Anabaena xisF gene encodes a developmentally regulated site-specific recombinase

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Two DNA elements are excised from the chromosome during *Anabaena* heterocyst differentiation. We have identified the gene *xisF* which encodes the site-specific recombinase responsible for the excision of a 55-kb element from within the *fdxN* gene. The cloned *xisF* gene is sufficient to cause site-specific rearrangement of an artificial substrate in *Escherichia coli*. Inactivation of *xisF* in the *Anabaena* chromosome prevents excision of the *fdxN* element and growth in nitrogen-deficient medium but does not alter the development of heterocysts. Forced transcription of *xisF* in vegetative cells did not result in excision of the *fdxN* element, suggesting that other factors may be involved in cell-type specificity. The predicted XisF protein shows significant similarity to the *Bacillus subtilis* SpoIVCA recombinase.

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Programmed DNA rearrangements occur in both eukaryotes and prokaryotes. Several examples of genome rearrangements associated with development in eukaryotes are known and include the immunoglobulin and T-cell receptor genes in the vertebrate immune system (Gellert 1992; Oettinger 1992); chromatin diminution in nematodes (Tobler et al. 1992); formation of the macronucleus in hypotrichous ciliates (Prescott 1992); and yeast mating-type switching (Haber 1992). In prokaryotes, developmentally regulated genome rearrangements have been found in two organisms (Haselkorn 1992). During *Bacillus subtilis* sporulation, a 42-kb element named *skin* is deleted from within the *sigK* gene in the terminally differentiating mother cell (Kunkel et al. 1990). The rearrangement is the result of site-specific recombination and requires the *spoIVCA* gene, which is located on the excised element and which shows homology to the Tn3 resolvase family of site-specific recombinases (Kunkel et al. 1990; Sato et al. 1990; Stark et al. 1992). During heterocyst differentiation in *Anabaena* sp. strain PCC 7120, at least two DNA rearrangements occur that involve the deletion of DNA elements from the chromosome by conservative site-specific recombination (Golden et al. 1985, 1987).

Anabaena sp. strain PCC 7120 is a filamentous, heterocystous cyanobacterium capable of oxygen-evolving photosynthesis and nitrogen fixation. Development of the nitrogen-fixing heterocysts is repressed in the presence of a source of combined nitrogen in the environment. Under nitrogen-limiting conditions *Anabaena* grows as a simple multicellular organism composed of two interdependent cell types: vegetative cells and het-

erocysts. Heterocysts differentiate from about every tenth vegetative cell along a filament producing a one-dimensional semiregular pattern (Wolk 1989). Heterocysts are highly specialized, terminally differentiated cells that reduce atmospheric dinitrogen to ammonia (Wolk 1982). The fixed nitrogen is exported to neighboring vegetative cells as glutamine, and in exchange, heterocysts are provided with carbohydrate products of photosynthesis.

Heterocyst differentiation in PCC 7120 is accompanied by at least two developmentally regulated DNA rearrangements that affect the *nifD* and the *fdxN* genes. Both rearrangements occur during the late stages of differentiation, at about the same time that the nitrogen-fixation genes begin to be transcribed (Golden et al. 1985, 1991). The *nifD* rearrangement is the excision of an 11-kb element from within the *nifD* open reading frame and allows for the formation of the complete *nifD* gene and the correct expression of the *nifHDK* operon (Haselkorn et al. 1986). Excision occurs by site-specific recombination between two 11-bp direct repeats that border the element and requires the *xisA* gene, which is present on the element (Lammers et al. 1986; Golden and Wiest 1988; Brusca et al. 1990). *xisA* is necessary and sufficient to cause rearrangement of the *nifD* element in PCC 7120 and of an artificial substrate in *E. coli*. However, XisA does not show significant similarity to either the integrase or resolvase family of site-specific recombinases (Stark et al. 1992).

The *fdxN* rearrangement is the excision of a 55-kb element from within the *fdxN* open reading frame by conservative site-specific recombination between 5-bp di-

rectly repeated sequences (Golden et al. 1987, 1988; Mulligan et al. 1988). Excision of the *fdxN* element results in the formation of the *fdxN* gene and the *nifB-fdxN-nifS-nifU* operon on the heterocyst chromosome (Mulligan and Haselkorn 1989).

