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The *devR* Gene Product Is Characteristic of Receivers of Two-Component Regulatory Systems and Is Essential for Heterocyst Development in the Filamentous Cyanobacterium *Nostoc* sp. Strain ATCC 29133

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Strain UCD 311 is a transposon-induced mutant of *Nostoc* sp. strain ATCC 29133 that is unable to fix nitrogen in air but does so under anoxic conditions and is able to establish a functional symbiotic association with the hornwort *Anthoceros punctatus*. These properties of strain UCD 311 are consistent with previous observations that protection against oxygen inactivation of nitrogenase is physiologically provided within *A. punctatus* tissue. Upon deprivation of combined nitrogen, strain UCD 311 clearly differentiates heterocysts and contains typical heterocyst-specific glycolipids; it also makes apparently normal akinetes upon phosphate starvation. Sequence analysis adjacent to the point of the transposon insertion revealed an open reading frame designated *devR*. Southern analysis established that similar sequences are present in other heterocyst-forming cyanobacteria. *devR* putatively encodes a protein of 135 amino acids with high similarity to the receiver domains of response regulator proteins characteristics of two-component regulatory systems. On the basis of its size and the absence of other functional domains, DevR is most similar to CheY and Spo0F. Reconstruction of the mutation with an interposon vector confirmed that the transposition event was responsible for the mutant phenotype. The presence of wild-type *devR* on a plasmid in strain UCD 311 restored the ability to fix nitrogen in air. While *devR* was not essential for differentiation of akinetes, its presence in *trans* in *Nostoc* sp. strain ATCC 29133 stimulated their formation to above normal levels in aging medium. On the basis of RNA analysis, *devR* is constitutively expressed with respect to the nitrogen source for growth. The *devR* gene product is essential to the development of mature heterocysts and may be involved in a sensory pathway that is not directly responsive to cellular nitrogen status.

All nitrogenase enzymes are highly O_2 sensitive; thus, diazotrophic prokaryotes have adapted a variety of mechanisms to protect these enzymes from O_2 inactivation, ranging from avoidance to metabolic consumption of O_2 by respiration to cellular compartmentalization (16, 17). Specialized cells termed heterocysts are an example of compartmentalization; they are formed by certain filamentous oxygenic photoautotrophic cyanobacteria under conditions of combined nitrogen deprivation (40). Heterocysts lack the photosystem II O_2-producing reaction, have an increased rate of respiratory O_2 uptake, and are enclosed by an extra outer wall layer consisting of unique glycolipids and polysaccharides, which together impede the entry of O_2 (40). Heterocysts that form under anoxic incubation conditions are structurally incomplete and are unable to protect nitrogenase from inactivation by O_2 when cultures are shifted to ambient oxic conditions (32).

The formation of heterocysts from vegetative cells clearly involves differential gene expression (16, 40). However, the mechanisms regulating differential gene expression of any metabolic or developmental system in these organisms are unknown. A primary sigma subunit of RNA polymerase, as well as two alternate sigma subunits, all belonging in the same family (similar to the sigma-70 family of *Escherichia coli*), has been identified (4). Cultures with defects in both of the alternative sigma subunits continue to differentiate heterocysts and fix N_2 (4). A DNA-binding protein, NtcA, that shows similarity to the cyclic AMP receptor class of regulatory proteins and that influences heterocyst differentiation and transcription of several diverse enzymes involved in nitrogen assimilation has been identified (14, 37). A gene product, HetR, that catalyzes an unknown reaction is essential for the initiation of heterocyst differentiation (5). The interactions between any of these factors in the initiation and continuation of heterocyst differentiation is unresolved. Genes with identity to those encoding sensor kinase and response regulator proteins of two-component regulatory systems have been characterized that control genes for phosphate assimilation of a unicellular cyanobacterium (27), but none that influence nitrogen metabolism have been identified. A gene product, PatA, that has similarity to the receiver domain of response regulator proteins influences the pattern of heterocyst differentiation in filaments of *Anabaena* sp. strain PCC 7120 (21).

Nostoc sp. strain ATCC 29133 was isolated from a corraloid root of the cycad *Macrozamia* sp. (30). It is a unique experimental organism for developmental studies, because in addition to heterocysts and under appropriate environmental conditions, vegetative cells of *Nostoc* sp. strain ATCC 29133 can also differentiate into spore-like akinetes or into hormogonia, which are gliding filaments that exist transiently as part of a

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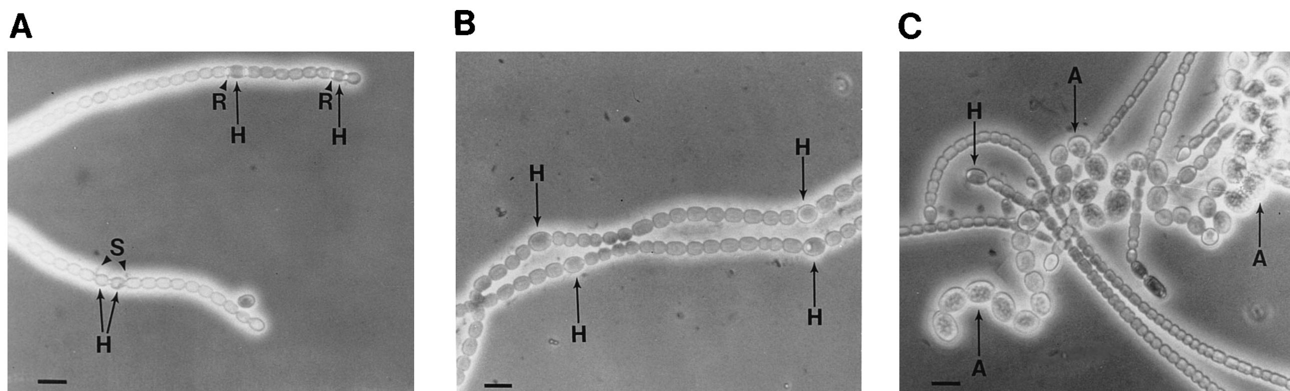

