MPMI Vol. 10, No. 2, 1997, pp. 280–289. Publication no. M-1997-0127-03R.

A Hormogonium Regulating Locus, *hrmUA*, of the Cyanobacterium *Nostoc punctiforme* Strain ATCC 29133 and its Response to an Extract of a Symbiotic Plant Partner *Anthoceros punctatus*

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Received 12 August 1996. Accepted 13 November 1996.

Transposon-generated mutant strain UCD 328 of *Nostoc punctiforme* strain ATCC 29133 has a phenotype of an increased sensitivity to a hormogonium-inducing factor exuded by a symbiotic plant partner, *Anthoceros punctatus*, and an initial increased hormogonium-dependent infection of the plant. Sequence analysis showed that the transposition site in strain UCD 328 lies within a 1,251-bp open reading frame (ORF), designated *hrmA*, that displays no significant similarity to known database sequences. A second, 837-bp ORF (*hrmU*) ends 2 bp 5′ from the start of *hrmA* and has the signature sequences belonging to a family of NAD(P)H-dependent oxidoreductases. Strains having insertional mutations in *hrmU* or *hrmA* reproduce the strain UCD 328 phenotype. Transcriptional fusions of *luxAB* to *hrmU* or *hrmA* show an 8- to 10-fold peak increase in luciferase activity 13 to 20 h after the start of incubation in the presence of an aqueous extract of *A. punctatus*. A promoter induced by the extract was deduced to be between 2.0 to 3.4 kb from the translational start of *hrmU*. A multicopy plasmid that contains *hrmUA* within a 6.2-kb fragment conferred an increased infection phenotype on wild-type *N. punctiforme* 29133. This plasmid and another plasmid containing 4.4 kb of DNA 5′ of the transposition site prevented extract-dependent induction of *hrmA-luxAB* transcription in strain UCD 328, implicating titration of some *trans*-activator(s) by the cloned fragments. We hypothesize a role for *hrmUA* in the inhibition of hormogonium formation by the metabolism of an unknown hormogonium-regulating metabolite.

Additional keywords: hormogonium differentiation, multicopy inhibition, *Nostoc* sp. strain ATCC 29133, omega-*npt* cassette, plant-cyanobacterial signaling, symbiotic cyanobacterium.

Cyanobacteria of the genus *Nostoc* form nitrogen-fixing symbiotic associations with representatives of all major groups within the phylogenetic spectrum of plants (Rai 1990). The plant partners include the bryophyte, *Anthoceros punctatus*, where the *Nostoc* spp. establish macroscopically visible colonies within preformed cavities of the plant gametophyte thallus (Ridgeway 1967; Rodgers and Stewart 1977; Enderlin and Meeks 1983).

Plant control over *Nostoc* developmental alternatives is critical in the establishment and maintenance of the symbiotic association (Meeks 1990). These developmental alternatives include the formation of hormogonia, motile (gliding) filaments that serve as the infective units in establishing the association, and heterocysts, cells specialized for nitrogen fixation. When starved for combined nitrogen, gametophyte tissue of *A. punctatus* enhances *Nostoc* infection by exuding a hormogonium-inducing factor (HIF) (Campbell and Meeks 1989). The capacity of a *Nostoc* strain to form hormogonia in response to HIF is necessary, but not singularly sufficient, for symbiotic competence (Enderlin and Meeks 1983). Hormogonium-inducing activity has also been observed from exudates of an angiosperm symbiotic partner of *Nostoc* spp., *Gunnera manicata* (Rasmussen et al. 1994) and of wheat (Gantar et al. 1993). In addition to plant derived HIF, environmental conditions such as light quality and excess nutrients can induce hormogonium formation (Herdman and Rippka 1988). Cells in the process of hormogonium formation undergo cell division without DNA replication or an increase in cell mass (Herdman and Rippka 1988). Nascent hormogonial filaments fragment at the junctions between vegetative cells and heterocysts; during this process heterocysts are released from the filaments and, thus, hormogonia cannot fix nitrogen (Campbell and Meeks 1989). The rates of ammonium assimilation and photosynthetic CO_2 fixation by hormogonia also decline substantially (Campbell and Meeks 1989). Several genes involved in the regulation of heterocyst differentiation have been identified (Wolk et al. 1994), however, comparatively little is known of the genetic regulatory mechanisms that underlie hormogonium development (Tandeau de Marsac 1994).

Once inside the symbiotic cavities, the differentiation of heterocysts is favored over non-nitrogen fixing developmental alternatives of *Nostoc*, including hormogonia. *A. punctatus* induces the *Nostoc* filaments within the cavities to differentiate approximately 45% of their total cells into heterocysts (Enderlin and Meeks 1983), which release more than 80% of their fixed nitrogen as ammonium into the cavity for uptake

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Nucleotide and/or amino acid sequence data have been submitted to GenBank as accession number L37087.

by the plant (Meeks et al. 1985). In contrast, heterocysts in free-living *Nostoc* filaments comprise 3 to 10% of the total cells and release their fixed nitrogen as glutamine to neighboring vegetative cells (Thomas et al. 1977).

The mechanisms used by the plant to influence the development of symbiotically associated *Nostoc* are unknown, but could conceivably involve the mimicry or metabolism of *Nostoc* autogenic regulatory factors. Autogenic factors have been implicated in the induction (Lazaroff and Vishniac 1961; Robinson and Miller 1970) and inhibition (Herdman and Rippka 1988; Lazaroff 1973; Lazaroff and Vishniac 1964) of hormogonium formation, and in the inhibition of heterocyst differentiation (Black and Wolk 1994).

Transposon mutagenesis of *Nostoc punctiforme* strain ATCC 29133 (PCC 73102) generated a mutant, strain UCD 328, that is more responsive to HIF in co-cultures with *A. punctatus* and subsequently infects the plant cavities at a higher frequency (Cohen et al. 1994). We report here that the transposition site of strain UCD 328 is within a genetic locus whose transcription is increased in response to incubation with an *A. punctatus* aqueous extract. We hypothesize that this locus encodes an enzyme complex which functions in the synthesis or modification of a hormogonium-regulating metabolite to inhibit hormogonium formation.

RESULTS

The phenotype of strain UCD 328.

