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Heterocyst-Specific Excision of the *Anabaena* sp. Strain PCC 7120 *hupL* Element Requires *xisC*

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In nitrogen-limiting conditions, approximately 10% of the vegetative cells in filaments of the cyanobacterium *Anabaena* (*Nostoc*) sp. strain PCC 7120 differentiate into nitrogen-fixing heterocysts. During the late stages of heterocyst differentiation, three DNA elements, each embedded within an open reading frame, are programmed to excise from the chromosome by site-specific recombination. The DNA elements are named after the genes that they interrupt: *nifD*, *fdxN*, and *hupL*. The *nifD* and *fdxN* elements each contain a gene, *xisA* or *xisF*, respectively, that encodes the site-specific recombinase required for programmed excision of the element. Here, we show that the *xisC* gene (alr0677), which is present at one end of the 9,435-bp *hupL* element, is required for excision of the *hupL* element. A strain in which the *xisC* gene was inactivated showed no detectable excision of the *hupL* element. *hupL* encodes the large subunit of uptake hydrogenase. The *xisC* mutant forms heterocysts and grows diazotrophically, but unlike the wild type, it evolved hydrogen gas under nitrogen-fixing conditions. Overexpression of *xisC* from a plasmid in a wild-type background caused a low level of *hupL* rearrangement even in nitrogen-replete conditions. Expression of *xisC* in *Escherichia coli* was sufficient to produce rearrangement of an artificial substrate plasmid bearing the *hupL* element recombination sites. Sequence analysis indicated that XisC is a divergent member of the phage integrase family of recombinases. Site-directed mutagenesis of *xisC* showed that the XisC recombinase has functional similarity to the phage integrase family.

In prokaryotes, developmentally regulated DNA rearrangements have been identified in several organisms including members of the genera *Bacillus*, *Clostridium*, *Anabaena*, and *Nostoc*. In all cases studied so far, DNA elements between approximately 10 and 60 kb interrupt the open reading frame (ORF) of genes that are not expressed in the undifferentiated vegetative cell type. The elements are programmed to excise from the chromosome by site-specific recombination during cellular differentiation, which restores the structure of the interrupted gene or operon. In *Bacillus subtilis*, the SpoIVCA recombinase is required for excision of the 48-kb *skin* element from the mother cell chromosome during sporulation (23, 37). This recombination restores the *sigK* ORF, which is required for transcription of genes specific to the mother cell (23). A strain in which the *skin* element was deleted did not show any detectable sporulation defects, indicating that the DNA rearrangement is not essential for sporulation (23). In *Clostridium difficile*, developmentally regulated site-specific excision of a $skin^{Cd}$ element is similarly required for sporulation because the element interrupts the gene for the σ^K sigma factor. Unlike that of *B. subtilis*, the *C. difficile* rearrangement is required for normal temporal regulation of *sigK* expression, because mutants in which the element is removed are defective in sporulation (21).

Several strains of filamentous cyanobacteria undergo developmentally regulated DNA rearrangements programmed to occur during the differentiation of heterocysts (7, 11, 22). Heterocysts are differentiated cells that turn off oxygen-evolving photosynthesis, stop growth and cell division, and become specialized for nitrogen fixation (17, 30, 41). In nitrogen-limiting growth conditions, heterocysts are produced in a semiregularly spaced pattern such that there are about 10 to 15 vegetative cells between each single heterocyst and the next. The filaments grow as a simple multicellular organism containing two interdependent cell types: reproductive photosynthetic vegetative cells and heterocysts, which supply fixed nitrogen to nearby vegetative cells.

During heterocyst differentiation in *Anabaena* (*Nostoc*) sp. strain PCC 7120, three programmed DNA rearrangements have been identified. All three occur late during the differentiation process, at approximately the same time that transcription of the nitrogenase genes begins, about 18 h after nitrogen step-down (11, 15). The cyanobacterial DNA elements are named for the genes they reside within: *nifD*, which encodes the alpha subunit of dinitrogenase (14); *fdxN*, which encodes a heterocyst-specific ferredoxin (12, 13, 32, 33); and *hupL*, which encodes the large subunit of uptake hydrogenase (5). For all three rearrangements, the DNA element is excised from the chromosome by site-specific recombination between two short directly repeated sequences that flank the element. In each case, the interrupted genes are not expressed in vegetative cells

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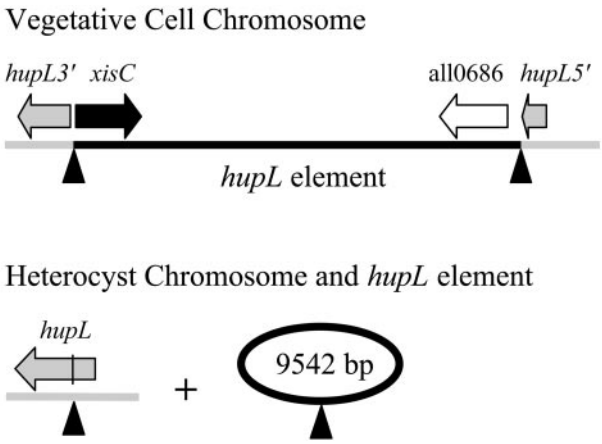

FIG. 1. *hupL* rearrangement in *Anabaena* sp. strain PCC 7120. The vegetative chromosome containing the interrupted *hupL* gene is shown above. The heterocyst chromosome and excised *hupL* element are shown below. Only open reading frames of interest are shown. Solid triangles, recombination sites.

but are turned on in heterocysts, where they are required for normal heterocyst function. Precise excision of each element restores the gene structure in heterocyst chromosomes.

At least eight strains of *Anabaena* or *Nostoc* contain a *nifD* element, and three strains are known to contain an *fdxN* element (7, 22). The *nifD* and *fdxN* elements in *Anabaena cylindrica* and a Fox^+ revertant of *Nostoc* strain Mac were shown to undergo rearrangement during heterocyst differentiation (7). For the *hupL* element, DNA hybridization studies suggested that the element was present in half of the filamentous strains tested (40); however, the *hupL* element is absent from two strains for which genome sequences are available, *Anabaena variabilis* (20) and *Nostoc punctiforme* (31).