Although the *nifD* and *fdxN* elements share some general characteristics, they are not directly related to each other. The elements do not show cross-hybridization using low-stringency Southern analysis (C.D. Carrasco and J.W. Golden, in prep.). The two elements are not always found together in cyanobacterial strains. Of several *Anabaena* and *Nostoc* strains examined using low-stringency hybridizations, three strains contained both elements while five others contained only the *nifD* element (C.D. Carrasco and J.W. Golden, in prep.). The repeated sequences involved in site-specific recombination differ for the two elements (Golden et al. 1987), and the *xisA* gene on the *nifD* element is not required for excision of the *fdxN* element in heterocysts (Golden and Wiest 1988). Under certain microaerobic heterocyst-inducing conditions, the *fdxN* element is excised from the chromosome while the *nifD* element is not (Golden et al. 1988). These observations indicate that at least some of the genes required for the developmentally regulated excision of the *fdxN* element are specific for that element.

We report the identification of the *xisF* gene, which is present on the *fdxN* element. *xisF* is shown to be required for excision of the *fdxN* element from the heterocyst chromosome. *xisF* is sufficient to cause rearrangement of an artificial substrate plasmid in *E. coli*, but forced *xisF* expression in PCC 7120 vegetative cells does not cause excision of the *fdxN* element, suggesting that other factors are required for cell-type specificity. XisF appears to be homologous to the *Bacillus subtilis* developmentally regulated recombinase SpoIVCA.

Results

Identification of the *xisF* gene

The 11-kb *nifD* element and the 55-kb *fdxN* element are located near each other around map position 1.7 Mb (Kurtz et al. 1993) on the PCC 7120 vegetative cell chromosome in a region containing several *nif* operons (Fig. 1A). The *fdxN* element interrupts the *fdxN* open reading frame in the *nifB-fdxN-nifS-nifU* operon. One of our approaches to identify the gene(s) required for excision of the *fdxN* element was to determine the DNA sequence of the ends of the element. We suspected that like most other mobile genetic elements, the gene encoding the site-specific recombinase was likely to be located near one of the recombination sites. Sequence analysis showed a large open reading frame located near the *nifS*-proximal border of the *fdxN* element, which we have named *xisF* (Fig. 1A). Figure 1B shows 1700 bp of DNA sequence that contains *xisF*. The *xisF* start codon is 47 bp from the 5-bp sequence involved in site-specific recombination that marks the border of the element. The 1545-bp *xisF* open reading frame could encode a 515-amino-acid polypeptide with a calculated molecular weight of 58,473 and a pI of 9.83.

We were unable to detect *xisF* transcription by Northern analysis. We suspect that *xisF* is transcribed at low levels and probably only transiently during heterocyst differentiation.

Sequence similarity between XisF and SpoIVCA

Comparison of the predicted XisF amino acid sequence with data bases revealed similarities with the resolvase family of site-specific recombinases. Members of this family include the resolvases from a number of transposons including Tn3, and the invertases related to Hin and Gin (Stark et al. 1992). All but one member of this class of proteins are ~20 kD in size. The exception is the *B. subtilis* SpoIVCA recombinase, which contains 500 amino acids and has a calculated molecular weight of 57,481 (Sato et al. 1990). The *spoIVCA* gene is required for excision of the 42-kb *skin* element in mother cells during *B. subtilis* sporulation. The amino acid sequences of XisF and SpoIVCA can be aligned along their entire length and show 49% similarity and 27% identity (Fig. 2). The amino-terminal region of SpoIVCA is homologous with the resolvase family of site-specific recombinases (Sato et al. 1990). An analysis using the GCG LINEUP program showed that the amino-terminal region of XisF can be aligned with this family of recombinases and that several amino acid positions are highly conserved in all of the proteins (Fig. 2).

Although XisF and SpoIVCA show significant similarity, their target sequences are quite different from each other (Golden et al. 1987; Stragier et al. 1989). SpoIVCA has been shown to bind to sequences flanking the *skin* element recombination sites and to cause DNA bending (Popham and Stragier 1992). It was also shown that a truncated form of SpoIVCA that contains only the amino-terminal region corresponding to the resolvase family retains its ability to bind DNA but is unable to carry out the *skin* excision *in vivo*. XisF could be expected to have similar properties.

