Genome Rearrangement and Nitrogen Fixation in *Anabaena* Blocked by Inactivation of *xisA* Gene

JAMES W. GOLDEN* AND DEBORAH R. WIEST

Two genome rearrangements involving 11- and 55-kilobase DNA elements occur during the terminal differentiation of an *Anabaena* photosynthetic vegetative cell into a nitrogen-fixing heterocyst. The *xisA* gene, located on the *nifD* 11-kilobase DNA element, was inactivated by recombination between the chromosome and a copy of the *xisA* gene that was mutated by inserting an antibiotic gene cassette. Site-directed inactivation of the *Anabaena xisA* gene blocked rearrangement of the 11-kilobase element and nitrogen fixation, but did not affect rearrangement of the 55-kilobase element, heterocyst differentiation, or heterocyst pattern formation.

ANABAENA SP. STRAIN PCC 7120 IS a filamentous, heterocystous cyanobacterium capable of oxygenic photosynthesis and nitrogen fixation. In the absence of a source of combined nitrogen such as ammonia, approximately every tenth photosynthetic vegetative cell undergoes a terminal differentiation to become a heterocyst, a nondividing cell specialized for nitrogen fixation (*1*). Heterocysts receive products of photosynthesis from neighboring vegetative cells and provide the filament with fixed nitrogen as glutamine. Two different DNA rearrangements occur near the nitrogen fixation (*nif*) genes during heterocyst differentiation: (i) the deletion of an 11-kb element from within the *nifD* gene and (ii) the deletion of a 55-kb element from within the *fdxN* gene (*2–6*). Both rearrangements are the result of site-specific recombination between short, directly repeated sequences present at the ends of the elements, but the repeated sequences and the regulation of the rearrangements differ for the two elements (*3, 6*).

The *xisA* gene, which is located on the *nifD* 11-kb element, is thought to encode the site-specific recombinase necessary for excision of the element from the heterocyst chromosome. This gene was shown to be required for rearrangement of the *Anabaena* 11-kb element in *Escherichia coli* (*7*). The element was found to rearrange at a low frequency when it was cloned into a plasmid vector and propagated in *E. coli*. Transposon mutagenesis of *xisA* abolished this rearrangement. We now report the site-directed inactivation of the *xisA* gene in the *Anabaena* sp. strain PCC 7120 chromosome. Although the *xisA* mutant produces heterocysts that correctly excise the *fdxN* 55-kb element, they fail to excise the *nifD* 11-kb element and are unable to fix nitrogen. This shows that *xisA* is required for the *nifD* rearrangement in *Anabaena* heterocysts and that the DNA rearrangement is necessary for proper expression of the nitrogen fixation genes.

The *xisA* gene was inactivated in vitro by inserting a gene cassette containing a gene encoding streptomycin- and spectinomycin-resistance (Sm^r/Spc^r) into the coding region. The Sm^r/Spc^r Ω fragment from pHP45Ω is flanked by transcription and translation termination signals (*8*). The Ω fragment was modified by destroying two Ava I restriction sites and flanking the fragment with inverted multiple cloning sites (*9*). The resulting plasmid, pDW9, allows the modified Sm^r/Spc^r Ω fragment to be isolated by using all of the restriction sites present in the pUC18/pUC19 multiple cloning site except Sph I (Fig. 1). To determine the level of drug resistance provided by the modified Sm^r/Spc^r Ω fragment in *Anabaena* sp. strain PCC 7120, we cloned the fragment into a conjugal shuttle vector to produce pDW10 (*10*). The plasmid pDW10 was conjugated into *Anabaena* and provided resistance to spectinomycin at 100 μg/ml, whereas wild-type *Anabaena* is sensitive to spectinomycin at 1 μg/ml (*11–13*).