The *Anabaena* sp. strain PCC 7120 *nifD* (11,289-bp) and *fdxN* (59,428-bp) elements each encode a site-specific recombinase, XisA (25) or XisF (8), respectively, which is required for excision of the element during heterocyst differentiation. Forced expression of the *xisA* gene in vegetative cells can result in the excision and loss of the *nifD* element (3). For efficient excision of the *fdxN* element in vegetative cells, expression of *xisF* plus two additional genes present on the *fdxN* element, *xisH* and *xisI*, is required (36). The XisF recombinase is homologous to the developmentally regulated *B. subtilis* SpoIVCA recombinase, and both belong to a subset of the resolvase family of site-specific recombinases (8).

The *xisC* gene (alr0677) (Fig. 1) is present at one end of the *hupL* element and is suspected to encode the site-specific recombinase for the element (5). The 9,542-bp *hupL* element interrupts the *hupL* gene, which is expressed only after filaments are induced to undergo heterocyst development by nitrogen deprivation (5). Uptake hydrogenases, which have been found in all nitrogen-fixing cyanobacteria, function to catalyze the consumption of the molecular hydrogen produced as a by-product of nitrogen fixation (39). The predicted XisC amino acid sequence shows strong similarity to cyanobacterial XisA recombinases as well as weak similarity to the phage integrase family of phage site-specific recombinases (6, 34). The phage integrase family contains a large number of genes found widely distributed in many different microorganisms (34). The family is characterized by a highly conserved tetrad, R-H-R-Y, in which the tyrosine is the catalytic residue and the R-H-R triad is on the DNA-interaction surface (24). However, XisC and XisA are missing the conserved histidine in the triad.

In this report, we describe the genetic analysis of the *Anabaena* sp. strain PCC 7120 *xisC* gene. We show that *xisC* is required for the programmed excision of the *hupL* element and that *xisC* expression is sufficient to cause site-specific recombination of an artificial substrate plasmid in *Escherichia coli*. Site-directed mutagenesis of *xisC* was used to show functional similarity to the phage integrase family of recombinases.

MATERIALS AND METHODS

***Anabaena* growth conditions and genetics.** *Anabaena* sp. strain PCC 7120 was cultured and induced to form heterocysts as described previously (15). Conjugation of shuttle vectors into *Anabaena* sp. strain PCC 7120 was performed essentially as described previously (8, 9, 16). Selection of double recombinants on medium containing 5% sucrose was performed essentially as described previously (28).

DNA manipulations. *Anabaena* sp. strain PCC 7120 total DNA was prepared from vegetative cells, purified heterocysts, and induced filaments as previously described (15). Restriction enzyme digests and other DNA-modifying reactions followed the manufacturer's suggested protocols. Southern analysis was performed as described previously (14, 15). DNA was transferred to MagnaCharge Plus membrane with 50 mM NaOH, 1 M NaCl. DNA fragments for hybridization probes were labeled with a random primer kit (Boehringer Mannheim).

Plasmid constructions. *E. coli* host strain DH10B (Invitrogen Life Technologies) was used for plasmid maintenance; growth conditions were as previously described (15). pAM1757 was used to inactivate the *xisC* gene and was constructed in two steps. Plasmid pAM1311, which contains the *xisC* gene on a 3.4-kb HindIII fragment (5), was digested with XbaI, blunted, and ligated with a blunted spectinomycin/streptomycin-resistant omega (Sp^r/Sm^r Ω) cassette to produce pAM1384. The 5.0-kb *xisC* fragment containing the Sp^r/Sm^r Ω cassette was released with XhoI-SmaI and cloned into the XhoI-ScaI sites of pRL278 (4), producing pAM1757.

The *xisC* open reading frame on a blunted DdeI fragment from pAM1311 was cloned into the StuI site of the expression vector pSE380 (2) to produce pAM1609, which allows isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible expression of *xisC* in *E. coli*.

The rearrangement substrate plasmid, pAM1500, was constructed by placing the *E. coli lacZ* gene between DNA fragments containing the recombination sites that flank the *hupL* element in their normal, directly repeated, orientation. A 1.9-kb HpaI fragment, containing the *xisC*-distal recombination site from pAM1268 (5), was cloned into the SmaI site of pBluescript II SK(+) to make pAM1283. The 0.62-kb AluI fragment from pAM1283 was cloned into the HincII site of pUC1819RI (8), resulting in pAM1755, and then released as a HindIII fragment that was then cloned into the HindIII site of pACYC*lacZ* (8). An 0.69-kb AluI fragment containing the *xisC*-proximal recombination site was cloned into the HincII site of pUC1819H3 (16), resulting in pAM1756, and then released as a BamHI fragment. This 0.69-kb BamHI fragment was then cloned into the BamHI site of the pACYC*lacZ* intermediate containing the distal recombination site to make pAM1500.

Hydrogen evolution measurements. Hydrogen evolution was measured with an H_2 electrode as previously described (26, 35). Essentially, *Anabaena* sp. strain PCC 7120 or *xisC* mutant strain AMC414 filaments from a 900-ml culture with known chlorophyll *a* (Chl_a) content were added to the electrode chamber, the light was turned on, and hydrogen evolution was recorded. The electrode was calibrated by adding standard amounts of H_2-saturated water, containing 774 nmol H_2 ml^{-1} at 30°C, to the electrode chamber.

DNA mutagenesis. Site-directed DNA mutagenesis of the *xisC* gene on pAM1609 was performed as described in the QuikChange site-directed mutagenesis kit (Stratagene). The complementary primer pairs for each *xisC* mutation were as follows, with the changed bases underlined: for mutation R306C (arginine 306 replaced with cysteine), oligonucleotides AMO-257 (GTTCCCTTGG GCTTAACCCAAATGTCG) and AMO-258 (CGACATTTGGGTTAAGCCC AAGGGAAC); for Y433F, AMO-255 (GAACATACAAAAACCTTTCAAAG ATGG) and AMO-256 (CCATCTTTGAAAGGTTTTTGTATGTTC); for the F433Y back mutation, AMO-265 (CCATCTTTGATAGGTTTTTGTATGTTC

