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Transposon mutagenesis of *Nostoc* sp. strain ATCC 29133, a filamentous cyanobacterium with multiple cellular differentiation alternatives

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***Nostoc* sp. strain ATCC 29133 (PCC 73102; *Nostoc* 29133) is a symbiotically-competent, facultatively heterotrophic, diazotrophic cyanobacterium with the capacity to differentiate specialized cells such as heterocysts, akinetes and hormogonial filaments. We have optimized several methods for physiological and molecular genetic analysis of *Nostoc* 29133. By use of a Tn*5* derivative, Tn*5*-1063 (Km^r Bm^r Sm^r), delivered by conjugation from *Escherichia coli*, antibiotic-resistant mutants of *Nostoc* 29133 were generated at a frequency of approximately 1×10^{-6}, 0·4% of which expressed a nitrogen fixation (heterocyst) defective phenotype. Mutant strain UCD 328 was isolated after co-culture of 86 *Nostoc* 29133::Tn*5*-1063 clones with the symbiotic plant partner, *Anthoceros punctatus*; strain UCD 328 expressed a symbiotic phenotype of increased frequency of hormogonia-dependent infection. The transposon and flanking genomic DNA was recovered from strain UCD 328, the mutation and phenotype reconstructed by homologous recombination in *Nostoc* 29133, and the transposition site identified from a *Nostoc* 29133 genomic library. Transposon mutagenesis has thus provided the means for isolation and identification of developmental and symbiotic-specific genes of *Nostoc* 29133.**

Keywords: *Nostoc* sp. ATCC 29133, diazotrophic cyanobacterium, symbiotic cyanobacterium, transposon mutagenesis, homologous recombination

INTRODUCTION

Cyanobacteria are a morphologically diverse group of eubacteria, united by their characteristic oxygenic photoautotrophic mode of growth. In certain filamentous cyanobacterial genera classified in Sections IV and V (*sensu* Rippka *et al.*, 1979), such as *Nostoc*, vegetative cells can mature in four developmental directions, depending upon the environmental growth conditions. First, they can divide upon reaching a critical size and perpetuate the vegetative growth cycle. Second, the vegetative cells can divide uncoupled from biomass increase or DNA replication to form transient hormogonial filaments (Damerval *et al.*, 1991). Hormogonia formation can be induced by environmental conditions which include light quality and excess nutrients (Herdman & Rippka, 1988). Third, a few or the entire population of vegetative cells can differentiate into akinetes (spores) which function in survival under environmental extremes (Herdman, 1987). A variety of environmental conditions, including nutrient (especially phosphate) deprivation, and autogenic production of a sporulation factor (Hirosawa & Wolk, 1979), stimulate their formation. Fourth, under conditions of combined nitrogen deprivation a small fraction (3–10%) of the vegetative cells will, in a defined spatial pattern along the filaments, differentiate into heterocysts which are specialized for nitrogen fixation in an oxic environment (Wolk, 1982). Thus, these filamentous cyanobacteria provide an experimental system for the study of how cells perceive, integrate and respond to environmental signals in the context of differentiation processes.

Techniques have been developed for genetic analysis of heterocyst differentiation and function in *Anabaena* sp.

Abbreviations: Ap, ampicillin; ATCC, American Type Culture Collection; Bm, bleomycin; Chl *a*, Chlorophyll *a*; Cm, chloramphenicol; Em, erythromycin; Fix, nitrogen fixation; Fox, aerobic nitrogen fixation; Km, kanamycin sulfate; MCR, deficient in restriction of DNA containing methylated adenine and cytosine residues; MDRS, methylation dependent restriction systems; Nm, neomycin sulfate; PCC, Pasteur Culture Collection; Sm, streptomycin; Sp, spectinomycin; Suc, sucrose; Sym, symbiotic competence.

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics*	Reference(s)
E. coli		
DH5α	*recA1 hsdR17* (r_K^- m_K^-) *endA1*	Grant *et al.* (1990)
DH5α-MCR	Genotype of DH5α and *mcrA* Δ(*mrr hsdMRS mcrBC*)	Grant *et al.* (1990)
HB101	*recA13 hsdS20* (r_B^- m_B^-)	
Nostoc		
ATCC 29133 (PCC 73102)	Fix^+ Sym^+	Rippka *et al.* (1979); Enderlin & Meeks (1983)
UCD 7801	Fix^+ Sym^+	Enderlin & Meeks (1983)
UCD 328	Superinfective *Nostoc* 29133::Tn*5*-1063 strain, Fix^+	This study
UCD 339	pSCR3 integrated by single recombination in the genome	This study
UCD 340	Reconstruction of the UCD 328 mutation	This study
Plasmids		
pDUCA7	Cosmid shuttle vector with RK2 and pDU1 *oriV*s, Km^r	Buikema & Haselkorn (1991)
pRK212.1	RK2 Mu phage insertion/deletion derivative, Km^s	Figurski *et al.* (1976)
pRK2013	RK2 derivative with ColE1 *oriV*, Km^r	Figurski & Helinski (1979)
pRK2073	pRK2013::Tn*7*, Km^s Sp^r	Better & Helinski (1983)
pRL6	Shuttle vector with pMB1 and pDU1 *oriV*s	Wolk *et al.* (1984)
pRL443	Spontaneous Km^s mutant of RP4	Elhai & Wolk (1988a)
pRL479	S.A2/L.EHE2/C.C2, Ap^r Cm^r	See Elhai & Wolk (1988b) for nomenclature
pRL1063a	Tn*5*-1063, RK2 *oriT*, Bm^r Km^r Sm^r	Wolk *et al.* (1991)
pRL1075	Used for reconstructing Tn*5*-1063 insertions, Cm^r Em^r Suc^s	Black *et al.* (1993)
pSCR1	Recovered 19 kb *Eco*RV Tn*5*-1063 bearing fragment from UCD 328, Bm^r Km^r Sm^r	This study
pSCR2	pSCR1 with *Not*I site filled in, Bm^r Km^r Sm^r	This study
pSCR3	pSCR2 with positive selection cassette from pRL1075, Bm^r Cm^r Em^r Km^r Sm^r Suc^s	This study
pSCR4	Subclone of 11 kb *Eco*RV fragment into pRL479	This study
pSCR5	Subclone of 6·2 kb *Spe*I fragment into pUC18	This study
pUC18	pBR322-derived vector	Vieira & Messing (1982)