The entire *nifD* 11-kb element, including the *xisA* gene, is contained on a 17-kb Eco RI fragment cloned into pBR322 (*14*). This plasmid, pAn207, contains three Sca I sites: (i) one is in the pBR322 vector, (ii) one is 0.5 kb downstream from the *xisA* gene, and (iii) one is within the *xisA* open reading frame 10 bp downstream from the ATG start codon (*7*). The plasmid pDW12 (Fig. 1) was constructed from pAn207 by placing the modified Sm^r/Spc^r Ω fragment into the Sca I site within the *xisA* gene.

The wild-type *xisA* gene on the *Anabaena* chromosome was replaced with the inactivated gene by homologous recombination (*15*). The plasmid pDW12 was conjugated into *Anabaena* sp. strain PCC 7120 and exconjugants expressing Sm^r/Spc^r were obtained (*16*). Because pDW12 cannot replicate in *Anabaena*, cells expressing Sm^r/Spc^r should contain the Sm^r/Spc^r Ω fragment integrated into the chromosome by homologous recombination. DNA blot analysis showed that all eight of the initial Sm^r/Spc^r exconjugants analyzed were the result of single recombination events that integrated pDW12 into the chromosome. These single recombinants were screened for segregation of colonies unable to grow on N_2 in which a second recombination event removed the wild-type *xisA* gene and the pDW12 vector from the chromosome (*17*). Our results

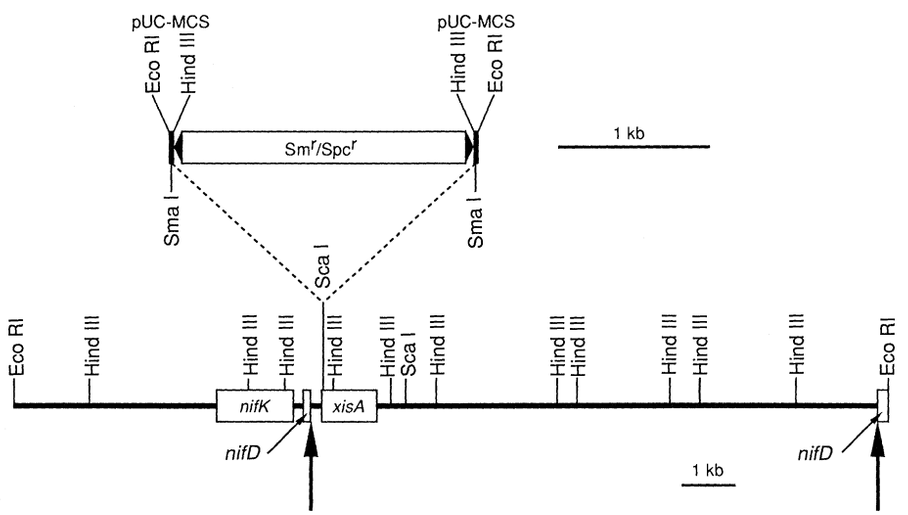

Fig. 1. Map of pDW12 containing the *xisA* gene inactivated with the modified Sm^r/Spc^r Ω fragment. The Sm^r/Spc^r Ω fragment was isolated with Sma I from pDW9 and inserted into pAn207 linearized by partial digestion with Sca I; the pBR322 vector is not shown. The *nifD* 11-kb element (borders indicated with large arrows) from the vegetative cell chromosome is on a 17-kb Eco RI fragment. The *nifK* gene is to the left of the element and the *nifD* gene is interrupted by the element and extends to the right beyond the Eco RI site. The *xisA* gene is located on the left end of the element. The Sm^r/Spc^r Ω fragment is bordered by transcription termination signals (black triangles) and inverted multiple cloning sites (pUC-MCS).

Department of Biology, Texas A&M University, College Station, TX 77843.

*To whom correspondence should be addressed.

differ from those obtained with transformable unicellular cyanobacteria, which almost exclusively produce double recombination or gene conversion events after transformation with a suicide plasmid (*18*). The underlying mechanism responsible for these differences is not known.

DNA blot analysis of two single (DW12-2.5 and DW12-7.1) and two double (DW12-2.2 and DW12-8.1) recombinants is shown in Fig. 2. The single recombinants contain both the wild-type and the inactivated *xisA* genes, as well as the pDW12 vector sequences (legend to Fig. 2). The double recombinants contain only the inactivated copy of *xisA*.

