(s) for nitrogen fixation (*nif* gene) from wild-type nitrogen-fixing *Nostoc muscorum* to a non-nitrogen-fixing mutant of the same alga.

Until recently most of the studies on genetic transfer in blue-green algae were confined to the transfer of markers between mutants of the same species. However, Devilly and Houghton (1977) have reported intergeneric transformation of drug-resistance marker between two Chroococcalean algae *Gloeocapsa alpicola* and *Anacystis nidulans*. Intergeneric transfer of markers between different blue-green algae is of considerable significance in the context of current attempts to transfer *nif* gene from nitrogen-fixing organisms to those which do not fix nitrogen.

In the present paper we describe the transfer of streptomycin-resistance marker between two widely different genera of blue-green algae, viz., *A. doliolum* (Nostocales) and *A. nidulans* (Chroococcales).

2. Materials and methods

2.1. *Growth conditions*

Unialgal and clonal cultures of the nitrogen-fixing, filamentous, sporulating blue-green alga *A. doliolum* Bharadwaja and the non-nitrogen-fixing unicellular blue-green alga *A. nidulans* (Myers' strain) were used. The two algae were grown in mineral salts medium of *Hughes et al* (1958) with the modification that $NaNO_3$ was used at 0·01 M concentration. The same medium without nitrate was designated as the " nitrogen-free medium ". The organisms were grown in liquid medium or on medium solidified with 1% Bacto Difco agar, and were incubated under a light intensity of 1200–1500 lux, at $25 \pm 1°C$.

Growth of the algae was measured by taking absorbance in a Bausch and Lomb Spectronic 20 spectrocolourimeter at 665 nm. *A. doliolum*, used as a recipient cell, was harvested during the late exponential phase of growth, because the recombination in bacteria is more under these conditions. Streptomycin sulphate was obtained from Sigma Chemical Company, USA, and was used after sterilisation by membrane filtration.

Sensitivity levels of the two algae towards different concentrations of streptomycin were determined separately in liquid cultures as well as on agar plates.

Plates supplemented with different concentrations of streptomycin were inoculated with a thin suspension of the alga and the colonies were counted after 20 days of incubation. Plates without any antibiotic supplementation served as controls. The minimal inhibitory concentration of streptomycin for each alga was determined.

To determine the minimal inhibitory concentration in liquid cultures, the two algae were inoculated separately in tubes containing 10 ml of medium supplemented with different concentrations of the antibiotic. Controls without streptomycin were also kept.

2.2. *Isolation of donor streptomycin-resistant A. nidulans*

Several strains of *A. nidulans* resistant to streptomycin were isolated at a frequency of 1×10^{-9} by plating a dense suspension of exponentially growing cells on agar plates supplemented with 5·0 μg streptomycin/ml. Later, a strain resistant to 50 μg streptomycin/ml was selected. This streptomycin-resistant strain designated as *str*^{r} *A. nidulans*, was subcultured in the absence of the antibiotic several times without losing its resistance and was maintained routinely in streptomycin-supplemented medium.

2.3. *Isolation of streptomycin-resistant A. doliolum*

Several attempts were made to isolate a streptomycin-resistant strain of *A. doliolum*, either spontaneously occurring or after treatment with mutagenic agents like ultraviolet radiation or N-methyl-N'-nitro-N-nitrosoguanidine (NTG).

For isolating a spontaneously occurring streptomycin-resistant mutant of *A. doliolum*, germinating spores as well as filaments from late exponential phase of growth were plated several times on plates containing nitrogen-free medium or nitrate-containing medium supplemented with 5 μg streptomycin/ml. Each time

30 plates were kept and experiments were carried out by plating 10^6 to 5 x 10^7 colony forming units/plate.

Germinating spores as well as filaments from late exponential phase of growth were treated with NTG (100 μg/ml) for 30 min (this treatment permits about 50% survival). For UV treatment, the algal suspension was exposed to a UV lamp (main emittance at 253 nm) at a distance of 48 cm. The treatment was given for 2 min and 1 ml aliquot samples were plated on medium supplemented with 5 μg streptomycin/ml. The plates were incubated in the dark for 24 h so as to avoid photoreactivation and then incubated in the presence of light (1200–1500 lux) at 25°C.

This wild type streptomycin-sensitive nitrogen-fixing *A. doliolum*, grown either in nitrogen-free or nitrate containing medium, was used as the recipient in recombination experiments.

