TRANSFER OF NITROGEN-FIXING (NIF) GENES IN THE BLUE-GREEN ALGA NOSTOC MUSCORUM

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ABSTRACT: Mutants of the nitrogen-fixing blue-green alga Nostoc muscorum have been isolated which do not fix nitrogen or reduce acetylene, and which are resistant to streptomycin (1000 $\mu g\ ml^{-1}$). One such mutant (nif$^-$st-R) was crossed with the wild-type nitrogen-fixing streptomycin-sensitive parent (nif$^+$st-S) and under conditions which counterselected the latter, recombinants (nif$^+$st-R) were obtained at a frequency of up to 4.6 in 10^5 colonies. The frequency of spontaneous mutations or revertants of each parent growing alone was 1 in 10^7 or less. The higher yield of new genotypes from mixed cultures is interpreted as evidence of nif gene transfer in Nostoc muscorum.

INTRODUCTION: The blue-green algae are prokaryotic organisms which, in addition to being able to fix nitrogen, have a photosynthetic machinery similar to that found in the chloroplasts of higher plants (1, 2). There has been no previous report of the transfer of nitrogen-fixing (nif) genes in these organisms, although evidence for sexuality (3) and the transfer of auxotrophic and drug-resistant markers through transformation (4, 5, 6) have been reported in the non-nitrogen-fixing unicellular Anacystis nidulans. In addition nif gene transfer has been achieved in free-living heterotrophic bacteria (7, 8, 9, 10, 11). Here we report the isolation of stable non-nitrogen-fixing (nif$^-$) mutants of the wild-type nitrogen-fixing (nif$^+$) blue-green alga Nostoc muscorum and show that it is possible to transfer nif genes from the wild type (nif$^+$) to a nif$^-$ mutant. Physiological and morphological features of some of the recombinants are also considered.

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MATERIALS & METHODS

Nostoc muscorum, obtained initially from the Botany Department, Iowa State University, was used in axenic clonal culture. It was grown in modified Chu No. 10 medium (12) with, or without, combined nitrogen (1.0 mM NH_4Cl) in liquid culture, or solidified with 1.5% (w/v) agar. The light intensity was continuous at 3,000 lux and the temperature was 26°C. Acetylene reduction was assayed as described previously (13). N-methyl-N-nitro-N-nitroso-guanidine (NTG) was supplied by Sigma Ltd., London and was sterilised by filtration. Other chemicals were used at the highest purity available from the British Drug Houses, Poole.

RESULTS & DISCUSSION

The Isolation of Nif^- Mutants

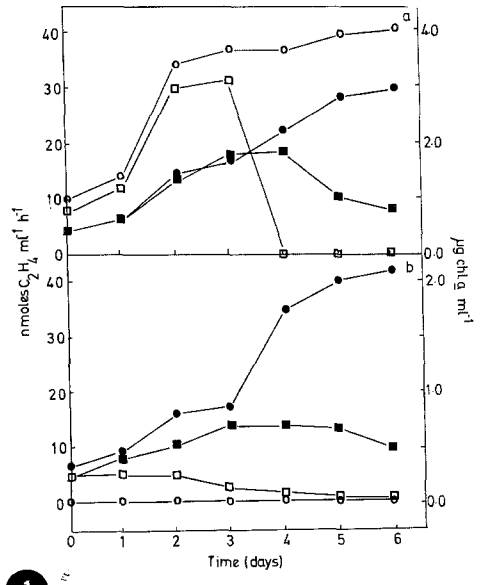
Nitrogenase-less (*nif*$^-$) mutants of *Nostoc muscorum* were obtained by taking log-phase cultures of wild-type nitrogen-fixing (*nif*$^+$) *Nostoc muscorum* which had been grown in batch culture in liquid nitrogen-free medium, and treating these with NTG (100 µg ml^{-1}) for 60 min at 37°C. This treatment permitted approximately 20% survival. After removal of NTG by centrifugation and washing, the mutagenised suspensions were inoculated into medium containing NH_4Cl and grown for 3 days. The algae were then washed free of combined nitrogen and subcultured into fresh nitrogen-free medium containing 100 µg ml^{-1} of penicillin. This selectively killed filaments which were potentially capable of growing in nitrogen-free medium, that is *nif*$^+$ algae, and thus led to an enrichment of *nif*$^-$ mutants. After 48 h in the presence of penicillin, the algal material was washed free of penicillin and inoculated

onto solid medium containing NH_4Cl. Pieces of colonies which developed were picked off and tested for growth in nitrogen-free medium. Strains which failed to grow after at least 2 - 3 weeks in nitrogen-free medium, were regarded as presumptive *nif*$^-$ mutants. Their inability to reduce acetylene to ethylene (13) and to grow (14) in nitrogen-free liquid culture under aerobic and microaerobic conditions was also established.

