JOURNAL OF BACTERIOLOGY, July 1990, p. 3925–3931
0021-9193/90/073925-07$02.00/0

Vol. 172, No. 7

Expression of the *Anabaena* sp. Strain PCC 7120 *xisA* Gene from a Heterologous Promoter Results in Excision of the *nifD* Element

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An 11-kilobase-pair element interrupts the *nifD* gene in vegetative cells of *Anabaena* sp. strain PCC 7120. The *nifD* element normally excises only from the chromosomes of cells that differentiate into nitrogen-fixing heterocysts. The *xisA* gene contained within the element is required for the excision. Shuttle vectors containing the *Escherichia coli tac* consensus promoter fused to various 5′ deletions of the *xisA* gene were constructed and conjugated into *Anabaena* sp. strain PCC 7120 cells. Some of the expression plasmids resulted in excision of the *nifD* element in a high proportion of vegetative cells. Excision of the element required deletion of an *xisA* 5′ regulatory region which presumably blocks expression in *Anabaena* sp. strain PCC 7120 vegetative cells but not in *E. coli*. Strains lacking the *nifD* element grew normally in medium containing a source of combined nitrogen and showed normal growth and heterocyst development in medium lacking combined nitrogen. The *xisA* gene was shown to be the only *Anabaena* gene required for the proper rearrangement in *E. coli* of a plasmid containing the borders of the *nifD* element.

Anabaena sp. strain PCC 7120 is a heterocyst-forming filamentous cyanobacterium. When this strain is deprived of a source of fixed nitrogen, approximately every tenth cell along the filament differentiates into a heterocyst, a thick-walled cell in which nitrogen fixation occurs. Heterocysts act in a cooperative way with the rest of the filament by providing reduced nitrogen to the vegetative cells, which are the sites of photosynthesis (7).

The *nifHDK* operon of *Anabaena* sp. strain PCC 7120 encodes nitrogenase reductase and the two subunits of the dinitrogenase enzyme (13). In vegetative cells, the *nifD* open reading frame is interrupted by an 11-kilobase-pair (kb) region known as the *nifD* element (5). The *nifD* element is excised from the chromosome of heterocysts late in the differentiation process. The *nifD* element contains the *xisA* gene, which is necessary for excision of the element when the element is subcloned into *Escherichia coli* (9). If the *xisA* gene is inactivated in the *Anabaena* sp. strain PCC 7120 chromosome, the mutant strain can neither excise the element nor produce functional dinitrogenase (6). The *xisA* gene contains an open reading frame with two in-frame start codons 117 base pairs (bp) apart and can code for a protein with a molecular weight of 46,000 if translated from the first start codon or 41,500 if translated from the second start codon.

We sought to learn more about the *nifD* element and the function of the *xisA* gene in *Anabaena* sp. strain PCC 7120. We constructed shuttle vector plasmids that expressed the *xisA* gene in vegetative cells. These plasmids caused the *nifD* element in vegetative cell chromosomes to rearrange and allowed the isolation of cells that lacked the *nifD* element. The experiments identified a regulatory region upstream of the *xisA* gene. We have also shown that the *xisA* gene is sufficient to cause rearrangement of the *nifD* element in *E. coli*.

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MATERIALS AND METHODS

Bacterial strains and conjugation. *E. coli* DH5α [F⁻ *endA1 recA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1* λ⁻ *gyrA96 relA1*] was purchased from Bethesda Research Laboratories, Gaithersburg, Md. *E. coli* XL1-Blue {*recA1 endA1 gyrA96 thi-1 hsdR17* (r_K^- m_K^+) *supE44 relA1* λ⁻(Lac⁻) [F′ *proAB lacI*^{q} *ZΔM15* Tn*10* (Tet^r)]} was purchased from Stratagene, La Jolla, Calif. All conjugations were performed as previously described (6). Briefly, shuttle vectors were transformed into *E. coli* DH5α containing plasmids pRL528 and pRL443. Plasmid pRL528 (4) is a ColK derivative that carries the *mob* region and genes for chloramphenicol resistance, *Eco* 47II methylase, and *Ava*I methylase. Plasmid pRL443 (4) is an RP4 derivative that does not express kanamycin resistance but retains genes for ampicillin and tetracycline resistance. Colonies resistant to kanamycin, chloramphenicol, and ampicillin were selected and conjugated to *Anabaena* sp. strain PCC 7120 and then selected with neomycin. *Anabaena* sp. strain PCC 7120 was grown in 100-ml liquid BG-11 cultures as previously described (6). Our BG-11 medium differs from the medium of Allen (1) by the omission of sodium silicate, ferric citrate, and citric acid and the addition of 0.012 g of ferric ammonium citrate per liter. BG-11 medium without fixed nitrogen is prepared by omitting the sodium nitrate added to complete BG-11. *E. coli* strains were grown and analyzed as previously described (3).