strain PCC 7120 (Buikema & Haselkorn, 1993; Wolk, 1991). However, *Anabaena* 7120 has limitations as an experimental system in developmental processes since it does not differentiate hormogonia or akinetes (Rippka *et al.*, 1979).

Nostoc sp. strain ATCC 29133 (PCC 73102; *Nostoc* 29133) displays all of the cellular differentiation processes described above (Rippka *et al.*, 1979). Unlike *Anabaena* 7120, *Nostoc* 29133 is also capable of photo- and dark-heterotrophic growth (Rippka *et al.*, 1979). Moreover, *Nostoc* 29133 was originally isolated from symbiotic association with the cycad *Macrozamia* sp. (Rippka *et al.*, 1979) and, via infection by hormogonia, can reconstitute a symbiotic

association with the bryophyte *Anthoceros punctatus* (Campbell & Meeks, 1989; Enderlin & Meeks, 1983) and the angiosperm *Gunnera manicata* (Johansson & Bergman, 1994). When in association with *A. punctatus*, *Nostoc* spp. differentiate heterocysts at a frequency of about 45% of the total cells (Enderlin & Meeks, 1983; Meeks, 1990), fix N_2 under photo- and dark-heterotrophic conditions at a rate that is at least three- to fivefold higher than free-living cultures (Steinberg & Meeks, 1991) and release approximately 80% of the fixed nitrogen as ammonium for growth of the associated eukaryotic tissue (Meeks *et al.*, 1985).

We are interested in the interactions between *Nostoc* spp. and *Anthoceros punctatus* that regulate symbiotic cyanobacterial gene expression, enzyme activity and cellular differentiation, and whether such response systems differ from those operative in the free-living growth state. A screen to identify mutants with altered symbiotic competence, for which there is no positive selection, requires a population composed only of clones already known to harbour a mutation. Transposon mutagenesis has an advantage over chemical and UV mutagenesis since it meets this requirement by yielding an antibiotic-resistant-marked mutant population. We report here parameters for mutagenesis of *Nostoc* 29133 using a transposon, Tn*5*-1063, which contains a promoterless *luxAB* as a reporter gene, developed by Wolk *et al.* (1991) for use in *Anabaena* 7120. From a *Nostoc* 29133::Tn*5*-1063 strain having an increased initial infection frequency of *A. punctatus*, the transposon and flanking genomic sequences were recovered and the mutation reconstructed by homologous recombination of the recovered DNA.

METHODS

Cultures and media. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were routinely cultured in Luria–Bertani (LB) broth (Sambrook *et al.*, 1989) and cell concentrations determined by measuring the optical density of cultures at 600 nm (OD_{600}). Sources and culture conditions for symbiont-free and *Nostoc*-associated *Anthoceros punctatus*, and all free-living *Nostoc* strains, were described previously (Enderlin & Meeks, 1983). The basal medium for growth of *Nostoc* was that of Allen & Arnon (1955) (AA) supplemented with 5 mM MOPS, pH 7·8 (−N), and, when specified, 2·5 mM NH_4Cl (+N); it was used at full strength when solidified with 1% (w/v) agar (purified using the methods of Braun & Wood, 1962) or diluted fourfold in liquid culture (AA/4). Chlorophyll *a* (Chl *a*) in methanolic extracts was quantified as described previously (Meeks *et al.*, 1983). To select for resistance conferred by the *npt* gene, kanamycin (Km; 12·5 or 25 μg ml^{-1}) was used for *E. coli* and neomycin (Nm; 10 μg ml^{-1}) was used for *Nostoc* 29133.

DNA isolations and manipulations. Small-scale preparations of plasmid DNA from *E. coli* were done using standard methods. Large-scale plasmid purifications from *E. coli* were carried out using a commercial kit (Qiagen). DNA restriction enzymes were obtained from New England Biolabs. Hybridization of radiolabelled probes to DNA immobilized on nylon membranes (Gene Screen Plus; Dupont NEN Products) was done as described by Sambrook *et al.* (1989) using 50% (v/v) formamide. Hybridization probes were prepared by random priming (5 Prime – 3 Prime, Inc.) using [α-^{32}P]dCTP (Dupont, NEN).