Streptomycin resistance was then introduced into a *nif*$^-$ mutant as follows. A suspension of a *nif*$^-$ clone which, like wild-type (*nif*$^+$) material, was killed by 0.3 ug ml^{-1} streptomycin, was plated onto medium containing NH_4Cl and streptomycin (10 ug ml^{-1}) and screened for streptomycin-resistant colonies. The observed frequency of streptomycin-resistant clones (*st-R*) was approximately 1 in 10^9. This is a similar frequency to that at which streptomycin-resistant strains form after NTG treatment in *Anacystis* (3). A strain was then selected which could grow consistently in the presence of 1000 ug ml^{-1} streptomycin and which also maintained its resistance to streptomycin when grown on streptomycin-free medium. This non-nitrogen-fixing streptomycin-resistant mutant (*nif*$^-$*st-R*) was used in subsequent work and was maintained routinely in NH_4Cl-containing medium in the presence of 1000 ug ml^{-1} streptomycin.

Nif *Transfer from* nif$^+$st-S *Colonies to* nif$^-$st-R *Colonies*

In previous studies on gene transfer in bacteria a commonly used technique (15), which has also been used in the case of *nif* transfer in bacteria (7), has been the crossing of prototrophs sensitive to streptomycin with streptomycin-



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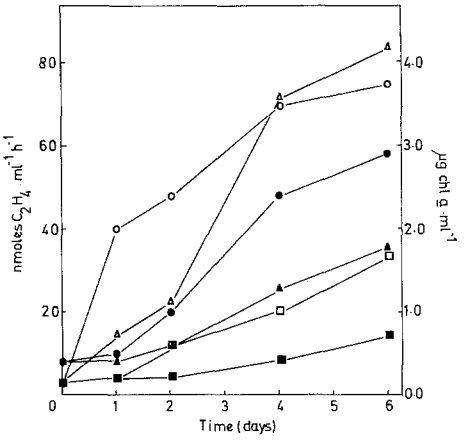

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Figure 1a Growth (measured as chlorophyll a) (●–●) and acetylene reduction (O–O) by wild-type (nif+st-S) Nostoc muscorum in nitrogen-free medium over a 6-day period in the absence of streptomycin, and growth (■–■) and acetylene reduction (□–□) by the same organism in a second series to which streptomycin (1000 µg ml^{-1}) was added after 3 days. Each value is the mean of triplicate determinations.

1b Growth of the parent (nif⁻st-R) on NH_4Cl-containing (●–●) and in nitrogen-free medium (□–□) in air over a 6-day period in the presence of streptomycin (1000 µg ml^{-1}). In a second series in which growth (■–■) and acetylene reduction (O–O) were measured, the NH_4Cl-containing medium was changed to nitrogen-free medium after three days. Nitrogenase activity was never detected. Each treatment was carried out in triplicate. The entire experiment detailed above was repeated under microaerobic conditions instead of in air and similar results were obtained

Figure 2. Growth (measured as chlorophyll a) and acetylene reduction by: wild-type Nostoc muscorum, (●–●, growth; O–O, acetylene reduction), by Recombinant 1 (▲–▲, growth; △–△, acetylene reduction) and by Recombinant 2 (■–■, growth; □–□, acetylene reduction) in nitrogen-free medium. Each value is the mean of triplicate determinations.

resistant auxotrophs under conditions which counterselected the prototrophic streptomycin-sensitive donor. The same basic technique was used here with Nostoc muscorum.

Before attempting genetic recombination experiments, routine tests were carried out to re-check the characteristics of the parents. The wild type parent (nif$^+$st-S), which had been growing in NH_4Cl-containing medium, was transferred to nitrogen-free streptomycin-free medium and checked for its ability to grow in this medium. As Fig. 1a shows, the alga grows and reduces C_2H_2 in nitrogen-free medium over the 6-day period in the absence of streptomycin. However, when streptomycin (1000 μg ml^{-1}) is added to a second series after 3 days, acetylene reduction is rapidly inhibited and growth ceases. Morphologically, this parent (nif$^+$st-S) is filamentous with numerous heterocysts.

