Genetic Transfer in a Nitrogen-fixing Filamentous Cyanobacterium

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Genetic transfer has been studied between wild-type *Nostoc muscorum* and a multiply marked mutant strain. Experiments conducted with heat-killed donor and live recipient cells indicated that the transfer occurred in both directions and may have been the result of transformation rather than conjugation. This conclusion was supported by the sensitivity of the process to DNAase. There was some indication of linkage between *het* and *eth*.

INTRODUCTION

Genetic recombination in mixed cultures of cyanobacterial mutants has been described in the unicellular *Anacystis nidulans* (Kumar, 1962; Bazin, 1968) and the filamentous *Cylindrospermum majus* (Singh & Sinha, 1965), *Anabaena doliolum* (Singh, 1967) and *Nostoc muscorum* (Stewart & Singh, 1975; Padhy & Singh, 1978). Genetic transformation in cyanobacteria mediated by purified DNA, has been described only for unicellular forms (Shestakov & Khyen, 1970; Herdman, 1973; Orkwiszewski & Kaney, 1974; Devilly & Houghton, 1977). Transformation mediated by nucleic acids excreted by the cells of *Anacystis nidulans* into the culture medium has also been reported (Herdman, 1973).

Nostoc muscorum is a heterocystous filamentous N₂-fixing organism which can be conveniently manipulated. Single cells can be easily obtained and the organism also forms discrete colonies which are convenient for genetic analysis. In the present investigation, auxotrophic and drug-resistant mutants have been utilized to study genetic recombination in *N. muscorum* further. A system of transformation, operating in mixed cultures of parental cells, is described.

METHODS

Organism and growth conditions. Wild-type *Nostoc muscorum* was kindly supplied by the late Professor R. N. Singh, Banaras Hindu University, Varanasi, India. The minimal medium (MM) used throughout the study was medium BG-11 (Allen, 1968) with NaNO₃ omitted. Axenic cultures were maintained in MM in a culture room at 27 °C, with cool white fluorescent tubes producing about 2500 lx at the culture surface. The culture flasks (125 ml) were shaken at hourly intervals in order to prevent clumping and wall growth. For colony growth 0.1 ml of a diluted culture (200 cells ml⁻¹) was spread on MM agar plates. Following incubation, single colonies were transferred with sterile glass capillaries to culture tubes containing 10 ml of sterile liquid MM. The resulting clonal populations were used throughout. Cell counts were made in a Neubauer counting chamber.

Mutagenesis. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) was used as mutagen. Exponentially growing cells were harvested by membrane filtration (pore size 0.8 µm), washed with 0.05 M-Tris/maleic acid buffer, pH 8.0 (Adelberg *et al.*, 1965) and resuspended in 15 ml of Tris/maleic acid buffer, pH 5.0. Freshly prepared saturated NTG solution (in water) was added to 10 ml of cell suspension to give a final concentration of about 100 µg ml⁻¹. The suspension was stirred on a magnetic stirrer for 20 min, filtered on membrane filters and washed five times with MM (pH 8). The cells were then suspended in MM, and 0.1 ml portions were spread on MM agar plates

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supplemented with ethionine (0.04 mg ml^{-1}) and incubated for 7 to 10 d. The surviving colonies were scored as ethionine-resistant (Eth^R) mutants and were isolated and re-tested for their resistance. The Eth^R mutant employed in further studies was non-heterocystous (Het^-).

A valine auxotroph (Val^-) was derived from this $\text{Eth}^R \text{ Het}^-$ mutant by further mutagenesis with NTG, and was isolated by the penicillin enrichment technique developed by Davis (1948) and adapted by Ingram *et al.* (1972) and Herdman & Carr (1972) for use with cyanobacteria. Following mutagenesis, cells were inoculated into a flask containing MM plus penicillin ($150 \mu\text{g ml}^{-1}$). Seven transfers were made at 48 h intervals to enrich the mutant population. The cells were then centrifuged, suspended in fresh MM and inoculated into a series of test tubes containing MM plus valine. These tubes were incubated in the light at 27°C and growth was observed. Alternatively, the cells were plated on MM plus valine, the resulting colonies were replica plated on to MM, and Val^- mutants were identified by their growth response. The Val^- mutant studied required $20 \mu\text{g valine ml}^{-1}$ for optimum growth.

Recombination experiments. All recombination experiments were started with suspensions of single cells, obtained by shaking with sterilized glass beads (which gave 98% survival). The cells were washed two or three times with sterile distilled water and MM by centrifuging at $3000 \text{ rev. min}^{-1}$. Suspensions of each strain in MM ($3 \times 10^6 \text{ cells ml}^{-1}$) were mixed together and agitated continuously on a magnetic stirrer, or slowly by hand, at 27°C . Recombination procedures were as reported by Singh *et al.* (1965). Recombinants were selected on agar plates, counterselecting the parental types, and were scored repeatedly for stability of characters. Het^- recombinants were identified microscopically. Frequencies of recombinants were calculated in relation to the total cell population plated.

DNAase treatment. DNAase ($20 \mu\text{g ml}^{-1}$) and MgCl_2 (2 mM) were added to a mixed culture of cells and the mixture was incubated for 4 or 8 h at $27 \pm 2^\circ\text{C}$, pH 7.5.

Chemicals. NTG was obtained from Fluka, Buchs, Switzerland, and DNAase from Sigma. Analytical grade chemicals, obtained from BDH, were used throughout.