FIG. 1. Photomicrographs of derivatives of *Nostoc* sp. strain ATCC 29133. (A) Mutant strain UCD 311 after 24 h of deprivation for combined nitrogen. (B) Mutant strain UCD 311 containing pSCR208 (*devR*) in *trans* in exponential culture with N_2 as the nitrogen source. (C) *Nostoc* sp. strain ATCC 29133 containing pSCR208 (*devR*) in *trans*, cultured for 29 days with N_2 as the nitrogen source. Symbols: A, akinetes; H, heterocysts; S, spur-like projections at the poles of heterocysts in strain UCD 311; R, refractive material at the poles of the heterocysts (polar bodies). Bars = 5 μm.

cellular life cycle (30). *Nostoc* sp. strain ATCC 29133 is also capable of dark heterotrophic growth, which facilitates studies in carbon catabolism (35), and it forms a symbiotic association in axenic culture with the experimentally manipulable bryophyte *Anthoceros punctatus* (12). Two of the *Nostoc* spp. differentiation alternatives are influenced by *A. punctatus*. First, when deprived of combined nitrogen, *A. punctatus* produces extracellular chemical factors stimulating *Nostoc* spp. to differentiate motile hormogonia, which serve as the infective units in establishing the symbiotic association (7). Second, nitrogen-limited *A. punctatus* produces chemical or environmental signals that stimulate heterocyst differentiation by symbiotically associated *Nostoc* spp. to be elevated to about 45% of the total population, compared with 3 to 8% in the free-living growth state (12, 24). Nitrogenase activity is increased (34) and ammonium assimilation is decreased (19) in symbiotically associated *Nostoc* spp., relative to free-living cultures; thus, the excess fixed nitrogen, as ammonium, is made available for the growth of *A. punctatus* tissue (25).

We are interested in the genetic and molecular mechanisms influencing cellular differentiation of *Nostoc* spp. in the symbiotic growth state. We have previously identified a Fox^- (unable to fix nitrogen in air) mutant (strain UCD 223) of *Nostoc* sp. strain ATCC 29133 that was derived by chemical mutagenesis and that formed a functional N_2-fixing symbiotic association (Sym^+) with *A. punctatus* (8). Microelectrode analysis subsequently established that the symbiotic cavities occupied by *Nostoc* spp. in *A. punctatus* gametophyte tissue contained little or no O_2 (8). However, we were unable to isolate the gene responsible for the Fox^- Sym^+ phenotype of strain UCD 223. We have since developed methods for transposon mutagenesis of *Nostoc* sp. strain ATCC 29133 and have found it to be a genetically facile organism by using many of the same tools that were developed for *Anabaena* sp. strain PCC 7120 (9). We report here the characterization and complementation of a transposon-derived Fox^- Sym^+ mutant of *Nostoc* sp. strain ATCC 29133 and the gene responsible for the described phenotype.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and physiological measurements. *Nostoc* sp. strain ATCC 29133 (PCC 73102, type strain of *Nostoc punctiforme* [31]), its mutant derivatives, and the cyanobacteria that yielded genomic DNA for Fig. 6 were grown in buffered (5 mM 3-[*N*-morpholino]propanesulfonic acid [MOPS], pH 7.8) liquid and agar-solidified medium as described previously (9). For non-nitrogen-fixing cultures, the medium was supplemented with 2.5 mM NH_4Cl. Antibiotic concentrations used for derivatives of *Nostoc* sp. strain ATCC 29133 were neomycin (Nm) at 10 μg ml^{-1}, ampicillin (Ap) at 5 μg ml^{-1}, and erythromycin (Em) at 3 μg ml^{-1} in liquid and 15 μg ml^{-1} on plates. Plates were incubated at 28°C under continuous illumination from cool white fluorescent lamps at 8 W m^{-2} and 1% (vol/vol) CO_2 enrichment. Mutagenesis with the transposon Tn*5-1063* (39) and isolation of the Fox^- mutants and their preliminary characterization were described previously (9). Oxic and anoxic acetylene reduction assays as measures of nitrogenase activity were performed, and chlorophyll *a* content was determined, as previously described (8). Coculture with *A. punctatus* in combined-nitrogen-free medium was used to determine symbiotic competence (12). Heterocyst glycolipids were extracted from cultures with chloroform-methanol (2:1, vol/vol), concentrated under nitrogen gas, spotted on a silica gel G thin-layer chromatography plate (Analtech), and separated by chromatography with chloroform-methanol-acetic acid-water (170:30:20:7.4, by volume) as previously described (26).

All *E. coli* strains were grown in Luria-Bertani broth (33) and supplemented with antibiotics at the following concentrations: kanamycin (Km) at 50 μg ml^{-1}, ampicillin at 100 μg ml^{-1}, and chloramphenicol (Cm) at 30 μg ml^{-1}. *E. coli* DH5α-MCR (Gibco-BRL) was used for all standard cloning experiments, including the subcloning of appropriate fragments, and standard protocols were observed (33).

Photomicrographs of *Nostoc* sp. strain ATCC 29133 and its derivatives, as filaments mounted on agar-coated slides, were obtained with a Zeiss Universal photomicroscope equipped with phase contrast objectives.

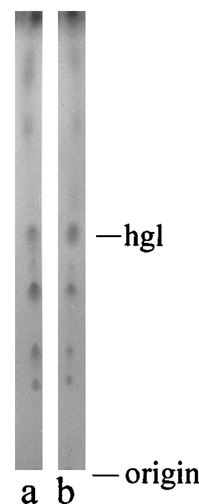

FIG. 2. Thin-layer chromatograms of chloroform-methanol extracts from cultures of *Nostoc* sp. strain ATCC 29133 grown with N_2 (lane a) and strain UCD 311 extracted after 24 h of deprivation for combined nitrogen (lane b). The position of heterocyst glycolipid III (hgl) is indicated.