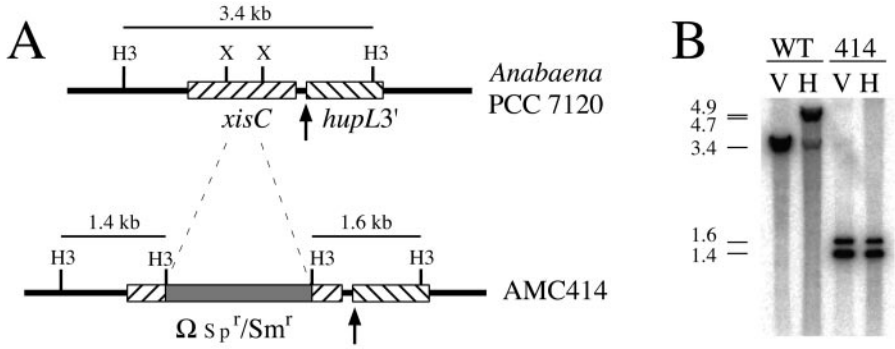

FIG. 2. Inactivation of *xisC*. (A) An internal 506-bp XbaI fragment of *xisC* was replaced by an Sp^r/Sm^r Ω cassette introduced on the conjugal suicide plasmid pAM1757. Double homologous recombination resulted in integration of the interrupted *xisC* gene into the chromosome, producing strain AMC414. (B) HindIII-digested genomic DNA from *Anabaena* sp. strain PCC 7120 (WT) and AMC414 vegetative cells (V) and purified heterocysts (H) was subjected to Southern analysis. The blot was hybridized with a radiolabeled 3.4-kb HindIII fragment from pAM1311, which spans the *hupL3′* recombination site. Fragment sizes are shown in kilobase pairs. H3, HindIII; X, XbaI; vertical arrows, recombination sites.

ATC) and AMO-266 (GATGAACATACAAAAACCTATCAAAGATGG); for Y398H, AMO-261 (GAGTTTCAACCCCACGTATTTGCGTC) and AMO-264 (GACGCAAATCGTGGGGTTGAAACTC); for Y398F, AMO-311 (GCATG ACGCAAATCGAAGGGTTGAAACTCAATTCC) and AMO-312 (GGAATT GAGTTTCAACCCTTCGATTTGCGTCATGC); and for Y398A, AMO-346 (GCATGACGCAAATCCGCGGGTTGAAACTCAATT) and AMO-347 (GG AATTGAGTTTCAACCCGCGGATTTGCGTCATGC).

Detection of genome rearrangement. PCR was performed in a MiniCycler (MJ Research) with *Taq* DNA polymerase according to standard protocols. Primers AMO-221 (CGTTCCAAAGAACAACCC) and AMO-215 (GCTTCGACTAA CTTCTG) were used to detect the presence of the *xisC*-proximal recombination site on the vegetative cell chromosome. Primers AMO-251 (GAGTTTAGACG ATTTTGGGG) and AMO-247 (GTTCGCCATTGACC) were used to detect the presence of the *xisC*-distal recombination site on the vegetative cell chromosome. Primers AMO-247 and AMO-221 were used to detect the presence of the recombination site on the rearranged heterocyst chromosome after excision of the *hupL* element.

Rearrangement assay in *E. coli*. Plasmid pAM1609, which carries wild-type *xisC*, and plasmids carrying site-directed *xisC* mutations were tested for their ability to cause rearrangement of the substrate plasmid pAM1500 in *E. coli*. Rearrangement of pAM1500 excises the *lacZ* gene from between the two flanking directly repeated recombination sites. *E. coli* DH10B cells harboring the rearrangement substrate pAM1500 were transformed by electroporation with pAM1609 or plasmids carrying the site-directed *xisC* mutations. Colonies were grown on LB agar plates containing both ampicillin (100 μg/ml) to select for the *xisC*-containing plasmids, chloramphenicol (17 μg/ml) to select for pAM1500, and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) to confirm the presence of unrearranged pAM1500. Individual blue colonies were inoculated into 1-ml cultures of LB medium with 0.01 mM IPTG to induce expression of *xisC*. Following overnight growth, plasmid DNA was extracted with a QIAGEN Miniprep kit and then transferred by electroporation into *E. coli* strain DH10B to screen individual pAM1500 plasmids for rearrangement. Transformant colonies were selected for chloramphenicol (17 μg/ml) resistance provided by pAM1500 and scored for blue or white color in the presence of X-Gal. Colonies containing unrearranged pAM1500 appeared blue, while those that had undergone rearrangement appeared white.

RESULTS

Inactivation of *xisC* blocks *hupL* rearrangement. To determine whether *xisC* is required for rearrangement of the *hupL* element, the *xisC* gene was inactivated in the chromosome of *Anabaena* sp. strain PCC 7120 by double reciprocal recombination with pAM1757 to make strain AMC414 (Fig. 2). pAM1757 was constructed from the conjugal vector pRL278, which is not able to replicate in *Anabaena* sp. strain PCC 7120 and contains the *B. subtilis sacB* gene. *sacB*, a conditionally lethal gene, facilitates positive selection of double recombinants in medium containing sucrose (4). pAM1757 carries a copy of the *xisC* gene interrupted by an Ω cassette encoding spectinomycin and streptomycin resistance. pAM1757 was transferred into wild-type *Anabaena* sp. strain PCC 7120 by conjugation, and exconjugants were selected for double recombinants on sucrose-containing medium.

Southern analysis of genomic DNA confirmed that the wild-type *xisC* gene had been replaced by the disrupted *xisC* allele from pAM1757 (Fig. 2B and data not shown). One strain, AMC414, was chosen for further analysis. Additional Southern analysis of vegetative cell and heterocyst genomic DNA showed that AMC414 did not have detectable levels of *hupL* rearrangement (Fig. 2B). For the wild type, the probe hybridized to itself in vegetative cell DNA and to 4.9- and 4.7-kb fragments (unresolved in Fig. 2B) in heterocyst DNA after excision of the element. The AMC414 *xisC* mutant showed the expected 1.6- and 1.4-kb fragments in vegetative cell DNA but no evidence of the expected chromosomal 4.9-kb band that would be generated by excision of the *hupL* element. Similar Southern analyses of AMC414 DNA with *nifD* and *fdxN* element probes show that inactivation of *xisC* did not affect rearrangement of these elements in heterocyst DNA (data not shown). PCR was used in an attempt to detect possible low levels of *hupL* rearrangement in DNA isolated from AMC414 heterocysts, but no evidence of rearrangement was detected (data not shown), indicating that the XisC product is essential for site-specific excision of the *hupL* element.

AMC414 grew normally on BG-11 medium, which contains sodium nitrate, and showed normal heterocyst development and diazotrophic growth after nitrogen step-down. AMC414 vegetative cells and heterocysts were morphologically normal. Therefore, failure to excise the *hupL* element during heterocyst differentiation did not produce obvious developmental defects.