RESULTS AND DISCUSSION

Recombinant formation with equal numbers of wild-type and mutant cells. Equal numbers of cells ($3 \times 10^6 \text{ ml}^{-1}$) of the wild-type and the $\text{Het}^- \text{Eth}^R \text{Val}^-$ mutant were mixed together. At intervals, cells were plated on MM plus ethionine, thereby selecting against both of the parental strains and selecting for $\text{Val}^+ \text{Eth}^R$ recombinants. The maximum frequency of recombinant formation occurred when the cells were incubated together for 4 h, the frequency decreasing (perhaps due to extracellular digestion of genetic material) with further increase in the incubation time (Table 1). Although all the recombinants were able to grow in MM, 30 to 37.5% of them were Het^- . It is not clear how the latter were able to grow aerobically at the expense of N_2 . The appearance of $\text{Het}^+ \text{Eth}^R$ recombinants indicated that ethionine resistance and the ability to form heterocysts are controlled by two different genes and that mutation in both had been induced simultaneously.

Effect of varying proportions of wild-type and mutant cells on the frequency of gene transfer. When the cell density of the wild-type was varied (from 1×10^2 to $6 \times 10^6 \text{ cells ml}^{-1}$) and that of the mutant ($\text{Het}^- \text{Eth}^R \text{Val}^-$) was kept constant ($3 \times 10^6 \text{ cells ml}^{-1}$), the frequency of recombinants increased with increasing cell density of the wild-type (Table 2). However, the frequency of $\text{Het}^- \text{Eth}^R \text{Val}^+$ colonies among the recombinants was always more than 50%, which indicated the possible linkage of *het* and *eth*.

When the concentration of the mutant cells was varied and that of the wild-type kept constant, the frequency of recombinants did not show any appreciable change except at very low concentrations of the mutant strain (Table 3). The frequency of $\text{Het}^- \text{Eth}^R \text{Val}^+$ colonies was always more than 58%, again indicating possible linkage between *het* and *eth*.

Effect of heat killing of cells on gene transfer. To study the nature of the gene transfer process further, each of the strains was killed by heat treatment (100°C for 10 min) and then mixed with the appropriate live strain in equal proportions ($3 \times 10^6 \text{ cells ml}^{-1}$). The viability of heat-killed cells was less than 1×10^{-6} . When the wild-type cells were killed the maximum frequency of $\text{Eth}^R \text{Val}^+$ recombinants was 0.07% after incubation with living $\text{Het}^- \text{Eth}^R \text{Val}^-$ cells for 2 to 8 h. This value is quite close to those obtained after mixing live strains (Table 1)

Table 1. *Effect of duration of contact on recombinant formation in N. muscorum*

Equal volumes of suspensions (3×10^6 cells ml⁻¹) of the wild-type and the Het⁻ Eth^R Val⁻ mutant were mixed together and incubated for the periods shown before plating on MM plus ethionine. The spontaneous frequency of mutation to ethionine resistance was 2×10^{-7} ; in each experiment 2.4×10^6 cells were plated.

Duration of contact before plating (h)	No. of recombinants (Val ⁺ Eth ^R)	Frequency of recombinants (%)
4	2523	0.10
8	2164	0.09
14	1452	0.06
24	487	0.02

Table 2. *Effect of cell density of the wild-type on recombinant formation in N. muscorum*

Recombination experiments were performed as described in Methods, with the cell density of the Het⁻ Eth^R Val⁻ mutant kept constant at 3×10^6 cells ml⁻¹ and that of the wild-type varied as shown.

No. of wild-type cells ml ⁻¹	Total no. of cells plated	Frequency of recombinants (%)
6×10^6	1.1×10^6	0.12
5×10^6	1.0×10^6	0.10
4×10^6	1.75×10^6	0.10
3×10^6	1.5×10^6	0.10
2×10^6	1.25×10^6	0.09
1×10^6	1.0×10^6	0.08
1×10^5	7.5×10^5	0.06
1×10^4	1.5×10^5	0.02
1×10^3	1.5×10^5	0.006
1×10^2	1.5×10^5	0.001

Table 3. *Effect of cell density of the mutant on recombinant formation in N. muscorum*

Recombination experiments were performed as described in Methods, with the cell density of the wild-type kept constant at 3×10^6 cells ml⁻¹ and that of the Het⁻ Eth^R Val⁻ mutant varied as shown.

No. of mutant cells ml ⁻¹	Total no. of cells plated	Frequency of recombinants (%)
6×10^6	1.1×10^6	0.10
5×10^6	1.0×10^6	0.10
4×10^6	1.75×10^6	0.10
3×10^6	1.5×10^6	0.10
2×10^6	1.25×10^6	0.10
1×10^6	1.0×10^6	0.10
1×10^5	7.5×10^5	0.10
1×10^4	7.5×10^5	0.05
1×10^3	7.5×10^5	0.02
1×10^2	7.5×10^5	0.004

and indicated that genes are transferred from the wild-type to the mutant. When the mutant strain was heat-killed, the maximum frequency of recombinants was 0.018%. Thus, gene transfer from the mutant to the wild-type was also taking place. These results demonstrated that both strains were able to donate and receive genetic material, and that transfer did not require a live donor strain.

Effect of DNAase on gene transfer. DNAase treatment reduced recombinant formation by at least 97%, the frequency of recombinants decreasing to 0.003% and 0.002% following incubation with DNAase for 4 h and 8 h, respectively. This suggests that gene transfer was the result of transformation. The residual transfer observed following DNAase treatment may

result from incomplete digestion of the DNA, or from an alternative gene transfer system which operates at low frequency.

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