Rearrangement of *pJG1A* in *E. coli*

The location of the *xisF* gene adjacent to one border of the *fdxN* element and the sequence similarity between XisF and other site-specific recombinases, especially SpoIVCA, strongly suggested to us that *xisF* encoded the recombinase for the *fdxN* element. To determine whether *xisF* was sufficient to cause site-specific recombination of an artificial substrate molecule, an *E. coli*-based rearrangement assay was designed. The approach was similar to that used for the identification of the *xisA* gene on the *nifD* element (Lammers et al. 1986). A rearrangement substrate plasmid, *pJG1A* (Fig. 3A), was constructed by positioning a *lacZ* gene between the restriction fragments An154.2 and An155 (Fig. 1), which contain the two *fdxN* element recombination sites (Golden et al. 1987, 1988). Site-specific recombination between the directly repeated recombination sites will result in the deletion of the *lacZ* gene from the plasmid and allow the rearrangement to be detected by the appearance of

Figure 1. Map of the PCC 7120 chromosomal region containing the *nifD* and *fdxN* elements, and the nucleotide sequence of *xisF*. (A) Map of the vegetative cell chromosomal region containing the *nifD* and *fdxN* elements. The *nifD* element interrupts the *nifHDK* operon, which encodes the nitrogenase polypeptides. The *fdxN* element interrupts the *nifB-fdxN-nifS-nifU* operon. The *fdxH* gene encodes a heterocyst-specific ferredoxin, and the *rbcLS* operon encodes the large and small subunits of ribulose-1,5-bisphosphate carboxylase. *xisA* and *xisF* encode the *nifD* and *fdxN* element site-specific recombinases, respectively. All genes read from right to left except for *xisA*, *xisF*, and *rbcLS*. The *nifD* and *fdxN* recombination sites are indicated by gray and black arrows, respectively. The positions of subcloned DNA fragments used in this study are shown below the map. (B) Nucleotide and predicted amino acid sequence of *xisF*. The *nifS*-proximal recombination site of the *fdxN* element is marked with a gray box; sequences to the left are from the *fdxN* open reading frame. The *DraIII* site (underlined) and the 154R1 internal PCR primer (open box marks the complementary sequence) were used in plasmid constructions for the expression of *xisF*. The two *Sau3AI* sites (underlined) mark the internal fragment used to inactivate the *xisF* gene. The GenBank accession number for the sequence reported in this paper is L23220.

white colonies on X-gal indicator plates. DNA fragments to be tested for the production of recombinase are introduced on a compatible expression vector into *E. coli* har-

boring pJG1A, and cells containing both plasmids are plated onto indicator plates.

Expression of *xisF* in *E. coli* caused the rearrangement

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Figure 4. *xisF* expression plasmids. (A) The plasmid pAM894 contains the *xisF* gene on a 2.9-kb restriction fragment cloned into the *E. coli* expression vector pSE380 (gray). The vector contains a *P_{trc}* promoter and a ribosome binding site (rbs) upstream from an *NcoI* site. The 2.9-kb fragment contains a 1.54-kb open reading frame (hatched box) with 375 bp of upstream and 1 kb of downstream sequence. The plasmid pAM1150 was constructed by replacing the 5' end of *xisF* with a synthetic fragment generated by PCR that lacks all upstream sequences. pAM1151 was derived from pAM1150 by removing the 102-bp *NcoI* fragment. Expression of *xisF* from pAM1151 utilizes the *P_{trc}* promoter and the ribosomal binding site on the pSE380 vector. (B) *xisF* PCR primer. The primer used for amplification of the *xisF* 5' end contained 13 bases of the *xisF*-coding region. The upstream sequence was designed to include *BamHI*, *HaeIII*, and *NcoI* restriction sites. The position of the *BamHI* site produced an imperfect ribosomal binding site. This PCR primer, along with the internal 154RI primer (Fig. 1), was used to amplify a fragment containing restriction sites that allowed the construction of pAM1150, pAM1151, and pAM1152.

that the low frequency of pJG1A rearrangement might be the result of inefficient *xisF* expression in *E. coli* caused by the upstream sequences present in pAM894.

In an effort to increase the efficiency of *xisF* expression in *E. coli*, PCR was used to modify the 5' end of the *xisF* gene. A DNA primer was designed to contain the first 13 bp of the *xisF* open reading frame and the restriction sites for *BamHI*, *HaeIII*, and *NcoI* (Fig. 4B). The *BamHI* site was positioned so that it could produce a partial ribosomal binding site. This 5' primer and an internal *xisF* gene-specific primer were used to amplify a fragment

containing the *xisF* 5' region from a pAn154 template. The amplified product was cloned as a *BamHI*–*DraIII* fragment, along with a *DraIII*–*XbaI* *xisF* fragment from pAM894, into pSE380 to produce pAM1150 (Fig. 4A). Removal of the small *NcoI* fragment from pAM1150 created pAM1151 (Fig. 4A), in which the *xisF* gene is correctly positioned to utilize the pSE380 promoter and ribosome binding site. pAM1150 and pAM1151 were tested for their ability to cause rearrangement of pJG1A. Strains containing either plasmid and pJG1A produced approximately 1% white colonies in overnight liquid cultures. The percentage of white colonies was quite variable because of the expected fluctuation when a cell was produced that contained only rearranged pJG1A during the growth of the culture. Although the rearrangement frequency was significantly higher than that obtained with the original pAM894 plasmid, the failure to obtain higher frequencies indicates that the conditions present in the *E. coli* assay are significantly different than those present in a differentiating heterocyst.