A Tn*5*-1063 transposition into a 6.2-kb *Spe*I fragment of the *N. punctiforme* 29133 genome generated mutant strain UCD 328, which has the phenotype of an increased initial infection frequency of *A. punctatus* (Table 1; Cohen et al. 1994) and converted more than 80% of its vegetative filaments to hormogonia in response to medium containing HIF from *A. punctatus* (Table 1). Co-culture of equal amounts of wild-type *N. punctiforme* 29133 and strain UCD 328 cells neither reduced nor increased the resultant infection frequency of *A. punctatus* by strain UCD 328 (Table 1). The epiphytic growth of strain UCD 328 was severely reduced relative to wild-type *Nostoc* strains when it was co-cultured with the plant (Cohen et al. 1994) and the strain UCD 328 population consisted mostly of hormogonia or short hormogonia-like filaments and clusters of heterocysts unattached to vegetative filaments. Following an initial burst of infection in co-cultures of strain UCD 328 with *A. punctatus*, there was a decline in the frequency of new infections so that, when coupled with the growth of *A. punctatus* tissue, the number of symbiotic colonies per mg dry weight of plant tissue decreased from 1.7 after 2 weeks of co-culture (Table 1) to 0.35 after 7 weeks. By 7 weeks after reconstitution with strain UCD 328, the margins of the *A. punctatus* gametophyte tissue had begun to bleach, presumably due to the lack of fixed nitrogen in the growing region, thus analysis was routinely terminated at that time. Conversely, in a co-culture of wild-type *N. punctiforme* 29133 with *A. punctatus*, the number of symbiotic colonies per mg of plant tissue increased from 0.15 after 2 weeks to a steady-state level of approximately 0.3 after 10 weeks. The 10-week time point after reconstitution of wild-type associations with *A. punctatus* was previously established as having reached steady state and such tissue was used in standard physiological experiments (Meeks 1990).

Sequence analysis of the DNA near the transposition site of strain UCD 328.

Sequence analysis of 2.4 kb around the transposition site of strain UCD 328 revealed the presence of two open reading frames (ORF). The translational stop site of an 837-bp ORF, termed *hrmU*, is followed just 2 bp downstream by a 1,251-bp ORF, designated *hrmA*, which contains the strain UCD 328::Tn*5*-1063 insertion site (Fig. 1A). The derived amino acid sequence of *hrmA* shows no significant similarity to database sequences, nor any characteristic motif. There are three direct repeats at residues 15 to 27, 49 to 61 and 81 to 93 of HrmA having the consensus sequence $QIXXL(X)_7R$. The derived amino acid sequence of *hrmU* has a putative NAD(P)H coenzyme-binding domain (Scrutton et al. 1990) near its N-terminus, a putative oxidoreductase catalytic domain (Fig. 1B; Chen et al. 1993) and additional similarity to proteins belonging to a large family of NAD(P)H-dependent oxidoreductases. It bears no greater than 35% identity to the amino acid sequence of any given member of the family.

Insertional mutagenesis of *hrmA* and *hrmU*.

Previously, reconstruction of the strain UCD 328 transposon mutation by double recombination of pSCR3 in wild-type *N. punctiforme* 29133 yielded strain UCD 340, which reproduced the mutant phenotype (Table 1; Cohen et al. 1994). An Ω-cassette was utilized for insertional mutagenesis of other sites within the *hrmUA* locus of *N. punctiforme* 29133. Strong transcriptional terminators prevent transcripts from exiting either end of an Ω-cassette (Prentki and Krisch 1984). Since the Ω-Sm^r/Sp^r cassette of pRL453 (Elhai and Wolk 1988) does not confer sufficient antibiotic resistance in *N. punctiforme* 29133,

Table 1. Infection frequency of *Anthoceros punctatus* gametophyte tissue by various *Nostoc* strains and their response to HIF

Nostoc strain[a]	Infection frequency[b]	HIF induced hormogonia formation (%)[c]
ATCC 29133	0.15 ± 0.06 (n = 6)	<30
UCD 328 (*hrmA*::Tn*5*-1063)	1.70 ± 0.40 (n = 6)	>80
UCD 328 + ATCC 29133[d]	2.0	NM
UCD 393 (*hrmA*::Ω-*npt*, SR)	0.05 ± 0.01 (n = 3)	<30
UCD 347 (*hrmA*:: Ω-*npt*, DR)	1.30 ± 0.10 (n = 2)	>80
UCD 357 (*hrmU*:: Ω-*npt*, SR)	1.50 ± 0.40 (n = 2)	>80
UCD 392 (*hrmU*:: Ω-*npt*, DR)	1.7	>80
UCD 346 (*hrmU-luxAB*, SR)	1.4	>80
UCD 328/pSCR7	1.6	>80
ATCC 29133/pSCR7	0.84 ± 0.09 (n = 3)	>80
UCD 328/pSCR18	1.5	>80

[a] Abbreviations: SR, single recombinant; DR, double recombinant.

[b] Infection frequencies were measured 14 days after the start of co-culture. Values are the mean number ± SE of symbiotic colonies per mg of tissue dry weight per unit of *Nostoc* cells added (as µg of Chl*a*) with the number of replicates of separate co-cultures in parentheses. Values without errors are results of single experiments.

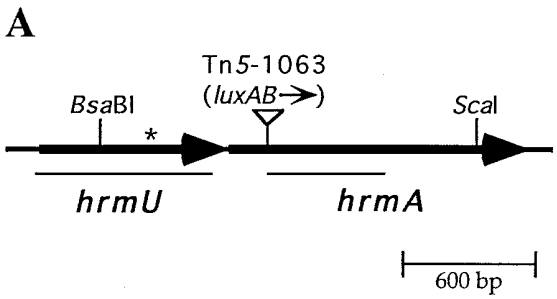
[c] Induction of hormogonia by HIF (*A. punctatus* combined nitrogen-minus growth-conditioned medium) was carried out as described previously (Cohen et al. 1994). Results were recorded as the total percentage of hormogonia in a population of filaments after overnight incubation in HIF. For the purpose of quantification, the cells between two heterocysts within a vegetative filament are counted as a single vegetative filament since each one of these units can potentially give rise to a single hormogonial filament. NM, not measured.

[d] Equal amounts of cells of strain UCD 328 and strain ATCC 29133 were added. The infection frequency was calculated relative to the Chl *a* added from only one of the strains.

we constructed a vector (pSCR9) that carries a neomycin resistance marker (*npt*) in place of the Sm^r/Sp^r gene of pRL453 (see Materials and Methods).

To interrupt *hrmA*, the Ω-*npt* cassette from pSCR9 was cloned into the *Sca*I site (Fig. 1A) of the 6.2-kb *N. punctiforme* 29133 *Spe*I fragment (pSCR7, Fig. 2B), combined with the *sacB*-containing portion of pRL271 and the resulting plasmid (pSCR10) transferred into a wild-type *N. punctiforme* 29133. A single recombination upstream of the Ω-*npt* insertion yielded the neomycin-resistant (Nm^r), sucrose-sensitive (Suc^s) strain UCD 393. Strain UCD 393, which has the sequence of the insertion vector, pRL271, 3.4 kb 5′ from the wild-type copy of *hrmUA* (Fig. 2A), infected *A. punctatus* at a lower frequency than wild-type *N. punctiforme* 29133 (Table 1). Upstream single recombinants of other *hrmUA*-insertion vectors having 2 kb or less of *N. punctiforme* 29133 DNA 5′ to the wild-type copy of *hrmUA* have a high infection phenotype (see below). A second recombination downstream of the *hrmA::* Ω-*npt* insertion yielded the Nm^r Suc^r strain UCD 347, which had an increased frequency of infection of *A. punctatus* and formed more than 80% hormogonia in response to HIF (Table 1).