The *xisC* mutant strain evolves hydrogen. Molecular hydrogen is a by-product of nitrogenase activity, and its production would result in a loss of potential energy for organisms during diazotrophic growth in the absence of uptake hydrogenases (39). It was previously shown that *hupL* transcripts are present at increased levels after the induction of heterocyst development by nitrogen step-down, and it is assumed that the *hupSL*

operon encodes a heterocyst-specific [NiFe] uptake hydrogenase (5). As a consequence of the failure to excise the *hupL* element during heterocyst differentiation, we expected that the *xisC*-inactivated strain AMC414 would be defective for [NiFe] uptake hydrogenase activity. Therefore, we measured hydrogen uptake from the medium in the wild type and AMC414. The wild type showed light-dependent hydrogen uptake of -0.79 nmol H_2 h^{-1} μg Chl_a^{-1} in filaments grown on nitrate-containing medium. Hydrogen uptake was enhanced to -3.9 nmol H_2 h^{-1} μg Chl_a^{-1} in heterocyst-containing filaments grown in diazotrophic conditions. AMC414 showed hydrogen uptake of -0.73 nmol H_2 h^{-1} μg Chl_a^{-1} in filaments grown on nitrate, which was similar to the wild type. However, not only were heterocyst-containing filaments of AMC414 grown in diazotrophic conditions on BG-11_0 medium defective for hydrogen uptake, but they evolved hydrogen at $+0.14$ nmol H_2 h^{-1} μg Chl_a^{-1}. Therefore, the failure to excise the *hupL* element in heterocysts results in a loss of [NiFe] uptake hydrogenase activity, and we conclude that the *hupSL* operon encodes the primary uptake hydrogenase in *Anabaena* sp. strain PCC 7120 heterocysts. A more extensive analysis of hydrogen photoproduction by AMC414 has been published elsewhere (27).

***xisC* complementation restores *hupL* recombination.** In AMC414, *xisC* was inactivated by deletion of an internal fragment of the open reading frame and insertion of a 2-kb Sp^r/Sm^r Ω cassette. These changes are relatively close to one of the *hupL* element recombination sites, and there was the possibility that the failure to undergo site-specific recombination was due to a *cis* effect, or to an unknown second-site mutation. To determine if the loss of the XisC product alone was responsible for defective excision of the *hupL* element, the AMC414 strain was complemented with a plasmid-borne copy of the *xisC* gene. The conjugal plasmid pAM2239 contains *xisC* expressed from the *rbcL* promoter. The *rbcL* promoter is not expressed well in *E. coli* (38), the conjugal donor, and therefore helps avoid potential deleterious effects of expressing a site-specific recombinase in the *E. coli* host. The *rbcL* promoter is strongly expressed in *Anabaena* sp. strain PCC 7120 vegetative cells (10, 43). Following conjugation of pAM2239 into AMC414, neomycin-resistant colonies were analyzed by PCR to determine if *hupL* rearrangement had been restored (Fig. 3 and data not shown). Several independent colonies contained rearranged chromosomes, which confirmed that the *hupL* rearrangement defect in AMC414 was due to *xisC* inactivation and that expression of *xisC* in vegetative cells was sufficient to cause excision of the *hupL* element.

We attempted to obtain a strain completely lacking the *hupL* element by screening colonies of AMC414 containing pAM2239 for loss of spectinomycin and streptomycin resistance. Although expression of *xisC* by the *rbcL* promoter on pAM2239 allowed complementation of the *xisC* mutant and produced rearranged chromosomes, we failed to isolate any clones that had entirely lost the *hupL* element. After screening several thousand colonies, we identified a few Sp/Sm-sensitive clones, which were examined with PCR for the presence of the *hupL* element. Wild-type vegetative cell chromosomes containing the *hupL* element were present in all clones tested, although one clone apparently contained a deletion or sequence change that caused a defect in the sequences recognized by primer 3 (Fig. 3, clone 5). Southern analysis of these clones was performed to determine the proportion of chromosomes that had lost the *hupL* element. Although PCR clearly detected the presence of rearranged chromosomes, bands representing the *hupL* rearrangement were not easily detected by Southern analysis (data not shown), indicating that only a small proportion of chromosomes had excised the *hupL* element. It is not clear why pAM2239 did not produce higher levels of *hupL* excision or why we were unable to isolate a strain cured of the *hupL* element.

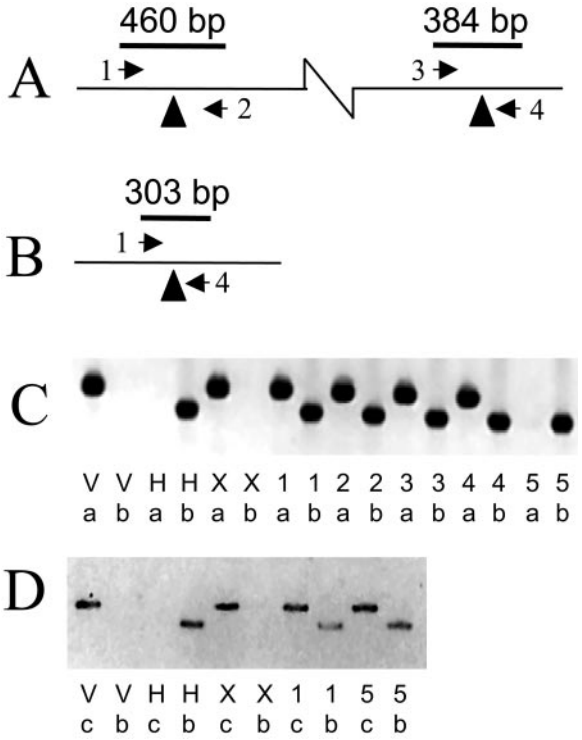

FIG. 3. Detection of *hupL* rearrangement after complementation of the *xisC* mutant AMC414. (A) Diagram showing PCR primers used to detect the unrearranged *hupL* element borders on vegetative cell chromosomes. The *xisC*-proximal recombination site is shown on the left as in Fig. 1. Predicted PCR product sizes are shown in base pairs. The oligonucleotide primers (arrows) are labeled as follows: 1, AMO-221; 2, AMO-215; 3, AMO-251; and 4, AMO-247. Recombination sites, solid triangles. (B) *hupL* recombination site on the heterocyst chromosome after excision of the *hupL* element. Labeling as in panel A. (C and D) PCR products were analyzed by gel electrophoresis. Lane labels indicate the DNA template (top) and the PCR primer pairs (bottom). Template DNA samples are labeled as follows: V, vegetative cell; H, heterocyst; X, *xisC* mutant AMC414; 1 to 5, five independent exconjugant complementation strains. Primer pairs are labeled as follows: a, primers 3 and 4; b, primers 1 and 4; and c, primers 1 and 2. (C) The upper band is the 384-bp product from the *xisC*-distal vegetative cell recombination site, and the lower band is the 303-bp product shown in panel B. (D) The upper band is the 460-bp product from the *xisC*-proximal vegetative cell recombination site, and the lower band is the 303-bp product shown in panel B.

