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Site-Specific Recombination in the Cyanobacterium *Anabaena* sp. Strain PCC 7120 Catalyzed by the Integrase of Coliphage HK022[▽]

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The integrase (Int) of the λ-like coliphage HK022 catalyzes the site-specific integration and excision of the phage DNA into and from the chromosome of its host, *Escherichia coli*. Int recognizes two different pairs of recombining sites *attP* × *attB* and *attL* × *attR* for integration and excision, respectively. This system was adapted to the cyanobacterium *Anabaena* sp. strain PCC 7120 as a potential tool for site-specific gene manipulations in the cyanobacterium. Two plasmids were consecutively cointroduced by conjugation into *Anabaena* cells, one plasmid that expresses HK022 Int recombinase and the other plasmid that carries the excision substrate P_{glnA}-*attL*-T1/T2-*attR*-*lacZ*, where T1/T2 are the strong transcription terminators of *rrnB*, to prevent expression of the *lacZ* reporter under the constitutive promoter P_{glnA}. The Int-catalyzed site-specific recombination reaction was monitored by the expression of *lacZ* emanating as a result of T1/T2 excision. Int catalyzed the site-specific excision reaction in *Anabaena* cells when its substrate was located either on the plasmid or on the chromosome with no need to supply an accessory protein, such as integration host factor and excisionase (Xis), which are indispensable for this reaction in its host, *E. coli*.

The site-specific recombination mechanism of the temperate coliphage HK022 is similar, if not identical, to that of phage λ. To lysogenize its *Escherichia coli* host, circular phage DNA recombines via a site-specific recombination reaction between the nonidentical *attP* and *attB* sites of the phage and host genomes, respectively. This reaction results in the integrated prophage that is flanked by the recombinant *attL* and *attR* sites, which are the sites for phage excision. The phage-encoded site-specific integrase (Int), which belongs to the Tyr recombinase family, catalyzes phage integration and excision. Both reactions require the presence of the host-encoded DNA-bending heterodimeric accessory protein integration host factor (IHF); excision requires in addition the phage-encoded excisionase (Xis), whose activity can be partially replaced by the host-encoded FIS (*f*actor for *i*nversion *s*timulation). The bacterial *attB* consists of a 21-base-pair (bp) core sequence (*BOB′*) that is composed of 7 central bp, defined as the overlap *O*, which is identical in all four *att* sites and serves as the site of DNA exchange. *O* is flanked by two 7-bp inverted repeats (*B* and *B′*) that serve as weak binding sites for Int. The phage *attP* site is composed of a similar core (*COC′*) that is flanked by two long arms (*P* and *P′* of 142 and 85 bp, respectively) carrying tight binding sites for Int and for the accessory proteins (IHF, Xis, and FIS). The accessory proteins, bound to their sites on the arms of *attP*, regulate and facilitate arm-bound Int monomers access to the core, the site of the reaction. Subsequently, this proteo-DNA complex (the intasome) captures *attB* to execute the recombination. Each of the recombinant sites *attL* (*BOP′*) and *attR* (*POB′*) that flank the integrated prophage is composed of the recombined core and one of the arms. Since the integration/excision reactions each use a different pair of *att* sites and a different composition of the accessory proteins, they are not completely reversible (reviewed in references 1, 25, and 37).

Wild-type Int of λ-like coliphage HK022 catalyzes its site-specific integrative and excisive reactions in mammalian cells and in *Arabidopsis* plants when supplied with the relevant *att* sites located on a plasmid or on the chromosome (14, 16, 21). In both reactions, Int does so without the need to supply the accessory proteins IHF and Xis that are required for these reactions in *E. coli*, though supplementation of the accessory factors improves the reaction (22). Int of λ can do likewise but only if IHF-independent mutants are active (4). Owing to its versatility in target specificity (*attB* plus *attP* and *attL* plus *attR*), the Int system may have an advantage over the common site-specific recombination systems Cre-*lox* of coliphage P1, FLP-*FRT* of *Saccharomyces cerevisiae*, and Int of *Streptomyces* phage ϕC31 that are already in use for site-specific gene manipulations (11, 15, 27, 32, 34, 40). Here, we introduced the site-specific recombination system of HK022 into the nitrogen-fixing filamentous cyanobacterium *Anabaena* sp. strain PCC 7120 and demonstrated that Int catalyzes site-specific excision whether its substrate is located on a plasmid or on the chromosome. This system can thus be used as a novel model for simple and efficient genome manipulations in cyanobacteria, in particular, the removal of genes conferring antibiotic resistance, which are unacceptable for applied purposes in transgenic organisms (26).