Construction of plasmids. Plasmid pJB4 (Fig. 1) was based on shuttle vector pRL405-*lux* (6). The *xisA* gene in pJB4 was obtained from plasmid pY6-1 (W. Buikema, unpublished data), which contains a 5′ deletion of the *xisA* region inserted into pUC18 (17) that begins approximately 50 bp 5′ of the first translational start of the *xisA* gene and ends 750 bp beyond the end of the open reading frame. Plasmid pCP106 contains a 411-bp fragment containing the *Anabaena* sp. strain PCC 7120 *glnA* promoter (15) cloned into the *Hin*dIII site of pUC18 (P. Lammers, unpublished data). The insert of pY6-1 was excised as a *Sal*I-*Kpn*I fragment and ligated between the *Sal*I and *Kpn*I sites of pCP106. The *Pvu*II fragment containing the *glnA* promoter fused to the 5′ end of the *xisA* gene was then inserted into the unique *Eco*RI site of pRL405-*lux* whose recessed 3′ end had been filled in and

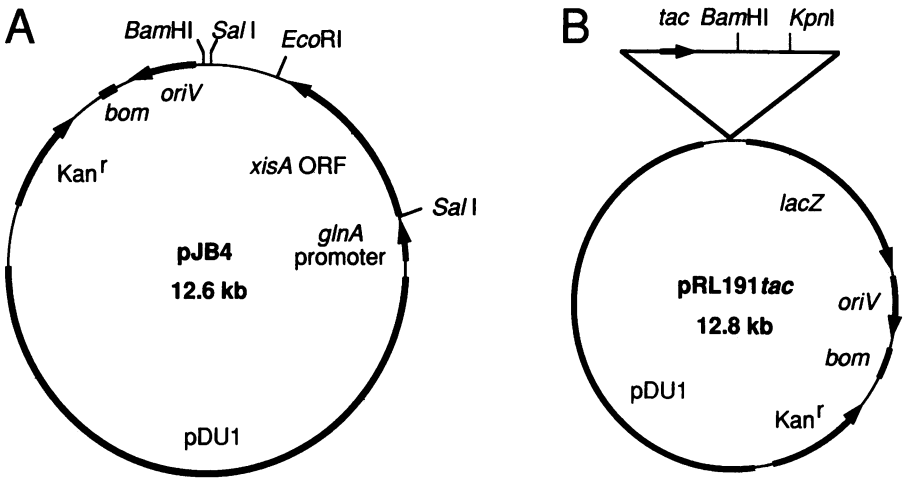

FIG. 1. Map of shuttle vectors pJB4 and pRL191*tac*. The pDU1 region is derived from a *Nostoc* sp. strain PCC 7524 plasmid and contains an origin of replication that is functional in *Anabaena* sp. strain PCC 7120. The *bom* region required for transfer is derived from pBR322 (16). (A) Shuttle vector pJB4, containing the *xisA* gene transcribed by the *glnA* promoter. The *Bam*HI and *Eco*RI sites are unique. ORF, Open reading frame. (B) Shuttle vector pRL191*tac*. The *tac* promoter is directed towards unique *Bam*HI and *Kpn*I sites.

treated with calf intestinal alkaline phosphatase. Both orientations of the *glnA-xisA* fusion relative to the pRL405-*lux* vector were obtained. One orientation, pJB4, is shown in Fig. 1. Both orientations behaved identically in the experiments described in this paper.

Plasmid pRL191*tac* (Fig. 1) was derived from pRL191 (J. Elhai, unpublished data). Plasmid pRL191 is derived from earlier shuttle vectors (16) and contains a promoterless *lacZ* gene downstream of a unique *Bam*HI site. First, plasmid pDR540 (14), containing the *E. coli* consensus promoter *tac*, was digested with *Bam*HI, and the recessed 3′ ends were filled in. The plasmid was then digested with *Hin*dIII, and the fragment containing the *tac* promoter was cloned into the multiple cloning site of pUC18 between a *Hin*dIII site and a *Pst*I site whose protruding 5′ end had been digested by T4 polymerase in the presence of all four deoxyribonucleotide triphosphates to produce pAM228. To allow for easier selection of recombinants when moving the *tac*-multiple cloning site into pRL191, a spectinomycin resistance cartridge (11) was inserted into pAM228. The spectinomycin resistance cartridge was removed from pDW9 (6) with *Hin*dIII, the recessed 3′ ends were filled in, and the fragment was ligated to *Bam*HI linkers. Following digestion with *Bam*HI, the fragment was inserted into the *Bam*HI site of pAM228. The *tac*-multiple cloning site was then removed by digestion with *Eco*RI and *Hin*dIII, the recessed 3′ ends were filled in, and the fragment was inserted into the unique *Bam*HI site of pRL191 after repair of the recessed 3′ ends. *E. coli* transformants were selected for spectinomycin resistance. The orientation with the *lacZ* gene downstream of the *tac* promoter was selected after determination by restriction endonuclease site mapping. The spectinomycin cartridge was subsequently deleted by digestion with *Bam*HI followed by a ligation to yield pRL191*tac*. Next, the 2.5-kb *Hin*cII-*Kpn*I fragment of pAn207.62 (9) that contains the *nifK* proximal border of the *nifD* element and the complete *xisA* gene was subcloned between the *Hin*cII and *Kpn*I sites of pBluescript KS$^+$ (Stratagene). This plasmid was cut with *Pst*I and *Hin*cII, and 5′ deletions of the *xisA* gene were generated by the ExoIII-S1 nuclease method with an Erase-A-Base kit according to the instructions of the vendor (Promega Biotec, Madison, Wis.). Double-stranded DNA was prepared by an alkaline lysis-polyethylene glycol precipitation procedure (8), and the vector-insert junctions of the deletions were sequenced with a Sequenase kit (United States Biochemical Corp., Cleveland, Ohio) and [α-^{32}P]dATP according to the instructions of the vendor. Appropriate deletions were then excised as *Bam*HI-*Kpn*I fragments and inserted between the unique *Bam*HI and *Kpn*I sites of pRL191*tac*. This directional cloning places the *xisA* gene downstream of the *tac* promoter in pRL191*tac*. All recessed 3′ ends were filled in with the Klenow fragment of DNA polymerase I (10).

