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 Tn5 derivative, Tn5-1063 (Kmr Bmr Smr), delivered by conjugation from Escherichia coli, antibiotic-resistant mutants of Nostoc 29133 were generated at a frequency of approximately $1 \times 10^{-4}$, 0.4% of which expressed a nitrogen fixation (heterocyst) defective phenotype. Mutant strain UCD 328 was isolated after coculture of 86 Nostoc 29133::Tn5-1063 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 photosynthetic, restricted 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 anoxic 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.
<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics*</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli DH5α</td>
<td>recA1 hsdR17 (rK mK) endA1</td>
<td>Grant et al. (1990)</td>
</tr>
<tr>
<td>E. coli DH5α-MCR</td>
<td>Genotype of DH5α and mcrA Δ(mrr hsdMR3 mcrBC)</td>
<td>Grant et al. (1990)</td>
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<tr>
<td>HB101</td>
<td>recA13 hsdS20 (rK mK)</td>
<td>Grant et al. (1990)</td>
</tr>
<tr>
<td>Nostoc ATCC 29133 (PCC 73102)</td>
<td>Fix+ Sym+</td>
<td>Rippka et al. (1979); Enderlin &amp; Meeks (1983)</td>
</tr>
<tr>
<td>UCD 7801</td>
<td>Fix+ Sym+</td>
<td>Enderlin &amp; Meeks (1983)</td>
</tr>
<tr>
<td>UCD 328</td>
<td>Superinfective Nostoc 29133::Tn5-1063 strain, Fix+</td>
<td>This study</td>
</tr>
<tr>
<td>UCD 339</td>
<td>pSCR3 integrated by single recombination in the genome</td>
<td>This study</td>
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<tr>
<td>UCD 340</td>
<td>Reconstruction of the UCD 328 mutation</td>
<td>This study</td>
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<tr>
<td>Plasmids</td>
<td></td>
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<tr>
<td>pDUCA7</td>
<td>Cosmid shuttle vector with RK2 and pDU1 oriV's, Km'</td>
<td>Buikema &amp; Haselkorn (1991)</td>
</tr>
<tr>
<td>pRK212.1</td>
<td>RK2 Mu phage insertion/deletion derivative, Km'</td>
<td>Figurski et al. (1976)</td>
</tr>
<tr>
<td>pRK2013</td>
<td>RK2 derivative with ColE1 oriV', Km'</td>
<td>Figurski &amp; Helinski (1979)</td>
</tr>
<tr>
<td>pRK2073</td>
<td>pRK2013::Tn7, Km' Sp'</td>
<td>Better &amp; Helinski (1983)</td>
</tr>
<tr>
<td>pRL6</td>
<td>Shuttle vector with pMB1 and pDU1 oriV's</td>
<td>Wolk et al. (1984)</td>
</tr>
<tr>
<td>pRL479</td>
<td>Subclone of 11 kb EcoRV fragment from UCD 328, Bm' Km' Sm'</td>
<td>Elhai &amp; Wolk (1988b) for nomenclature</td>
</tr>
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<td>pRL1063a</td>
<td>Tn5-1063, RK2 oriT, Bm' Km' Sm'</td>
<td>Wolk et al. (1991)</td>
</tr>
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<td>pRL1075</td>
<td>Used for reconstructing Tn5-1063 insertions, Cm' Em' Suc'</td>
<td>Black et al. (1993)</td>
</tr>
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<td>pSCR1</td>
<td>Recovered 19 kb EcoRV Tn5-1063 bearing fragment from UCD 328, Bm' Km' Sm'</td>
<td>This study</td>
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<tr>
<td>pSCR2</td>
<td>pSCR1 with NdeI site filled in, Bm' Km' Sm'</td>
<td>This study</td>
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<td>pSCR3</td>
<td>pSCR2 with positive selection cassette from pRL1075, Bm' Cm' Em' Km' Sm' Suc'</td>
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<td>pSCR4</td>
<td>Subclone of 11 kb EcoRV fragment into pRL479</td>
<td>This study</td>
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<tr>
<td>pSCR5</td>
<td>Subclone of 6.2 kb Spel fragment into pUC18</td>
<td>This study</td>
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<tr>
<td>pUC18</td>
<td>pBR322-derived vector</td>
<td>Vieira &amp; Messing (1982)</td>
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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 Macrogamia 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* sp. differentiate heterocytes at a frequency of about 45% of the total cells (Enderlin & Meeks, 1983; Meeks, 1990), fix N₂ 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, Tn5-1063, which contains a promoterless lacZ as a reporter gene, developed by Wolk et al. (1991) for use in *Anabaena* 7120. From a *Nostoc* 29133::Tn5-1063 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 (OD600). Sources and culture conditions for sambroakit-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 & Amon (1955) (AA) supplemented with 5 mM MOPS, pH 7.8 (−N), and, when specified, 25 mM NH₄Cl (+N); it was used at full strength when solidified with 1.5% (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-³²P]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/Cl⁻, 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 T₁ (Bethesda Research Laboratories).