MATERIALS AND METHODS

Bacterial strains, plasmids, and primers. The bacterial strains, plasmids, and primers used in this study are listed in Tables 1 and 2. *E. coli* XL-Blue MRF′

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description[a]	Source or reference
Strains		
E. coli XL-Blue MRF′	F′ *proAB lacI*^{q}ZΔM15 Tn*10*(Tet^r)	Stratagene (1993)
E. coli DH10B	AM1359 cells for conjugation; carries pRL623 and pRL433	X. Wu
Anabaena sp. strain PCC 7120	Filamentous N_2-fixing, internal heterocysts	38
Plasmids		
pKH3	*int* cloned under P_{tac} on vector pKK233-2	Lab collection
pRL443	Derivative of conjugal plasmid RP4; Ap^r Tet^r Km^s	9
pRL528	Helper plasmid; M.Eco47II, M.AvaI; *ColK*; Cm^r	7
pRL623	Derivative of pRL528; M.Eco47II, M.AvaI, M.AvaIII	9
pRL488p	Derivative of pRL488 (8) with P_{psbA} from pRL435K	39
pRL488p-*int*	pRL488p carrying *int* under P_{psbA}; P_{tac}	This study
pLD205	Carrying the recombination reporter substrate *attR*-T1/T2-*attL-lacZ*	6
pRL2831a	Sm^r Sp^r; P_{glnA}; RSF1010-based cloning vector	10
pRL2833b	Cm^r Em^r; glutamine synthetase promoter P_{glnA}	10
pRSLZ	Derivative of pRL2831b (P_{glnA} is in the orientation opposite that in pRL2831a), *attR*-T1/T2-*attL-lacZ* cloned into KpnI-SphI sites of pRL2831b	This study
pOMs	Derivative of pRL2833b in which the MluI-MluI fragment was replaced with an intact copy of all3924 and the SphI-XhoI fragment was replaced with the SphI-XhoI fragment (carrying P_{glnA} *attR*-T1/T2-*attL-lacZ*) of pRSLZ	This study
pBZ	Derivative of pRSLZ carrying *attB-lacZ* instead of *attR*-T1/T2-*lacZ*	This study

[a] Tet^r, tetracycline resistance; Ap^r, ampicillin resistance; Km^s, kanamycin sensitivity.

(Stratagene, La Jolla, CA) was used for plasmid constructions. *E. coli* donor strain DH10B (kindly provided by X. Wu), carrying helper plasmid pRL623 (derivative of pRL528), conjugal plasmid pRL443 (derivative of RP4) (7), and cargo plasmid (either pRL488p-*int*, pRSLZ, or pOMs), was used for the biparental conjugation (7). *Anabaena* sp. strain PCC 7120 was employed as the cyanobacterial model system.

The shuttle vector pRL488p (39) was used for cloning *int* of bacteriophage HK022 into *E. coli* and *Anabaena*. The *int* gene was amplified from plasmid pKH3 by PCR with the primer pair *intKpn*I and *intSac*I, and the amplicon was ligated to the KpnI and SacI sites of pRL488p.

Plasmid pRL2831b was constructed by religation of an EcoRI-EcoRI fragment carrying P_{glnA} in the opposite orientation into pRL2831a. The recombination