Plasmid pAM461 contains the left and right borders of the *nifD* element and serves as a substrate for the excision reaction in *E. coli*. Plasmid pAM461 was constructed by inserting the 771-bp *Hin*dIII-*Sca*I fragment of pAn207.6 (13), which contains the *nifK* proximal border of the *nifD* element, between the *Hin*dIII and the *Hin*cII sites of pUC18. The 440-bp *Eco*RI-*Sau*3AI fragment of pAn256 (13) containing the *nifD* proximal border of the *nifD* element was then inserted between the *Bam*HI and the *Eco*RI sites of the vector. The resulting plasmid contains both borders of the *nifD* element in their normal orientation with respect to each other. Each border contains about 250 bp of *nifD* element sequences.

Plasmid pJB6 contains the 5′ region of the *xisA* gene extending 45 bp past the second in-frame translational start. This plasmid was used for generation of in vitro-labeled RNA transcripts. pJB6 was constructed by digesting pAn207.6 with *Hin*dIII and *Taq*I, gel purifying the 806-bp fragment containing the 5′ region of the *xisA* gene, and cloning the fragment between the *Acc*I and *Hin*dIII sites of pBluescript KS$^+$.

Isolation of nucleic acids. Total vegetative-cell DNA from *Anabaena* sp. strain PCC 7120 was prepared as follows. Fifteen milliliters of a stationary-phase culture was pelleted and suspended in 1/4 volume (3.75 ml) of protoplast buffer containing 15 mM Tris hydrochloride (pH 8.0), 0.45 M sucrose, 8 mM EDTA, and 1 mg of freshly added lysozyme per ml. The cells were incubated at room temperature for 15 min and pelleted. The pellet was suspended in 0.5 ml of buffer containing 10 mM Tris hydrochloride (pH 8.0), 0.1 mM EDTA, 1% sodium dodecyl sulfate, 0.7 M sodium chloride, and 1% cetyltrimethylammonium bromide. The mixture was incubated at 65°C for 10 min and extracted with

1 volume of chloroform-isoamyl alcohol 24:1. Fifty microliters of a solution containing 10% cetyltrimethylammonium bromide and 0.7 M sodium chloride was added, and the mixture was extracted successively with chloroform, phenol, and chloroform. The nucleic acids were then precipitated with ethanol and suspended in 50 μl of TE buffer containing 10 mM Tris hydrochloride (pH 8.0) and 1 mM EDTA.

Total RNA was prepared as previously described for heterocyst DNA (5), except that the initial lysozyme treatment was omitted and the RNA was purified through a 5.7 M cesium chloride cushion (10).

Southern blots. Approximately 1 μg of DNA was digested with restriction enzymes and resolved by agarose gel electrophoresis in 0.5× Tris-borate-EDTA buffer (10). The gel was treated with 0.125 N hydrochloric acid for 15 min and blotted to GeneScreen Plus (Dupont, NEN Research Products, Boston, Mass.) in 0.4 N sodium hydroxide (12). The blots were probed with DNA labeled by a random primer labeling kit (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Hybridization conditions were 5× SSPE (1× SSPE is 0.15 M sodium chloride, 10 mM monobasic sodium phosphate, and 0.1 mM EDTA [pH 7.4] [10])–1% sodium dodecyl sulfate at 65°C. The blots were washed in 0.5× SSPE–0.1% sodium dodecyl sulfate at 65°C.

RNA transcript analysis. RNA probes were prepared by in vitro transcription of pJB6 digested with *Eco*RI with T3 RNA polymerase by using a kit supplied by Stratagene according to the instructions of the vendor. This results in an 843-base transcript that can hybridize to *xisA* mRNA. Formaldehyde Northern (RNA) blot, primer extension, and mung bean nuclease and RNase mapping analyses were performed as described by Ausubel et al. (2). RNA probes for primer extension and RNase mapping were gel purified before use by electrophoresis in gels containing 3% NuSieve agarose (FMC Bioproducts, Rockland, Maine).