Figure 3B shows the *HindIII* restriction pattern produced by pAM1151, pJG1A, and the plasmids isolated from a blue and a white colony in the rearrangement assay. The pJG1A plasmid contains three *HindIII* restriction enzyme sites that produce fragments of 6.1, 5.0, and 3.3 kb. Rearrangement of pJG1A removes one *HindIII* site and results in two fragments of 5.0 and 2.3 kb. DNA sequence analysis of a rearranged plasmid confirmed that it was generated by site-specific recombination between the directly repeated sequences present at the borders of the *fdxN* element (data not shown). Control experiments with pJG1A alone or in the presence of the pSE380 vector produced a few rare white colonies, but none of these contained plasmids that showed a restriction pattern consistent with site-specific recombination.

We found that a few of the *E. coli* cultures started from either blue or white colonies had lost the *xisF*-containing plasmid. For example, the plasmid sample from the blue colony shown in Figure 3B contains pJG1A but does not contain the 5.6- and 1.3-kb *HindIII* fragments of pAM1151 that are present in the plasmid sample from the white colony. The loss of these ampicillin-resistant (*Ap^r*) plasmids is possible because of the destruction of the antibiotic relatively early during the growth of the culture. Loss of the *xisF*-containing plasmids suggests that overexpression of XisF causes plasmid instability, an effect presumably related to its recombinase activity. This phenomenon was not studied further; however, a similar effect was observed with *xisA* (Lammers et al. 1986).

Inactivation of *xisF* in PCC 7120

To determine whether *xisF* is required for rearrangement of the *fdxN* element during heterocyst differentiation, we inactivated the *xisF* gene in the PCC 7120 chromosome. An 864-bp *Sau3AI* fragment internal to the *xisF* open reading frame was cloned into the vector pAM580 to create pAM882 (Fig. 5). The *Sau3AI* fragment lacks 492 bp from the 5' end of *xisF* and 189 bp from the 3' end.

Figure 5. Inactivation of *xisF* in the strain KSR9. The plasmid pAM882 contains an internal 864-bp fragment of *xisF* (gray bar) cloned into the conjugal vector pAM580. The *xisF* fragment is missing 492 bp of 5' sequence ($\Delta 5'$) and 189 bp of 3' sequence ($\Delta 3'$). A single homologous recombination event between pAM882 and the *xisF* gene on the PCC 7120 chromosome results in integration of the plasmid into the chromosome and creation of two incomplete copies of the *xisF* gene. The *Hind*III sites that produce a 6.9-kb fragment in KSR9 are shown. (Ap) Ampicillin resistance gene; (Sp) spectinomycin resistance gene.

pAM882 was transferred into PCC 7120 by conjugation and exconjugants resistant to streptomycin (Sm) and spectinomycin (Sp) were obtained. Because the vector is unable to replicate in the cyanobacterial cells, these exconjugants were expected to contain pAM882 integrated into the chromosome by a single homologous recombination event (Fig. 5). The recombination event results in two incomplete copies of the *xisF* gene, thereby inactivating the gene. Southern blot analysis confirmed the integration of pAM882 into the *xisF* gene, and one isolate, KSR9, was selected for further study (data not shown).

KSR9 grew normally on medium containing nitrate, but it was unable to sustain growth in the absence of a source of combined nitrogen. KSR9 produced heterocysts that appeared morphologically normal by light micros-

copy and showed nearly normal heterocyst frequency and pattern formation. These characteristics are consistent with a defect in the excision of the *fdxN* element from the *nifB-fdxN-nifS-nifU* operon.