To interrupt *hrmU*, the Ω-*npt* cassette was cloned into the *Bsa*BI site (Fig. 1A) of a 3.0-kb *N. punctiforme* 29133 genomic DNA fragment (pSCR15), combined with the *sacB*-containing portion of pRL271 and the resulting plasmid (pSCR12) transferred into a wild-type *N. punctiforme* 29133 by conjugation. A single recombination upstream of the Ω-*npt* insertion site yielded strain UCD 357, which has 1.0 kb of *Nostoc* DNA 5′ to the wild-type copy of *hrmUA* (Fig. 2A). A second recombination generated the Nm^r Suc^r strain UCD 392. Both strains UCD 357 and UCD 392 expressed the mutant phenotypes of increased infection frequency of *A. punctatus* and increased hormogonium formation in response to HIF (Table 1).



B

HrmU	172	Tyr	Ser	Ala	Ala	Lys	Ala Gly
HetN	155	Tyr	Ser	Ala	Ser	Lys	Ala Gly
NodG	152	Tyr	Cys	Ala	Ser	Lys	Ala Gly
ActKR	157	Tyr	Ser	Ala	Ser	Lys	His Gly
FabG	152	Tyr	Ser	Ala	Ala	Lys	Ala Gly
PGDH	150	Tyr	Cys	Ala	Ser	Lys	His Gly
ADH	152	Tyr	Ser	Gly	Thr	Lys	Ala Ala

Fig. 1. A, The 2.4-kb sequenced region of *Nostoc punctiforme* 29133 DNA (GenBank L37087) showing the transposition site in strain UCD 328 and the restriction sites used in the construction of recombinant strains. Tn*5*-1063 (7.8 kb) is not drawn to scale. The DNA fragments used as *hrmU* and *hrmA* specific hybridization probes are shown below their respective genes. The putative catalytic domain of HrmU, whose position is indicated by the asterisk in panel **A**, is aligned in **B** from residue 172 to 178 with the corresponding sequence of HetN from *Anabaena* (*Nostoc*) sp. strain PCC 7120 (Black and Wolk 1994); NodG from *Rhizobium meliloti* (Debellé and Sharma 1986); ActKR, ActIII ketoreductase from *Streptomyces coelicolor* (Hallam et al. 1988); FabG from *Escherichia coli* (Rawlings and Cronan 1992); PGDH, 15-hydroxyprostaglandin dehydrogenase (Krook et al. 1990); and ADH, alcohol dehydrogenase from *Drosophila melanogaster* (Thatcher and Sawyer 1980).

Response of *Nostoc* strains to plant extract.

The *luxAB* of Tn*5*-1063 in strain UCD 328 formed a transcriptional fusion to *hrmA* upon transposition (Fig. 1A). Luciferase expression from strain UCD 328 was monitored in response to several treatments and the preliminary results indicated that some plant factor(s), not HIF, can induce an increase in *hrmA* transcript levels. Cells of strain UCD 328 were suspended in a filtered extract of macerated *A. punctatus* tissue which had been cultured in medium containing combined nitrogen. Within 13 to 20 h after the start of incubation, luciferase activity increased by approximately 8- to 10-fold, when normalized to biomass (Chl *a*; Table 2, Fig. 3A) or total protein synthesis (Fig. 3B), respectively, of strain UCD 328. Extracts made from *A. punctatus* tissue previously incubated for 7 days in the absence of combined nitrogen had similar *hrmA-luxAB* inducing activities (data not shown). A dilution series of the extract showed a linear decline in the *hrmA-luxAB* inducing activity (Fig. 4). Luciferase activity of strain UCD 339, containing a single recombination of a *hrmA::*Tn*5*-1063 (*hrmA-luxAB*) reconstruction vector (pSCR3) and its double recombinant derivative, strain UCD 340, were both induced by incubation in the presence of plant extract (Table 2). The relatively higher levels of uninduced and extract-induced luciferase activity from strain UCD 339 are presumably due to read-through transcription from a chloramphenicol resistance gene of pSCR3 located upstream of the *hrmA-luxAB* fusion. A single recombinant, strain UCD 394, of a different *hrmA*::Tn*5*-1063 reconstruction vector (pSCR19), which does not have vector sequence upstream of *hrmA-luxAB*, shows uninduced and extract-induced levels of luciferase activity similar to strains UCD 328 and UCD 340 (Table 2). In a control experiment, incubation in plant extract did not increase the luciferase activity of strain UCD 372 (Table 2), which has a transcriptional fusion of *luxAB* to *zwf*, the gene encoding glucose-6-phosphate dehydrogenase (Summers et al. 1995).

Unlike the HIF from *A. punctatus* (Campbell and Meeks 1989), the factor(s) responsible for the *hrmA-luxAB* inducing activity of the extract does not appear to be excreted into the medium, tolerates autoclaving (Fig. 4) and long-term storage, and is not complexed by the addition of polyvinylpyrrolidone (PVPP; data not shown). Treatments known to have an effect on hormogonium formation in some *Nostoc* strains, such as simple dilution into fresh medium (Fig. 3), incubation in HIF, or 50 mM glucose, and exposure to red or green light, had no effect on the expression of *hrmA-luxAB* (data not shown). While dilution into fresh medium induced hormogonium formation in *N. punctiforme* 29133 and strain UCD 328, none of the other treatments were effective in either strain. The pres-

ence of plant extract completely repressed the HIF-dependent formation of hormogonia by wild-type *N. punctiforme* 29133, but not by strain UCD 328. Thus, strain UCD 328 had apparently lost the wild-type ability to respond to some hormogonium repressing factor(s) in the extract.

To determine if incubation with the extract also increased the expression of *hrmU*, a non-replicating vector (pSCR13) having a *hrmU-luxAB* transcriptional fusion was transferred into a wild-type *N. punctiforme* 29133 by conjugation. The resulting single recombinant, strain UCD 346, which retains vector sequence 2 kb upstream of the wild-type copy of *hrmUA* (Fig. 2A), was monitored for luciferase expression. Both the intensity (Table 2) and duration (data not shown) of its response to plant extract were like that from the *hrmA-luxAB* fusion of strain UCD 328. Strain UCD 346 showed a high frequency of infection of *A. punctatus* and increased formation of hormogonia in response to HIF (Table 1).

Effect of multiple copies of the *hrmUA* locus in *Nostoc* strains.

The 6.2-kb *Spe*I fragment of *N. punctiforme* 29133 DNA which encompasses *hrmUA* (pSCR7, Fig. 2B; Cohen et al. 1994) was subcloned into pSCR202, a multicopy shuttle vector (Summers et al. 1995) and the resulting plasmid (pSCR7) transferred into strain UCD 328 by electrotransformation. The presence of pSCR7 in strain UCD 328 did not change its infection frequency nor its frequency of hormogonium formation in the presence of HIF (Table 1) and, thus, did not complement the strain UCD 328 mutant phenotype. However, the presence of pSCR7 in strain UCD 328 prevented an increase in luciferase activity in response to plant extract (Table 2). In a control experiment, the presence of unmodified pSCR202 in strain UCD 328 did not repress the expression of luciferase in response to plant extract (Table 2). A wild-type *N. punctiforme* 29133 was electrotransformed with pSCR7; the resulting strain had an approximately fivefold increase in the infection frequency of *A. punctatus* and an increased frequency of hormogonium formation in response to HIF (Table 1).