XisC catalyzes site-specific recombination in *E. coli*. To determine if XisC alone was sufficient to cause site-specific recombination, we expressed *xisC* in an *E. coli*-based assay similar to that used previously for the *xisA* and *xisF* site-specific recombinases (8, 25). A plasmid, pAM1609, with *xisC* under control of an IPTG-inducible promoter was constructed.

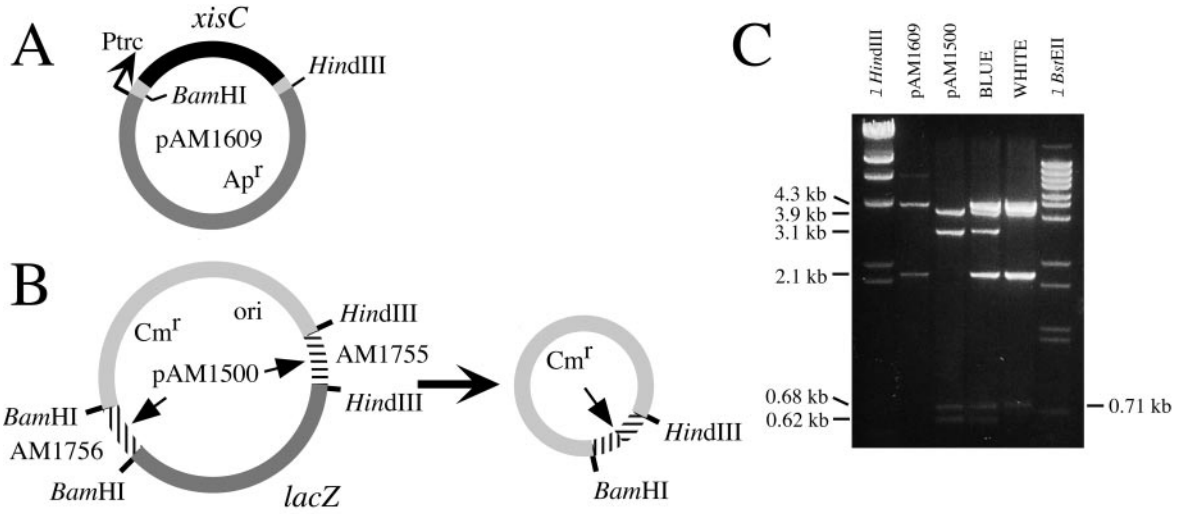

FIG. 4. *hupL*-element rearrangement assay in *E. coli*. (A) pAM1609 contains *xisC* cloned into an *E. coli* expression vector under the control of the IPTG-inducible promoter P_{trc}. (B) Artificial substrate plasmid pAM1500 before and after loss of the fragment containing the *lacZ* gene by site-specific recombination. A BamHI fragment from pAM1756 contains the *xisC*-distal recombination. A HindIII fragment from pAM1755 contains the *xisC*-proximal recombination site. Arrows mark recombination sites. (C) Ethidium bromide-stained electrophoretic gel of plasmid DNA digested with BamHI and HindIII. Lanes λ HindIII and λ BstEII are size markers. Lanes pAM1609 and pAM1500 are digests of these plasmids for reference. Lanes BLUE and WHITE contain plasmid DNA from representative blue and white colonies produced in a rearrangement assay after IPTG induction on plates containing X-Gal. pAM1609 produces fragments of 4.3 and 2.1 kb. pAM1500 produces fragments of 3.9, 3.1, 0.68, and 0.62 kb. The rearranged pAM1500 produces fragments of 3.9 and 0.71 kb.

pAM1609 was moved by electroporation into a strain of *E. coli* harboring pAM1500, which serves as an artificial rearrangement substrate (Fig. 4). pAM1500 carries a *lacZ* gene between two restriction fragments of *Anabaena* sp. strain PCC 7120 DNA that each contain one of the two directly repeated *hupL* recombination sites, such that site-specific recombination will excise the *lacZ* gene from the plasmid. Rearrangement was assayed by screening for blue or white colony color on plates containing the indicator X-Gal. After incubation with 1 mM IPTG, strains containing both pAM1609 and pAM1500 produced about 10% white colonies. Restriction analysis of plasmid DNA from blue and white colonies was performed to confirm rearrangement of pAM1500 (Fig. 4). DNA sequence analysis of rearranged plasmids from white colonies showed that the expected site-specific recombination of the artificial substrate had occurred. Therefore, XisC is sufficient to catalyze the *hupL* element site-specific recombination in *E. coli*.

***xisC* shows functional similarity to the phage integrase family.** XisC is 47% identical and 65% similar to XisA, and both *Anabaena* sp. strain PCC 7120 proteins show amino acid sequence similarity to conserved regions of members of the phage integrase family of site-specific recombinases (34). The hallmark of the family is a catalytic tetrad composed of an arginine-histidine-arginine triad and a tyrosine, which forms a transient covalent bond to target DNA (24). However, the conserved histidine residue in the catalytic pocket (18) is a tyrosine residue in both XisC and XisA. Of the characterized phage integrase family members, there is only one other example of a tyrosine at this position, in the SLP1 element of *Streptomyces coelicolor* (34). Lysine, arginine, and asparagine have also been shown to be functional at this position in other rare variants of the family (34).

To further characterize XisC and provide evidence that it is functionally similar to the phage integrase family, site-directed mutagenesis was performed on *xisC* in pAM1609. Site-directed mutations were made to change the catalytic tyrosine (residue 433) and two highly conserved amino acids in the catalytic pocket (arginine residue 306 and tyrosine residue 398). Each site-directed *xisC* mutant was tested in the *E. coli*-based rearrangement assay using the pAM1500 artificial substrate plasmid (Fig. 4).