2.4. *Incubation of mixed cultures*

A. nidulans (donor) and *A. doliolum* (recipient) strains were mixed in 1 : 5 ratio, based on absorbance. They were incubated for seven days with 2 ml of either nitrogen-free or nitrate containing medium. Controls with only *A. doliolum* or *A. nidulans* were also kept. Each experiment was repeated twice.

Mixed cultures were washed with nitrogen-free medium to remove traces of nitrate. After crushing with sterilised glass beads they were plated on medium supplemented with 5 μg streptomycin/ml. They were also plated on nitrogen-free medium with no streptomycin to determine the viability of the recipient. The plating efficiency of the alga was found to be 90–93%.

On a nitrogen-free medium containing streptomycin, cells of *A. nidulans*, whether streptomycin-sensitive or streptomycin-resistant, would not grow because they are unable to fix nitrogen. The *str*^{s} *A. doliolum* cells would also be killed because they are sensitive to this concentration of streptomycin. But any mutant or recombinants resistant to streptomycin would grow on this medium.

The *str*^{r} *A. doliolum* colonies (recombinants) were picked up, grown in liquid medium, and were checked for their resistance to streptomycin. Their growth behaviour in nitrogen-free medium and in nitrate-containing medium supplemented with different concentrations of streptomycin was studied in liquid cultures.

3. Results

3.1. *Sensitivity of the two algae towards streptomycin*

A. nidulans tolerated upto 0·1 μg/ml of streptomycin in both liquid and agar medium (table 1); 0·2 μg/ml streptomycin was lethal. The generation time of the alga increased with increase in antibiotic concentration.

A. doliolum, on the other hand, showed differential tolerance to streptomycin on agar and liquid medium as well as in nitrogen-free and nitrate containing media. On plates, it survived up to 0·5 μg streptomycin/ml. In liquid (nitrogen-free) medium, it was more sensitive to streptomycin (0·01 μg/ml) whereas in nitrate-containing medium it grows in the presence of 0·05 μg/ml, higher concentrations being lethal (table 1).

Table 1. Generation time* (h) of wild-type alga and of streptomycin-resistant strains of *A. nidulans* and of *A. doliolum* at different concentrations of streptomycin.

Strains	Streptomycin concentration (μg/ml)								
	0·0	0·001	0·01	0·05	0·10	0·50	1·0	5·0	50·0
Wild-type:									
A. nidulans	39·0	42·0	52·0	61·3	82·3	—	—	—	—
A. doliolum (in nitrogen-free medium)	58·0	58·5	65·0	—	—	—	—	—	—
A. doliolum (in nitrate-containing medium)	52·0	56·0	61·0	68·0	—	—	—	—	—
Resistant:									
A. nidulans	38·5	42·0	41·0	42·5	42·0	42·0	42·0	42·5	42·5
A. doliolum (in nitrogen-free medium)	58·0	58·0	59·0	59·5	62·0	—	—	—	—
A. doliolum (in nitrate-containing medium)	53·5	55·6	56·0	58·0	58·5	58·5	58·5	59·0	—

— indicates no growth

* Value given (average of triplicates) is the time taken by the growing algal culture to double its original absorbance in exponential phase of growth and is determined directly from the growth curve.

The experiment was repeated at least five times but no streptomycin-resistant colonies appeared even after six weeks of incubation on any of these plates.

The reason for the lack of occurrence of spontaneous streptomycin resistant *A. doliolum* in nitrogen-free medium or in nitrate-containing medium is not clear; either the alga is very difficult to mutate to drug resistance or the chance of occurrence of streptomycin resistant mutant is extremely low i.e., less than 1 in 10^{10} colony forming units.

3.2. *Transfer of streptomycin-resistance character from Str^r A. nidulans to str^s A. doliolum*

Control plates, on which cells of *A. nidulans* or *A. doliolum* obtained from single cultures were plated, did not show any appearance of nitrogen-fixing *A. nidulans* or *str^r A. doliolum*. But the plates on which inocula from mixed cultures were plated showed the appearance of a few *str^r A. doliolum* colonies as judged from their morphology.

Table 2 shows the number of colony forming units added per plate, the number of recombinant colonies appeared and the recombination frequencies. The frequency determinations are based on viable cell counts. The observed frequency of these *str^r A. doliolum* (which is virtually the result of transfer, establishment in recipient and expression of streptomycin-resistance character) was higher when the donor and recipient were incubated in nitrate-containing medium. Since in the control series

Table 2. Frequency of appearance of streptomycin-resistant recombinants.