Southern analysis of DNA isolated from KSR9 vegetative cells and heterocysts showed that inactivation of the *xisF* gene prevented excision of the *fdxN* element but did not affect excision of the *nifD* element. Figure 6 shows the Southern analysis of *Hind*III-digested DNA isolated from vegetative cells and heterocysts of wild-type PCC 7120, KSR9, and KSR11. Strain KSR11 is KSR9 complemented with an *xisF* gene on a shuttle vector (see below). The blots were probed with DNA fragments that contain the recombination sites for the *fdxN* and *nifD* elements (for probe locations, see Fig. 1A). The *Hind*III fragment An154.2, which contains the *nifS*-proximal recombina-

Figure 6. Rearrangement of the *fdxN* and *nifD* elements in KSR9 and KSR11. Southern analyses of *Hind*III-digested DNA isolated from wild-type PCC 7120 (WT), KSR9, and KSR11 vegetative cells (V) and heterocysts (H). The DNA samples were fractionated by electrophoresis on a 0.7% agarose gel and then transferred to a nylon membrane. The blot was hybridized with radiolabeled probes generated from restriction fragments An154.2 and An155, which span the *fdxN*-element recombination sites, and An256, which spans one of the *nifD*-element recombination sites (see Fig. 1).

tion site of the *fdxN* element, hybridized with itself at 3.2 kb in wild-type vegetative cell DNA and with 4.6- and 2.2-kb fragments in heterocyst DNA, which are the products of rearrangement. KSR9 vegetative cell DNA showed 3.2- and 6.9-kb fragments with the An154.2 probe because of the integration of pAM882. The KSR9 heterocyst DNA showed no sign of rearrangement with the An154.2 probe. The *EcoRI* fragment An155, which contains the *nifB*-proximal recombination site, hybridized with a 3.6-kb *HindIII* fragment in wild-type vegetative cell DNA and with 4.6- and 2.2-kb fragments in heterocyst DNA. A faint 11-kb *HindIII* fragment, An265 (Golden et al. 1987), also hybridized with this probe. The KSR9 heterocyst DNA showed no sign of rearrangement with the An155 probe. The larger 6.9- and 11-kb fragments described above are underrepresented in the heterocyst DNA samples because of partial degradation of the DNA that occurs during the isolation procedure.

The Southern blots were also probed with the *HindIII* fragment An256, which contains the *nifH*-proximal recombination site of the *nifD* element, to determine whether the *nifD* element is excised normally in KSR9 (Fig. 6). Wild-type PCC 7120 vegetative cellular DNA showed hybridization to the 2.9-kb An256 fragment, and heterocyst DNA showed 2.1- and 1.8-kb fragments characteristic of the rearranged *nifD* element. DNA from KSR9 vegetative cells and heterocysts showed the same pattern as the wild type. These results show that the *nifD* rearrangement is not affected by inactivation of the *xisF* gene.

Expression of *xisF* in KSR9 and PCC 7120

The *xisF* gene was cloned into a conjugal expression vector to accomplish two goals. One was to complement the *xisF* mutation in KSR9 to confirm that the phenotype of KSR9 was attributable to the inactivation of *xisF*. The other was to determine whether forced expression of *xisF* in vegetative cells would cause excision of the *fdxN* element. The shuttle vector pAM692 contains the PCC 7120 *rbcL* and *glnA* promoters in tandem upstream of a cloning site (T.S. Ramasubramanian, unpubl.). The *rbcL* and *glnA* promoters both drive transcription in vegetative cells while the *glnA* promoter also allows for expression of genes in heterocysts (Tumer et al. 1983; Nierzwicki-Bauer et al. 1984). The *xisF* gene from pAM1150 (Fig. 4A) was cloned as a *Bam*HI-*Kpn*I fragment into the same unique restriction sites of pAM692 creating pAM1152. pAM1152 was transferred by conjugation into the strain KSR9, creating strain KSR11, and into wild-type PCC 7120, creating strain 692*xisF*.

Southern blot analysis of KSR11 is shown in Figure 6. The An154.2 and An155 probes showed that the *xisF* gene in pAM1152 was able to complement the *xisF* mutation in KSR9. Both probes showed the presence of DNA fragments produced by the excision of the *fdxN* element. However, the KSR11 heterocyst DNA also showed significant amounts of unrearranged *fdxN* element, indicating that the *xisF* gene on pAM1152 was not able to fully complement the inactivated gene in the

chromosome. The 2.0-kb fragment identified with the An154.2 probe in KSR11 DNA represents the *xisF* gene in pAM1152. The An256 probe showed the same pattern of bands for KSR11 as for the wild type and KSR9, indicating that *xisF* had no influence on the rearrangement of the *nifD* element.