The *xisC* Y433F (tyrosine 433 replaced with phenylalanine) mutation of the putative catalytic tyrosine completely abolished XisC recombinase activity (Fig. 5). As a control, a reverse mutation, F433Y, which restored the tyrosine at this position, also restored recombinase activity. Similarly, an R306C mutation, which changed the first arginine in the conserved arginine-histidine-arginine triad, also showed no rearrangement of the artificial substrate. However, a Y398F mutation of the tyrosine in the catalytic pocket, which we expected to result in loss of activity, instead retained recombinase activity. Therefore, an additional mutation, Y398A, was made at this position, and this mutant form of *xisC* lacked all recombinase activity. Finally, we changed the position 398 tyrosine to a histidine so that *xisC* would contain the very highly conserved arginine-histidine-arginine triad present in nearly all other members of the phage integrase family. This Y398H mutation not only retained recombinase activity but showed slightly increased activity compared to the wild-type *xisC* in our *E. coli*-based assay.

DISCUSSION

Our results show that *xisC* is necessary and sufficient in *Anabaena* sp. strain PCC 7120 for the site-specific excision of the *hupL* element. Inactivation of *xisC* resulted in a strain that

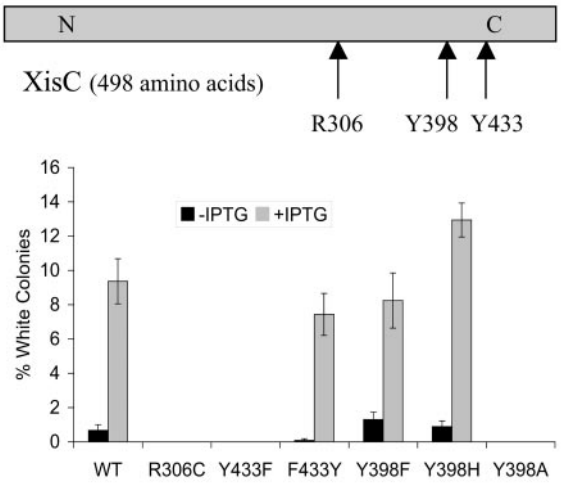

FIG. 5. *hupL*-element rearrangement assay of *xisC* wild type (WT) and site-directed point mutants. The diagram (above) represents the XisC protein with vertical arrows indicating the location of site-directed point mutations. The bar graph shows the percentage of white *E. coli* colonies containing rearranged artificial substrate plasmid pAM1500 produced in the rearrangement assay shown in Fig. 4. Wild-type and site-directed mutant *xisC* genes expressed from an IPTG-inducible promoter were introduced into *E. coli* cells containing the compatible substrate plasmid pAM1500. Each strain was tested in the assay in the absence or presence of IPTG. Each bar indicates the mean of at least three independent assays, and error bars indicate the standard deviations.

failed to excise the *hupL* element, and as a consequence, the strain lost uptake hydrogenase activity under diazotrophic growth conditions. Expression of *xisC* from a plasmid in the mutant strain restored site-specific recombination and excision of the *hupL* element, even in filaments grown in nitrogen-replete conditions. Furthermore, expression of *xisC* in *E. coli* was sufficient to cause rearrangement of an artificial substrate plasmid in the absence of *Anabaena*-specific cofactors. Analyses of *xisC* site-directed mutants provide experimental evidence that XisC belongs to the phage integrase family of site-specific recombinases.

The *xisC* mutant strain AMC414 failed to excise the *hupL* element during heterocyst differentiation and was deficient for uptake hydrogenase activity, but it did not show obvious defects in heterocyst morphogenesis or diazotrophic growth. Strains containing mutations in the *xisA* and *xisF* recombinase genes fail to excise the *nifD* and *fdxN* elements, respectively, and as a consequence, they are defective for nitrogen fixation and diazotrophic growth, but similar to the *xisC* mutant, the *xisA* and *xisF* mutant strains undergo normal heterocyst morphogenesis (8, 16). Therefore, although all three programmed DNA rearrangements are tightly controlled during *Anabaena* sp. strain PCC 7120 heterocyst differentiation, the rearrangements do not seem to control any downstream events required for morphogenesis. The sole consequence of their failure to excise from the chromosome appears to be limited to the genes and operons that the elements interrupt.

Overexpression of *xisC* from the vegetative cell-specific *rbcL* promoter produced only low levels of *hupL* rearrangement, suggesting that XisC provided in *trans* does not function well or that other heterocyst-specific factors may be required for efficient excision. We would expect that these putative accessory factors would be expressed only in differentiating heterocysts and absent in vegetative cells. A precedent for this was found for the *Anabaena* sp. strain PCC 7120 *xisF* recombinase, which was shown to require the *xisH* and *xisI* genes located downstream of *xisF* on the *fdxN* element (36). Overexpression of *xisF* alone cannot excise the *fdxN* element in vegetative cells. However, overexpression of both *xisH* and *xisI* in a strain containing wild-type *xisF* forced the *fdxN* rearrangement to occur in vegetative cells (36). The *hupL* element does not contain *xisH* or *xisI* homologs, and we would not expect any interaction between XisC and XisH/XisI because the XisC and XisF recombinases belong to different families of site-specific recombinases.

A 1.4-kb ORF, all0686, present at the opposite end of the *hupL* element from *xisC*, is apparently not required for excision of the element because a mutant in which this ORF was disrupted with a Sp^r/Sm^r Ω cassette showed normal heterocyst-specific excision of the *hupL* element (C. D. Carrasco, unpublished results). A small open reading frame, asl0678, which is immediately downstream of *xisC* and on the opposite strand, potentially encodes a 48-amino-acid protein that shows similarity to a region of the *xerD* family of tyrosine recombinases, but the significance of this putative gene is unclear.

We were unable to obtain a strain cured of the *hupL* element despite significant efforts to identify such a strain. Although this could be due to the relatively inefficient frequency of excision, it is also possible that the site-specific recombination reaction is biased towards insertion rather than excision in vegetative cells. It is also possible that the *hupL* element contains an unidentified addiction gene that results in postsegregational killing if the element is lost.