DNA blot analysis of the *nifD* 11-kb and the *fdxN* 55-kb heterocyst genome rearrangements in wild-type *Anabaena* sp. strain PCC 7120 and in DW12-2.5 and DW12-2.2 are shown in Fig. 3. All three strains exhibit normal rearrangement of the *fdxN* 55-kb element in heterocyst DNA (Fig. 3, An154.2 probe). Rearrangement of this element in DW12-2.2 demonstrates that the *xisA* gene is not required for excision of the *fdxN* 55-kb element.

Near normal rearrangement of the *nifD* 11-kb element occurred in heterocyst DNA from the single recombinant DW12-2.5 (Fig. 3, An256 probe). The 2.1-kb band representing the excised 11-kb element is in lower than stoichiometric amounts, and a few very faint larger fragments are visible. These results are presumably due to the unusual organization of the chromosome in the single recombinant created by the insertion of pDW12. Although the exact map of the single recombinants depends on the site of homologous recombination, the 17-kb Eco RI fragment containing the 11-kb element is duplicated on the chromosome, with the two copies separated by the pDW12 vector sequences. Therefore, the heterocyst site-specific recombination could occur between any two pairs of the four directly repeated copies of the 11-bp sequence that border the duplicated *nifD* 11-kb elements.

The double recombinant DW12-2.2 did not rearrange the *nifD* 11-kb element during heterocyst differentiation (Fig. 3, An256 probe). Therefore, the *xisA* gene is required for the *nifD* 11-kb rearrangement during *Anabaena* heterocyst differentiation. These experiments and previous results (*7*) show that the *xisA* gene is necessary for excision of the *nifD* 11-kb element in *Anabaena*, but it is not known whether *xisA* alone is sufficient to produce the rearrangement.

The phenotypes of the single recombinant DW12-2.5 and the double recombinant DW12-2.2 are shown in Table 1. DW12-2.5 was wild type for all of the characteris-