TABLE 2. Primers used in this study

Primer	Sequence[a]	Purpose
*int-Kpn*I	5′-GGCAGCCATCGGTACCTGTGGATTGGCTG-3′	Amplification of *int* (complementary to pKH3)
*int-Sac*I	5′-GGGTTATTGTCTCATGAGCTCATACATATTTG-3′	Amplification of *int* (complementary to pKH3)
S-*Kpn*I	5′-GGAATTGTGAGCGGGTACCAATTTCACACAGG	Amplification of the recombination substrate (*attR*-T1/T2-*attL-lacZ*)
S-*Sph*I	5′-ATAATGGAATTCCTTACGCGAAATACGGGCATGC ATGG-3′	Amplification of the recombination substrate (*attR*-T1/T2-*attL-lacZ*)
*3924*u*Mlu*I	5′-GTAAGACATTTTACGCGTGATGAGACGGC-3′	Amplification of all3924 from the genome
*3924*d*Mlu*I	5′-GATTTGACGCGTATGGTCAGATAATCGACTTC-3′	Amplification of all3924 from the genome
SeqS1	5′-GTTTGACCAATCACCCGTCGAAC-3′	Sequencing of *attR*-T1/T2-*attL* and *attB* (complementary to the end of P_{glnA})
SeqS2	5′-GGGATGGCTTGTAGATATGACGACAGGAAG-3′	Sequencing of *attR*-T1/T2-*attL*
SeqS3	5′-CTCTAGAAGCTTCGATCTGTGG-3′	Sequencing of *attR*-T1/T2-*attL*
SeqS4	5′-CCAAGCTTCTAGAGATCTTCCATAC-3′	Sequencing of *attR*-T1/T2-*attL*
SeqS5	5′-GGAAGAGTTTGGTAGAAACG-3′	Sequencing of *attR*-T1/T2-*attL*
SeqS6	5′-GCTTGGCGTAATCATGGTCATAGCTG-3′	Sequencing of *attR*-T1/T2-*attL*
SeqP	5′-GACTGGGAAAACCCTGGCGTTACCC-3′	Sequencing of *attB*
cl	5′-CATTTATACCCTGTGCTATTTGCGTTTTAG-3′	Amplification and sequencing to confirm integration into the genome
*3924*down2	5′-GTGGAAGATAATTATACATAG-3′	Sequencing for integration into the genome
pl	5′-GCCATTGCCTGTAGTACAGACGTTCTTAG-3′	Amplification and sequencing to confirm integration into the genome
downZ2	5′-GGTACATCTCAAAAAGCTGTC-3′	Sequencing for confirming integration into the genome
all(1)	5′-GTTAAGCTGTGACAAGTGTAATTCTATACCAC-3′	Sequencing of the rescued plasmid
all(2)	5′-CGGAGAAACCGTCAATGCCAACC-3′	Sequencing of the rescued plasmid
P*glnA*	5′-CATTATGGTGAAAGTTGGAACCTC-3′	Amplification and sequencing before and after excision of pRSLZ or pBZ
mid*lacZ*	5′-GTTCTGCTTCATCTAGAGGATATCCTGCACC-3′	Amplification and sequencing before and after excision of pRSLZ or pBZ
ex1	5′-GGAAACAAGATCTAACAATGACTCAACC-3′	Amplification and sequencing before and after excision from the chromosome and amplification and sequencing of the *attR* probe
ex2	5′-GTAACGCCAGGGAGCTCCCAGTCACGACG-3′	Amplification and sequencing before and after excision from chromosome
Ter	5′-CTTCTAGAGATCTGCAGTCACGCAATAATTAAC-3′	Amplification and sequencing of the *attR* probe
*lacZ*1772	5′-CGCTTTAATGATGATTTCAGCCGC-3′	Amplification of the *lacZ* probe
*lacZ*2275	5′-CACAGCGGATGGTTCGGATAATG-3′	Amplification of the *lacZ* probe

[a] The restriction sites are underlined.

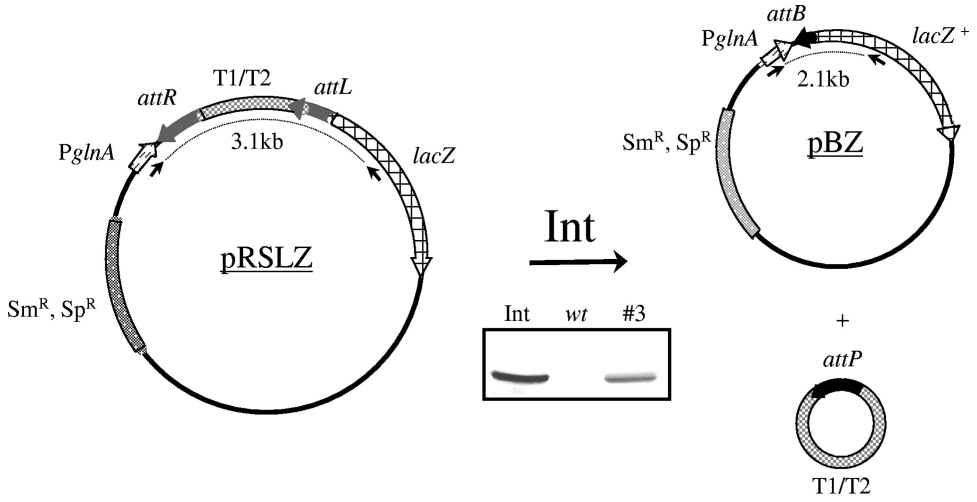

FIG. 1. The recombination reporter plasmid and anticipated products of the Int-catalyzed site-specific excision. (See text for details.) Genes conferring resistance to streptomycin and spectinomycin (Sm^r and Sp^r, respectively) are indicated. (Inset) Western blot analysis of the transgenic *Anabaena* clone (clone 3) carrying pRL488p-*int* (lane #3), *Anabaena* wild-type cells (lane *wt*), and *E. coli* cells expressing *int* from pKH3 (lane Int). Dotted arcs between arrows indicate amplicons generated with primers P*glnA* and mid*lacZ*, which are designated by the small black arrows.