The results from our site-directed mutagenesis of critical residues required for site-specific recombination confirm a functional similarity between XisC, XisA, and the phage integrase family of recombinases. Mutation of conserved residue R306 and the essential catalytic residue Y433 produced a complete loss of recombinase activity. However, it is notable that the catalytic pocket of the *Anabaena* sp. strain PCC 7120 recombinases contains a tyrosine residue (Y398) in a position where histidine is highly conserved for the majority of the integrase family (34). However, sequence databases now contain over a dozen putative recombinase genes containing a tyrosine at this position. There are now approximately 10 cyanobacterial members of the phage integrase family, and they all contain tyrosine residues at this position, as does the SLP1-element recombinase in *Streptomyces* species (1) and a putative recombinase in *Bacteroides thetaiotaomicron* (42). XisC, XisA, and these other putative site-specific recombinases represent a distinct subset of the phage integrase family. The site-directed XisC Y398H mutant protein, which replaced the tyrosine with the more conserved histidine residue, not only was functional but increased the percentage of rearranged substrate plasmids in our *E. coli*-based assay, possibly indicating that a histidine at this position functions better in the *E. coli* cytoplasm (Fig. 5). The presence of the tyrosine residue at this position in XisC, XisA, and all other cyanobacterial homologs could indicate that these recombinases are all derived from a common ancestor that appeared early in the cyanobacterial lineage, or there

may be some selective pressure for a tyrosine residue at this position for recombinases that function in cyanobacterial cells.

The *Anabaena* sp. strain PCC 7120 genome contains another member of the phage integrase family, ORF alr3224, which is approximately 50% similar to XisA and XisC. Like XisA and XisC, alr3224 contains a tyrosine residue instead of a histidine in the catalytic pocket. It is unknown if this putative recombinase gene is associated with a mobile DNA element or if it is regulated during heterocyst development similarly to the other programmed DNA rearrangements.

Although the excision of the *hupL* element is not essential for the differentiation of nitrogen-fixing heterocysts, it is required for reformation of the intact *hupL* gene and the production of uptake hydrogenase activity in diazotrophically growing filaments. Uptake hydrogenases are important for energy efficiency during nitrogen fixation, and understanding their expression and function is relevant to potential biotechnology applications (19, 39). Disruption of the *Anabaena* sp. strain PCC 7120 *hupL* gene, but not the bidirectional hydrogenase gene *hoxH*, resulted in hydrogen production as a byproduct of nitrogen fixation (29). Targeted inactivation of the *hupL* gene in *Nostoc punctiforme* ATCC 29133 resulted in H_2 evolution under nitrogen-fixing conditions in air (26). Similar increased rates of H_2 production had been found when the *hupSL* genes were inactivated in *Anabaena variabilis* (20). These studies provide a better understanding of the potential for nitrogenase-based photobiological hydrogen production. For the nitrogen-fixing organism, the escape of hydrogen in uptake hydrogenase mutants results in a loss of available chemical energy. We did not observe an obvious growth defect in diazotrophic growth conditions for the *xisC*-inactivated strain AMC414. However, Lindblad et al. showed that, in a competitive growth environment, the difference in energy efficiency favored the wild-type strain over AMC414 (27).