Expression of *xisF* in vegetative cells is not sufficient to cause excision of the *fdxN* element. The vector pAM692 can provide very high levels of expression of a *lacZ* reporter gene in PCC 7120 vegetative cells (T.S. Ramasubramanian, unpubl.). The plasmid pAM1152 should express *xisF* in vegetative cells, and Northern analysis of RNA isolated from wild-type cells harboring pAM1152 confirmed that *xisF* message was present in significant amounts (data not shown). However, Southern analysis of this strain showed no detectable rearrangement of the *fdxN* element in vegetative cells (data not shown). KSR11, which contains pAM1152, also showed no rearrangement of the element in vegetative cells with either the An154.2 or An155 probe (Fig. 6).

Discussion

The *Anabaena* sp. strain PCC 7120 *xisF* gene encodes a developmentally regulated site-specific recombinase required for excision of the *fdxN* element from the chromosome during heterocyst differentiation. Expression of *xisF* was sufficient to cause site-specific recombination of an artificial substrate in *E. coli*, and inactivation of *xisF* in PCC 7120 prevented rearrangement of the *fdxN* element in heterocysts. A plasmid-borne copy of *xisF* transcribed by a heterologous promoter complemented the *xisF* mutation but failed to force rearrangement of the *fdxN* element in vegetative cells. XisF shows significant sequence similarity to the developmentally regulated SpoIVCA recombinase from *B. subtilis* and to the resolvase family of site-specific recombinases.

The *xisF* mutant KSR9 fails to excise the *fdxN* element from the chromosome and cannot grow on media lacking a source of combined nitrogen; however, it forms morphologically normal heterocysts. These results mirror those obtained with *xisA* (Golden and Wiest 1988) and suggest that the *fdxN* element must be precisely excised from the chromosome to allow the correct expression of the *nifB*-*fdxN*-*nifS*-*nifU* operon. Although the *xisF* mutant shows that excision of the *fdxN* element is required to produce a functional heterocyst, no positive role for the element has been identified in vegetative cells or heterocysts. *xisF* is the only gene that has been identified on the *fdxN* element, and no region of the element shows detectable levels of transcription in either vegetative cells or heterocysts (Golden et al. 1988).

XisF may not be completely sufficient for site-specific recombination. Expression of *xisF* in *E. coli* from a strong promoter in the plasmids pAM894, pAM1150, and pAM1151 caused site-specific recombination and rearrangement of the pJG1A substrate but only at a low frequency.

Similarly, expression of *xisF* from the plasmid pAM1152 complemented the *xisF* mutation in KSR9

heterocysts but failed to rearrange the *fdxN* element in vegetative cells of KSR9 or wild-type PCC 7120. Although several trivial explanations could account for these results, we suspect that at least one accessory factor, which is not present in *E. coli* or PCC 7120 vegetative cells, may be required for efficient recombination. The accessory factor could act similarly to IHF or FIS by participating in the assembly of a higher-order nucleoprotein complex required for efficient recombination (Landy 1989; Finkel and Johnson 1992). If the accessory factor were heterocyst specific, then rearrangement would occur inefficiently in *E. coli* or PCC 7120 vegetative cells even when *xisF* was overexpressed.

Expression of *xisF* from the plasmid pAM1152 caused excision of only about half of the *fdxN* elements in KSR9 heterocysts. This incomplete complementation may indicate that the XisF protein does not diffuse through the cell efficiently; the resolvase family of proteins tend to be hydrophobic and form insoluble inactive aggregates when overproduced (Glasgow et al. 1989; Sherratt 1989). Alternatively, the insertional inactivation of the *xisF* gene in KSR9 may have a polar effect on the expression of downstream genes that might be required for efficient rearrangement.

The mechanism for heterocyst-specific regulation of the *fdxN* element excision is not known. In *Bacillus*, excision of the *skin* element requires the *spoIIID* gene, which encodes a mother cell-specific transcription factor (Kunkel et al. 1989). Our understanding of cell-type specificity for the *Anabaena nifD* and *fdxN* elements will require the identification of genes like *spoIIID* by use of genetic or biochemical approaches.

The *Anabaena fdxN* element and the *Bacillus skin* element have several similar features. The amino acid sequence similarity between their recombinases, XisF and SpoIVCA, respectively, indicates that the elements are evolutionarily related. Both elements are relatively large, 55 and 42 kb, respectively, and could encode a substantial number of genes. They both excise from the chromosome by conservative site-specific recombination that restores the coding sequences within which the elements reside. The genes that are interrupted by the elements are not required for vegetative growth, but they are required in the terminally differentiated cell type that undergoes rearrangement, the *sigK* gene in *Bacillus* mother cells, and the *nifB-fdxN-nifS-nifU* operon in *Anabaena* heterocysts. For both elements, failure to excise from the chromosome results in a nonfunctional cell. It will be interesting if other similarities exist between the *Bacillus* and *Anabaena* elements. For example, in neither case is it known what selective pressures might be provided by these elements that allows them to be maintained in their hosts.