reporter substrate *attR*-T1/T2-*attL-lacZ* was amplified from pLD205 (6) by PCR with primers S-KpnI and S-SphI and cloned between the KpnI and SphI sites of pRL2831b, creating plasmid pRSLZ (Fig. 1). T1/T2 are strong T1 and T2 transcription terminators from the *rrnB* gene (31).

The pOMs plasmid was constructed in two steps as follows: the 1.7-kb MluI fragment (carrying plasmid replication functions in *Anabaena*) from the pDUI portion of pRL2833b was replaced by the 2.6-kb *Anabaena* open reading frame all3924, encoding a probable penicillin amidase (see the CyanoBase website [http://bacteria.kazusa.or.jp/cyanobase/index.html]), amplified from the chromosome with the primer pair *3924*u*Mlu*I and *3924*d*Mlu*I. The 4,993-bp SphI-XhoI *attR*-T1/T2-*attL-lacZ* fragment was then transferred from pRSLZ to the corresponding sites of pRL2833b. pOMs was introduced into *Anabaena* cells by conjugation. Single recombinants were selected for erythromycin resistance.

Growth conditions. *E. coli* was grown in Luria-Bertani medium with the following antibiotics and final concentrations: ampicillin, 100 μg ml^{-1}; kanamycin, 15 μg ml^{-1}; streptomycin, 30 μg ml^{-1}; and chloramphenicol, 34 μg ml^{-1}.

Anabaena was grown in BG11 medium (38) at 28°C under cool white fluorescent light illumination with the following antibiotics and final concentrations: neomycin, 25 μg ml^{-1}; spectinomycin, 1.5 μg ml^{-1}; and erythromycin, 1 μg ml^{-1}.

Conjugation was performed as described by Elhai and Wolk (7).

PCR. Amplification was carried out with *Taq* DNA polymerase. The DNA in a standard reaction mixture was denatured at 94°C for 1 or 5 min (for plasmid or chromosomal DNA, respectively) and then subjected to 30 cycles of PCR, with 1 cycle consisting of 40 s at 94°C for amplicon denaturation, 40 s at 53°C or 70°C for annealing, and 186 s or 30 s at 72°C for elongation (in plasmid or chromosomal analyses, respectively).

β-Galactosidase assay. Cells were permeabilized by treatment with chloroform and sodium dodecyl sulfate, and debris was separated by centrifugation after the reaction was terminated as described previously (23). A_{420} was determined in the supernatant, and the specific activity of β-galactosidase was calculated in terms of A_{420} min^{-1} mg $chlorophyll^{-1}$.

Immunoblot analysis. Proteins (ca. 30 μg per lane) were separated by electrophoresis and electrotransferred onto Protran BA 83 cellulose nitrate filters (Schleicher & Schuell). The blots were exposed to antisera directed against wild-type integrase (20). Protein A-alkaline phosphatase conjugate was used as a primary antibody detector, and bands were visualized using the chromogenic substrate for alkaline phosphatase fast-5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium tablets (Sigma), diluted in 10 ml water.

Southern blot analysis. One microgram of *Anabaena* genomic DNA was cut with either EcoRI (to confirm substrate integration) or BssHII (to test excisional recombination) and separated on a 0.7% agarose gel. Alkaline transfer and hybridization were performed according to the GeneScreen manual (DuPont) using ^{32}P-labeled probes generated by the random primer method. The following probes were PCR amplified from pLD205: *attR* fragment with primers *ex1* and *Ter* and part of *lacZ* with primers *lacZ1772* and *lacZ2275*.

Plasmid rescue. Extracted genomic DNA was cleaved by ClaI, religated, and introduced into *E. coli*. Transformants were selected on plates containing chloramphenicol.

In vitro site-specific recombination. In vitro site-specific recombination with the reaction mixture containing Int, IHF, Xis, and pRSLZ was performed as previously described (13).