Nostoc total DNA was isolated by a method based on that of Ausubel *et al.* (1987). Cells were collected from a 50 ml AA/4+N culture having a density of ⩽ 5 μg Chl *a* ml^{-1} by centrifugation in a clinical centrifuge at 1000 ***g*** for 5 min. The supernatant was discarded and the cells washed twice with 5 M NaCl. The pellet from the final wash was resuspended in TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8) to give a total volume of 1 ml and the cells stored frozen overnight at −20 °C. After thawing by immersion in a water bath at room temperature, 1 ml of a 20 mg ml^{-1} lysozyme solution in TE was added to the cell suspension. Following incubation at 37 °C for 1 h with gentle shaking, 0·5 ml 0·5 M EDTA, pH 8·0, was added, and the cells were lysed by addition of 1 ml of a 2 mg ml^{-1} Proteinase K solution in TE, plus 0·1 ml 20% (w/v) SDS, followed by incubation as above for 1 h. After lysis, 0·6 ml 5 M NaCl was added and the suspension mixed gently; 0·45 ml of a 10% (w/v) solution of hexadecyltrimethylammonium bromide (CTAB) in 0·7 M NaCl was added, and the suspension was mixed and incubated for 10 min in a 65 °C water bath. The cell debris was pelleted for 5 min at 13000 ***g***. The supernatant solution was extracted once with chloroform and nucleic acids were precipitated with ethanol. The pellet was resuspended in 0·5 ml TE and extracted one to three times with phenol/chloroform until the interface was clear. The aqueous phase was precipitated and the pellet washed once with 70% (v/v) ethanol and dried under vacuum. The resulting pellet was resuspended in 50 μl TE containing 1 U $μl^{-1}$ RNase T_1 (Bethesda Research Laboratories).

Plasmids were isolated from 50 ml liquid cultures of *Nostoc* 29133 grown under Nm selection and having Chl *a* concentrations of approximately 5 μg ml^{-1}. *Nostoc* cells were harvested as before, washed twice with 5 M NaCl and then resuspended in 2 ml TE. Lysozyme was added to a final concentration of 5 mg ml^{-1}. After mixing, the tube was incubated with gentle shaking for 2 h at 37 °C then Triton X-100 (0·5 ml of a 20%, v/v, solution) was added to lyse the cells. The resulting solution was made up to a concentration of 1 M NaCl by the addition of cold 5 M NaCl and the suspension was stored overnight at 4 °C. The suspension was cleared by centrifugation at 1000 ***g*** for 10 min and the supernatant was extracted with phenol/chloroform as above. Nucleic acids in the final aqueous phase were precipitated with 0·6 vol. of 2-propanol followed by centrifugation at 12000 ***g*** for 10 min. The pellet was washed twice with 70% ethanol, vacuum dried, and resuspended in glass-distilled water.

Genomic library construction and screening. High-molecular-mass genomic DNA of *Nostoc* 29133 was sheared randomly and the ends were blunted by incubation with mung bean nuclease. *Eco*RI–*Xmn*I adapters were ligated to the ends and these adapted inserts were ligated into *Eco*RI-digested cosmid shuttle vector pDUCA7 (Buikema & Haselkorn, 1991). The ligated material was packaged into λ phage using the methods of Ish-Horowicz & Burke (1981) and transfected into *E. coli* strain DH5α-MCR. A total of 16200 clones were pooled and stored as a massed library. Restriction analysis of 23 randomly chosen clones showed an average insert size of 28 kb. A total of 2112 clones was stored individually in microtitre plates and patched onto nylon membranes for hybridization screening. The patched colonies were lysed on the membrane according to Sambrook *et al.* (1989) after overnight growth on LB agar containing 12·5 μg Km ml^{-1}.

Transposon mutagenesis. A protocol for transposon muta-

genesis of *Anabaena* 7120 (Wolk *et al.*, 1991) was adapted for use with *Nostoc* 29133. Tri-parental plate matings were conducted on sterile conjugation filters (Millipore HATF 082) lying on agar plates containing AA+N +0·5% (v/v) LB broth. In preparation for conjugation, *Nostoc* filaments were fragmented by sonic cavitation to a length of four to six cells per filament, allowed to recover by incubation under growth conditions for at least 6 h in 50 ml AA/4+N and were concentrated to approximately 75 μg Chl *a* ml^{-1} immediately before mating. *E. coli* was prepared for mating by combining in a centrifuge tube equal numbers of the donor strain (DH5α containing the transposon-bearing plasmid pRL1063a) and the conjugal strain (HB101 containing the conjugal plasmid pRK2013) both grown to concentrations of less than 1·0 OD_{600} in LB containing 25 μg Km ml^{-1}. The cells were pelleted by centrifugation at 2000 ***g*** for 10 min and resuspended to a final concentration of 9–10 OD_{600} units in LB. For each conjugation filter, 0·5 ml of the *E. coli* mixture was combined in a microfuge tube with 0·5 ml of the concentrated *Nostoc* 29133 cell suspension. The *E. coli*/*Nostoc* 29133 mixture was then centrifuged at 4000 ***g*** for 30 s. The supernatant was aspirated, leaving approximately 150 μl total volume in the microfuge tube. The pellet was resuspended and immediately spread on the filter. The filter-plates were incubated at 28 °C and low light intensity (1·5 W m^{-2}) in air plus 1% (v/v) CO_2 for 16–24 h to allow for conjugal mating. To allow the expression of antibiotic resistance, the filters were transferred to fresh AA+N 1% agar plates without LB and incubated at low light intensity for an additional 2 d and then moved to 8 W m^{-2} illumination for another 1–2 d incubation. The filters were then transferred to selective AA+N plates containing 10 μg Nm ml^{-1} and 1 μg Sm ml^{-1}. At approximately 1 week intervals the filters were transferred to fresh selective medium. Mutants appeared as colonies after 10–21 d. To screen for aerobic nitrogen-fixation mutants (Fox^-; *sensu* Ernst *et al.*, 1992), filters bearing mutant colonies were transferred to AA−N plates. Colonies that developed yellowing edges after about 7 d were patched on AA−N and AA+N media.

Quantification of conjugation frequency. For the purpose of estimating exconjugant frequencies that were dependent on the conjugal plasmid, matings were conducted as described above except that a 0·2 OD_{600} mixture of *E. coli* was used and filters were not transferred to selective medium. Instead, the filters were flooded with fresh liquid medium and the mixed *Nostoc* and *E. coli* cells were collected, centrifuged and resuspended in an exact volume of fresh medium. Dilutions of the cell suspensions were plated on both selective and non-selective media, and colonies counted after 2 weeks.