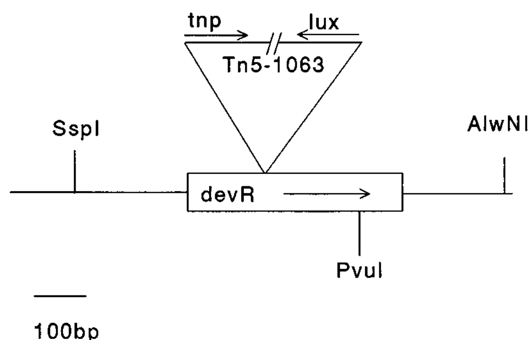

FIG. 3. Restriction map of the *devR* region in *Nostoc* sp. strain ATCC 29133 showing the approximate site and orientation of the insertion of the transposon Tn*5-1063*. tnp, transposase gene of Tn*5-1063*; lux, the bacterial luciferase reporter genes of Tn*5-1063*. Arrows indicate the direction of transcription. The 100-bp bar marker is relevant only for the chromosomal portion of the figure and does not reflect the actual size of the transposon.

Molecular genetic techniques. Genomic DNA isolations from cyanobacteria were performed as previously described (9). All DNA-modifying enzymes were purchased from New England Biolabs, Gibco-BRL, or Boehringer Mannheim and used according to the manufacturer's instructions. Recovery of the transposon with flanking genomic DNA in strain UCD 311 was done as previously described (9) by digesting mutant genomic DNA with *Eco*RV and then by ligation and electrotransformation into *E. coli* DH5α-MCR. The resulting plasmid, pSCR205, served as the template for sequencing with synthetic oligonucleotide primers by the dideoxy nucleotide method (with a kit purchased from United States Biochemical). Sequence homology searches were performed with the BLAST network service (2). To reconstruct the mutation, an interposon vector was constructed as follows. The *Not*I site within the transposase gene from plasmid pSCR205 was made blunt by Klenow and then ligated, which created a frame-shift mutation in *tnp*. A *Spe*I-*Eco*RV double digest removed 4.4 kb of *Nostoc* sp. strain ATCC 29133 genomic DNA, and in its place in the remaining 9.8-kb fragment was ligated a *Nae*I-*Spe*I-digested pRL271 fragment, which contains the *sacB* gene, to allow positive selection of double recombinants (6). The resulting 20-kb plasmid, pSCR206, was triparentally mated into *Nostoc* sp. strain ATCC 29133 with mobilizer pRL443 and helper plasmid pDS4101 as previously described (9). Single recombinants were grown on neomycin and erythromycin, and double recombinants were selected by determining resistance to 5% sucrose and neomycin. The genotypes of putative single and double recombinants were verified by Southern hybridization as described below.

Cosmids containing the wild-type sequence were identified by using the 10.5 kb of flanking genomic DNA in pSCR205 as a probe. The probe was labeled with [α-^{32}P]dCTP (DuPont, NEN) by the random priming method (with a kit from Gibco-BRL) and hybridized to clones from a randomly sheared genomic cosmid library of *Nostoc* sp. strain ATCC 29133 (9). A 3.4-kb *Xba*I fragment from cosmid pSCR207 was found to contain the sequences flanking both sides of the point of the Tn*5-1063* insertion and was subcloned into the *Xba*I site of the shuttle vector pSCR202 (35), resulting in plasmid pSCR208. Electroporation of *Nostoc* sp. strain ATCC 29133 or strain UCD 311 with pSCR208 was done as previously described (35).

A *Ssp*I-*Alw*NI digest of plasmid pSCR208 yielded an 862-bp fragment which contains all of *devR* and no other complete or partial open reading frames. This fragment was isolated by electrophoresis through agarose, purified with glass fines (Gene Clean, Bio 101), and used as the probe for hybridization to the RNA and DNA blots. RNA and DNA fragments were blotted to GeneScreen Plus nylon membranes as described previously (35). Hybridizations were performed at 42°C in 50% (vol/vol) formamide, and blots were washed at the highest stringency (65°C) as specified by the manufacturer (DuPont, NEN). Alignment of *devR* with other similar genes was done with the Pileup program of the University of Wisconsin Genetics Computer Group at default settings (10).

Nucleotide sequence accession number. The sequence of a 798-bp fragment containing *devR* is available from the National Center for Biotechnology Information under accession number L44605.

RESULTS

Phenotypic characteristics. We have previously reported the isolation of 14 Fox$^-$ mutants derived from mutagenesis of *Nostoc* sp. strain ATCC 29133 with the transposon Tn*5-1063* (9). On the basis of the restriction patterns of genomic DNA flanking the transposon, each mutant reflected transposition into a distinctly different site in the *Nostoc* sp. strain ATCC 29133 chromosome (data not shown). These Fox$^-$ clones represented approximately 0.4% of the antibiotic-resistant mutants recovered after segregation on NH_4^+-supplemented medium, which was permissive for any nitrogen fixation-defective phenotype. All of these Fox$^-$ mutants differentiated visibly normal akinetes following phosphate starvation, but only four exhibited a Sym$^+$ phenotype in the *A. punctatus* association.