RESULTS

Int-catalyzed excision from a plasmid. The reporter gene encoding β-galactosidase (*lacZ*) was constructed on a plasmid (pRSLZ, the recombination reporter) such that it is expressed only as a result of an Int-catalyzed site-specific excision reaction (Fig. 1). That is because the *Anabaena* constitutive promoter for glutamine synthetase (P_{glnA}) (5, 10) was separated from the reading frame of *lacZ* by the strong transcription terminators T1/T2 flanked by *attR* and *attL* in a tandem orientation (6). Int-catalyzed recombination between the two *att* sites excises the terminator, leading to formation of the 21-bp recombinant *attB* site and brings the P_{glnA} promoter closer to the reading frame of *lacZ*, thereby allowing the production of β-galactosidase (Fig. 1).

The first plasmid (pRL488p-*int*), carrying *int* under the tandem constitutive promoters P_{psbA} and P_{tac} (39), was introduced into *Anabaena* by biparental conjugation. Western blot analysis of extracts from an *Anabaena* exconjugant demonstrated *int* expression (Fig. 1, inset). The presence of pRL488p-*int* did not affect the growth rate of the culture (data not shown). Next, the compatible recombination reporter plasmid (pRSLZ) was introduced into the Int expression exconjugant. Int-catalyzed recombination resulted in two products (Fig. 1): *attB*-containing pBZ that expresses *lacZ* (12.8 kb) and a smaller (1-kb) nonreplicating T1/T2 circular DNA that carries the recombinant *attP* site.

The recombination activity of Int was monitored in six exconjugants by the expression of *lacZ* (Fig. 2A) along with cells that carry the substrate alone as a negative control (bar S) and cells transformed with the product alone (bar P). The product

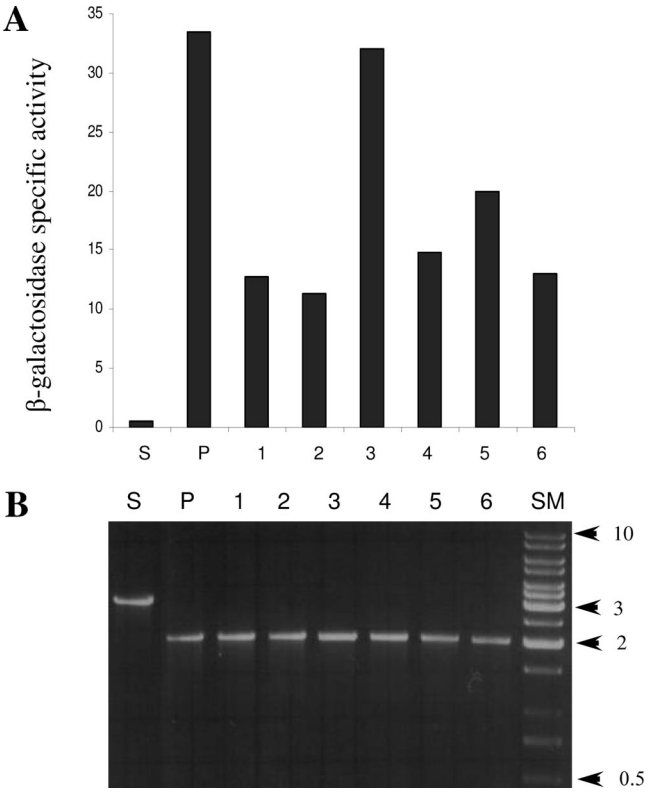

FIG. 2. (A) Specific activities of β-galactosidase (in A_{420} $min^{-1} \cdot mg$ $chlorophyll^{-1}$) in cultures of *Anabaena*. Clones 1 to 6 (bars 1 to 6) carry recombination substrate on pRSLZ and express *int* from pRL488p-*int*. Cells with pRSLZ alone (negative control) (bar S) and cells with pBZ product alone (positive control) (bar P) are shown. (B). PCR products from extracted plasmids of the same six transgenic *Anabaena* clones and controls as shown in panel A. The positions (in kilobases) of size markers (lane SM) are shown to the right of the gel.

(pBZ) was purified from an in vitro excision reaction of the reporter substrate (pRSLZ). Compared to the negative control (bar S), all six Int-treated conjugants expressed LacZ at various amounts, one of them (bar 3) as much as the positive control (bar P). The appropriate excision of the T1/T2 fragment was verified by PCR using plasmid extract from each culture (six recombinants and two controls) as template and oligomers *S-Kpn*I+*S-SphI* (depicted as arrows in Fig. 1) as primers. Figure 2B shows the expected 3.1-kb amplicon from cells with the substrate alone (lane 3) and the expected 2.1-kb amplicon of the recombination product in all other lanes. A sequence analysis (not shown) of all six amplicons obtained from the Int-treated cells confirmed the anticipated presence of the recombinant *attB* site. These results demonstrate that Int functions efficiently in vivo in excisional recombination in *Anabaena* cells on the episomal level.