Recovery of DNA flanking the transposon in strain UCD 328 and reconstruction of the mutation. The transposon Tn*5*-1063 contains an *oriV*(p15A) which allows for recovery of the transposon with the DNA flanking its insertion site in the *Nostoc* genome (Wolk *et al.*, 1991). pSCR1, containing 11 kb of *Nostoc* DNA contiguous with the 7·8 kb transposon, was recovered from an *Eco*RV digest of strain UCD 328 genomic DNA using the strategy of Black & Wolk (1994). To make pSCR2, a frameshift mutation was introduced into the transposase gene of pSCR1 by filling in a unique *Not*I site. pSCR3 was constructed by ligating an *Spe*I pSCR2 fragment, encompassing the transposon and 6·2 kb of *Nostoc* flanking DNA, to a pRL1075 *sacB*-containing *Spe*I fragment (Black *et al.*, 1993). To reconstruct the mutation, pSCR3 was mated into *Nostoc* 29133 cells using the conjugation method described above. Nm^r clones were grown in liquid, sonicated and plated on AA containing 10 μg Nm ml^{-1} and 5% (w/v) sucrose to select for double recombinants (Cai & Wolk, 1990).

Luciferase filter assay. Filters containing mutant colonies were placed on a 0·5 ml pool of liquid medium on a glass plate. This was overlaid with a thin glass plate streaked on the filter-facing side with 2 μl decyl aldehyde (Aldrich) per filter, leaving a 1 mm space between the two plates so as not to crush the *Nostoc* colonies. In a darkroom, pre-flashed X-ray film was placed on top of the apparatus and exposed for 3–5 h. To examine the Fox^- mutants for changes in luciferase activity induced by nitrogen deprivation, each mutant was patched to two filters and incubated under light for 7 d. One filter was then transferred to an AA−N plate and the other to fresh AA+N; the plates were incubated for an additional 2 d before subjecting them to the filter assay.

Cell differentiation assays. Cultures were induced to form akinetes by subculturing into AA/4+N without inorganic phosphate. To induce hormogonia, *Anthoceros punctatus* growth-conditioned medium was obtained by transferring approximately 5 g (fresh weight) of gametophyte tissue (washed twice with 100 ml AA/4) into 50 ml AA/4 without supplementation. Samples of 0·2 ml from *Nostoc* cultures were combined with 2·3 ml of *A. punctatus* conditioned medium in culture tubes and incubated at 100 r.p.m. at room temperature and 8 W m^{-2}. After 24–36 h, filaments were examined microscopically for hormogonia formation.

RESULTS AND DISCUSSION

Nostoc 29133 as an experimental strain

In addition to the three developmental alternatives, heterotrophic growth and the ability to establish a symbiotic association with *A. punctatus*, two other considerations influenced our choice of *Nostoc* 29133 as an experimental strain. First, *Nostoc* 29133 is reported to have no type II endonuclease activity (Lambert & Carr, 1984; Shestakov & Reaston, 1987). This deficiency is an advantage when attempting to transfer DNA into the strain, obviating the *in vivo* methylation used in *Anabaena* strains (Elhai & Wolk, 1988b). Second, the lack of endogenous plasmids in *Nostoc* 29133 (Houmard & Tandeau de Marsac, 1988) eases the identification and recovery of vector plasmids introduced into the strain.

Conjugation procedures

Initial attempts at introducing the transposon Tn*5*-1063 into *Nostoc* 29133, using the conjugation protocol designed for use in *Anabaena* 7120 (Elhai & Wolk, 1988b), yielded only one mutant out of three filter matings. Moreover, conjugation frequencies of only about 5×10^{-7} were observed using this same protocol to introduce a pDU1-based shuttle vector, pRL6, into *Nostoc* 29133 (Wallis, 1993). When *Nostoc* 29133 cell suspensions were mixed with *E. coli* suspensions at the concentration used with *Anabaena* 7120 (Elhai & Wolk, 1988b), the *Nostoc* 29133 bleached rapidly on non-selective medium; *Nostoc* 29133 by itself did not bleach. For conjugal transfer of pRL1063a, an approximately 20-fold reduction in the reported *E. coli* concentration gave the highest frequency of resultant *Nostoc* 29133::Tn*5*-1063 colonies. Control matings transferring pDUCA7 from *E. coli* donors to *E. coli* recipients showed that exponential cultures of *E. coli*

mated at a nearly 10-fold higher frequency than stationary-phase cultures (data not shown); the growth phase of *Nostoc* 29133 had no influence on the frequency of conjugation (Wallis, 1993). Therefore, for conjugal matings between *Nostoc* 29133 and *E. coli*, exponential-phase *E. coli* was used and the cells plated immediately after mixing.

An increase in conjugation frequency was also obtained by use of a different conjugal plasmid. The plasmid, pRK2013, contains the conjugal transfer functions of RK2 inserted into a derivative of plasmid ColE1, and is dependent on the ColE1 origin of replication (Figurski & Helinski, 1979). Control mating experiments between *E. coli* strains demonstrated that pDUCA7 was mobilized 50-fold more effectively by pRK2073, a Km^s derivative of pRK2013, than by plasmids pRL443 and pRK212.1, which use the RK2/RP4 origin of replication (data not shown). When *Nostoc* 29133 was the recipient, mating via pRK2073 yielded a frequency of exconjugants per viable c.f.u. ($5{\cdot}5 \times 10^{-4}$ Km^r per c.f.u.) that was at least 50-fold higher than the frequency using pRL443 ($< 9{\cdot}1 \times 10^{-6}$ Km^r per c.f.u.). The improved mobilization may be explained by the higher copy number of pRK2073 compared to the RK2/RP4-based conjugal plasmids, perhaps producing more of the proteins necessary for the conjugation process. Compatibility between the ColE1 and the RK2/RP4 origins of replication allows both the conjugal plasmid and the mobilized vector plasmid to reside in the same *E. coli* cell, which may also contribute to the observed increase in the frequency of transfer.