The other parent was tested for growth and acetylene reduction in the presence and absence of NH_4Cl, in medium containing 1000 ug ml^{-1} streptomycin. As Fig. 1b shows, it grows in the presence of streptomycin on combined nitrogen but dies in nitrogen-free medium, both under aerobic and microaerobic conditions (13). In none of the treatments was there any evidence of acetylene reduction. These results confirm that this parent, which forms short non-heterocystous filaments, lacks nitrogenase and is streptomycin-resistant.

The nitrogen-fixing streptomycin-sensitive wild-type parent (nif$^+$st-S) was then crossed with the nif$^-$st-R parent by mixing aliquots of each together in 10 ml of medium containing NH_4Cl plus 1000 ug ml^{-1} streptomycin. In this culture medium the wild-type (nif$^+$st-S) would be killed

Table I

Observed frequencies of spontaneous mutations, spontaneous revertants and recombinants of Nostoc muscorum

Algal material	Frequency of nif$^+$st-R colonies			Presumed method of formation of nif$^+$st-R colonies
	Expt. I.	Expt. II	Expt. III	
nif$^+$st-S alone	1 in 10^9	<1 in 10^7	<1 in 10^7	Spontaneous mutations
nif$^-$st-R alone	1 in 10^7	<1 in 10^7	<1 in 10^7	Spontaneous mutations
nif$^+$st-S and nif$^-$st-R mixture	2.2 in 10^5	4.6 in 10^5	5.0 in 10^6	Genetic transfer

Twenty replicate plates were available in each series of each experiment. The medium onto which the algal materials were inoculated contained 1.0 mM NH_4Cl and 1000 μg ml^{-1} streptomycin. In all experiments the frequencies of the spontaneous mutants were lower than that of the recombinants at the P = 0.05 significance level.

rapidly (see Fig. 1) while the other parent (nif⁻st-R) would grow normally. Three days later, 0.1 ml aliquots of the mixed algal suspension were plated onto nitrogen-free medium containing streptomycin and scored for potential recombinants after incubation for 15 days. Spontaneous mutants or revertants of nif⁺st-S and nif⁻st-R were also scored by plating 0.1 ml of either parent alone onto nitrogen-free medium containing streptomycin. The frequencies of colony-forming units in the mixed suspension and in suspensions of each parent growing separately were determined by plating 0.1 ml aliquots of 1000 times-diluted suspensions onto NH_4Cl-containing medium.

Data obtained in three separate experiments are presented in Table I. In none of the experiments was there any evidence of the wild-type (nif⁺st-S) cells mutating spontaneously to nif⁺st-R in a population size of 10^7 colony-forming units, although in experiment I nif⁺st-R colonies were obtained at a frequency of 1 in 10^9. The observed frequency of nif⁻st-R to nif⁺st-R revertants in the control series was 1 in 10^7 in experiment I and less than that in experiments II and III. On the other hand, when nif⁺st-S algae were mixed with nif⁻st-R algae, the frequency of nif⁺st-R isolates was as high as 4.6×10^5. This frequency is at least 400 times higher than that attributable to spontaneous mutations to nif⁺st-R and appears to be due to the transfer of nif genes from the wild-type, which is selected against, to the streptomycin-resistant parent. It is impossible nevertheless to rule out the possibility that what appears to be nif gene transfer may be due to the transfer of some factor which allows the expression of latent nif genes in the non-nitrogen-fixing mutant. Further studies on this aspect are in progress.

Fig. 2 compares growth and acetylene reduction by the wild-type and by two of the recombinants. One recombinant grows and reduces acetylene well, while the other does so more slowly. Both recombinants have heterocysts and show less tendency to form short filaments than the non-nitrogen-fixing parent. They also differ slightly from the parents in pigmentation. The various other nitrogen-fixing recombinants which we have studied show rates of growth and acetylene reduction which are intermediate between these two extremes and again all are heterocystous isolates. These findings suggest that in nitrogen-fixing heterocystous algae there is a close linkage between the heterocyst (*het*) and nitrogenase (*nif*) genes.

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