RESULTS

Low levels of *xisA* expression in developing heterocysts. In order to understand the regulation of the *xisA* gene and the rearrangement of the *nifD* element, an attempt was made to detect *xisA* transcripts in total RNA prepared from *Anabaena* sp. strain PCC 7120 filaments at different times after induction of heterocyst development. Northern gel analysis, mung bean nuclease protection analysis with in vitro-synthesized RNA probes, and primer extension analysis failed to detect *xisA* transcripts at any point in heterocyst development (data not shown). We conclude that *xisA* was expressed at very low levels, perhaps transiently, in the cells that were differentiating into heterocysts.

***xisA* expression in vegetative cells.** We wanted to determine if expression of the *xisA* gene in vegetative cells would result in rearrangement of the *nifD* element in those cells. We attempted to express the *xisA* gene by introduction of a shuttle vector containing the *xisA* gene fused to a strong promoter. Plasmid pJB4 contains the *Anabaena* sp. strain PCC 7120 *glnA* promoter fused to the intact *xisA* open reading frame (Fig. 1A). The *glnA* promoter functions in both vegetative and heterocyst cells (15). Northern blot analysis of RNA from *E. coli* containing pJB4 showed abundant transcripts from the *xisA* region. In contrast, Northern blots of RNA from pJB4-containing *Anabaena* sp. strain PCC 7120 grown in the presence (N^+ RNA) or absence (N^- RNA) of fixed nitrogen showed no hybridization to *xisA* probes. The *Anabaena* Northern blot did, however, detect chromosomal *glnA* transcripts in both N^- and N^+ RNA when hybridized with a *glnA* probe that did not contain homology to pJB4 (data not shown). Genomic Southern blots of vegetative-cell DNA from *Anabaena* sp. strain PCC 7120 containing pJB4 showed no evidence of excision of the *nifD* element.

Effect of *xisA* deletions on vegetative-cell DNA. Since the *glnA-xisA* gene fusion in pJB4 was not transcribed in *Anabaena* sp. strain PCC 7120, it is possible that sequences upstream of the *xisA* open reading frame were blocking expression of the gene. We generated a series of 5′ deletions of the *xisA* gene and inserted them into the shuttle vector pRL191*tac*, as described in Materials and Methods. The shuttle vector is shown in Fig. 1B, and maps of the deletions are shown in Fig. 2. These constructs were then conjugated into *Anabaena* sp. strain PCC 7120 and kept under continuous selection with 17.5 μg of neomycin per ml to retain the shuttle vectors.

DNA was prepared from *Anabaena* sp. strain PCC 7120 cells bearing each of the *xisA* deletion plasmids. The cultures were grown in complete BG-11 media and contained no heterocysts detectable by microscopic observation. An additional guard against contamination with heterocyst DNA is that heterocysts do not lyse under the conditions used for isolation of vegetative-cell DNA. The DNA was analyzed by Southern blot analysis after digestion with *Hin*dIII (Fig. 3). The blot was probed with pAn256, which contains the *nifD* proximal border of the *nifD* element on a 2.9-kb *Hin*dIII fragment (13). Plasmid pAn256 hybridizes to a 2.9-kb fragment in vegetative-cell DNA, while in heterocysts it detects a 1.8-kb chromosomal fragment and a 2.1-kb fragment from the excised 11-kb circle (5). The results of this blot are shown in Fig. 3. Deletions 261 through 265 showed no rearranged DNA. Deletion 266 showed a low level of rearranged DNA, and deletions 267 and especially 268 showed easily detectable amounts of properly rearranged chromosomal DNA. A Southern blot of the same *Hin*dIII-digested DNA was probed with pAn207.6 (13), which contains the *nifK* proximal border of the *nifD* element on a 1.0-kb *Hin*dIII fragment. The pAn207.6 probe, like pAn256, detects the 1.8- and 2.1-kb fragments in heterocyst DNA (5). This probe detected the corollary change from a 1.0- to a 1.8-kb chromosomal fragment in strains 266 through 268 (data not shown). This shows that the rearrangement induced by these plasmids in vegetative cells is the same as the rearrangement that occurs in wild-type heterocysts. DNA prepared from cultures of strains 266, 267, and 268 shortly after conjugation also contained detectable amounts of the excised circular *nifD* element in similar Southern blots. Strain 268 gave the highest ratio of rearranged to unrearranged chromosomal DNA. This ratio did not vary for any of the strains after prolonged culture. We have observed variable copy levels of the shuttle vectors in different DNA preparations of strains 261 through 385. The variable copy level of the shuttle vector did not affect the extent of rearrangement for any of the strains examined.

The *xisA* deletions define a region at the 5′ end of the gene that appears to prevent *xisA* expression in *Anabaena* sp. strain PCC 7120. This 5′ regulatory region is contained between the deletion endpoints of pAM264 and pAM268, a span of 127 bp. The region is centered around the first translational start codon of the *xisA* open reading frame, 117 bp upstream of the second translational start of *xisA*.