Nostoc 29133 DNA isolation and library construction

To obtain DNA for the construction of a genomic library and for the recovery of Tn*5*-1063 transposition sites, a method of total DNA isolation from *Nostoc* 29133 was devised that reliably gave high yields. The major modification of the DNA isolation method of Ausubel *et al.* (1987) was the introduction of two preliminary washes with 5 M NaCl to remove *Nostoc* exopolysaccharides, which appear to inhibit the extent of cell lysis in subsequent steps. The exopolysaccharide content of the starting cellular material can be reduced by harvesting dilute cultures ($\leqslant 5$ μg Chl *a* ml^{-1}) grown in ammonium-supplemented medium. This method routinely yielded approximately 1 μg DNA per 2 μg Chl *a*, and has been applied to some other *Nostoc* strains with similar success (Wallis, 1993).

Before introducing *Nostoc* 29133 DNA into *E. coli*, restriction of the DNA by the methylation-dependent restriction systems (MDRS) which are present in many *E. coli* K 12 strains was examined; MDRS restrict DNA introduced into *E. coli* cells only if that DNA is methylated at specific sequences (Raleigh, 1992). A method for isolating plasmid DNA from *Nostoc* 29133 was necessary to perform this experiment. Several published methods specific to cyanobacteria (Felkner & Barnum, 1988; Potts, 1989; Stuttzman & Gendel, 1985) failed to yield usable DNA, principally because few *Nostoc* 29133 cells were lysed. As with the total DNA isolation protocol, preliminary 5 M NaCl washes in the plasmid preparation protocol improved subsequent cell lysis. This protocol yielded 17 μg pDUCA7 DNA per μg Chl *a*.

Table 2. Effect of methylation dependent restriction systems on transformation frequency

Source of pDUCA7	*E. coli* strain transformed	Km^r colonies per μg DNA	Ratio DH5α/ DH5α-MCR
E. coli HB101	DH5α	$7{\cdot}1 \times 10^{-2}$	0·91
	DH5α-MCR	$7{\cdot}9 \times 10^{-2}$	
Nostoc 29133	DH5α	$< 1{\cdot}0 \times 10^{-5}$	< 0·005
	DH5α-MCR	$0{\cdot}2 \times 10^{-3}$	

For a comparison of transformation efficiencies, *E. coli* strains DH5α and DH5α-MCR were transformed with vector pDUCA7 isolated from *Nostoc* 29133. These *E. coli* strains are isogenic except that the loci coding for the MDRS have been deleted from strain DH5α-MCR (Grant *et al.*, 1990). In control experiments plasmid DNA isolated from *E. coli* HB101 transformed the two DH5α strains at nearly equal efficiencies. However, plasmid DNA isolated from *Nostoc* 29133 transformed DH5α-MCR with at least a 200-fold greater frequency than it transformed DH5α (Table 2). To bypass this apparent restriction barrier, a *Nostoc* 29133 genomic DNA library, constructed in pDUCA7 and packaged in λ phage, was transfected into *E. coli* strain DH5α-MCR. This genomic library has served as a source for wild-type DNA corresponding to the transposition sites of all *Nostoc* 29133::Tn*5*-1063 mutants so far recovered from the genome (unpublished observations), including that of strain UCD 328 discussed below.

Mutagenesis and mutant phenotypes

Tri-parental filter matings using the conjugal plasmid pRK2013 to introduce Tn*5*-1063 into *Nostoc* 29133 were conducted on AA+N to allow the growth of any resulting nitrogen fixation and nitrate assimilation mutants. The procedure resulted in a frequency of $1{\cdot}0$ $(\pm 0{\cdot}8) \times 10^{-6}$ [mean (±SD), $n = 7$] *Nostoc* 29133::Tn*5*-1063 colonies per viable *Nostoc* cell in the mating mixture (based on a value of $1{\cdot}75 \times 10^{-13}$ g Chl *a* per viable cell). The yield of resultant colonies per filter is about five times lower than that reported for Tn*5*-1063 mutagenesis of *Anabaena* 7120 (Wolk *et al.*, 1991).

Of $5{\cdot}1 \times 10^3$ mutant colonies screened, 14 Fox^- mutants, all with discernible heterocyst defects, were identified. Two Fox^- strains had a fragmented, short-filament phenotype in the presence or absence of combined nitrogen. Symbiotic competence was retained in four Fox^- strains; it has been shown previously that for strain UCD 223, a *Nostoc* 29133 Fox^- mutant generated by chemical mutagenesis, the microaerobic symbiotic cavities