Excision from the *Anabaena* chromosome. To introduce the same recombination substrate (P_{glnA}-*attR*-T1/T2-*attL*-*lacZ*) into the chromosome by homologous recombination, an *Anabaena* suicide plasmid, pOMs, that was disabled to replicate in *Anabaena*, was constructed such that it also carried the 2.6-kb all3924 sequence, a probable penicillin amidase-encoding gene of *Anabaena* (http://bacteria.kazusa.or.jp/cyanobase/index.html). pOMs was introduced into the *Anabaena* chromosome via erythromycin selection via a single homologous recombination exchange between its all3924 gene and that of the exconjugant (Fig. 3). The designed integration that results in contiguity of plasmid and genomic DNA was confirmed in recombinants by Southern blotting, PCR analyses, and plasmid rescue. In the blot, genomic DNAs from three erythromycin-recombinant (Em^r) recombinants and the wild-type control, each cleaved with EcoRI, were hybridized with an *attR* probe. Figure 3E shows the expected 12.8-kb fragment that spans between the inserted plasmid and genome DNA in the left junction and that is absent in the wild-type negative control. Next, the sequence (not shown) of a PCR amplicon obtained with primers *cl* and *pl* (Fig. 3C) confirmed the left-hand junction of the integrated plasmid. Finally, genomic DNA from one of the Em^r recombinants was cut with ClaI and ligated. A chloramphenicol-resistant (Cm^r) *E. coli* transformant of the reaction mixture carried an 11-kb rescue plasmid. Sequence analysis (not shown) of the rescued plasmid using primers *all(1)* and *all(2)* revealed the right-hand junction of the integrated plasmid.

The Int-expressing plasmid pRL488p-*int* was introduced into each of the three recombinant Em^r clones (Fig. 3E) by conjugation, and the Int-catalyzed excision reaction (Fig. 3C to 3D) was monitored by β-galactosidase activity in six exconjugants (Fig. 4A, bars 1 to 3, 5, 6, and 8). All six exconjugants displayed β-galactosidase activity levels about 50-fold higher than the level of a wild-type *Anabaena* culture included as a negative control (Fig. 4A, bar 10) and the three transgenic lines not transformed with Int (bars 4, 7, and 9).

Excision of the T1/T2 fragment was evident by PCR of genomic DNA (Fig. 4B) with primers *ex1* and *ex2* (Fig. 3C and D) in all 10 clones displaying the expected amplicons of 1,244 and 99 bp that represent unrecombined substrate and product, respectively. Sequence analysis (not shown) of the 99-bp amplicons of the Int-treated cultures confirmed the recombinant *attB* sequence. Finally, the expected 3- and 2-kb fragments of BssHII-cleaved genomic DNA prior to and following excision (Fig. 3C and D) were confirmed by Southern blot analysis (Fig. 3F, lanes 3 and 4, respectively) using a 508-bp *lacZ* probe. The additional, intense bands in lane 4 of Fig. 3F probably indicate incomplete digestion of the genomic DNA. These results show that Int efficiently catalyzed the excision of the substrate when located on a chromosome.

DISCUSSION

Expression of the coliphage HK022 *int* and the site-specific excisive recombination activity of its product in the cyanobacterium *Anabaena* sp. strain PCC 7120 are reported here for the first time. A similar study with Cre with two separately inserted chromosomal *lox* sites was reported earlier (40). Int was active with no need to import the accessory proteins IHF and Xis that are required for its activity in its natural *E. coli* host. The Int-catalyzed site-specific recombination, monitored by the expression of β-galactosidase production, was evident whether the recombination substrate was located on a plasmid (Fig. 1 and 2) or on the chromosome (Fig. 3 and 4). For the extrachromosomal reaction, *int* and the substrate were consecutively introduced into *Anabaena* cells on separate plasmids. In the case of the chromosomal reaction, the Int-encoding plasmid was introduced into transgenic strains carrying the recombina-

