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DNA-mediated Transformation in *Nostoc muscorum*, a Nitrogen-fixing Cyanobacterium

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Abstract

Genetic transformation of an auxotrophic valine-requiring (*val*) marker and a marker with resistance to *p*-fluorophenylalanine (*fpa*^{r}) as well has been demonstrated in *N. muscorum*. Transformation is primarily mediated by DNA and is insensitive to ribonuclease and proteinase. The kinetics of the frequency of transformation, which is dependent on the concentration of DNA, suggests a saturation phase. Transformants, though devoid of heterocysts, are able to grow in a medium lacking a combined nitrogen source.

Introduction

Genetic transformation occurs as an orderly continuum of physiological events. It begins with the onset of competence, followed by uptake of genetic material. Eventually there is molecular integration, replication and expression of the genome. Genetic transformation has already been reported in Cyanobacteria (Shestakov and Khyen 1970; Herdman 1973; Orkwiszewski and Kaney 1974; Stevens and Porter 1980). However, there is a lack of information on transformation in nitrogen-fixing heterocystous forms which could be important for understanding genetic regulation of nitrogen fixation and as a model system for fundamental studies in molecular biology and genetics. Cyanobacteria have a photosynthetic system similar to that found in the chloroplasts of higher plants (Stewart 1973). Genetic recombination following transfer of nitrogen-fixing genes has been reported in *Nostoc muscorum* (Stewart and Singh 1975). Evidence is presented in this paper for DNA-mediated transformation in *N. muscorum*.

Material and Methods

Culture Medium

N. muscorum was grown in medium BG-11 of Allen (1968), omitting nitrate. All cultures were maintained at 27±2°C in a culture chamber fitted with cool white fluorescent tubes, which gave illumination of approximately 2800 lux at the culture surface, and were repeatedly tested for purity using standard bacteriological methods.

Cloning

For cloning either spores (obtained in nutritionally exhausted cultures) or single cells (procured by sonically disrupting the suspended filaments) were employed. A suitably diluted suspension of cells (about 300 cells per millilitre) from axenic culture was aseptically spread on solid BG-11 medium (with plating efficiency of more than 60% as assessed by counting colonies). The plates were incubated

in the culture room at 27±2°C. Discrete colonies which appeared were subsequently transferred with sterilized pointed glass capillaries to culture tubes containing 10 ml of sterilized liquid medium. Populations were grown from these culture tubes and cloned again as described above. Finally colonies which appeared after three such clonings were used for the genetic investigations.

Growth Measurements

Growth experiments were carried out in 100-ml Erlenmeyer flasks fitted with cotton wool plugs. Growth was monitored by changes in optical density with a Bausch and Lomb Spectronic 20 spectrophotometer (1 cm path length) at 670 and 400 nm, to confirm growth both in terms of pigment synthesis and cell division, respectively. Growth rates are expressed as doublings per 24 h.

DNA Extraction

The techniques of Marmur (1961) and Stanier *et al.* (1971) were used to extract DNA from the appropriate donor strain. After the final precipitation with ethanol the DNA was dissolved in saline–citrate (0·15 M sodium chloride+0·015 M sodium citrate) and stored at 6°C. The diphenylamine test of Burton (1968) was used to estimate DNA, calf thymus DNA being used to prepare the standard curve. Absence of RNA was confirmed by the orcinol reaction (Zamenhof 1961). The spectral properties of purified DNA were as reported by Marmur (1961) and its molecular weight, as determined by analysis on agarose gels, was estimated to range from 10×10^6 to 11×10^6.

Transformation Procedure

Two mutants were used for genetic transformation experiments: *fpa*^{r}, which was selected on medium fortified with *p*-fluorophenylalanine at a concentration of 50 μg/ml, and *val*, a valine auxotroph which lacked heterocysts and which was resistant to ethionine at a concentration of 40 μg/ml. The valine auxotroph was isolated as a second-step mutant by the penicillin-enrichment technique following mutagenic treatment of the *fpa*^{r} cells with *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (NTG) at a concentration of 100 μg/ml. The *val* mutant was able to grow as well as the wild-type *val*$^+$, even in the absence of combined nitrogen in medium BG-11.

Recipient cells were harvested by centrifugation and washed and suspended in BG-11 medium, the final concentration being kept at 3×10^6 per millilitre. DNA was added to the suspension at a final concentration of 50 μg/ml and the mixture incubated in light (2800 lux) for the required time at 27±2°C. After the appropriate period of treatment, deoxyribonuclease (50 μg/ml) and $MgCl_2$ (final concn 2 mM) were added to the mixture and shaken to degrade the extracellular donor DNA. Alternatively, DNA uptake was terminated by chilling the test tube containing the suspension in an ice–water bath. The cells were pelleted by centrifuging at 3100 *g* for 10 min and the pellet washed three times with ice-cold minimal medium (BG-11). Serial dilutions with BG-11 were made (about 200 cells per plate) and cells plated and incubated at 27±2°C for 4–8 days.