Northern gel analysis and RNase protection with in vitro-synthesized RNA probes were used to assess transcription of the *xisA* region in *Anabaena* sp. strain PCC 7120 and *E.*

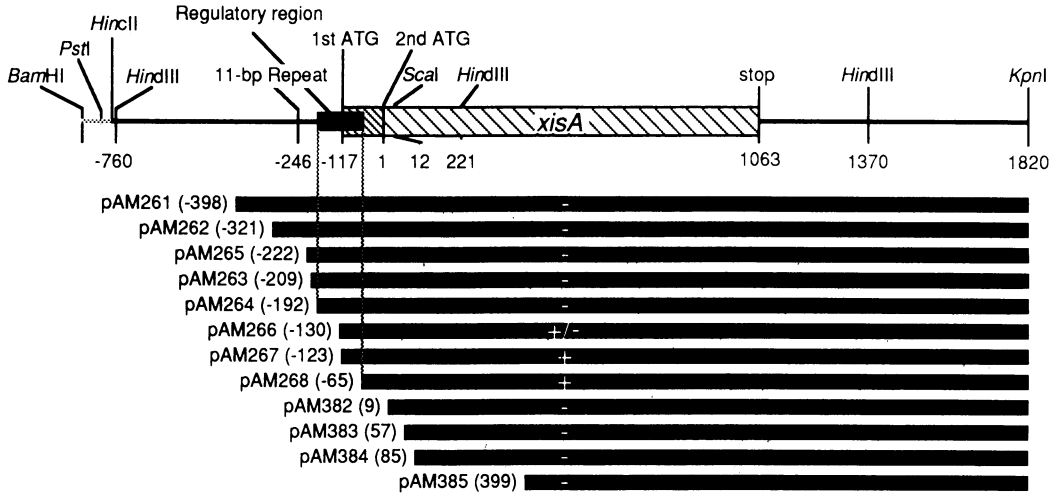

FIG. 2. 5′ deletions of the *xisA* gene. The map at the top shows the *Hin*cII-*Kpn*I fragment of pAn207.62. Nucleotide 1 has been assigned to the second translational start site of the *xisA* open reading frame, as in the work of Lammers et al. (9). The nucleotide numbers refer to the 5′ end of the indicated restriction endonuclease site, 11-bp repeat, or codon. Restriction endonuclease sites to the left of the *Hin*cII site are derived from the pBluescript KS^+ vector used in the construction of the plasmids. The bars below the map indicate the extent of the *xisA* region remaining in each plasmid after deletion. The numbers in parentheses indicate the position of the first nucleotide of the *xisA* region in each plasmid. The + and − symbols indicate the extent of rearrangement of the *nifD* element in *Anabaena* sp. strain PCC 7120 vegetative cells conferred by each plasmid. The 5′ regulatory region indicated by the black box in the map is defined by the borders of the deletions in pAM264 and pAM268.

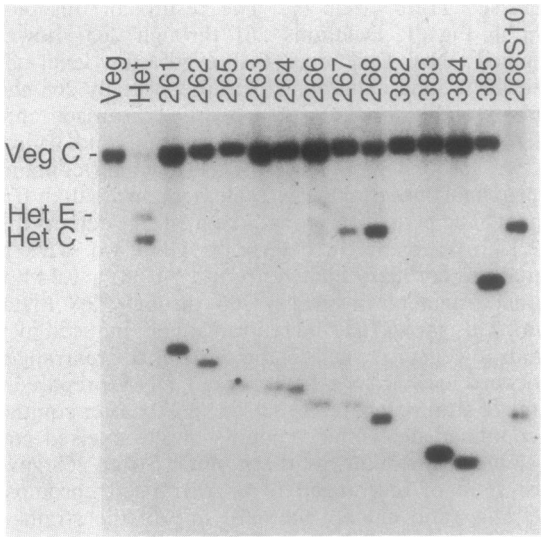

FIG. 3. Southern blot of *Anabaena* sp. strain PCC 7120 vegetative-cell DNA from cells containing shuttle vectors bearing 5′ deletions of the *xisA* gene. Wild-type vegetative (Veg) and purified heterocyst (Het) DNAs as well as vegetative-cell DNAs of strains containing the indicated plasmid were digested with *Hin*dIII and analyzed as described in Materials and Methods. The blot was probed with pAn256, which contains the *nifD*-proximal border of the *nifD* element. The positions of detected fragments derived from vegetative-cell chromosomes (Veg C), heterocyst chromosomes (Het C), and excised circles (Het E) are indicated at the left. The fragments at the bottom of the blot are derived from the shuttle vectors and are detected because of contaminating vector sequences in our probe. The small *Hin*dIII fragments contain the deletion point and form a ladder as the size of the deletion increases. Deletion pAM385 has deleted a *Hin*dIII site, causing the cross-hybridizing fragment to increase in size.

coli strains containing deletion plasmids 261 through 385. Although all of the plasmids produced easily detectable transcripts when present in *E. coli*, the levels of transcripts were very low and the relative levels of transcription could not be quantitated when the plasmids were in *Anabaena* sp. strain PCC 7120 (data not shown).