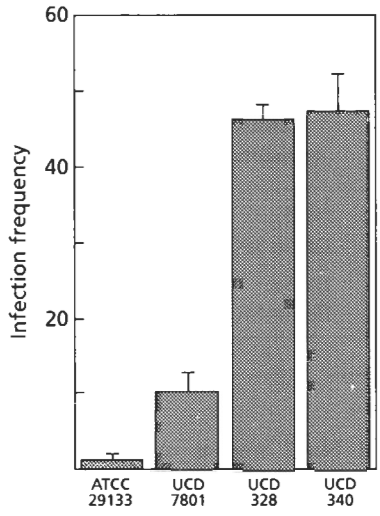

Fig. 1. Relative infection frequencies of *Anthoceros punctatus* by various *Nostoc* strains. *Nostoc* cells representing 30 μg or 60 μg Chl *a* were added to 2–3 g fresh weight of *A. punctatus* gametophyte tissue in 50 ml medium lacking combined nitrogen. After 14 d co-culture the plant tissue was washed and samples were removed for the quantification of symbiotic colonies. Infection frequencies were calculated as the mean number ± SD of symbiotic colonies per mg tissue dry weight per unit of *Nostoc* cells added (as μg Chl *a*) for: *Nostoc* ATCC 29133, $n = 3$; UCD 7801, $n = 2$; UCD 328, $n = 4$; and UCD 340, $n = 4$. The reported frequencies are normalized to the infection frequency of *Nostoc* ATCC 29133.

of *Anthoceros punctatus* can replace the physiological function of the heterocyst envelope (Campbell & Meeks, 1992). The heterocyst envelope polysaccharide of two *Anabaena* strains is apparently the same as that found in their respective akinetes (Cardemil & Wolk, 1976, 1981). We examined the akinetes of Fox⁻ *Nostoc* 29133::Tn*5*-1063 strains which had microscopically observable defects in their heterocyst envelopes, but found no corresponding defects in the continuity of their akinete envelopes.

The promoterless bacterial luciferase genes, *luxAB*, of Tn*5*-1063 can form transcriptional fusions to the *Nostoc* 29133 genome upon transposition. To screen for transpositions within transcribed regions of the genome, several filters containing mutant colonies were assayed for luciferase activity by exposure to X-ray film. Light emission was detected from 30% (313/1063) of the mutant colonies when grown on minimal medium with or without NH_4^+. The 14 Fox⁻ mutant colonies were separately screened for changes in luciferase activity after 2 d of ammonium deprivation; one group of seven mutants showed increased levels of expression, a second group of five mutants showed no change and a third group of two mutants showed a decrease.

Of 86 randomly chosen Fix⁺ *Nostoc* 29133::Tn*5*-1063 strains tested for symbiotic competence, one (strain UCD 328) was found to have a high initial frequency of infection. After 2 weeks of co-culture, strain UCD 328 infected *Anthoceros punctatus* at about a 50-fold higher frequency than the parental *Nostoc* 29133 strain and fivefold higher than *Nostoc* sp. strain UCD 7801 (Fig. 1), our original isolate from *A. punctatus* (Enderlin & Meeks, 1983). Strain UCD 328 was found to be more sensitive than wild-type *Nostoc* 29133 to a hormogonia-inducing factor in *A. punctatus* growth-conditioned medium (M. F. Cohen & J. C. Meeks, unpublished). Furthermore, unlike other *Nostoc* strains, which may grow to a high density in the medium surrounding the plant (Enderlin & Meeks, 1983), epiphytic growth of strain UCD 328 was severely reduced; strain UCD 328 showed no obvious growth defect in the free-living state.

Reconstruction of the strain UCD 328 mutation

Southern blots of total *Eco*RV-digested DNA from strain UCD 328, probed with *Hin*cII-digested pRL1063a, showed a single 19 kb band of hybridizing DNA (data not shown), implying that the genome bears only one copy of the transposon. The mutation was reconstructed by insertion mutagenesis to confirm that the phenotype of strain UCD 328 was a result of the Tn*5*-1063 insertion. The positive selection plasmid, pSCR3, containing Tn*5*-1063 with an inactivated transposase gene in a 6·2 kb *Nostoc* 29133 *Spe*I fragment (Fig. 2), was introduced into *Nostoc* 29133 by conjugation. Single recombination of pSCR3 into the *Nostoc* 29133 genome gave rise to a Nm^r Em^r Suc^s phenotype. A second recombination on the opposite side of the transposon resulted in a Nm^r Em^s Suc^r phenotype and was selected by plating on sucrose-containing medium (Cai & Wolk, 1990). Southern analysis confirmed single recombination (strain UCD 339) and double recombination (strain UCD 340) of pSCR3 at the same locus as the transposition site in strain UCD 328 (Fig. 3). Strain UCD 340 was co-cultured with *Anthoceros punctatus* and found to have an initial infection frequency near that of strain UCD 328 (Fig. 1). A 2 kb fragment of DNA flanking the transposition site of strain UCD 328

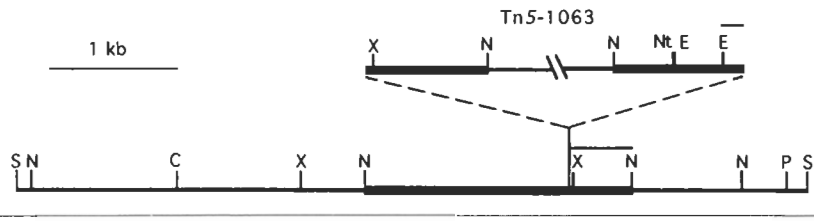

Fig. 2. Partial restriction map of the 6·2 kb *Spe*I genomic fragment bearing the Tn*5*-1063 insertion in strain UCD 328. Restriction sites are for *Cla*I (C), *Eag*I (E), *Nhe*I (N), *Not*I (Nt), *Pvu*II (P), *Spe*I (S) and *Xba*I (X). Bold lines indicate the *Nhe*I fragments which hybridize to the *Eag*I–*Nhe*I pSCR1 fragment (overlined) that was used to probe the Southern blot shown in Fig. 3.