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REFERENCES

1. **Brasch, M. A., G. S. Pettis, S. C. Lee, and S. N. Cohen.** 1993. Localization and nucleotide sequences of genes mediating site-specific recombination of the SLP1 element in *Streptomyces lividans.* J. Bacteriol. **175:**3067–3074.
2. **Brosius, J.** 1989. Superpolylinkers in cloning and expression vectors. DNA **8:**759–777.
3. **Brusca, J. S., C. J. Chastain, and J. W. Golden.** 1990. Expression of the *Anabaena* sp. strain PCC 7120 *xisA* gene from a heterologous promoter results in excision of the *nifD* element. J. Bacteriol. **172:**3925–3931.
4. **Cai, Y., and 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.
5. **Carrasco, C. D., J. A. Buettner, and J. W. Golden.** 1995. Programmed DNA rearrangement of a cyanobacterial *hupL* gene in heterocysts. Proc. Natl. Acad. Sci. USA **92:**791–795.
6. **Carrasco, C. D., J. S. Garcia, and J. W. Golden.** 1998. Programmed DNA rearrangement of a hydrogenase gene during *Anabaena* heterocyst development, p. 203–207. *In* R. Zaborsky (ed.), BioHydrogen. Plenum Press, New York, N.Y.
7. **Carrasco, C. D., and J. W. Golden.** 1995. Two heterocyst-specific DNA rearrangements of *nif* operons in *Anabaena cylindrica* and *Nostoc* sp. strain Mac. Microbiology **141:**2479–2487.
8. **Carrasco, C. D., K. S. Ramaswamy, T. S. Ramasubramanian, and J. W. Golden.** 1994. *Anabaena xisF* gene encodes a developmentally regulated site-specific recombinase. Genes Dev. **8:**74–83.
9. **Elhai, J., A. Vepritskiy, A. M. Muro-Pastor, E. Flores, and C. P. Wolk.** 1997. Reduction of conjugal transfer efficiency by three restriction activities of *Anabaena* sp. strain PCC 7120. J. Bacteriol. **179:**1998–2005.
10. **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.
11. **Golden, J. W.** 1997. Programmed DNA rearrangements in cyanobacteria, p. 162–173. *In* F. J. de Bruijn, J. R. Lupski, and G. Weinstock (ed.), Bacterial genomes: physical structure and analysis. Chapman & Hall, New York, N.Y.
12. **Golden, J. W., C. D. Carrasco, M. E. Mulligan, G. J. Schneider, and R. Haselkorn.** 1988. Deletion of a 55-kilobase-pair DNA element from the chromosome during heterocyst differentiation of *Anabaena* sp. strain PCC 7120. J. Bacteriol. **170:**5034–5041.
13. **Golden, J. W., M. E. Mulligan, and R. Haselkorn.** 1987. Different recombination site specificity of two developmentally regulated genome rearrangements. Nature **327:**526–529.
14. **Golden, J. W., S. J. Robinson, and R. Haselkorn.** 1985. Rearrangement of nitrogen fixation genes during heterocyst differentiation in the cyanobacterium *Anabaena.* Nature **314:**419–423.
15. **Golden, J. W., L. L. Whorff, and D. R. Wiest.** 1991. Independent regulation of *nifHDK* operon transcription and DNA rearrangement during heterocyst differentiation in the cyanobacterium *Anabaena* sp. strain PCC 7120. J. Bacteriol. **173:**7098–7105.
16. **Golden, J. W., and D. R. Wiest.** 1988. Genome rearrangement and nitrogen fixation in *Anabaena* blocked by inactivation of *xisA* gene. Science **242:**1421–1423.
17. **Golden, J. W., and H. S. Yoon.** 2003. Heterocyst development in *Anabaena.* Curr. Opin. Microbiol. **6:**557–563.
18. **Grainge, I., and M. Jayaram.** 1999. The integrase family of recombinase: organization and function of the active site. Mol. Microbiol. **33:**449–456.
19. **Hansel, A., and P. Lindblad.** 1998. Towards optimization of cyanobacteria as biotechnologically relevant producers of molecular hydrogen, a clean and renewable energy source. Appl. Microbiol. Biotechnol. **50:**153–160.
20. **Happe, T., K. Schutz, and H. Bohme.** 2000. Transcriptional and mutational analysis of the uptake hydrogenase of the filamentous cyanobacterium *Anabaena variabilis* ATCC 29413. J. Bacteriol. **182:**1624–1631.
21. **Haraldsen, J. D., and A. L. Sonenshein.** 2003. Efficient sporulation in *Clostridium difficile* requires disruption of the σ^K gene. Mol. Microbiol. **48:**811–821.
22. **Henson, B. J., L. E. Watson, and S. R. Barnum.** 2005. Characterization of a 4 kb variant of the *nifD* element in *Anabaena* sp. strain ATCC 33047. Curr. Microbiol. **50:**129–132.
23. **Kunkel, B., R. Losick, and P. Stragier.** 1990. The *Bacillus subtilis* gene for the developmental transcription factor σ^K is generated by excision of a dispensable DNA element containing a sporulation recombinase gene. Genes Dev. **4:**525–535.
24. **Kwon, H. J., R. Tirumalai, A. Landy, and T. Ellenberger.** 1997. Flexibility in DNA recombination—structure of the lambda integrase catalytic core. Science **276:**126–131.
25. **Lammers, P. J., J. W. Golden, and R. Haselkorn.** 1986. Identification and sequence of a gene required for a developmentally regulated DNA excision in Anabaena. Cell **44:**905–911.
26. **Lindberg, P., K. Schutz, T. Happe, and P. Lindblad.** 2002. A hydrogen-producing, hydrogenase-free mutant strain of *Nostoc punctiforme* ATCC 29133. Int. J. Hydrogen Energy **27:**1291–1296.
27. **Lindblad, P., K. Christensson, P. Lindberg, A. Fedorov, F. Pinto, and A. Tsygankov.** 2002. Photoproduction of H_2 by wildtype *Anabaena* PCC 7120 and a hydrogen uptake deficient mutant: from laboratory experiments to outdoor culture. Int. J. Hydrogen Energy **27:**1271–1281.
28. **Liu, D., and J. W. Golden.** 2002. *hetL* overexpression stimulates heterocyst formation in *Anabaena* sp. strain PCC 7120. J. Bacteriol. **184:**6873–6881.
29. **Masukawa, H., M. Mochimaru, and H. Sakurai.** 2002. Disruption of the uptake hydrogenase gene, but not of the bidirectional hydrogenase gene, leads to enhanced photobiological hydrogen production by the nitrogen-fixing cyanobacterium *Anabaena* sp. PCC 7120. Appl. Microbiol. Biotechnol. **58:**618–624.
30. **Meeks, J. C., and J. Elhai.** 2002. Regulation of cellular differentiation in filamentous cyanobacteria in free-living and plant-associated symbiotic growth states. Microbiol. Mol. Biol. Rev. **66:**94–121.
31. **Meeks, J. C., J. Elhai, T. Thiel, M. Potts, F. Larimer, J. Lamerdin, P. Predki, and R. Atlas.** 2001. An overview of the genome of *Nostoc punctiforme*, a multicellular, symbiotic cyanobacterium. Photosynth. Res. **70:**85–106.
32. **Mulligan, M. E., W. J. Buikema, and R. Haselkorn.** 1988. Bacterial-type ferredoxin genes in the nitrogen fixation regions of the cyanobacterium *Anabaena* sp. strain PCC 7120 and *Rhizobium meliloti.* J. Bacteriol. **170:**4406–4410.
33. **Mulligan, M. E., and R. Haselkorn.** 1989. Nitrogen-fixation (*nif*) genes of the cyanobacterium *Anabaena* sp. strain PCC 7120: the *nifB-fdxN-nifS-nifU* operon. J. Biol. Chem. **264:**19200–19207.
34. **Nunes-Duby, S. E., H. J. Kwon, R. S. Tirumalai, T. Ellenberger, and A. Landy.** 1998. Similarities and differences among 105 members of the Int family of site-specific recombinases. Nucleic Acids Res. **26:**391–406.

35. **Oxelfelt, F., P. Tamagnini, R. Salema, and P. Lindblad.** 1995. Hydrogen uptake in *Nostoc* strain PCC 73102: effects of nickel, hydrogen, carbon and nitrogen. Plant Physiol. Biochem. **33:**617–623.
36. **Ramaswamy, K. S., C. D. Carrasco, T. Fatma, and J. W. Golden.** 1997. Cell-type specificity of the *Anabaena fdxN*-element rearrangement requires *xisH* and *xisI.* Mol. Microbiol. **23:**1241–1250.
37. **Sato, T., and Y. Kobayashi.** 1998. The *ars* operon in the *skin* element of *Bacillus subtilis* confers resistance to arsenate and arsenite. J. Bacteriol. **180:**1655–1661.
38. **Schneider, G. J., J. D. Lang, and R. Haselkorn.** 1991. Promoter recognition by the RNA polymerase from vegetative cells of the cyanobacterium *Anabaena* 7120. Gene **105:**51–60.
39. **Tamagnini, P., R. Axelsson, P. Lindberg, F. Oxelfelt, R. Wunschiers, and P. Lindblad.** 2002. Hydrogenases and hydrogen metabolism of cyanobacteria. Microbiol. Mol. Biol. Rev. **66:**1–20.
40. **Tamagnini, P., J. L. Costa, L. Almeida, M. J. Oliveira, R. Salema, and P. Lindblad.** 2000. Diversity of cyanobacterial hydrogenases, a molecular approach. Curr. Microbiol. **40:**356–361.
41. **Wolk, C. P.** 2000. Heterocyst formation in *Anabaena*, p. 83–104. *In* Y. V. Brun and L. J. Shimkets (ed.), Prokaryotic development. American Society for Microbiology, Washington, D.C.
42. **Xu, J., M. K. Bjursell, J. Himrod, S. Deng, L. K. Carmichael, H. C. Chiang, L. V. Hooper, and J. I. Gordon.** 2003. A genomic view of the human-*Bacteroides thetaiotaomicron* symbiosis. Science **299:**2074–2076.
43. **Yoon, H.-S., and J. W. Golden.** 1998. Heterocyst pattern formation controlled by a diffusible peptide. Science **282:**935–938.