To help determine whether the effect of pSCR7 was due to the titration of a *trans*-activating factor of *hrmUA* transcription or due to production of a possible pSCR7-encoded repressor of *hrmUA* (see Discussion), a fragment of *N. punctiforme* 29133 DNA from the Tn*5*-1063 insertion in *hrmA*, 4.4-kb upstream to the *Spe*I site, was subcloned into pSCR202 (Fig. 2B). The resulting plasmid (pSCR18) was electrotransformed into strain UCD 328 and, like pSCR7, did not complement the strain UCD 328 mutant phenotype (Table 1), but did prevent induction of luciferase activity (Table 2).

DISCUSSION

Disruption of the *hrmUA* locus of *N. punctiforme* 29133 results in an increased initial infection frequency of *A. punctatus* that correlates with an increased sensitivity to HIF exuded by

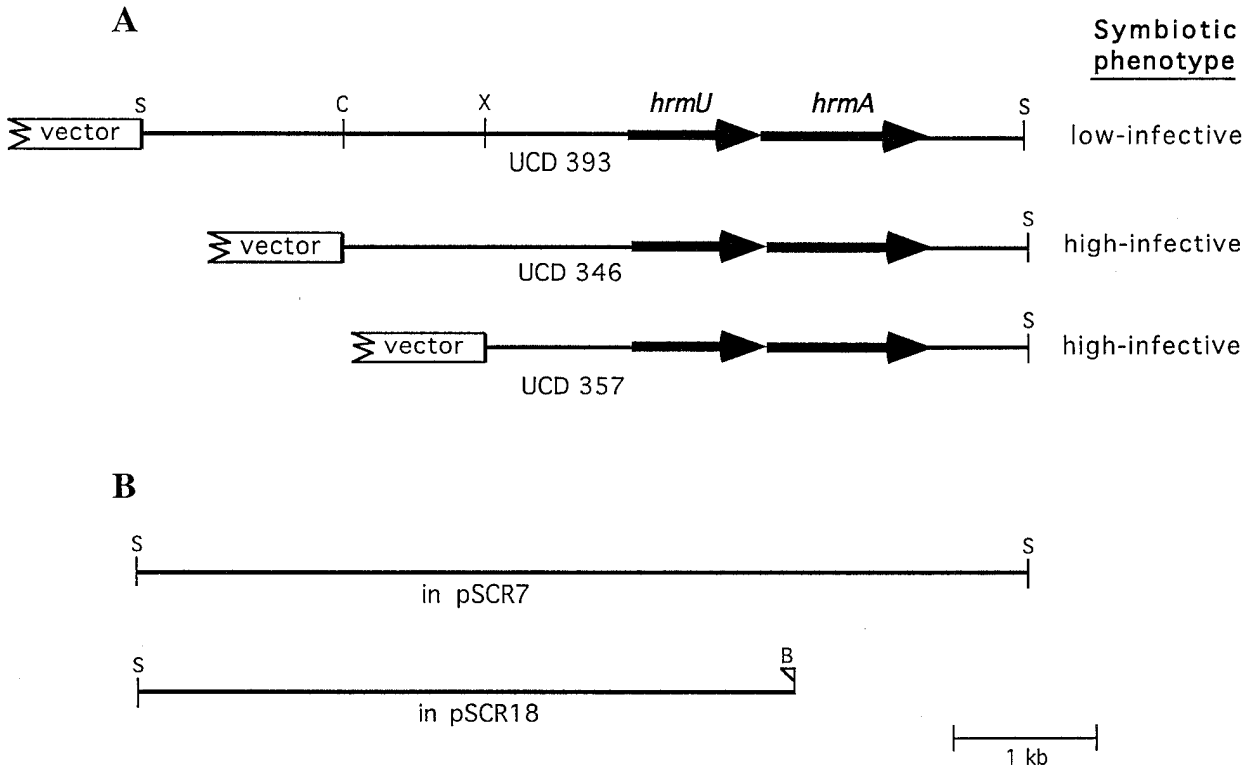


Fig. 2. Aligned maps of *hrmUA* DNA found in the chromosome of selected *Nostoc punctiforme* 29133 single recombinant strains and in two multicopy plasmids. **A,** Effects of truncations in the DNA 5´ to the wild-type copy *hrmUA* sequence on the symbiotic phenotype of single recombinant strains where the initial recombination occurred upstream of the insertion of Ω-*npt* (strains UCD 393 [*hrmA*] and 357 [*hrmU*]) or *luxAB* (strain UCD 346 [*hrmU*]). Sequences upstream of the identified vector position contains the interrupted gene copy and various amounts of DNA 3´ of the interrupted gene. **B,** Fragments of *Nostoc* DNA carried in the multicopy plasmids pSCR7 and pSCR18. Restriction sites are for *Bam*HI, B (originally from the *lux* end of Tn*5*-1063); *Cla*I, C; *Spe*I, S; and *Xba*I, X.

A. punctatus (Table 1). Epiphytic filaments of strain UCD 328 in co-culture with *A. punctatus* do form heterocysts and, thus, they do exit the hormogonial state. However, as evidenced by the large clusters of individual detached heterocysts, newly formed heterocysts do not remain attached to the filaments. Thus, strain UCD 328 is unable to grow to a high density in co-cultures with N_2 as the sole nitrogen source. Apparently, because of its sensitivity to HIF, strain UCD 328 re-enters the hormogonium differentiation mode more often than its parental and other wild-type *Nostoc* strains. The high continuous production of hormogonia, a non-growth state, accounts for low epiphytic growth yield and the reduced frequency of infections by strain UCD 328 in long term co-cultures with *A. punctatus.* The growth advantage of the *hrmUA* locus to *N. punctiforme* 29133 appears to be its role in inhibiting the response of vegetative cells to hormogonium induction thereby allowing filaments to accumulate biomass before re-entering the hormogonial state.

The *hrmU* gene has extensive similarity to a superfamily of oxidoreductases that includes members which function in the synthesis and degradation of signaling molecules of bacteria, plants and animals (Baker 1992). Southern blot analysis of 10 different filamentous cyanobacteria and one unicellular cyanobacterium shows that bands hybridizing to both *hrmU* and *hrmA* are not restricted to hormogonium-producing strains and that *hrmU* and *hrmA*, when present, appear to be linked; the only surveyed cyanobacterium lacking a detectable hybridization band to *hrmU* and/or *hrmA* was *Anabaena* (*Nostoc*) sp. strain PCC 7120 (Cohen 1996). Transcription of *hrmU* and *hrmA* in *N. punctiforme* 29133, as determined from transcriptional fusions to *luxAB*, begins soon after exposure to *A. punctatus* extract and peaks within 13 to 20 h (Fig. 3). A dilution series of the extract shows that the *hrmUA*-inducing factor(s) is not saturating at the concentration (92%) of extract routinely tested (Fig. 4). This factor(s) is not HIF since it is present under nitrogen-replete conditions, is not excreted in significant amounts into the medium, is not bound by PVPP and is heat stable. HIF has a half-life of about 6 days in liquid at room temperature, whereas the activity of the *hrmUA* inducing factor(s) does not decline during this duration (data not shown).