Strain 268S10 lacks the *nifD* element. Strain 268 was gently sonicated to single cells, and individual colonies were screened by Southern blot analysis for the extent of their chromosomal rearrangement. Of the 26 colonies screened, 15 (58%) lacked the *nifD* element. A representative clone lacking the *nifD* element, 268S10, is shown in the last lane in Fig. 3. This strain was phenotypically normal. Heterocyst development was suppressed and growth was normal in media containing fixed nitrogen. Strain 268S10 induced heterocysts and exhibited normal growth in media lacking fixed nitrogen.

***xisA* expression in *E. coli*.** Proper rearrangement of the *nifD* element in *E. coli* has been previously demonstrated with plasmids containing the entire *nifD* element (9). Insertional inactivation of the *xisA* gene blocks rearrangement of these plasmids, showing that a functional *xisA* gene is necessary for the deletion of the *nifD* element. We sought to determine whether the *xisA* gene was not only necessary but also sufficient to catalyze the rearrangement in *E. coli*. To test this, a plasmid that contained the DNA sequences required to serve as a substrate for the *xisA*-mediated rearrangement but lacked intact *Anabaena* sp. strain PCC 7120 genes was constructed. This plasmid, pAM461 (Fig. 4), contains the left and right borders of the *nifD* element and does not contain an intact *Anabaena* sp. strain PCC 7120 open reading frame larger than 180 bp. pAM461 does not rearrange in the absence of a complementing plasmid expressing the *xisA* gene. We then tested the ability of *E. coli* cells expressing the *xisA* gene to rearrange pAM461.

E. coli DH5α cells containing the *xisA* deletions in pRL191*tac* grew poorly. This may be because DH5α cells

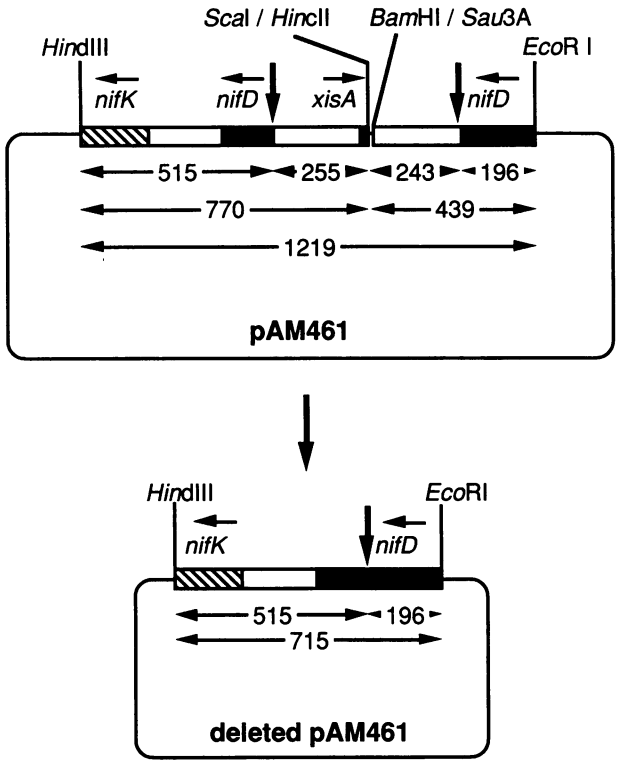

FIG. 4. Map of pAM461. The upper map shows pAM461 before excision at the borders of the *nifD* element. The *Sca*I-*Hin*cII and *Bam*HI-*Sau*3AI junctions were generated during cloning and are no longer complete restriction enzyme sites. The orientations of the portions of the genes in pAM461 (→) and the position of the 11-bp repeats that are the recombinational sites of the *nifD* element (↓) are indicated. The lower map shows the plasmid after excision of the region between the recombinational junctions. All sizes are in base pairs.

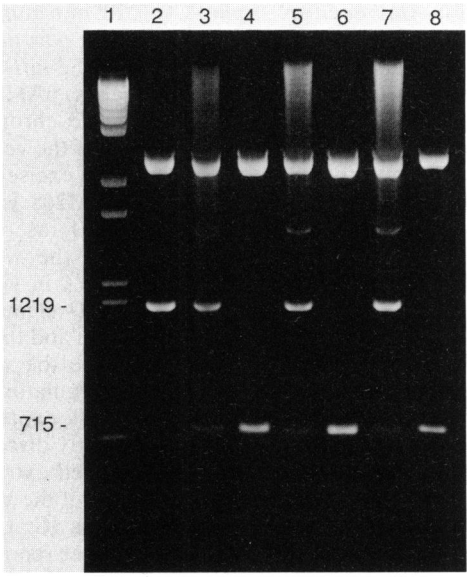

FIG. 5. Ethidium bromide-stained gel of pAM461 after transformation into *xisA*-expressing strains of *E. coli*. The DNA was electrophoresed on a 1.2% agarose gel. Lane 1, lambda DNA digested with *Bst*EII as a size marker; lanes 2 through 8, DNA digested with *Bam*HI and *Hin*dIII. Lane 2, pAM461 DNA; lanes 3, 5, and 7, DNA of pAM461 after transformation into cells containing pAM261, pAM264, and pAM268, respectively; lanes 4, 6, and 8, DNA of excised pAM461 subcloned from the DNA of lanes 3, 5, and 7, respectively. The positions of the 1,219-bp unrearranged pAM461 insert and the 715-bp deleted insert are shown at the side of the gel.