Mutant strain UCD 311 was derived from this round of mutagenesis and had an acetylene-reducing activity of 138 nmol of C_2H_4 formed min^{-1} (mg of chlorophyll *a*)$^{-1}$ under anoxic conditions; it had no acetylene-reducing activity when assayed in air. For comparison, *Nostoc* sp. strain ATCC 29133 had an acetylene-reducing activity of 129 nmol of C_2H_4 formed min^{-1} (mg of chlorophyll *a*)$^{-1}$ in air. Strain UCD 311 clearly differentiated heterocysts upon deprivation of combined nitrogen, although these heterocysts appeared not to have a contiguous-wall layer; material in a spur-like pattern was often seen extending from the polar regions of the wall, and many heterocysts contained unusually large amounts of refractile material at the cell poles (Fig. 1A). Heterocyst glycolipids were present at typical concentrations in combined-nitrogen-deprived cultures of strain UCD 311, and they showed no unusual migration patterns during thin-layer chromatography, relative to wild-type cultures (Fig. 2).

Strain UCD 311 established symbiotic colonies within *A. punctatus* gametophyte tissue and supported N_2-dependent growth of the association. However, strain UCD 311 did not reinfect the growing marginal meristematic regions of the gametophyte tissue during prolonged coculture, as do wild-type *Nostoc* strains (12); thus, the association eventually ceased to grow with N_2 as the sole nitrogen source. A lack of reinfection has been previously observed with another Fox$^-$ Sym$^+$ mutant (8).

Identification and expression of the altered gene. The transposon and 10.5 kb of flanking genomic DNA (2.9 kb on the *lux* side and 7.6 kb on the *tnp* side) were recovered from strain UCD 311 as an *Eco*RV fragment, which was ligated to form pSCR205. Sequence analysis using pSCR205 as the template revealed that a 9-bp sequence (GTCCAGAAG, at positions 407 to 415 in the wild-type nucleotide sequence) was repeated at the site of transposition; 9-bp repeats are characteristic of

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        1          *                                            50
 DevR   ....MKTVLI VEDDLINARV FSKILSKRGG LGVKHTENVE EVIKIAQSGE
 CheY   MADKELKFLV VDDFSTMRRI VRNLLKELGF NNVEEAEDGV DALNKLQAGG
Spo0F   ..MMNEKILI VDDQYGIRIL LNEVFNKEGY .QTFQAANGL QALDIVTKER
 NtrC   ..MQRGIVWV VDDDSSIRWV LERALAGAGL .TCTTFENGN EVLAALASKT
 PhoB   ...MARRILV VEDEAPIREM VCFVLEQNGF .QPVEAEDYD SAVNQLNEPW
 OmpR   .MQENYKILV VDDDMRLRAL LERYLTEQGF .QVRSVANAE QMDRLLTRES

        51    *                                                100
 DevR   ADLILMDVSL SRSVYQGKSV DGIKITQMLK SDPKTANLPV ILVTAHAMEG
 CheY   YGFVISDWNM P.......NM DGLELLKTIR ADGAMSALPV LMVTAEAKKE
Spo0f   PDLVLLDMKI P.......GM DGIEILKRMK VIDEN..IRV IIMTAYGELD
 NtrC   PDVLLSDIRM P.......GM DGLALLKQIK QRHPM..LPV IIMTAHSDLD
 PhoB   PDLILLDWML P.......GG SGIQFIKHLK RESMTRDIPV VMLTARGEEE
 OmpR   FHLMVLDLML P.......GE DGLSICRRLR SQS..NPMPI IMVTAKGEEV

        101                 *                                  150
 DevR   DRENFLKQSG ADGYISKPVV DHQQFVDQIL ALLPTDGQSZ ..........
 CheY   NIIAAA.QAG ASGYVVKP.F TPATLEEKLN KIF.....EK LGM.......
Spo0f   MIQESK.ELG ALTHFAKP.F DIDEIRDAVK KYLPLKSN.. ..........
 NtrC   AAVSAY.QQG AFDYLPKP.F DIDEAVALVE RAISHYQEQQ QPRNIEVNGP
 PhoB   DRVRGL.ETG ADDYITKP.F SPKELVARIK AVM.....RR ......ISPM
 OmpR   DRIVGL.EIG ADDYIPKP.F NPRELLARIR AVL.....RR QANELPGAPS
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FIG. 4. Amino acid alignment of DevR and other response regulator proteins. The two conserved aspartate residues and the conserved lysine residue that compose the acid pocket as defined by CheY are indicated by asterisks above the residues. The putative site of phosphorylation is the aspartate residue at position 57 with respect to the CheY numbering. DevR is from *Nostoc* sp. strain ATCC 29133, Spo0F is from *B. subtilis*, NtrC is from *Salmonella typhimurium*, and CheY, OmpR, and PhoB are from *E. coli.*

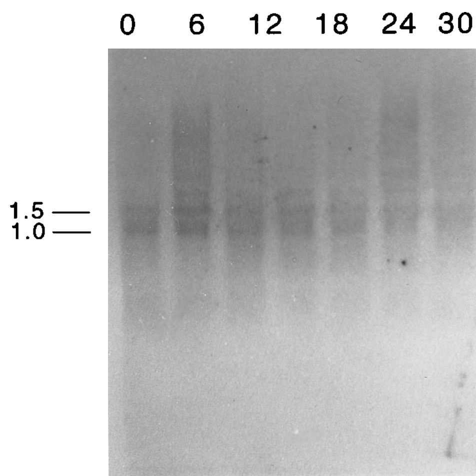

FIG. 5. Hybridization to an RNA blot with a *Ssp*I-*Alw*NI fragment containing all of *devR* as the probe. RNA was isolated from *Nostoc* sp. strain ATCC 29133 following nitrogen stepdown at the hours indicated at the top of the figure. Sizes of hybridizing bands (noted at the left) are in kilonucleotides and were determined relative to RNA standards that were electrophoresed in the same gel and stained with ethidium bromide.