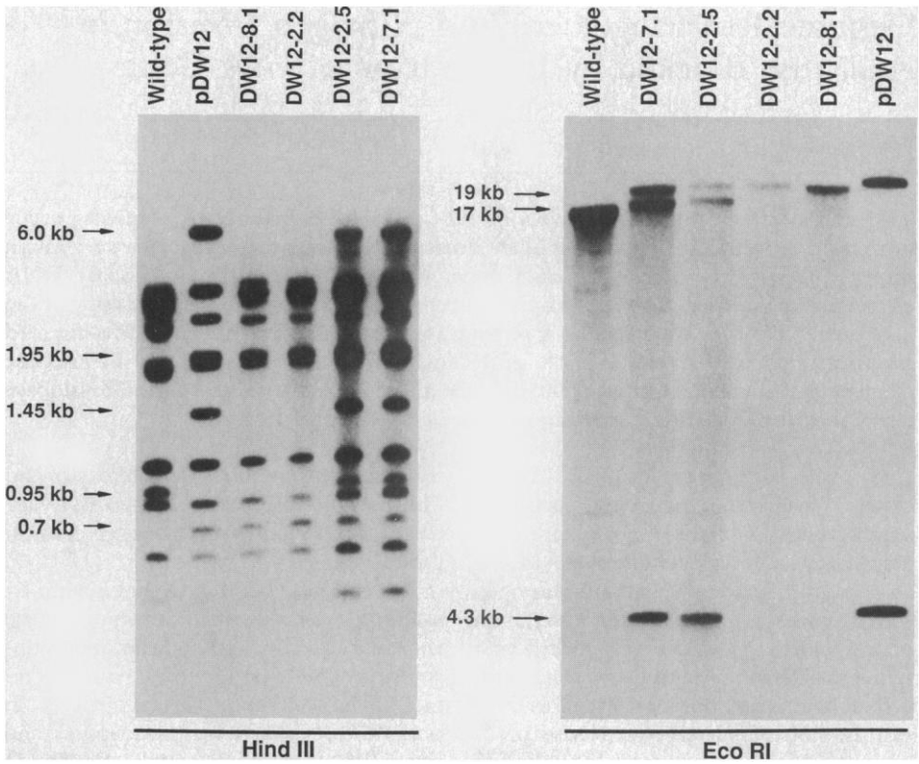

Fig. 2. DNA blots showing single and double recombinants of *Anabaena* sp. strain PCC 7120. DNA blots of genomic DNA from the indicated strains or the plasmid pDW12 digested with Hind III or Eco RI were probed with labeled pDW12 DNA. DNA from wild-type *Anabaena* sp. strain PCC 7120 and from the plasmid pDW12 were included as markers. The strains DW12-2.5 and DW12-7.1 are single recombinants that contain pDW12 integrated into the chromosome. The strains DW12-2.2 and DW12-8.1 are double recombinants that have lost the pDW12 vector sequences and replaced the wild-type *xisA* gene in the chromosome with the Sm^r/Spc^r Ω fragment–inactivated *xisA* gene. In the Hind III–digested samples the wild-type *xisA* gene is seen as a 0.95-kb band and the inactivated *xisA* gene is split into 0.7- and 0.25-kb fragments (the 0.25-kb fragment is not seen in this exposure). The Sm^r/Spc^r Ω fragment is present on a 1.95-kb fragment and fragments unique to the pDW12 vector are seen as 1.45- and 6.0-kb bands. In the Eco RI–digested samples, the wild-type *xisA* gene is present on a 17-kb fragment, and insertion of the Sm^r/Spc^r Ω fragment produces a 19-kb fragment. The 4.3-kb band represents the pBR322 vector.

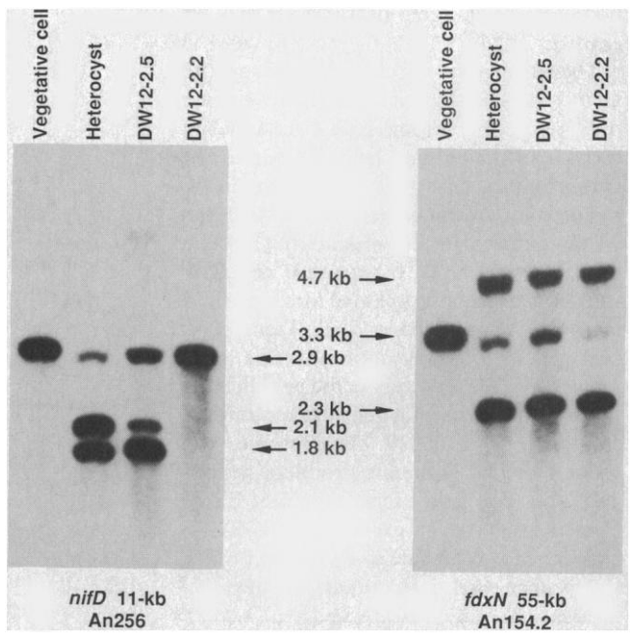

Fig. 3. DNA blots showing the two heterocyst-specific DNA rearrangements in wild-type *Anabaena* sp. strain PCC 7120 and in the single and double recombinants. DNA blots of genomic DNA digested with Hind III were probed with the Hind III fragment An256, which identifies the *nifD* 11-kb rearrangement (represented by the 2.9-kb band in vegetative cell DNA and the 2.1- and 1.8-kb bands in heterocyst DNA), or with the Hind III fragment An154.2, which identifies the *fdxN* 55-kb rearrangement (represented by the 3.3-kb band in vegetative cell DNA and the 2.3- and 4.7-kb bands in heterocyst DNA) (*2*). Genomic DNA samples are from wild-type vegetative cells and purified heterocysts, and purified heterocysts from the single (strain DW12-2.5) and double (strain DW12-2.2) recombinants. Purified heterocysts were prepared as previously described (*2, 6*) and contain a small percentage of vegetative cells.