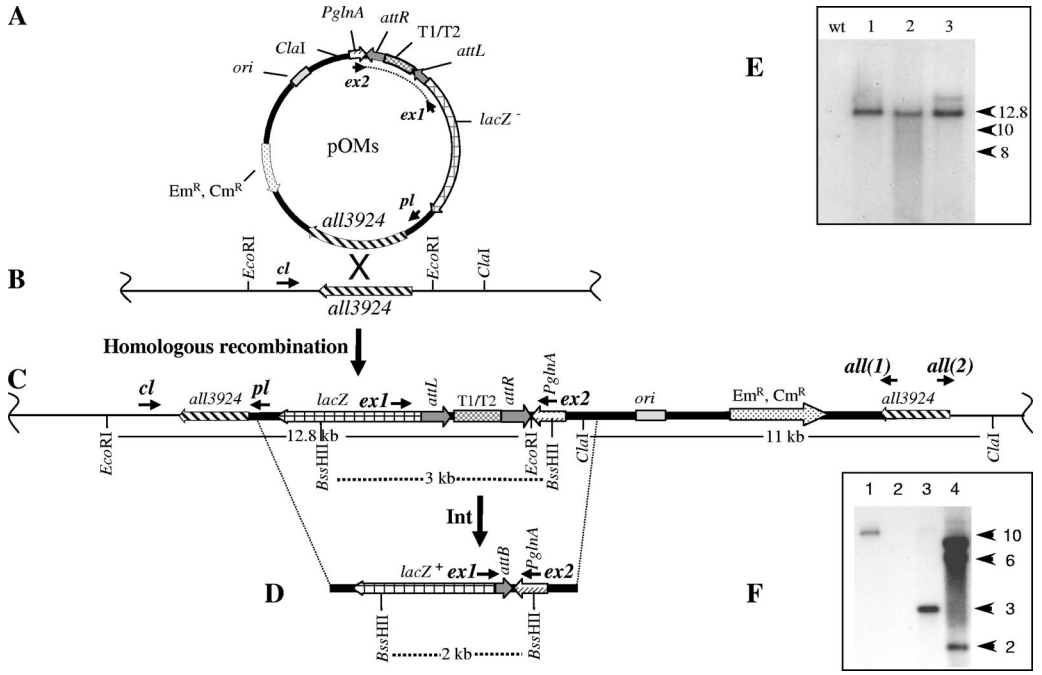

FIG. 3. (A to D) Schematic representation of the pOMs plasmid (A), the *Anabaena* chromosome (B), their homologous recombination product (C), and the following site-specific excision product (D). *ori*, replication origin in *E. coli*. Genes conferring resistance to erythromycin and chloramphenicol (Em^r and Cm^r, respectively) are indicated. The small black arrows indicate primers used in PCRs and sequencing. (E) Southern blot of three Em^r recombinant *Anabaena* clones (lanes 1 to 3) and of the wild-type (wt) *Anabaena* as a negative control. Hybridization was against ^{32}P-labeled *attR* probe. (F) Southern blot of wild-type *Anabaena* DNA (lane 2), a clone that carries the chromosomal recombination substrate that was not treated with Int (lane 3), and a similar Int-treated clone (lane 4). Lane 1 contains a linearized plasmid carrying the recombination substrate as a positive control. A *lacZ* fragment was used as the probe. The positions of molecular size markers (in kilobases) are indicated by black arrowheads to the right of the gels in panels E and F. Further details are described in the text.

tion substrate. The site-specific excision was confirmed in both cases by the formation of the recombined *attB* site. All six recombinant clones obtained by either the extrachromosomal or chromosomal reaction showed only the product and no substrate cassette (Fig. 2B and 4B), indicating a high frequency of recombination. This is in contrast to the relatively lower activity of Int observed in mammalian and plant cells (14, 16). In the case of the extrachromosomal reactions, this high frequency could be explained by the fact that the exconjugants were selected in succession, for the Int-encoding plasmid (pRL488p-*int*) first, followed by the low-copy-number plasmid encoding the recombination substrate (pRSLZ) (Fig. 1). Since *int* had been constitutively expressed during many generations of cell growth before Int activity was tested, the presence of Int in cells with the newly introduced substrate may be the reason for the high frequency of recombination. Also, in the chromosomal excision, where the Int-encoding plasmid was introduced to transgenic cells containing the substrate on the chromosome, many generations of selection elapsed before the reaction was examined. However, it should also be noticed that in the chromosomal reaction, the size of the excision product of 99 bp was about 12.5-fold smaller than of the substrate (1,244 bp; Fig. 4B), such that the former may have preferentially replicated. The same can also partially hold for the extrachromosomal PCR analysis (Fig. 2B). Likewise, the Southern blot analysis (Fig. 3F) demonstrates that the clone expressing *int* has exposed only the 2-kb product but not the 3-kb substrate, supporting the assumption that the excisional recombination is effective in *Anabaena*.