Tn*5* insertion sites (3). The transposon interrupted a 405-bp open reading frame which potentially encodes a protein of 135 amino acids (Fig. 3). We have designated this open reading frame *devR*, for developmental regulation, because strain UCD 311 failed to develop mature heterocysts, but it is capable of synthesizing components of the heterocyst envelope. The deduced amino acid sequence of *devR* shows a high level of relatedness to response-regulator proteins of two-component regulatory systems, with 54 and 51% similarity to CheY of *E. coli* and Spo0F of *Bacillus subtilis*, respectively (Fig. 4). DevR contains the highly conserved aspartate and lysine residues (Fig. 4) that constitute the acid pocket where phosphorylation of Asp-57 (numbering relative to CheY) takes place in the receiver domain of these proteins (28).

Since the transposon had been inserted with *luxAB* oriented antiparallel to the *devR* transcriptional direction (Fig. 3), luciferase expression could not be used as a reporter to monitor *devR* transcriptional activity in strain UCD 311 in response to environmental changes. Figure 5 shows a blot of RNA isolated from *Nostoc* sp. strain ATCC 29133 prior to and at various times following deprivation of combined nitrogen. When probed with an 862-bp genomic fragment encompassing *devR*, a potential doublet band can be seen migrating at about 1,000 nucleotides and a larger band is apparent at 1,500 nucleotides. Since rRNA also migrates in the 1,500-nucleotide range, we are unsure whether this band reflects a *devR* transcript or nonspecific hybridization. The 1,000- and 1,500-nucleotide bands were present in all samples; thus, *devR* appeared to be constitutively transcribed with respect to culture nitrogen status (Fig. 5).

Hybridization of the *devR* probe to a Southern blot indicated that all other filamentous, heterocyst-forming cyanobacteria tested contained similar nucleotide sequences, and in many cases multiple hybridizing bands were present in each sample (Fig. 6). Hybridizing bands were absent in the lane containing DNA from the unicellular cyanobacterium *Synechococcus* sp. strain PCC 7942 (Fig. 6, lane b), whereas hybridization was observed in this lane with other probes.

Mutant reconstruction and complementation. The causal relationship between the site of transposition and the observed phenotype of the mutant was confirmed by double recombination of the interposon vector, pSCR206. Although the presence of other less intensely hybridizing bands can be seen in all lanes of the Southern blot in Fig. 7 when probed with *devR*, the wild-type lane shows a strongly hybridizing 2.4-kb *Eco*RI band (Fig. 7, lane a). This probe identifies a strongly hybridizing *Eco*RI band of approximately 10 kb in strain UCD 311 (Fig. 7, lane b), as well as in the putative double recombinant (Fig. 7, lane c), which is consistent with the insertion of a 7.8-kb transposon into the 2.4-kb *Eco*RI wild-type fragment containing *devR*. A putative single recombinant strain had both the 2.4- and 10-kb bands characteristic of the wild-type and transposon-interrupted fragments, respectively (Fig. 7, lane d). The single recombinant strain had the wild-type Fox$^+$ phenotype, whereas the double recombinant strain had the same Fox$^-$ Sym$^+$ phenotype as strain UCD 311. The wild-type Fox$^+$ phenotype could be restored in strain UCD 311 by the presence in *trans* of pSCR208, which contains a 3.4-kb *Xba*I fragment encompassing *devR*. The complemented strain formed normal-appearing heterocysts in the spacing pattern typical of wild-type filaments (Fig. 1B). The presence of the complementing fragment in cells of *Nostoc* sp. strain ATCC 29133 resulted in the production of akinetes both in ammonium-supplemented cultures after approximately 10 days of incubation and in N_2-grown cultures (Fig. 1C). Wild-type *Nostoc* sp. strain ATCC 29133 does not normally produce akinetes in NH_4^+-supplemented medium, and they are less than 1% of the total cells in aging N_2 cultures.

DISCUSSION

The Tn*5-1063*-induced mutant strain UCD 311 has a Fox$^-$ Sym$^+$ phenotype similar to that of a previously characterized chemically induced mutant of *Nostoc* sp. strain ATCC 29133 (8). We have now identified and characterized the gene re-

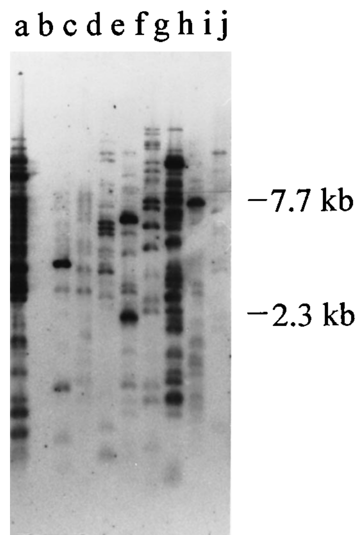

FIG. 6. Southern hybridization with the *Ssp*I-*Alw*NI fragment containing all of *devR* as the probe to a blot of *Hin*dIII-digested genomic DNA isolated from *Nostoc* sp. strain ATCC 27896 (lane a), *Synechococcus* sp. strain PCC 7942 (lane b), *Nostoc* sp. strain Mac (lane c), *Nostoc* sp. strain UCD 124 (lane d), *Anabaena* sp. strain PCC 7120 (lane e), *Nostoc* sp. strain UCD 120 (lane f), *Anabaena variabilis* ATCC 29413 (lane g), *Nostoc* sp. strain UCD 7801 (lane h), *Nostoc* sp. strain ATCC 29133 (lane i), and *Nostoc* sp. strain ATCC 29106 (lane j). *Synechococcus* sp. strain PCC 7942, *Nostoc* sp. strain Mac, *Anabaena variabilis*, and *Anabaena* sp. strain PCC 7120 are nonsymbiotic; all other strains infect *A. punctatus*. Molecular size markers are noted at the right.