Plasmids were isolated from 50 ml liquid cultures of *Nostoc* 29133 grown under Nm selection and having Chl a concentration 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 Trition 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 aqueous phase was washed twice with phenol/chloroform until the interface was clear. 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% (v/v) 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 nuclelease. *EcoRI–XmnI* adapters were ligated to the ends and these adapted inserts were ligated into *EcoRI*-digested cosmids 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 OD₆₀₀ 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 OD₆₀₀ 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 for 16–24 h to allow for conjugal mating. To allow 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⁻ 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, Anabaena 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 Tn5-1063 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::Tn5-1063 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<sup>a</sup> 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. (5.5 x 10<sup>-4</sup> Km<sup>a</sup> per c.f.u.) that was at least 50-fold higher than the frequency using pRL443 (< 9.1 x 10<sup>-6</sup> Km<sup>a</sup> 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.

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

<table>
<thead>
<tr>
<th>Source of DNA</th>
<th>E. coli strain transformed</th>
<th>Km&lt;sup&gt;a&lt;/sup&gt; colonies per µg DNA</th>
<th>Ratio DH5a/ DH5a-MCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>pDUCA7</td>
<td>EH101</td>
<td>7.4 x 10&lt;sup&gt;-2&lt;/sup&gt;</td>
<td>0.91</td>
</tr>
<tr>
<td><em>Nostoc</em> 29133</td>
<td>DH5a</td>
<td>7.9 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>0.005</td>
</tr>
</tbody>
</table>

For a comparison of transformation efficiencies, *E. coli* strains DH5a and DH5a-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 DH5a-MCR (Grant et al., 1990). In control experiments plasmid DNA isolated from *E. coli* HB101 transformed the two DH5a strains at nearly equal efficiencies. However, plasmid DNA isolated from *Nostoc* 29133 transformed DH5a-MCR with at least a 200-fold greater frequency than it transformed DH5a (Table 2). To bypass this apparent restriction barrier, a *Nostoc* 29133 genomic DNA library, constructed in pDUCA7 and packaged in λ phage, was transferred into *E. coli* strain DH5a-MCR. This genomic library has served as a source for wild-type DNA corresponding to the transposition sites of all *Nostoc* 29133::Tn5-1063 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 Tn5-1063 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 1.0 ± 0.8) x 10<sup>-8</sup> [mean ± SD, n = 7] *Nostoc* 29133::Tn5-1063 colonies per viable *Nostoc* cell in the mating mixture (based on a value of 1.75 ± 1.8 g Chl a per viable cell). The yield of resultant colonies per filter is about five times lower than that reported for Tn5-1063 mutagenesis of *Anabaena* 7120 (Wolk et al., 1991).

Of 5.1 x 10<sup>8</sup> mutant colonies screened, 14 Fox<sup>-</sup> mutants, all with discernible heterocyst defects, were identified. Two Fox<sup>-</sup> strains had a fragmented, short-filament phenotype in the presence or absence of combined nitrogen. Symbiotic competence was retained in four Fox<sup>-</sup> strains; it has been shown previously that for strain UCD 223, a *Nostoc* 29133 Fox<sup>-</sup> mutant generated by chemical mutagenesis, the microaerobic symbiotic cavities
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.

Reconstruction of the strain UCD 328 mutation

Southern blots of total EcoRV-digested DNA from strain UCD 328, probed with *HincII*-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 Tn5-1063 insertion. The positive selection plasmid, pSCR3, containing Tn5-1063 with an inactivated transposase gene in a 6.2 kb *Nostoc* 29133 SpeI fragment (Fig. 2), was introduced into *Nostoc* 29133 by conjugation. Single recombination of pSCR3 into the *Nostoc* 29133 genome gave rise to a Nmr Emr Sucs phenotype. A second recombination on the opposite side of the transposon resulted in a Nmr Em Sucs phenotype and was selected by plating on sucrose-containing medium (Chai & 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 without NH₄⁺. 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::Tn5-1063 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.

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Of 86 randomly chosen Fix⁺ *Nostoc* 29133::Tn5-1063 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 EcoRV-digested DNA from strain UCD 328, probed with *HincII*-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 Tn5-1063 insertion. The positive selection plasmid, pSCR3, containing Tn5-1063 with an inactivated transposase gene in a 6.2 kb *Nostoc* 29133 SpeI fragment (Fig. 2), was introduced into *Nostoc* 29133 by conjugation. Single recombination of pSCR3 into the *Nostoc* 29133 genome gave rise to a Nmr Em Sucs phenotype. A second recombination on the opposite side of the transposon resulted in a Nmr Em Sucs phenotype and was selected by plating on sucrose-containing medium (Chai & 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

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**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.

**Fig. 2.** Partial restriction map of the 6.2 kb SpeI genomic fragment bearing the Tn5-1063 insertion in strain UCD 328. Restriction sites are for Clal (C), Eagl (E), Nhel (N), NotI (Nt), PvuII (P), SpeI (S) and Xbal (X). Bold lines indicate the Nhel fragments which hybridize to the Eagl–Nhel pSCR1 fragment (overlined) that was used to probe the Southern blot shown in Fig. 3.
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 EcoRV fragment common to all three cosmids. Restriction mapping of a 6.2 kb SphI fragment (Fig. 2), internal to the 11 kb EcoRV 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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