Materials and methods

Strains and growth conditions

Anabaena sp. strain PCC 7120 and derived strains were grown as described except that some large-scale liquid cultures were

grown in BG-11 medium instead of AA/8 medium (Golden et al. 1991). Heterocyst induction and isolation were essentially as described with minor modifications (Golden et al. 1991). Heterocysts were purified away from vegetative cells by passing the filaments through a Stansted cell disruptor at 12,000 psi. This procedure produces complete breakage of vegetative cells while leaving most of the heterocysts intact.

E. coli strains HB101, DH5 α MCR, and DH10B were used for plasmid maintenance and cyanobacterial conjugations and were grown in LB or 0.5 $\times$ TB medium with appropriate antibiotics as described (Golden et al. 1991).

DNA manipulations

DNA manipulations and recombinant DNA techniques were performed by standard procedures (Ausubel et al. 1987). Plasmid DNA was isolated from *E. coli* essentially as described in the GeneClean Kit (Bio 101). PCC 7120 vegetative cell and heterocyst DNA was isolated as described (Golden et al. 1991). Restriction endonucleases and other DNA-modifying enzymes were used according to the manufacturer's recommendations or standard protocols. For Southern analysis, DNA was transferred from agarose gels to MagnaCharge membranes (MSI) with 50 mM NaOH, 1 M NaCl. DNA probes were labeled using a random primer kit (Boehringer Mannheim), and Southern hybridizations were performed as described (Golden et al. 1991).

DNA sequencing

Double-stranded DNA sequencing was done using the Sequenase II sequencing kit (U.S. Biochemical) and [α - 32 P]dATP (DuPont, NEN Research Products). Sequencing reactions were primed with custom-made oligonucleotides. Sequencing data were analyzed using the sequence analysis software package of the Genetics Computer Group (GCG) (Devereux et al. 1984) and the NCBI GenBank BLAST e-mail server (Altschul et al. 1990).

PCR

PCR reactions were carried out in a MiniCycler (MJ Research) with *Taq* polymerase (U.S. Biochemical) under the following conditions—denaturation at 94°C for 1 min; annealing at 40°C for 2 min; polymerization at 72°C for 3 min—and cycled 30 times. The *xisF* internal primer sequence is identified in Figure 1, and the 5' primer is shown in Figure 4. The cloned PCR product was confirmed by DNA sequencing.

Plasmid constructions

The *xisF* open reading frame was cloned as a *Hae*II (blunted)–*Xba*I fragment from pAn154 into the *Sma*I and *Xba*I sites of pSE380 to produce pAM894. The plasmid pAM1150 was constructed with a synthetic *xisF* 5' end produced by PCR. The primers used for the PCR were an internal 154R1 primer (Fig. 1) and a 5' primer (Fig. 4) that had all upstream sequences replaced with appropriate cloning sites. The PCR product was digested with *Bam*HI and *Dra*III and ligated along with the *Dra*III–*Xba*I fragment from pAM894 into the *Bam*HI and *Xba*I sites of pSE380. For shuttle vector constructions, the *xisF* gene was obtained from pAM1150 as a *Bam*HI–*Kpn*I fragment. The *xisF* gene was placed under the control of the P_{trc} promoter and ribosome binding site of pSE380 by removing the 102-bp *Nco*I fragment from pAM1150 to produce pAM1151.

The plasmid pAM882 was used to inactivate the *xisF* gene in the PCC 7120 chromosome. A *xisF* internal 864-bp *Sau*3AI fragment (Fig. 1) was cloned into one of the two *Bam*HI sites of

pAM580 to make pAM882. pAM580 was constructed by inserting a Sp^r/Sm^r Ω cassette into the *Bam*HI (partial) and *Hind*III sites of the conjugal suicide vector pDW6 (Golden et al. 1991).

The *xisF* gene was cloned into the shuttle vector pAM692 (T.S. Ramasubramanian, unpubl.) for expression in vegetative cells and heterocysts. The plasmid pAM1152 was constructed by ligating a *Bam*HI–*Kpn*I fragment released from pAM1150 into the corresponding sites of pAM692.