Table 1. Summary of phenotypes for *Anabaena* sp. strain PCC 7120 wild type, DW12-2.5, and DW12-2.2. The single recombinant DW12-2.5 contains pDW12 recombined into the chromosome and contains both a wild-type and inactivated *xisA* gene. The double recombinant DW12-2.2 contains only the inactivated *xisA* gene as a result of a second recombination event that removed the pDW12 vector sequences and the wild-type *xisA* gene from the chromosome. (+) Wild type, (−) mutant (loss of function).

Phenotype	7120 wild type	DW12-2.5	DW12-2.2
Growth on 2.5 m*M* NH_4^+	+	+	+
Heterocyst repression by NH_4^+	+	+	+
Growth on N_2	+	+	−
Nitrogenase activity*	+	+	−
Heterocyst formation	+	+	+
Heterocyst spacing pattern	+	+	+
Heterocyst DNA rearrangement			
nifD 11-kb element	+	+	−
fdxN 55-kb element	+	+	+

*Measured by an acetylene reduction assay (*21*).

tics tested. The *xisA* mutant DW12-2.2 was unable to grow on N_2 as the sole source of nitrogen and showed no nitrogenase activity, although morphological heterocyst development was normal. Transfer of DW12-2.2 vegetative cells to medium lacking a source of combined nitrogen produced a normal time course of differentiation and normal heterocyst frequency and pattern formation. These cultures showed little change after several days at low light levels (30 to 50 $\mu E\ s^{-1}\ m^{-2}$) but quickly yellowed under normal growth conditions (*11*).

In summary, the *xisA* gene is required for rearrangement of the *nifD* 11-kb element and correct expression of the *nif* genes, but is not required for heterocyst development, pattern formation, or rearrangement of the *fdxN* 55-kb element. These results are consistent with the *xisA* gene encoding the site-specific recombinase involved in the *nifD* 11-kb rearrangement, and demonstrate that excision of the *nifD* 11-kb element is required for proper expression of the nitrogen fixation genes.

Our results are in agreement with previous observations that the two rearrangements differ in their mechanism and regulation. The sequences at the recombination sites differ for the two elements, indicating that they may be recognized by different site-specific recombinases (*3*). The two rearrangements can occur independently of one another. Although both rearrangements are detected at the same developmental time during normal heterocyst differentiation (*2*), under microaerobic heterocyst-inducing conditions only the *fdxN* 55-kb rearrangement occurs, whereas the *nifD* 11-kb element does not rearrange (*6*). It may be that the elements are completely unrelated to each other and that their common property of excision from the chromosome during heterocyst differentiation is a coincidence.

These experiments demonstrate that site-directed inactivation of chromosomal genes is possible in *Anabaena* sp. strain PCC 7120. It should also be possible to inactivate genes by a single recombination between the chromosome and a cloned internal fragment of a gene (*19*). Site-directed gene inactivation is particularly useful in identifying a gene's function with reverse genetics as we have now shown. The related technique of ectopic mutagenesis has been used to obtain tagged mutations in the cyanobacterium *Synechococcus* sp. strain PCC 7002 (*20*); a similar procedure could presumably be applied to *Anabaena* sp. strain PCC 7120.