The factor(s) in the extract appears to prevent the entry of a vegetative filament into the hormogonium differentiation mode, at least in part, by its upregulation of *hrmUA*. We have termed it a hormogonium repressing factor (HRF). Since we have not tested extracts of other organisms for HRF activity, including other symbiotic plant partners of *N. punctiforme* 29133 such as cycads (Rippka and Herdman 1992) or *Gunnera* sp. (Rasmussen et al. 1994), we cannot conclude the activity is specific for *A. punctatus*. HRF can override HIF activity only in strains where *hrmUA* is functional, since the extract eliminates HIF-stimulated hormogonium formation by wild-type *N. punctiforme* 29133, but has no effect on the HIF response of strain UCD 328. *A. punctatus* is able to induce symbiotically associated *Nostoc* filaments to form a high frequency of heterocysts even though N_2-derived ammonium is in excess in the symbiotic plant cavities (Meeks et al. 1985). It seems possible that plant-produced HRF could have a role in the symbiotic cavities in maintaining the high heterocyst fre-

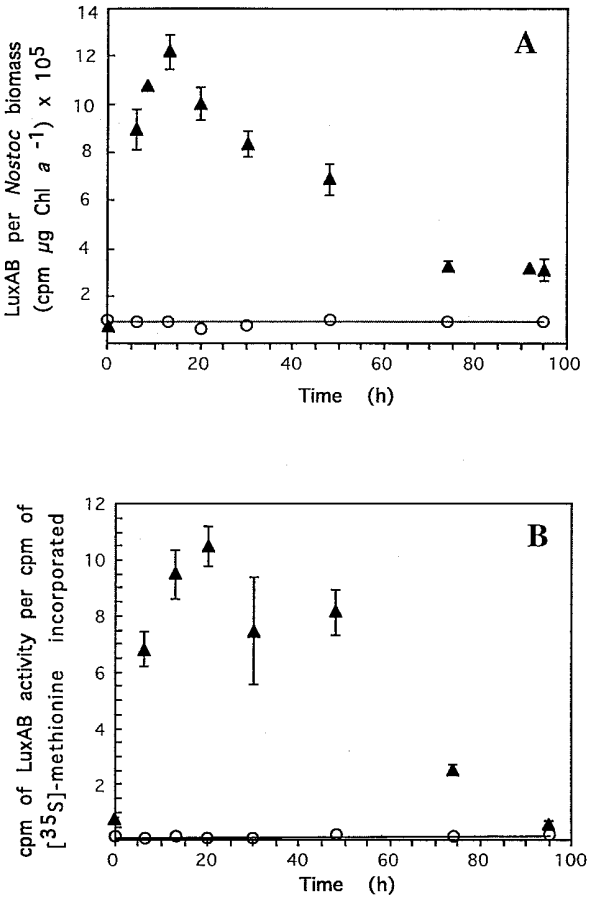


Fig 3. Effect of *Anthoceros punctatus* aqueous extract on *hrmA-luxAB* activity. Results of an experiment showing strain UCD 328 luciferase (LuxAB) activity (cpm) normalized to (**A**) unit of *Nostoc punctiforme* strain biomass (μg Chl *a*), or (**B**) the rate of strain UCD 328 protein synthesis (measured as total cpm of ^{35}S-methionine incorporated following a 30-min incubation) during a 95-h time course. Triangles represent the mean average ± SD (**A**, n = 3; **B**, n = 2) from cells incubated in *A. punctatus* extract; open circles represent a single reading from cells incubated in minimal medium.

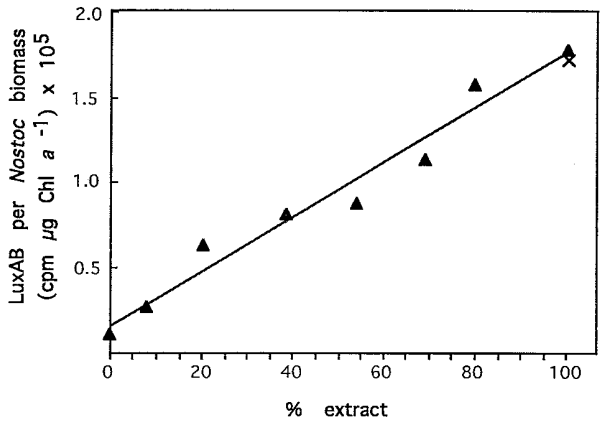


Fig. 4. Effects of extract dilution and autoclaving on *hrmA-luxAB* expression. Triangles represent data points for luciferase (LuxAB) activity (cpm) from cells of strain UCD 328 per unit of *Nostoc* biomass (μg Chl *a*), as a function of *A. punctatus* extract concentration. The X symbol represents a reading from cells incubated in extract that had previously been autoclaved. All readings were made after a 13-h incubation with extract.

quency by preventing the formation of metabolically unproductive hormogonia. However, HRF is not required for the formation of an effective symbiotic *Nostoc* colony since a strain not responsive to HRF, such as strain UCD 328, is able to support growth of the surrounding plant tissue. Alternatively, HRF may have a role in maintaining a long-term association between the plant and *Nostoc* in the environment. The *hrmUA* inducing activity of HRF was first observed from conditioned medium having a bronze-colored tint characteristic of the presence of polyphenols, indicating that HRF can be released by autolysis of the *A. punctatus* tissue. Growth of *A. punctatus* in nature is perennial. Release of a stable hormogonium-repressing factor upon death of the plant tissue may encourage persistence of *Nostoc* in the immediate environment while the *A. punctatus* is in the sporophyte stage. Upon germination to form the gametophyte thallus, *Nostoc* would be readily available to establish new infections.

It is likely that *hrmU* and *hrmA* are transcribed from the same promoter since they are so closely spaced and their transcriptional fusions to *luxAB* show similar activity (Table 2). Since mRNA hybridizing to *hrmU* or *hrmA* probes could not be detected in RNA preparations of cells from a variety of steady-state growth and hormogonium-induced incubation conditions (Cohen 1996), we were unable to identify transcript sizes or relative abundance. However, as discussed below, it is likely that additonal genes are present 5′ of *hrmU* that may contribute to the *hrmUA* phenotype.