Table 1. Mutagenesis of *N. muscorum* using *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (NTG)

Type of mutant isolated	Induced mutation frequency[A]	Spontaneous mutation frequency	Reversion frequency	Characteristics
fpa^r	4×10^{-5}	3×10^{-9}	2×10^{-9}	Resistant to *p*-fluorophenylalanine
val	3×10^{-6}	2×10^{-9}	$1{\cdot}2\times10^{-9}$	Requiring valine; also resistant to ethionine and lacking heterocysts

[A] Treatment of samples containing 3×10^5 cells per millilitre for 20 min with NTG resulted in 3–4% survival.

Results

The mutation frequency for the *fpa*^{r} and *val* mutants is shown in Table 1. The *fpa*^{s} strain was used as a recipient and the *fpa*^{r} mutant was the donor. Uptake of DNA was screened through one complete cell cycle (0·50 doublings per day) as

confirmed by absorption maxima at 400 and 670 nm. The maximum number of transformants appeared in exponentially growing cultures during the 8–28 h period. (Table 2). At 48 h, the number of transformants decreased to 90% of the maximum value, indicating a gradual disappearance of competence.

Table 2. Genetic transformation of *p*-fluorophenylalanine resistance in *N. muscorum*

DNA (40 μg/ml) from *fpa*^{r} cells was added to a 72-h *fpa*^{s} culture once and aliquots were then assayed at intervals of 4 h for one complete cell division. The transformants (*fpa*^{r}) were observed for stability of the acquired gene for 20 generations. The total number of cells plated for each treatment was $1{\cdot}5 \times 10^7$

Age of recipient culture (h)	Total No. of colonies appearing	$10^5 \times$ transformation frequency	Age of recipient culture (h)	Total No. of colonies appearing	$10^5 \times$ transformation frequency
0	0	0	28	825	5·5
4	376	2·5	32	676	4·5
8	828	5·5	36	526	3·5
12	901	6·0	40	229	1·5
16	901	6·0	44	77	0·5
20	976	6·5	48	75	0·5
24	900	6·0			

Transfer of val$^+$

The spontaneous reversion of *val* to *val*$^+$ was $0{\cdot}33 \times 10^{-9}$. *val*$^+$ DNA was used for the *val* recipient mutant and transformants were selected on minimal medium (BG-11) fortified with ethionine (40 μg/ml), thus counterselecting *val*$^+$.

Table 3. Transformation of *val* to *val*$^+$ and of *fpa*^{s} to *fpa*^{r} as a function of increasing DNA concentration

Different sources of DNA were used for selecting *val*$^+$ and *fpa*^{r} transformants

DNA concn (μg/ml)	$10^7 \times$ Transformation frequency *val*$^+$	*fpa*^{r}	DNA concn (μg/ml)	$10^7 \times$ Transformation frequency *val*$^+$	*fpa*^{r}
10	0·8	250	60	3·4	540
20	2·0	380	70	3·5	550
30	3·0	420	80	3·5	560
40	3·5	530	90	3·4	550
50	3·4	550	100	3·4	550

The DNA concentration used to select *val*$^+$ transformants ranged from 10 to 100 μg/ml. The transformation process was saturated at a DNA concentration of 40–50 μg/ml (Table 3). The maximum number of transformants appeared when the cultures were grown for 16–20 h (Fig. 1). In the final stages of cell division, transformants were not found. Moreover, in cultures grown for 0–8 h, the transformation frequency was nearly 70% less than the value at 16 h of growth. At 40 h of growth the values were 98% less than the maximum value during one complete cell division. About 10% (200 colonies) of the transformants were observed microscopically and heterocyst differentiation was apparently not transferred during transformation. The transformants, like the recipient mutants, could grow in nitrate-free medium. All the transformants tested showed resistance to ethionine. The transformation was

highly sensitive to deoxyribonuclease, with the frequency falling to zero after 15 min (Table 4), and it was insensitive to ribonuclease and proteinase.

The genetic stability of transformants was observed for several cell cycles. These stable transformants were used for extraction and purification of DNA and used for

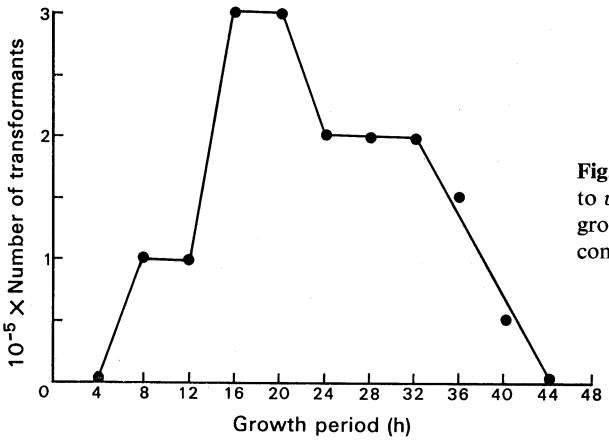

Fig. 1. Genetic transformation of *val* to *val*$^+$ in *N. muscorum* as a function of growth stage of recipient cells. DNA concentration is 50 μg/ml.