are constitutive for expression from *lac* or *tac* promoters on multicopy plasmids. Because of this, DH5α cells containing the *xisA* deletion plasmids can accumulate a high level of XisA protein, which may be toxic to *E. coli*. *E. coli* XL1-Blue contains a *lacI*^{q} gene that overproduces the *lac* repressor and allows only limited transcription from the *tac* promoter on multicopy plasmids. XL1-Blue cells containing the *xisA* deletion plasmids grew normally, presumably because the strains contained low levels of XisA protein.

Competent XLI-Blue *E. coli* bearing pAM261, pAM264, or pAM268 were prepared by the calcium chloride technique (10). The cells were transformed with pAM461 and plated on ampicillin plates to select for the incoming plasmid. Five colonies from each transformation were selected, and plasmid DNA from the colonies was digested with *Eco*RI plus *Hin*dIII to excise the pAM461 insert. The insert in the multiple cloning site of pAM461 is 1,219 bp. A deletion between the two *nifD* element borders reduces the insert size to 715 bp. Results of electrophoresis of plasmid DNA from representative colonies are shown in Fig. 5. Every colony derived from cells containing pAM261, pAM264, and pAM268 contained at least some rearranged pAM461. Although antibiotic selection for the shuttle vectors ended at the time of the pAM461 transformation, a low level of the shuttle vectors persisted in the DNA samples. All three plasmids rearranged pAM461 to approximately the same extent. Rearranged pAM461 was subcloned from all three minipreps. *Eco*RI-plus-*Hin*dIII digests of these purified rearranged plasmids are shown in Fig. 5. pAM461-transformed cells were also grown in the presence of ampicillin, kanamycin, and 0.1 mM isopropyl-β-D-thiogalactopyranoside to retain the shuttle vector and permit continued expression of the *xisA* gene after plating. These conditions gave generally higher levels of pAM461 rearrangement. These results show that pAM461 contains all the DNA sequences required to act as a substrate for the *nifD* element rearrangement. In addition, the ability of the *xisA*-expressing plasmids to rearrange pAM461 shows that the XisA protein is sufficient to cause the site-specific rearrangement in *E. coli*. Since pAM268 lacks the first in-frame translational start site of the *xisA* open reading frame, translation from the second start site is sufficient to produce a functional protein.

The plasmids pAM261, pAM264, and pAM268 contain about 750 bp downstream of the end of the *xisA* open reading frame. Only the first 364 bp of this region has been sequenced. The longest open reading frame within the sequenced region is 30 codons. The distal unsequenced region could code for a maximum of 181 amino acids. We do not believe that this region contains a gene involved in the rearrangement, since it can be deleted without abolishing rearrangement in *E. coli* (9).

DISCUSSION

We have been able to force expression of the *xisA* gene in vegetative cells of *Anabaena* sp. strain PCC 7120 by deleting the 5′ end of the gene and fusing it to the strong *E. coli tac* promoter in a shuttle vector. Transcription of the *xisA* gene could not be directly monitored because of the low levels of mRNA produced by the constructs. This might be due to rapid degradation of *xisA* mRNA, poor activity of the *tac*

promoter in *Anabaena* sp. strain PCC 7120, or a combination of these effects. Expression of the *xisA* gene was monitored by the ability of XisA to cause excision of the *nifD* element in the chromosomal DNA of vegetative cells. pAM264 does not induce detectable rearrangement of the chromosome, while pAM268 induces approximately 50% of the vegetative-cell chromosomes in a population of cells to excise the *nifD* element. The 5′ end of the insert in pAM268 is 127 bp downstream of the 5′ end of the pAM264 insert. These deletions define a regulatory region 5′ of the *xisA* open reading frame that blocks expression of *xisA* in vegetative cells. This 127-bp region is within the *nifD* element. The relationship between the 5′ regulatory region and the endogenous *xisA* promoter is not known because the *xisA* promoter has not yet been mapped. The 5′ regulatory region may interact with the *xisA* promoter to block expression of the gene in vegetative cells. We have recently discovered a DNA-binding factor present in vegetative-cell extracts that binds specifically to the 5′ regulatory region of the *xisA* gene as well as to the *glnA* and *rbcL* promoters (C. Chastain, submitted for publication). This suggests that repression of *xisA* activity in vegetative cells may be due to a transcriptional block mediated by binding of this factor to the upstream regulatory region. We cannot, however, eliminate the possibility that the 5′ regulatory region acts at a post-transcriptional level. Deletion of the 5′ regulatory region might force translation to begin at the second in-frame start codon in deletions pAM266 and pAM267. Translation of deletion pAM268 must begin at the second start codon since the first start codon is deleted. The resulting shorter form of the XisA protein may catalyze rearrangement, while the longer form translated from the first start codon may be inactive.