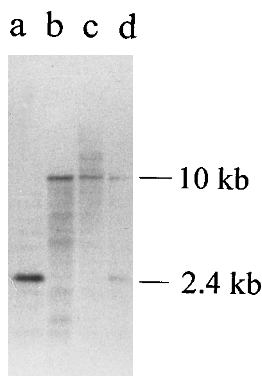

FIG. 7. Southern blot of *Eco*RI-digested genomic DNA from *Nostoc* sp. strain ATCC 29133 (lane a), strain UCD 311 (lane b), the double-recombinant strain resulting from mating pSCR206 into *Nostoc* sp. strain ATCC 29133 (lane c), and the single-recombinant strain resulting from mating pSCR206 into *Nostoc* sp. strain ATCC 29133 (lane d). The blot was probed with the *Ssp*I-*Alw*NI fragment containing all of *devR*. Molecular size markers are noted at the right.

sponsible for the strain UCD 311 mutation, as well as reconstructed the mutation and complemented it in *trans*. These latter results verify that the mutation was caused by the transposition event and confirm identity of the gene responsible for the phenotype. The characteristics of the previously described chemically induced mutant implied that mutations which lead to defective heterocyst walls should not affect symbiotic competence, because the micro-oxic environment of the symbiotic cavities serves to prevent inactivation of nitrogenase (8). The properties of strain UCD 311 confirm this initial observation. However, only 28% of the *Nostoc* sp. strain ATCC 29133::Tn*5-1063* Fox$^-$ mutants retained symbiotic competence. Therefore, the biochemical and genetic basis of a Fox$^-$ Sym$^+$ phenotype is more complicated than implied by our initial assumption.

Heterocyst development has been observed to proceed in stages (1, 38). A major transition is the maturation of proheterocysts into heterocysts, which can be blocked in wild-type cultures by the absence of O_2 (32) and by inhibition of DNA replication (1). Elhai and Wolk (11) presented evidence that synthesis of the Mo-Fe nitrogenase complex occurred only in heterocysts (defective or not) of *Anabaena* sp. strain PCC 7120 and related strains. They suggested that initiation of transcription of these nitrogenase genes was in response to developmental rather than environmental (nitrogen status) signals. Thiel et al. (36) identified two gene clusters encoding Mo-Fe nitrogenase complexes in *Anabaena variabilis* and some free-living *Anabaena* sp. isolates cultured from the water fern *Azolla* sp.; expression of the *nif1* cluster was confined to heterocysts under oxic and anoxic conditions and was under developmental control, while the *nif2* cluster appeared to be expressed in vegetative cells only under anoxic conditions and was under the control of environmental factors. We have no evidence for the presence of a *nif2*-like gene cluster or alternative nitrogenase complex in *Nostoc* sp. strain ATCC 29133. Proheterocysts may be the earliest developmental stage in which the heterocyst-specific nitrogenase can be synthesized, even under anoxic conditions in cyanobacteria such as *Anabaena* sp. strain PCC 7120 or *Nostoc* sp. strain ATCC 29133.

Ernst et al. (13) identified three classes of Fox$^-$ mutants of *Anabaena* sp. strain PCC 7120 and suggested that these classes also reflect stages of heterocyst development. Mutants with the phenotype Fox$^-$ Het$^+$ Fix$^+$ differentiate heterocysts (Het$^+$, whether the heterocysts are defective or not) and fix N_2 (Fix$^+$) but only under anoxic or micro-oxic conditions (Fox$^-$). Het$^+$ strains that by light microscopy possess defective envelopes were designated Hen$^-$. Hen$^-$ strains were further subdivided by diaminobenzidine staining; those unable to reduce diaminobenzidine were considered to have protoplasm that could not mature physiologically and to be unable to establish a reducing environment. The temporal ordering of developmental events of these Fox$^-$ Het$^+$ mutants following initiation of differentiation was suggested to be Hen$^-$ Dab$^-$ $<$ Hen$^-$ Dab$^+$ $<$ Hen$^+$ Dab$^+$ (13). On the basis of light microscopy results, strain UCD 311 should be characterized as Hen$^-$ (Fig. 1A). However, we were unable to observe diaminobenzidine staining in heterocysts of *Nostoc* sp. strain ATCC 21933 and its derivatives, even though we duplicated staining in *Anabaena* sp. strain PCC 7120 as previously described (reference 13, with corrections in reference 22). Thus, the comparable stage at which heterocyst development and complete formation of the O_2 protective mechanism is blocked in strain UCD 311 has not been determined. Since strain UCD 311 was capable of synthesizing heterocyst glycolipids (Fig. 2) and polysaccharides (see below) and of fixing N_2, heterocyst development was presumably blocked prior to the organization of the heterocyst envelope but well after differentiation had begun, proheterocysts developed, and nitrogenase was synthesized. A mutant of *Anabaena* sp. strain PCC 7120 that was unable to develop mature heterocysts from proheterocysts and is Dab$^-$ (13) also pleiotrophically failed to make heterocyst glycolipids or polysaccharides (22). The gene, *devA*, defining this developmental phenotype encodes a protein with similarity to ATP-dependent permeases (22) rather than a putative regulatory protein, as apparently does *devR* (see below). Thus, *devA* and *devR* apparently influence different aspects of the formation of a mature heterocyst envelope.

It is of interest that the gene responsible for the Fox$^-$ phenotype in strain UCD 311 has similarity to response regulator proteins of two-component regulatory systems. DevR most closely resembles Spo0F and CheY in that these three proteins consist of only the receiver domains of a response regulator. Other response regulators such as NtrC, PhoB, or Spo0A have the phosphorylation site of the receiver domain within the N-terminal portion of the protein and, minimally, an output domain in the C-terminal region of the protein (reference 28 and references therein). Many of these two-component regulatory systems have been characterized in eubacteria, and in all cases the response regulator proteins are part of a sensory pathway which relays information about the environment detected by the sensor kinase to effect an intracellular change (28). The highly conserved aspartate residue in the receiver domains of response regulator proteins can accept the transfer of phosphate from a histidine residue in the sensor kinase proteins. Phosphorylation of the receiver domain is proposed to control the output activity of the response regulator protein, in most cases to activate transcription of specific genes. PatA, which is involved in heterocyst pattern formation of *Anabaena* sp. strain PCC 7120, also has similarity to response regulator proteins (21). However, the putative receiver domain of PatA is located in the C-terminal portion of the protein while the N-terminal region has an unknown function.