The rearrangement substrate plasmid pJG1A was constructed by placing a *lacZ* gene between the *fdxN* element recombination sites. A *Bam*HI–*Hind*III fragment containing the *lacZ* gene from pDX11-1871 (M. Benedik, unpubl.) was cloned into the same sites of pACYC184 to make pACYClac [chloramphenicol resistant (Cm^r)]. The 3.2-kb *Hind*III fragment An154.2 (Fig. 1) was cloned into the *Hind*III site of pACYClac to obtain pACYClac154.2A, which has *lacZ* on the *fdxN*-element side of An154.2. The 3.5-kb *Eco*RI fragment An155 (Fig. 1) was cloned into the *Eco*RI site of pUC1819RI and then released as a *Bam*HI fragment. pUC1819RI was constructed by ligating the large *Sca*I–*Eco*RI fragment from pUC18 to the small *Sca*I–*Eco*RI fragment from pUC19 producing a unique *Eco*RI site flanked by an inverted repeat of the multiple cloning site. The An155 *Bam*HI fragment was cloned into the *Bam*HI site of pACYClac154.2A to make pJG1A, which has *lacZ* on the *fdxN*-element side of An155.

Strain constructions

Conjugation of shuttle vectors and the pAM882 suicide vector into PCC 7120 were performed essentially as described except for minor modifications (Golden and Wiest 1988). The *E. coli* donor strain was AM512, which is DH5 α MCR containing the plasmids pRL528 and pRL443 (Elhai and Wolk 1988). The strain KSR9 was constructed by the integration of pAM882 into the *xisF* gene on the PCC 7120 chromosome by homologous recombination. Filaments from a growing 100-ml PCC 7120 culture were collected by centrifugation and suspended in 4.5 ml of BG-11. Cells from a 5-ml overnight culture of AM512 containing pAM882 were collected by centrifugation, washed once to remove antibiotics, and suspended in 1 ml of LB medium. The PCC 7120 cells were mixed with 500 μ l of the *E. coli* donor cells and incubated overnight without shaking in a 50-ml flask at 30°C with 150 μ E/m² per sec light intensity. The filaments were collected by centrifugation and evenly distributed among five 90-mm petri plates containing 40 ml of BG-11 agar medium supplemented with *Sm* (2 μ g/ml) and *Sp* (2 μ g/ml). The plates were incubated at 30°C with 150 μ E/m² light intensity; exconjugant colonies appeared in 10–14 days. Two exconjugant colonies from independent conjugations were analyzed by Southern hybridization to confirm that they were the result of a single recombination event that integrated pAM882 into the chromosome. One clone, KSR9, was selected for further analysis.

The strains 692*xisF* and KSR11 were constructed by conjugating pAM1152 into PCC 7120 and KSR9, respectively. An overnight culture (1.5 ml) of the *E. coli* donor was pelleted and resuspended in the original volume of LB medium. Filaments from 1.5 ml of a growing culture of the PCC 7120 recipient were collected by centrifugation and resuspended in 1 ml of BG-11. Washed donor cells (100 μ l) were added to the recipient filaments in a 1.5 ml microcentrifugation tube, pelleted, and resuspended with BG-11 to ~90 μ l. LB (10 μ l) was added, and the cells were incubated overnight as above. Exconjugants were selected on BG-11 agar petri plates containing neomycin (Nm) (25 μ g/ml) for the PCC 7120 recipient or neomycin (15 μ g/ml), *Sp* (2 μ g/ml), and *Sm* (2 μ g/ml) for KSR9. The presence of pAM1152 was confirmed by Southern analysis.

Rearrangement assay

The rearrangement assay was based on the loss of *lacZ* from pJG1A by site-specific recombination in *E. coli*. Essentially, *E. coli* cells harboring pJG1A and a compatible expression vector containing a DNA fragment to be tested for the *fdxN*-element recombinase gene were grown overnight and plated onto indicator plates to detect loss of the *lacZ* gene from the plasmid. *xisF* expression plasmid pAM894, pAM1150, or pAM1151, along with the rearrangement substrate pJG1A, was introduced into DH5 α MCR cells by electroporation and plated onto LB medium containing Ap, Cm, and 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal). Two independent blue colonies were each used to inoculate two 5-ml cultures of LB medium supplemented with Ap and Cm. One culture was supplemented with 1 mM isopropyl- β -D-thiogalactoside (IPTG). After growth at 37°C for 24 and 48 hr, 25 μ l of a 10^{–3} dilution of each culture was plated onto LB agar containing IPTG, X-gal, Ap, and Cm. White colonies were selected for restriction analysis of the pJG1A plasmid. In several experiments a sample was also plated onto LB agar containing Cm alone to estimate the frequency of loss of the *xisF* expression plasmid.

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