When plasmids containing the complete *nifD* element were passaged in *E. coli*, insertional inactivation of the *xisA* gene blocked excision of the *nifD* element (9). These experiments demonstrated that the *xisA* gene product is essential for the proper rearrangement of the *nifD* element. It was not clear, however, if the *xisA* gene product alone was sufficient to catalyze the rearrangement.

We have shown that pAM268 containing only the *xisA* gene is able to rearrange pAM461, which contains only small fragments of the borders of the *nifD* element. In prior experiments, expression of *xisA* relied on either the *xisA* promoter itself or a fortuitous readthrough from vector sequences (9). The higher efficiency of pAM461 rearrangement is likely to be due to efficient expression of the *xisA* gene by the strong *E. coli tac* promoter. Our results show that the *xisA* gene product is able to properly rearrange the *nifD* element in the absence of any other *Anabaena* sp. strain PCC 7120 gene product. The simplest interpretation of this is that *xisA* codes for a recombinase that catalyzes the excision of the *nifD* element. It may be that the XisA protein cooperates with other *Anabaena* sp. strain PCC 7120 proteins during the rearrangement and that *E. coli* proteins are able to provide the same function in the *E. coli* experiments. An in vitro rearrangement system may be needed to examine this possibility. The rearrangement of the *nifD* element in *E. coli* is not RecA dependent, since *E. coli* DH5α used in these experiments contains a *recA1* mutation.

We do not know why the ratio of unrearranged to rearranged chromosomes in *Anabaena* sp. strain PCC 7120 containing plasmids pAM266 through pAM268 remains constant. The different levels of rearrangement in these strains imply different levels of *xisA* activity. If higher levels of *xisA* activity increase the amount of rearrangement, it is not clear why the level of rearrangement remains constant after prolonged culture of the strains. One explanation for this may be that the excision of the *nifD* element is reversible. Since we see no evidence for replication of the excised circle in vegetative cells, however, it would be expected that an equilibrium between integrated and excised states could not be maintained. If the excision is reversible, heterocysts may contain accessory factors that prevent integration of the excised element, since the element is completely excised in heterocysts. Analysis of the reversibility of the excision may best be addressed through an in vitro rearrangement system.

A strain completely lacking the *nifD* element was cloned from cells containing pAM268. This strain, 268S10, showed no obvious phenotypic difference from wild-type *Anabaena* sp. strain PCC 7120 under laboratory conditions in medium with and without a combined nitrogen source. The normally Fix$^-$ *Nostoc* sp. strain MAC (PCC 8009) also contains an element similar to the *nifD* element. This strain appeared to excise the *nifD* element at a low rate during all growth conditions. In addition, a spontaneously arising nitrogen-fixing revertant of *Nostoc* sp. strain MAC that has lost the *nifD* element, inclusive of the *xisA* gene, has been isolated. This *Nostoc* sp. strain MAC isolate grows normally under laboratory conditions (J. C. Meeks, personal communication). Whether the *nifD* element confers some benefit to cells grown in a natural environment is not known. Although the *xisA* protein is not essential in the *Nostoc* revertant, we do not know whether the pAM268 plasmid that persists in strain 268S10 plays a role in maintaining a normal phenotype by supplying a source of the *xisA* protein. We have not been able to cure the 268S10 strain of pAM268 to check this possibility.

The ability of plasmids containing the *xisA* gene to induce excision of the *nifD* element in *Anabaena* sp. strain PCC 7120 vegetative cells shows that the XisA protein is the limiting factor needed for excision in vegetative cells. Since *xisA* mRNA levels in those plasmids that induced chromosomal rearrangement were very low, it appears that only low levels of XisA protein are required to excise the *nifD* element. This, in turn, suggests that the repression of *xisA* activity in vegetative cells must be extremely efficient, because spontaneous excision of the *nifD* element in *Anabaena* sp. strain PCC 7120 grown in the presence of fixed nitrogen has never been detected by Southern blot analysis. In addition, no spontaneous mutants of *Anabaena* sp. strain PCC 7120 that lack the *nifD* element are known. Heterocyst development and the consequent excision of the *nifD* element in *Anabaena* sp. strain PCC 7120 are never seen when cells are maintained in media containing a source of fixed nitrogen. Yet, upon induction of heterocyst development, the chromosomes of a population of heterocysts appear to rearrange completely. The regulation of *xisA* expression and heterocyst development must therefore be intimately related.

ACKNOWLEDGMENTS

We acknowledge the assistance of J. W. Lee in the transcriptional analysis of the *xisA* region. We thank R. Young, J. Meeks, and members of our laboratory for their many helpful suggestions during the preparation of the manuscript.

This work was supported by Public Health Service grant GM36890 from the National Institutes of Health, National Science Foundation grant DMB-8553185 and equipment grant BBS-8703784, and a grant from the Searle Scholars Program of the Chicago Community Trust.

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