Donor	Recipient	Medium	Number of C.F.U. of recipient alga inoculated per plate	Number of recombinant colonies ***	Frequency of appearance of recombinants
A. nidulans str^r	*A. doliolum str*^{s} (nitrogen-fixing)	Nitrogen-free medium	$1{\cdot}5 \times 10^8$	3*	$6{\cdot}6 \times 10^{-8}$
,,	,,	Nitrate-containing medium	$1{\cdot}0 \times 10^8$	8*	$2{\cdot}6 \times 10^{-7}$
A. doliolum str^r (nitrogen-fixing)	*A. nidulans str*^{s}	Nitrate-containing medium	$9{\cdot}0 \times 10^8$	68**	$2{\cdot}5 \times 10^{-7}$

C.F.U. Colony forming units

*Str^r *A. doliolum* screened by plating the mixed culture of donor and recipient on nitrogen-free agar plates supplemented with 5 μg streptomycin/ml

Str^r A. nidulans selected and screened by plating the mixed culture of donor and recipient on nitrate-containing agar plates supplemented with 5 μg streptomycin/ml. Donor *str^r A. doliolum* colonies appear at 90-92% plating efficiency and colonies of *str^r A. nidulans* recombinants were differentiated over the background colonies of donor *srt^r A. doliolum* by their morphology under the binocular microscope.

***Average from two experiments, each having 30 plates.

no resistant colonies appeared, it may be inferred that these colonies arose as a result of recombination and not mutation. The other class of possible recombinants, viz., nitrogen-fixing *A. nidulans*, was not detected on any of the plates though the possibility of this transfer cannot be entirely ruled out.

3.3. *Growth of str*^{r} *A. doliolum in different concentrations of streptomycin*

A colony which showed a growth rate similar to wild-type *str^r A. doliolum* was selected from the several *str^r A. doliolum* colonies isolated. This strain was grown in antibiotic-free medium for several generations without losing its resistance, thus showing the stable nature of the character.

Its growth behaviour in nitrogen-free medium and nitrate-containing medium supplemented with different concentrations of streptomycin was studied. Table 1 shows the growth of wild-type as well as of the recombinants in terms of generation time.

The recombinant showed greater resistance in nitrate-containing medium as compared to nitrogen-free medium. In nitrogen-free liquid medium it could grow in the presence of up to 0·1 μg streptomycin/ml thus showing a ten-fold increase in resistance over the wild-type. However, in nitrate-containing medium, whereas the parent could tolerate up to 0·05 μg streptomycin/ml, the recombinant tolerated up to 5 μg/ml, thus showing a 100-fold increase in its resistance.

3.4. *Transfer of streptomycin-resistance character from str^r A. doliolum to str^s A. nidulans*

Table 2 gives the recombination frequencies observed. The frequency of appearance of *str^r* recombinant *A. nidulans*, though low, was higher than that of spontaneous *str^r* mutants of *A. nidulans*.

4. Discussion

Kumar (1964) has studied the effect of two antibiotics on growth and pigment production in *A. nidulans*. He successfully isolated streptomycin-resistant strains of *A. nidulans*. Kumar and Kaushik (1971) also studied the effect of certain antibiotics on growth and development of *A. doliolum* and *Fischerella mucicola*; however, they used a very low concentration of streptomycin (0·001 μg/ml). This concentration brought about an increase in lag phase of the alga as well as a decrease in its growth rate, but no attempts were made to isolate a streptomycin-resistant mutant of any of the algae. Singh (1973) has reported an increase in the spontaneous mutation frequency of a non-sporulating mutant of *A. doliolum* in the presence of combined nitrogen. But in this case no streptomycin-resistant *A. doliolum* colonies appeared even in nitrate containing medium.

The results presented here indicate perceptibly frequent intergeneric transfer of drug-resistance marker between two blue-green algae. The recombinants appeared only when the donor and recipient were incubated together in nitrate-supplemented medium. Also the recombinant showed greater resistance to the antibiotic in nitrate-containing medium. The explanation for this is, however, not clear.

In various nitrogen-fixing bacteria, the localisation of *nif* gene and drug-resistance markers into certain extrachromosomal determinants (plasmids) has been confirmed; but in blue-green algae we do not know anything about the locus of *nif* gene marker or of drug-resistance markers. Kumar *et al* (1967) have indicated the localisation of high level streptomycin-resistance character on plasmids in *A. nidulans*.

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