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9. General cloning methods were as described in various manuals [T. Maniatis, E. F. Fritsch, J. Sambrook, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982); F. M. Ausubel *et al.*, *Current Protocols in Molecular Biology* (Greene and Wiley-Interscience, New York, 1987)] or as recommended by suppliers. Construction of pDW9 was as follows. The Hind III Sm^r/Spc^r Ω fragment from pHP45Ω (*8*) was placed into pDW6 (pBR322 with the Ava I site removed by mung bean nuclease treatment), and the two Ava I sites were destroyed by filling in the unpaired bases with the Klenow fragment of *E. coli* DNA polymerase I. The resulting Hind III Sm^r/Spc^r Ω fragment was cloned into pUC1819H3, which consists of the large Sca I–Hind III fragment of pUC18 ligated to the small Sca I–Hind III fragment of pUC19 [C. Yanisch-Perron, J. Vieira, J. Messing, *Gene* **33**, 103 (1985)] and results in an inverted duplication of the multiple cloning site with a single Hind III site in the middle.
10. Construction of pDW10 was as follows. The Eco RI Sm^r/Spc^r Ω fragment from pDW9 was cloned into the Eco RI site of pRL405-*lux*, which consists of pRL405 with the Bam HI fragment containing the *lux* genes removed (J. Elhai and C. P. Wolk, unpublished data).
11. General cyanobacterial growth conditions were as described previously (*2, 6, 7*). Cyanobacterial cultures were incubated in lighted (100 to 150 $\mu E\ s^{-1}\ m^{-2}$) chambers at 30°C. Conjugation of shuttle vectors into *Anabaena* sp. strain PCC 7120 were done as follows [modified as in (*12, 13*)]. The *E. coli* donor was produced by introducing a shuttle vector into strain AM187 (strain HB101 containing plasmids pRL443 and pRL528 (J. Elhai and C. P. Wolk, *Methods Enzymol.*, in press) by transformation. An overnight culture of the *E. coli* donor strain grown in the presence of the appropriate antibiotics was pelleted and resuspended in the original volume of Lennox broth (LB) medium. An *Anabaena* sp. strain PCC 7120 culture (1.5 ml) [the age of the culture is probably not very important (*13*)] was pelleted in a 1.5-ml microcentrifuge tube, and the supernatant was removed, leaving the pelleted cells in 90 μl of BG-11 medium. Washed donor cells (10 μl) were mixed in with the *Anabaena* cells and the tube was incubated overnight. The next day the cells were spread onto the surface of a 90-mm petri plate filled with BG-11 agar containing the appropriate antibiotics [25 μg of neomycin per milliliter for pRL shuttle vectors (*12*), 25 μg of spectinomycin or streptomycin per milliliter for the Sm^r/Spc^r Ω fragment on a pRL shuttle vector], and incubated for 5 to 10 days.
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16. Since suicide plasmids produce lower frequencies of exconjugants and provide lower levels of antibiotic resistance than the autonomously replicating shuttle vectors, the standard conjugation protocol (*11–13*) was modified by using *Anabaena* cells from a 100-ml culture suspended in 4.5 ml of BG-11 medium and donor *E. coli* cells from a 2.5-ml culture suspended in 0.5 ml of LB medium. After overnight incubation, the cells were distributed onto five BG-11 agar plates containing spectinomycin (2 μg/ml) and streptomycin (2 μg/ml). Exconjugant colonies became visible after incubation for 10 to 14 days. The plasmid pDW12 produced 6 to 10 exconjugant colonies per plate. Exconjugants were maintained in liquid or on agar-solidified BG-11 medium containing spectinomycin (2 μg/ml) and streptomycin (2 μg/ml). In initial experiments, media contained 10 μg of spectinomycin per milliliter, but this higher level of a single antibiotic resulted in the occurrence of spontaneous antibiotic resistant mutants and selected for insertion of multiple copies of the Sm^r/Spc^r Ω fragment.
17. Double recombinants were obtained by screening for the ability to grow on N_2 as the sole nitrogen source. Filaments from single recombinant clones grown for 3 to 4 days in BG-11 liquid cultures were treated in an ultrasonic bath to obtain filaments with an average length of two or three cells. These short filaments were plated onto BG-11 agar medium to obtain colonies that were then tested for their ability to grow in liquid or solid medium lacking a source of combined nitrogen. Seven out of 48 tested colonies were unable to grow on N_2 and were shown to be double recombinants by DNA blot analysis.
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22. We thank C. P. Wolk and J. Elhai for supplying vectors and strains, D. Zuberer for performing the nitrogenase assays, and S. Golden for helpful advice. This work was supported by NIH grant GM36890, NSF grant DMB-8553185, NSF equipment grant BBS-8703784, and a grant from Searle Scholars Program of the Chicago Community Trust.

18 July 1988; accepted 30 September 1988