Analysis of the phenotypes of the single recombinant strains have helped to narrow the location of promoter(s) driving *hrmUA* transcription. If the primary recombination of a vector for insertional mutagenesis occurs upstream of the insertion site, then the uninterrupted copy of the gene in the single recombinant strain will be preceded upstream by vector sequence. If the promoter for the gene is not within the portion of the *Nostoc* DNA up to the vector sequence, then the primary single recombination of the vector would be sufficient to prevent expression of the gene (assuming that transcripts from the native promoter are terminated within the vector). Thus, the increased infection frequency of the single recombinant strains UCD 357 and UCD 346, in which the vector sequence lies 1.0 and 2.0 kb, respectively, upstream of the uninterrupted *hrmUA* (Fig. 2A), implies that a *hrmUA* promoter is greater than 2.0 kb from the *hrmU* translational start site. Both the low infection frequency phenotype of strain UCD 393 (Table 1) and the plant extract-dependent induction of luciferase activity in strain UCD 339 (Table 2) imply that a *hrmUA* promoter is less than 3.4 kb from the *hrmU* start site. (The low infection frequency of strain UCD 393 may be due to higher expression of the wild-type copy of *hrmUA* due to transcriptional readthrough from the Cm^r gene. Read-through from the same Cm^r gene into *hrmA-luxAB* presumably causes the slightly higher overall expression of luciferase activity of strain UCD 339).

We have shown that loss of function caused by the insertion of Tn*5*-1063 within *hrmA* is responsible for the mutant phenotype of strain UCD 328. Because mutations in *hrmU* are expected to be polar to *hrmA*, we can not unequivocally rule out the possibility that it is only the lack of synthesis of HrmA that determines the phenotype of strains UCD 328 or UCD 392. Two lines of evidence rule out the possibility that a transposon promoter causes the mutant phenotype of strain UCD 328 by transcribing a downstream gene (or portion of *hrmA*) encoding a repressor of *hrmUA* transcription. First, both strain UCD 347, which terminates transcription within the C-terminal encoding region of *hrmA*, and strain UCD 392, which terminates transcription upstream of *hrmA* within *hrmU*, have the same mutant phenotype (Table 1). An Ω-cassette was chosen for use in insertional mutagenesis to avoid the possibility of transcription exiting the insert, thereby confounding mutant analysis. Transcription starting from the *psbA* promoter in Ω-*npt* was shown by Northern blot analysis to terminate within the cassette in both strain UCD 347 and UCD 357 (Cohen 1996) and, therefore, transcription emanating from the Ω-*npt* insertions cannot be responsible for the expression of a repressor gene. Secondly, pSCR18, which lacks DNA downstream of the UCD 328 transposition site, has the same effect as pSCR7 in preventing the induction of luciferase expression from strain UCD 328 bearing the plasmid. Thus, the region responsible for the phenotype conferred by these plasmids is upstream of the transposon insertion site in *hrmA*. It is possible that this region may titrate some *trans*-activator of the chromosomal promoter driving *hrmA-luxAB* transcription.

The observations that the presence in *trans* of pSCR7 failed to complement the strain UCD 328 high infection mutant phenotype, but yielded a high infection phenotype in wild-type *N. punctiforme* 29133, may also be explained by the titration of this putative *trans*-activator by the cloned fragment. Limited concentrations of a *trans*-activator coupled with multiple binding sites would prevent transcription of either the plasmid or chromosomal *hrmUA*-containing operon or related operons. A polar effect on transcription of an unidentified downstream gene by insertions in *hrmU* or *hrmA* could explain the lack of complementation of strain UCD 328. However, polarity is not

Table 2. Luciferase activity of *Nostoc punctiforme* strains containing *luxAB* transcriptional fusions in response to *Anthoceros punctatus* aqueous extract

	Luciferase activity[a] (cpm µg Chl $a^{-1} \times 10_5$) length of exposure to extract:	
***Nostoc* strain**	t = 0	t = 13 h
UCD 328 (*hrmA*::Tn*5*-1063)	1.1 ± 0.3 (n = 6)	9.4 ± 4.0 (n = 8)
UCD 339 (*hrmA*::Tn*5*-1063, SR)	2.6 ± 0.2 (n = 3)	12.3 ± 0.4 (n = 2)
UCD 340 (*hrmA*::Tn*5*-1063, DR)	1.2	8.7
UCD 394 (*hrmA*::Tn*5*-1063, SR)	1.2 ± 0.2 (n = 2)	7.2 ± 1.1 (n = 2)
UCD 372 (*zwf-luxAB*)	6.2 ± 0.2 (n = 2)	4.3 ± 0.1 (n = 2)
UCD 346 (*hrmU-luxAB*, SR)	1.2 ± 0.2 (n = 2)	7.9 ± 0.3 (n = 2)
UCD 328/pSCR7	1.2	1.4
UCD 328/pSCR202	1.1	9.7
UCD 328/pSCR18	1.4 ± 0.1 (n = 2)	1.9 ± 0.3 (n = 2)

[a] Values are means ± SE with the number of replicates of separate experiments in parentheses. Values without errors are results of single experiments.

consistent with the effect of pSCR7 on the wild-type (Table 1), or pSCR7 and pSCR18 on *luxAB* expression in strain UCD 328 (Table 2). The titration of a *trans*-activator by a plasmid, termed multicopy inhibition (Merrick 1992), has been observed with *nif* plasmids of *Klebsiella pneumoniae* (Riedel et al. 1983; Brown and Ausubel 1984) and *act* plasmids of *Streptomyces coelicolor* (Fernández-Moreno et al. 1992). Multicopy inhibition by *act* plasmids is of particular interest because the *act* gene cluster, which encodes for the production of a polyketide, contains *act*III, a member of the same oxidoreductase superfamily as *hrmU* (Fig. 1; Hallam et al. 1988). Clustering of the genes involved in the biosynthesis of secondary metabolites is common (Martín and Liras 1989) as is their regulation by *trans*-activators (Malpartida et al. 1990). We are currently sequencing the region 3.4 kb 5′ of *hrmU* and analyzing subclones for their effect when in trans on *luxAB* expression in strain UCD 328 and infection of *A. punctatus* in wild type *N. punctiforme* 29133.

Also within the above family of oxidoreductases is *hetN* from *Anabaena* (*Nostoc*) 7120 (Fig. 1; Black and Wolk 1994), a gene postulated to function in the production of a heterocyst inhibiting metabolite which diffuses within a filament. Recent evidence indicates that normal heterocyst development requires unrestricted flow through the microplasmodesmata of cell to cell junctions in filaments (Zahalak et al. 1995). Hormogonium differentiation progresses through all vegetative cells between two heterocysts (Damerval 1991; Cohen 1996). Therefore, hormogonium regulating factors would likewise be expected to diffuse through the microplasmodesmata. The function encoded by the *hrmUA* locus is most likely intrafilamentous since the strain UCD 328 mutation cannot be physiologically complemented by the addition of wild-type *N. punctiforme* 29133 (Table 1).