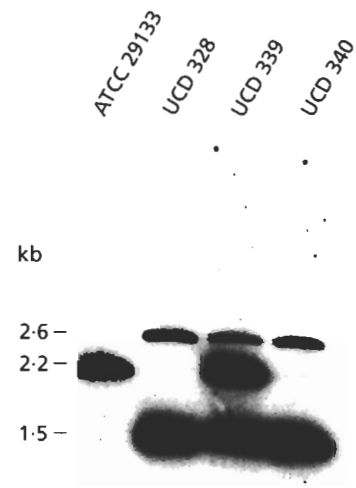

Fig. 3. Southern analysis of *Nostoc* 29133 strains. Markers indicate size of DNA in kb. A blot of total DNA (3–4 μg per lane) digested with *Nhe*I from wild-type *Nostoc* ATCC 29133, *Nostoc* 29133::Tn*5*-1063 (strain UCD 328); a *Nostoc* 29133::pSCR3 single recombinant (strain UCD 339); and a *Nostoc* 29133::pSCR3 double recombinant (strain UCD 340) was probed with a 660 bp *Eag*I–*Nhe*I fragment from pSCR1 having homology both to the transposon ends and to the *Nostoc* DNA flanking the right side of the transposon (Fig. 2). The uninterrupted 2·2 kb band has 540 bp of direct homology to the probe. Two bands, each containing a transposon end with flanking DNA, can be seen in strains UCD 328 and UCD 340; the 1·5 kb and the 2·6 kb bands respectively have 660 bp and 53 bp of direct homology to the probe. Strain UCD 339 contains both a wild-type 2·2 kb band and the two bands resulting from the transposon insertion.

hybridized strongly to three cosmid clones out of 2112 clones from the genomic library. Southern analysis revealed the hybridizing stretch of DNA to be on an 11 kb *Eco*RV fragment common to all three cosmids. Restriction mapping of a 6·2 kb *Spe*I fragment (Fig. 2), internal to the 11 kb *Eco*RV fragment, showed it to be identical to the *Nostoc* DNA flanking the transposition site in pSCR3.

This study demonstrates that transposon and insertion mutants of *Nostoc* 29133 can be generated. Such mutants will be useful in investigations of its developmental alternatives and its symbiotic interactions with plant tissues. We are currently characterizing the physiology and molecular genetics of strain UCD 328.

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REFERENCES

Allen, M. B. & Arnon, D. I. (1955). Studies on nitrogen-fixing blue-green algae. I. Growth and nitrogen fixation by *Anabaena cylindrica* Lemm. *Plant Physiol* **30**, 366–372.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (1987). *Current Protocols in Molecular Biology*. New York: Green Publishing Associates & Wiley-Interscience.

Better, M. & Helinski, D. R. (1983). Isolation and characterization of the *recA* gene of *Rhizobium meliloti*. *J Bacteriol* **155**, 311–316.

Black, T. A. & Wolk, C. P. (1994). Analysis of a Het⁻ mutation in *Anabaena* sp. strain PCC 7120 implicates a secondary metabolite in the regulation of heterocyst spacing. *J Bacteriol* **176**, 2282–2292.

Black, T. A., Cai, Y. & Wolk, C. P. (1993). Spatial expression and autoregulation of *hetR*, a gene involved in the control of heterocyst development in *Anabaena*. *Mol Microbiol* **9**, 77–84.

Braun, A. C. & Wood, H. N. (1962). On activation of certain essential biosynthetic systems in cells of *Vinca rosea* L. *Proc Natl Acad Sci USA* **48**, 1776–1782.

Buikema, W. J. & Haselkorn, R. (1991). Isolation and complementation of nitrogen fixation mutants of the cyanobacterium *Anabaena* sp. strain PCC 7120. *J Bacteriol* **173**, 1879–1885.

Buikema, W. J. & Haselkorn, R. (1993). Molecular genetics of cyanobacterial development. *Annu Rev Plant Physiol Plant Mol Biol* **44**, 33–52.

Cai, Y. & Wolk, C. P. (1990). Use of a conditionally lethal gene in *Anabaena* sp. strain PCC 7120 to select for double recombinants and to entrap insertion sequences. *J Bacteriol* **172**, 3138–3145.

Campbell, E. L. & Meeks, J. C. (1989). Characteristics of hormogonia formation by symbiotic *Nostoc* spp. in response to the presence of *Anthoceros punctatus* or its extracellular products. *Appl Environ Microbiol* **55**, 125–131.

Campbell, E. L. & Meeks, J. C. (1992). Evidence for plant-mediated regulation of nitrogenase expression in the *Anthoceros–Nostoc* symbiotic association. *J Gen Microbiol* **138**, 473–480.

Cardemil, L. & Wolk, C. P. (1976). The polysaccharides from heterocyst and spore envelopes of a blue-green algae. *J Biol Chem* **251**, 2967–2975.

Cardemil, L. & Wolk, C. P. (1981). Polysaccharides from the envelopes of heterocysts and spores of the blue-green algae *Anabaena variabilis* and *Cylindrospermum licheniforme*. *J Phycol* **17**, 234–240.

Damerval, T., Guglielmi, G., Houmard, J. & Tandeau de Marsac, N. (1991). Hormogonium formation in the cyanobacterium *Calothrix*: a photoregulated developmental process. *Plant Cell* **3**, 191–201.

Elhai, J. & Wolk, C. P. (1988a). A versatile class of positive-selection vectors based on the nonviability of palindrome-containing plasmids that allows cloning into long polylinkers. *Gene* **68**, 119–138.

Elhai, J. & Wolk, C. P. (1988b). Conjugal transfer of DNA to cyanobacteria. *Methods Enzymol* **167**, 747–754.