The similarity of DevR to other receiver proteins of two-component regulatory systems is evidence that a sensory pathway, responding to an environmental (perhaps oxygen) or developmental signal after the onset of nitrogen limitation, is essential to the continued development of mature heterocysts. It is unlikely that DevR is involved in signaling the nitrogen status within cells, because strain UCD 311 has a Het$^+$ Fix$^+$ phenotype, which is repressed by NH_4^+, similar to wild-type

cells. Since the pattern of heterocyst spacing is not altered in this mutant (Fig. 1A), DevR is not related to the function of PatA (21). While most response regulator proteins have output domains that interact with DNA and regulate transcription, CheY and Spo0F lack such output domains and interact directly with another protein (28). Phosphorylated CheY interacts with the switching protein of the flagellar motors (29), and phosphorylated Spo0F is a phosphodonor to Spo0A, the transcriptional activator (15). Spo0F is, therefore, part of a phosphorelay system that may allow integration of a number of environmental signals in the sporulation pathway (18). DevR has a putative domain organization similar to those of CheY and Spo0F (Fig. 4). We suggest DevR also may interact with another protein, either as part of a phosphorelay system similar to that of Spo0F or in the activation of an enzyme that is involved in the organization or assembly of the heterocyst outer envelope.

The presence of multiple-banding patterns in all of the heterocyst-forming strains we surveyed by probing with *devR* implies, first, that two-component regulatory systems, or related phosphoproteins (23), may be widely distributed among filamentous cyanobacteria and, second, that multiple bands within the same organism may reflect similar regulatory mechanisms for other metabolic or developmental processes. For example, heterocysts may have evolved from akinetes, or the two cell types may have evolved from a common ancestor; thus, they could share some biosynthetic and regulatory mechanisms. This hypothesis is supported by biochemical evidence that the polysaccharide layers are identical in the envelopes of both heterocysts and akinetes of the two strains analyzed (40). Genetic evidence of a common origin is provided by the observation that HetR is essential for both akinete and heterocyst formation in *Nostoc ellipsoporum* (20). The genes which define the divergence of heterocysts from akinetes would then logically be in a pathway separate from those which are common to the two cell types (i.e., those containing the structural genes for polysaccharide synthesis). DevR could have a role in akinete formation. However, since strain UCD 311 differentiated what microscopically appeared to be typical akinetes, we assume that (i) synthesis of polysaccharides that form the outer layer of heterocyst envelopes is unaltered and (ii) the *devR* gene product normally functions exclusively in heterocyst development. Nevertheless, the observation that *Nostoc* sp. strain ATCC 21933 atypically formed akinetes in aging medium in the presence or absence of ammonium when *devR* was present in *trans* (Fig. 1C) implies that akinete differentiation may be influenced by a similar phosphorelay mechanism and a demonstrable amount of cross talk may occur between the two differentiation processes.