As a working hypothesis, we propose that the *hrmUA* locus encodes proteins (most likely more than 2) whose function results in inhibition of hormogonium formation. Such a function, perhaps the synthesis of a hormogonium repressing metabolite or the catabolism of a hormogonium inducing factor, could act to maintain filaments in the vegetative state. A factor in *A. punctatus* extract, possibly released into the symbiotic cavities, may stimulate *hrmUA* transcription by inducing the expression of a *trans*-activator.

MATERIALS AND METHODS

Cultures, plasmids, and media.

The bacterial strains and plasmids used in this study are listed in Table 3. *Escherichia coli* strains were cultured in Luria-Bertani broth (Sambrook et al. 1989). Sources, media and culture conditions for symbiont-free and *Nostoc*-associated *A. punctatus*, and all free-living *Nostoc* strains were described previously (Enderlin and Meeks 1983). Chlorophyll *a* (Chl *a*) in methanolic extracts of *Nostoc* was quantified as described previously (Meeks et al. 1983). To select for resistance conferred by the *npt* gene, kanamycin (50 μg ml^{-1}) was used for *E. coli* and neomycin (10 μg ml^{-1}) was used for *Nostoc*.

Response of *Nostoc-luxAB* fusion strains to incubation with plant extract.

Nostoc strains containing *luxAB* transcriptional fusions were monitored for their response to various treatments, including incubation with *A. punctatus* extract. Extracts were prepared by grinding 60 to 80 mg fresh weight ml^{-1} of *A. punctatus* gametophyte tissue in buffered *Nostoc* basal medium or, for certain experiments, HIF-conditioned medium. Extracts were then filtered (Whatman No. 4) and 2.3 ml aliquots combined with 0.2 ml of *N. punctiforme* 29133 culture in glass tubes and incubated with shaking under light. At various time intervals a tube was removed from incubation, vortex mixed and sampled for measurement of luciferase activity and Chl *a* content. Luciferase activity was monitored by adding 50 μl of 2.65 mM *n*-decalaldehyde (Aldrich) to 50 μl of cells using a Model 6100 PICO-LITE luminometer (Packard Instruments Co.); photons emanating from the sample were counted for 1 min following a 1 min delay after injection. In one experiment to determine the rates of total protein synthesis, the remainder of the cell suspension in the tube was then labeled to a concentration of 2.5 μCi L-[^{35}S]methionine (1175.0 Ci $mmol^{-1}$; DuPont NEN) per ml (Borbély et al. 1985). After 30 min an 0.8-ml aliquot was TCA precipitated, washed and its radioactivity determined by scintillation counting.

For the extract dilution series, aliquots of the *Nostoc* culture were pelleted and resuspended to 1.0 ml in various concentrations of extract diluted with buffered *Nostoc* basal medium and incubated as above.

DNA methods and sequence analysis.

Total cyanobacterial DNA and plasmid DNA were isolated as described previously (Cohen et al. 1994). In vitro DNA manipulations were performed according to standard procedures (Ausubel et al. 1993). Restriction enzymes and T4 DNA ligase were purchased from Gibco-BRL or New England Biolabs. The loci of insertion for all recombinant strains were determined by restriction analysis and Southern blot hybridization.

To make plasmid templates for sequencing, a 3.2-kb *Bam*HI-*Cla*I fragment and a 2.0-kb *Eag*I-*Sal*I fragment were subcloned separately from the transposition site of pSCR1 into pBluescript II KS+, yielding pSCR15 and pSCR16, respectively. Nested deletion clones were produced using a commercial kit (Promega) and both strands sequenced using the dideoxy chain termination method (kit from United States Biochemical). A region of one strand which had no suitable nested deletion clone was sequenced using two 20-bp primers complementary to the previously sequenced strand of *Nostoc* DNA. The sequence was analyzed using several of the Genetics Computer Group set of programs (Devereux et al. 1984) and the National Center for Biotechnology Information (NCBI) BLAST Network Service alignment of translated sequences to the data base (Altschul et al. 1990). The GenBank accession number for the 2448-bp DNA sequence encompassing *hrmUA* is L37087.

Subcloning of *Nostoc* DNA around the strain UCD 328 transposition site.

A 6.2-kb *Sph*I-*Bam*HI fragment from pSCR5, containing *hrmUA*, was ligated to *Sph*I-*Bam*HI-digested pSCR202 to make pSCR7. A 6.5-kb *Sal*I-*Bam*HI fragment from pSCR3, containing 4.4 kb of *Nostoc* DNA and the chloramphenicol and erythromycin resistance genes of the vector, was ligated to a *Sal*I-*Bam*HI-digested pSCR202 to make pSCR18.

Recombinant insertions in *hrmU* and *hrmA*.

Ligation of *Spe*I-digested pSCR2 to *Spe*I-digested pRL1075 resulted in pSCR3 (Cohen et al. 1994) and also pSCR19, in which the *sacB*-containing fragment of pRL1075 is oriented in the opposite direction as found in pSCR3.

An Ω-cassette is a selectable marker flanked by short inverted repeats which each have a bacteriophage T4 rho-independent transcriptional terminator, translational stops in all three reading frames and a polylinker (Prentki and Krisch 1984). *N. punctiforme* 29133 containing the Sp^r/Sm^r gene from the pRL453 Ω-cassette in the chromosome does not express sufficient antibiotic resistance for selection (Summers 1995). Therefore a vector (pSCR9) was constructed having the Sp^r/Sm^r gene of pRL453 replaced with a *npt* gene driven by the *psbA* promoter of *Amaranthus hybridius*, a marker known to function when integrated into the *N. punctiforme*