Enderlin, C. S. & Meeks, J. C. (1983). Pure culture and reconstitution of the *Anthoceros–Nostoc* symbiotic association. *Planta* **158**, 157–165.

Ernst, A., Black, T., Cai, Y., Panoff, J.-M., Tiwari, D. N. & Wolk, C. P. (1992). Synthesis of nitrogenase in mutants of the cyano-

bacterium *Anabaena* sp. strain PCC 7120 affected in heterocyst development or metabolism. *J Bacteriol* **174**, 6025–6032.

Felkner, R. H. & Barnum, S. R. (1988). Plasmid content and homology of 16 strains of filamentous, nonheterocystous cyanobacteria. *Curr Microbiol* **17**, 37–41.

Figurski, D. H. & Helinski, D. R. (1979). Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in *trans*. *Proc Natl Acad Sci USA* **76**, 1648–1652.

Figurski, D., Meyer, R., Miller, D. S. & Helinski, D. R. (1976). Generation in vitro of deletions in the broad host range plasmid RK2 using phage Mu insertions and a restriction endonuclease. *Gene* **1**, 107–119.

Grant, S. G., Jesse, J., Bloom, F. R. & Hanahan, D. (1990). Differential plasmid rescue from transgenic mouse DNAs into *Escherichia coli* methylation-restriction mutants. *Proc Natl Acad Sci USA* **87**, 4645–4649.

Herdman, M. (1987). Akinetes: structure and function. In *The Cyanobacteria*, pp. 227–250. Edited by P. Fay & C. van Baalen. Amsterdam: Elsevier.

Herdman, M. & Rippka, R. (1988). Cellular differentiation: hormogonia and baeocytes. *Methods Enzymol* **167**, 232–242.

Hirosawa, T. & Wolk, C. P. (1979). Isolation and characterization of a substance which stimulates the formation of akinetes in the cyanobacterium *Cylindrospermum licheniforme* Kutz. *J Gen Microbiol* **114**, 433–441.

Houmard, J. & Tandeau de Marsac, N. (1988). Cyanobacterial genetic tools: current status. *Methods Enzymol* **167**, 808–847.

Ish-Horowicz, D. & Burke, J. F. (1981). Rapid and efficient cosmid cloning. *Nucleic Acids Res* **9**, 2989–2998.

Johansson, C. & Bergman, B. (1994). Reconstitution of the *Gunnera manicata* Linden symbiosis: cyanobacterial specificity. *New Phytol* **126**, 643–652.

Lambert, G. R. & Carr, N. G. (1984). Resistance of DNA from filamentous and unicellular cyanobacteria to restriction endonuclease cleavage. *Biochim Biophys Acta* **781**, 45–55.

Meeks, J. C. (1990). Cyanobacterial–bryophyte associations. In *Symbiotic Cyanobacteria*, pp. 43–63. Edited by A. N. Rai. Boca Raton, FL: CRC Press.

Meeks, J. C., Wycoff, K. L., Chapman, J. S. & Enderlin, C. S. (1983). Regulation of expression of nitrate and dinitrogen assimilation by *Anabaena* species. *Appl Environ Microbiol* **45**, 1351–1359.

Meeks, J. C., Enderlin, C. S., Joseph, C. M., Chapman, J. S. & Lollar, M. W. L. (1985). Fixation of $[^{13}N]N_2$ and transfer of fixed nitrogen in the *Anthoceros–Nostoc* symbiotic association. *Planta* **164**, 406–414.

Potts, M. (1989). Isolation of plasmids from cyanobacteria. *BRL Focus* **6**, 4–6.

Raleigh, E. A. (1992). Organization and function of the *mcrBC* genes of *Escherichia coli* K-12. *Mol Microbiol* **6**, 1079–1086.

Rippka, R., Deruelles, J., Waterbury, J. B., Herdman, M. & Stanier, R. Y. (1979). Generic assignments, strain histories and properties of pure cultures of cyanobacteria. *J Gen Microbiol* **111**, 1–61.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Shestakov, S. V. & Reaston, J. (1987). Gene transfer and host–vector systems of cyanobacteria. *Oxford Surv Plant Mol Cell Biol* **4**, 137–166.

Steinberg, N. A. & Meeks, J. C. (1991). Physiological sources of reductant for nitrogen fixation activity of *Nostoc* sp. strain UCD 7801 in symbiotic association with *Anthoceros punctatus*. *J Bacteriol* **173**, 6227–6233.

Stuttzman, K. & Gendel, S. M. (1985). A rapid method for identifying plasmids in cyanobacteria. *FEMS Microbiol Lett* **26**, 337–348.

Vieira, J. & Messing, J. (1982). The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene* **19**, 259–268.

Wallis, J. G. (1993). *Development of genetic tools for the cyanobacterium Nostoc sp. strain ATCC 29133.* PhD thesis, University of California, Davis.

Wolk, C. P. (1982). Heterocysts. In *The Biology of Cyanobacteria*, pp. 359–386. Edited by N. G. Carr & B. A. Whitton. Oxford: Blackwell Scientific Publications.

Wolk, C. P. (1991). Genetic analysis of cyanobacterial development. *Curr Opin Genet Dev* **1**, 336–339.

Wolk, C. P., Vonshank, A., Kehoe, P. & Elhai, J. (1984). Construction of shuttle vectors capable of conjugative transfer from *Escherichia coli* to nitrogen fixing filamentous cyanobacteria. *Proc Natl Acad Sci USA* **81**, 5355–5359.

Wolk, C. P., Cai, Y. & Panoff, J-M. (1991). Use of a transposon with luciferase as a reporter to identify environmentally responsive genes in a cyanobacterium. *Proc Natl Acad Sci USA* **88**, 5355–5359.

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