further transformation of the mutants. A serial-transformation experiment was designed and the DNA extracted from first cycle transformants (*val*$^+$-1) was used to transform the recipient mutant *val*. The transformants selected through the second cycle (*val*$^+$-2) were used for further extraction of DNA and transformation of the

Table 4. Control of DNA-mediated transformation in *N. muscorum* by deoxyribonuclease

Period of treatment (min)	Transformation frequency *fpa*^{r}	*val*$^+$	Period of treatment (min)	Transformation frequency *fpa*^{r}	*val*$^+$
0	$5{\cdot}5\times10^{-5}$	$3{\cdot}5\times10^{-7}$	10	$8{\cdot}2\times10^{-10}$	$7{\cdot}0\times10^{-10}$
2	$5{\cdot}4\times10^{-5}$	$3{\cdot}5\times10^{-7}$	12	$2{\cdot}1\times10^{-11}$	$6{\cdot}2\times10^{-11}$
5	$4{\cdot}0\times10^{-6}$	$2{\cdot}8\times10^{-8}$	15	0	0
8	$3{\cdot}8\times10^{-8}$	$1{\cdot}4\times10^{-9}$	20	0	0

recipient mutant *val*. Five such serial attempts, through five continuous cycles gave transformation frequencies of either $3\text{–}4\times10^{-7}$ (first, fourth and fifth cycles) or $3{\cdot}5\times10^{-7}$ (second and third cycles) and indicated that DNA-mediated transformation for valine occurred with almost the same frequency.

Discussion

Only about 40–50 μg of DNA seemed to be necessary for transformation in filamentous Cyanobacteria in the present investigation. Unicellular Cyanobacteria can be transformed with a relatively lower DNA concentration (Stevens and Porter 1980). Apparently transformation of auxotrophic mutants (*val*) occurs at a much lower frequency than that of *fpa*^{s}. There is at present no information on the valine operon system in Cyanobacteria. The transformants, like the recipient mutants, could grow in nitrate-free medium. It is not clear how these transformants were able to grow aerobically.

We were not able to transform *val* with DNA extracted from wild-type *Anacystis nidulans*, a unicellular non-nitrogen-fixing cyanobacterium. Apparently *N. muscorum* has a highly specific system for gene expression and its control. The DNA concentration-dependent transformation frequency curve indicates that there may be multiple sites for uptake of DNA which become saturated at a particular DNA concentration. However, it is not known how many sites are actually involved in this genetic transformation.

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References

Allen, M. M. (1968). Simple conditions for growth of unicellular blue-green algae on plates. *J. Phycol.* **4**, 1–4.

Burton, K. (1968). Determination of DNA concentration with diphenylamine. In 'Methods in Enzymology'. Vol. 12. Pt.B. pp. 163–6. (Academic Press: New York.)

Herdman, M. (1973). Mutations arising during transformation in the blue-green alga *Anacystis nidulans*. *Mol. Gen. Genet.* **120**, 369–78.

Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J. Mol. Biol.* **3**, 208–18.

Orkwiszewski, K. G., and Kaney, A. R. (1974). Genetic transformation of the blue-green bacterium *Anacystis nidulans*. *Arch. Microbiol.* **98**, 31–7.

Shestakov, S. V., and Khyen, N. T. (1970). Evidence for genetic transformation in the blue-green alga *Anacystis nidulans*. *Mol. Gen. Genet.* **107**, 372–5.

Stanier, R. Y., Kunisawa, R., Mandel, M., and Cohen-Bazire, G. (1971). Purification and properties of unicellular blue-green algae (Order Chroococcales). *Bact. Rev.* **35**, 171–205.

Stevens, S. E. Jr., and Porter, R. D. (1980). Transformation in *Agmenellum quadroplicatum*. *Proc. Natl Acad. Sci. U.S.A.* **77**, 6052–6.

Stewart, W. D. P. (1973). Nitrogen fixation by photosynthetic microorganisms. *Ann. Rev. Microbiol.* **27**, 283–318.

Stewart, W. D. P., and Singh, H. N. (1975). Transfer of nitrogen-fixing (*nif*) genes in the blue-green alga *Nostoc muscorum*. *Biochem. Biophys. Res. Commun.* **62**, 62–9.

Zamenhof, S. (1961). Preparation and essay of deoxyribonucleic acids from animal tissues. In 'Methods in Enzymology'. (Eds S. P. Colowick and N. O. Kaplan.) Vol. 3. pp. 696–704. (Academic Press: New York.)

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