Table 3. Bacterial strains and plasmids used in this study

Strains and plasmids	Relevant characteristics[a]	Source or reference
E. coli		
DH5α-MCR	Genotype of DH5α and *mcrA*Δ (*mrr hsdMRS mcrBC*)	Grant et al. 1990
HB101	*recA*13 *hsdS*20($r_B^- m_B^-$)	Boyer and Roulland-Dussoix 1969
Nostoc		
ATCC 29133 (PCC 73102)	Type strain of *Nostoc punctiforme*; Fix^+, Sym^+	Herdman and Rippka 1992; Enderlin and Meeks 1983
UCD 328	Highly infective *N. punctiforme*::Tn*5*-1063 strain; Bm^r Nm^r Sm^r	Cohen et al. 1994
UCD 339	Single recombinant of *hrmA*::Tn*5*-1063 (pSCR3); $Bm^rCm^rEm^rNm^rSm^rSuc^s$	Cohen et al. 1994
UCD 340	Reconstruction of the strain UCD 328 mutation, double recombinant derived from strain UCD 339; Bm^r Nm^r Sm^rSuc^r	Cohen et al. 1994
UCD 346	Single recombinant of *hrmU-luxAB* transcriptional fusion in the genome; Nm^r	This study
UCD 347	Double recombinant of *hrmA*::Ω-*npt* in the genome; Nm^r	This study
UCD 357	Single recombinant of *hrmU*:: Ω-*npt* in the genome; Nm^r	This study
UCD 372	*zwf-luxAB* in *N. punctiforme*; Nm^r	Summers 1995
UCD 392	Double recombinant of *hrmU::* Ω-*npt* in the genome; Nm^r	This study
UCD 393	Single recombinant of *hrmA*:: Ω-*npt* in the genome; Nm^r	This study
UCD 394	Single recombinant of *hrmA*::Tn*5*-1063 (*luxAB*) in the genome; $Bm^rCm^rEm^rNm^r$ Sm^rSuc^s	This study
Plasmid		
pBluescript II KS^+	pUC derivative cloning vector; Ap^r	Stratagene Inc.
pDS4101	ColK::Tn*1* conjugation helper for *bom* sites	Tacon et al. 1981
pRK2013	RK2 derivative with ColE1 *oriV*; Km^r	Figurski and Helinski 1979
pRL271	Conjugatable non-replicating suicide plasmid containing *sacB* for selection of double recombinants; Cm^r Em^r	Y. Cai and C. P. Wolk
pRL448	pUC18/19 + L.EHE1-derived containing P_{psbA}*nptII* cassette	Elhai and Wolk 1988
pRL453	pUC18/19 with Ω-Sm^r/Sp^r cassette	Elhai and Wolk 1988
pRL479	Cloning vector, S.A2/L.EHE2/C.C2; Ap^r Cm^r	see Elhai and Wolk 1988, for nomenclature
pRL561	For single recombination of *lux*AB transcriptional fusions; Nm^r	Elhai and Wolk 1990
pSCR1	Recovered 19-kb *Eco*RV Tn*5*-1063 bearing fragment from UCD 328; Bm^r Nm^r Sm^r	Cohen et al. 1994
pSCR2	pSCR1 with *Not*I site filled in; Bm^r Km^r Sm^r	Cohen et al. 1994
pSCR3	14-kb *Spe*I fragment from pSCR2 in *SpeI* site of pRL1075 so that the Cm^r gene faces *hrmA*; Bm^r Cm^r Em^r Nm^r Sm^r *SacB*; p15A *oriV*	Cohen et al. 1994
pSCR4	Subclone of 11-kb *Eco*RV genomic fragment in pRL479	Cohen et al. 1994
pSCR5	6.2-kb *N. punctiforme Spe*I fragment from pSCR4 in pUC18; Ap^r	Cohen et al. 1994
pSCR7	6.2-kb *Spe*I fragment from pSCR5 in pSCR202; Ap^r	This study
pSCR9	Sp^r/Sm^r gene of pRL453 replaced with P_{psbA}-*npt* from pRL448; Ω-*npt* element; Ap^r Nm^r	This study
pSCR10	Suicide vector (pRL271 based) for insertion of *hrmA::*Ω-*npt* into the *Nostoc* genome; Nm^r Cm^r Em^r, *sacB*	This study
pSCR11	Ω-*npt Ecl*136II fragment cloned into the *Bsa*BI site (*hrmU*) of pSCR15; Ap^r Nm^r	This study
pSCR12	Suicide vector (pRL271 based) for insertion of *hrmU::* Ω-*npt* into the *Nostoc* genome; Nm^rCm^r Em^r, *sacB*	This study
pSCR13	Suicide vector (pRL271 based) for single recombination of *hrmU-luxAB* into the *Nostoc* genome, Nm^rCm^r Em^r, *sacB*	This study
pSCR15	Subclone of a fragment of Tn*5*-1063 *luxAB* end with 3.2 kb flanking DNA from pSCR1, cloned into pBluscript II KS^+; Ap^r	This study
pSCR16	Subclone of a fragment of Tn*5*-1063 *tnp* end with 2.0 kb flanking DNA from pSCR1, cloned into pBluescript II KS^+; Ap^r	This study
pSCR18	Subclone of entire *Nostoc* sequence flanking *luxAB* end of pSCR3 into pSCR202; Ap^r Cm^r Em^r	This study
pSCR19	Same as pSCR3 except opposite orientation of pRL1075 so that Cm^r gene is not facing *hrmA*; Bm^r Cm^r Em^r Nm^r Sm^r, *sacB*	This study
pSCR22	Ω-*npt Ecl*136II fragment cloned into the *Sca*I (*hrmA*) site of pSCR5	This study
pSCR202	Shuttle vector, polylinker, Ap^r, transfer to *Nostoc* by electroporation	Summers et al. 1995

[a] Abbreviations: Ap, ampicillin; Bm, bleomycin; Cm, chloramphenicol; Em, erythromycin; Fix, nitrogen fixation; Nm, neomycin; r, resistance; s, sensitivity; Suc, sucrose; Sym, symbiotic.

29133 chromosome. To make pSCR9, *Bsa*AI-*Bsa*BI-digested pRL453 was ligated to a Klenow-blunt-ended *Xba*I fragment from pRL448 containing P_{psbA}*nptII* (Elhai and Wolk 1990).

A 1.8-kb *Ecl*136II Ω-*npt* fragment from pSCR9 was ligated to a partially *Sca*I-digested pSCR5 so that the *npt* gene inserted within *hrmA* facing the opposite direction, resulting in pSCR22. An 8-kb *Sal*I-*Sst*I fragment of pSCR22 was ligated to *Sst*I-*Xho*I-digested pRL271 to make pSCR10. A triparental mating (Cohen et al. 1994) using a conjugal *E. coli* strain (strain HB101 containing pRK2013) to transfer pSCR10 from the donor/helper strain (strain DH5α-MCR containing pDS4101) into *N. punctiforme* resulted in the single recombinant strain UCD 393 and the double recombinant strain UCD 347.

To insert the Ω-*npt* cassette in *hrmU*, the 1.8-kb *Ecl*136II fragment of pSCR9 was ligated to *Bsa*BI-digested pSCR15 to make pSCR11. *Xba*I-digested pSCR11 was ligated to *Spe*I-digested pRL271 to make pSCR12. The Cm^r gene is oriented in the opposite direction as *hrmA* in pSCR12. A triparental mating using a conjugal/helper strain (strain DH5α-MCR containing pRK2013 and pDS4101) to transfer pSCR12 from the donor strain (strain DH5α-MCR containing pSCR12) into *N. punctiforme* resulted in the single recombinant strain UCD 357 and the double recombinant strain UCD 392.

To make a *luxAB* transcriptional fusion to *hrmU*, a 2.4-kb *Kpn*I-*Xho*I fragment of pSCR11 was ligated to *Kpn*I-*Sal*I-digested pRL561 forming pSCR13. A triparental mating using a conjugal/helper strain (strain DH5α-MCR containing pRK2013 and pDS4101) to transfer pSCR13 from the donor strain (strain DH5α-MCR containing pSCR13) into *N. punctiforme* resulted in the single recombinant UCD 346.

ACKNOWLEDGMENTS

We thank Michael Syvanen for his conceptual advice and Elsie Campbell, Kari Hagen, Tom Hanson, and Francis Wong for critical review of the manuscript. This work was supported by the U. S. National Science Foundation (Grants IBN 92-06139 and IBN 95-14787) and a Floyd and Mary Schwall Fellowship in Medical Research to M. F. C.

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