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REFERENCES

1. **Adams, D. G., and N. G. Carr.** 1981. The developmental biology of heterocyst and akinete formation in cyanobacteria. Crit. Rev. Microbiol. **9:**45–100.
2. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. L. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
3. **Berg, C. M., and D. E. Berg.** 1987. Uses of transposable elements and maps of known insertions, p. 1071–1109. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
4. **Brahamsha, B., and R. Haselkorn.** 1992. Identification of multiple RNA polymerase sigma factor homologs in the cyanobacterium *Anabaena* sp. strain PCC 7120: cloning, expression, and inactivation of the *sigB* and *sigC* genes. J. Bacteriol. **174:**7273–7282.
5. **Buikema, W. J., and R. Haselkorn.** 1990. Characterization of a gene controlling heterocyst differentiation in the cyanobacterium *Anabaena* 7120. Genes Dev. **5:**321–330.
6. **Cai, Y., and C. P. Wolk.** 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.
7. **Campbell, E. L., and J. C. Meeks.** 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.
8. **Campbell, E. L., and J. C. Meeks.** 1992. Evidence for plant-mediated regulation of nitrogenase expression in the *Anthoceros-Nostoc* symbiotic association. J. Gen. Microbiol. **138:**473–480.
9. **Cohen, M. F., J. G. Wallis, E. L. Campbell, and J. C. Meeks.** 1994. Transposon mutagenesis of *Nostoc* sp. strain ATCC 29133, a filamentous cyanobacterium with multiple cellular differentiation alternatives. Microbiology **140:**3233–3240.
10. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **16:**387–395.
11. **Elhai, J., and C. P. Wolk.** 1990. Developmental regulation and spatial pattern of expression of the structural genes for nitrogenase in the cyanobacterium *Anabaena*. EMBO J. **9:**3379–3388.
12. **Enderlin, C. S., and J. C. Meeks.** 1983. Pure culture and reconstitution of the *Anthoceros-Nostoc* symbiotic association. Planta **158:**157–165.
13. **Ernst, A., T. Black, Y. Cai, J. M. Panoff, D. N. Tiwari, and C. P. Wolk.** 1992. Synthesis of nitrogenase in mutants of the cyanobacterium *Anabaena* sp. strain PCC 7120 affected in heterocyst development or metabolism J. Bacteriol. **174:**6025–6032.
14. **Frias, J. E., E. Flores, and A. Herrero.** 1994. Requirement of the regulatory protein NtcA for the expression of nitrogen assimilation and heterocyst development genes in the cyanobacterium *Anabaena* sp. PCC 7120. Mol. Microbiol. **14:**823–832.
15. **Grossman, A. D.** 1991. Integration of developmental signals and the initiation of sporulation in *B. subtilis*. Cell **65:**5–8.
16. **Haselkorn, R., and W. Buikema.** 1992. Nitrogen fixation in cyanobacteria, p. 166–190. *In* G. Stacy, R. H. Burris, and H. J. Evans (ed.), Biological nitrogen fixation. Chapman & Hall, New York.
17. **Hill, S.** 1992. Physiology of nitrogen fixation in free-living heterotrophs, p. 87–134. *In* G. Stacy, R. H. Burris, and H. J. Evans (ed.), Biological nitrogen fixation. Chapman & Hall, New York.
18. **Ireton, K., D. Z. Rudner, K. J. Siranosian, and A. D. Grossman.** 1993. Integration of multiple developmental signals in *Bacillus subtilis* through the Spo0A transcription factor. Genes Dev. **7:**283–294.
19. **Joseph, C. M., and J. C. Meeks.** 1987. Regulation of expression of glutamine synthetase in a symbiotic *Nostoc* strain associated with *Anthoceros punctatus*. J. Bacteriol. **169:**2471–2475.
20. **Leganés, F., F. Fernández-Piñas, and C. P. Wolk.** 1994. Two mutations that block heterocyst differentiation have different effects on akinete differentiation in *Nostoc ellipsosporum*. Mol. Microbiol. **12:**679–684.
21. **Liang, J., L. Scappino, and R. Haselkorn.** 1992. The *patA* gene product, which contains a region similar to CheY of *Escherichia coli*, controls heterocyst pattern formation in the cyanobacterium *Anabaena* 7120. Proc. Natl. Acad. Sci. USA **89:**5655–5659.
22. **Maldener, I. G. Fiedler, A. Ernst, F. Fernández-Piñas, and C. P. Wolk.** 1994. Characterization of *devA*, a gene required for the maturation of proheterocysts in the cyanobacterium *Anabaena* sp. strain PCC 7120. J. Bacteriol. **176:**7543–7549.
23. **Mann, N. H.** 1994. Protein phosphorylation in cyanobacteria. Microbiology **140:**3207–3215.
24. **Meeks, J. C.** 1990. Cyanobacteria-bryophyte associations, p. 43–65. *In* A. N. Rai (ed.), Handbook of symbiotic cyanobacteria. CRC Press, Boca Raton, Fla.
25. **Meeks, J. C., C. S. Enderlin, C. M. Joseph, J. S. Chapman, and M. W. L. Lollar.** 1985. Fixation of [^{13}N]N_2 and transfer of fixed nitrogen in the *Anthoceros-Nostoc* symbiotic association. Planta **164:**406–414.
26. **Murry, M. A., and C. P. Wolk.** 1989. Evidence that the barrier to the penetration of oxygen into heterocysts depends upon two layers of the cell envelope. Arch. Microbiol. **151:**469–474.
27. **Nagaya, M., H. Aiba, and T. Mizuno.** 1994. The *sphR* product, a two-component system response regulator protein, regulates phosphate assimilation in *Synechococcus* sp. strain PCC 7942 by binding to two sites upstream from the *phoA* promoter. J. Bacteriol. **176:**2210–2215.
28. **Parkinson, J. S., and E. C. Kofoid.** 1992. Communication modules in bacterial signaling proteins. Annu. Rev. Genet. **26:**71–112.
29. **Parkinson, J. S., S. R. Parker, P. B. Talbert, and S. E. Houts.** 1983. Interaction between chemotaxis genes and flagellar genes in *Escherichia coli*. J. Bacteriol. **155:**265–274.
30. **Rippka, R., J. Deruelles, J. B. Waterbury, M. Herdman, and R. Y. Stanier.** 1979. Generic assignments, strain histories and properties of pure cultures of cyanobacteria. J. Gen. Microbiol. **111:**1–61.
31. **Rippka, R., and M. Herdman.** 1992. Pasteur culture collection of cyanobac-

teria in axenic culture, p. 44–57. Institut Pasteur, Paris.
32. **Rippka, R., and R. Y. Stanier.** 1978. The effects of anaerobiosis on nitrogenase synthesis and heterocyst development by Nostocacean cyanobacteria. J. Gen. Microbiol. **105:**83–94.
33. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
34. **Steinberg, N. A., and J. C. Meeks.** 1991. Physiological sources of reductant for nitrogen fixation activity in *Nostoc* sp. strain UCD 7801 in symbiotic association with *Anthoceros punctatus*. J. Bacteriol. **173:**7324–7329.
35. **Summers, M. L., J. G. Wallis, E. L. Campbell, and J. C. Meeks.** 1995. Genetic evidence of a major role for glucose-6-phosphate dehydrogenase in diazotrophic and dark heterotrophic growth of the cyanobacterium *Nostoc* sp. strain ATCC 29133. J. Bacteriol. **177:**6184–6194.
36. **Thiel, T., E. M. Lyons, J. C. Erker, and A. Ernst.** 1995. A second nitrogenase in vegetative cells of a heterocyst-forming cyanobacterium. Proc. Natl. Acad. Sci. USA **92:**9358–9362.
37. **Wei, T.-F., T. S. Ramasubramanian, and J. W. Golden.** 1994. *Anabaena* sp. strain PCC 7120 *ntcA* gene required for growth on nitrate and heterocyst development. J. Bacteriol. **176:**4473–4482.
38. **Wilcox, M., G. J. Mitchison, and R. J. Smith.** 1973. Pattern formation in the blue-green alga, *Anabaena*. I. Basic mechanisms. J. Cell Sci. **12:**707–725.
39. **Wolk, C. P., Y. Cai, and J. M. Panoff.** 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.
40. **Wolk, C. P., A. Ernst, and J. Elhai.** 1994. Heterocyst metabolism and development, p. 769–823. *In* D. A. Bryant (ed.), The molecular biology of cyanobacteria. Kluwer Academic Publishers, Dordrecht, The Netherlands.