The variation in levels of β-galactosidase activity was much higher in the six clones in which the recombination substrate was on a plasmid (Fig. 2A) than in the six clones where it was chromosomally located (Fig. 4A). This variation may have resulted from a varying number of product (pBZ) copies per cell, whereas the frequency of the excised chromosome is stable. It is noteworthy that spectinomycin selection was partial: nonrecombinant cultures of *Anabaena* survived in the presence of the low drug concentrations used for the selection, albeit at a lower growth rate (data not shown).

Integration and excision catalyzed by Int of coliphage HK022 without the supplementary accessory proteins that are required in the natural host have already been demonstrated in mammalian cells (21) and *Arabidopsis* plants (14), where they may be substituted by endogenous, nonspecific DNA-bending proteins HMG1/2 (33). It is thus likely that proteins substituting for IHF and Xis are present in the cyanobacterium as well. However, sequences homologous to *E. coli*'s IHF-encoding subunit genes (*ihfA* and *ihfB*) and to *fis* do not exist in the *Anabaena* sp. strain PCC 7120 genome, leaving the apparent substitutes enigmatic.

The small basic *E. coli* protein HU (29) encoded by *hupA* or *hupB* serves as a chaperone to bend and loop DNA (2, 36). IHF binds to specific sequences by forming structural properties of the DNA, whereas HU binds to structural distortions in

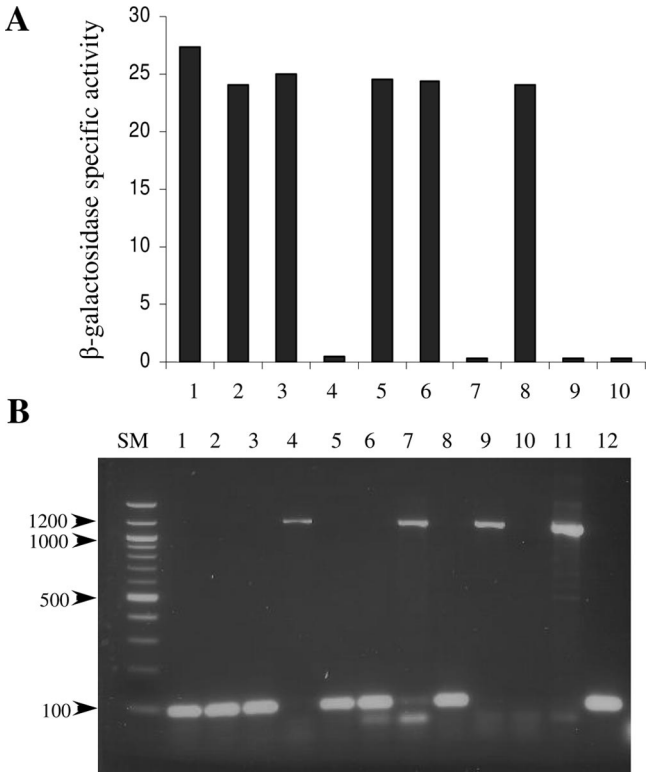

FIG. 4. (A) Specific activities of β-galactosidase (in A_{420} $min^{-1} \cdot mg$ $chlorophyll^{-1}$) in cultures of six transgenic *Anabaena* clones carrying the recombination substrate on the chromosome and expressing *int* from pRL488p-*int*. Exconjugants stemming from introduction of pRL488p-*int* into clone 1 of Fig. 3E (bars 1 to 3), exconjugants from clone 2 of Fig. 3E (bars 5 and 6), an exconjugant from clone 3 of Fig. 3E (bar 8), and clones 1 to 3 of Fig. 3E not treated with Int (negative controls) (bars 4, 7, and 9), and the wild-type *Anabaena* clone (bar 10) are shown. (B) PCR analysis of genomic DNA extracted from *Anabaena*. Lanes 1 to 10 show the amplicons from the respective transgenic clones as in panel A; lanes 11 and 12 contain pure pOMs (substrate [Fig. 3]) and pBZ (product [Fig. 1]); lane SM, 100-bp ladder (size markers).The primers were *ex1* and *ex2*. The positions (in base pairs) of molecular size markers are shown to the left of the gel by the black arrowheads.

DNA irrespective of sequence (30). Nevertheless, HU can replace IHF in λ site-specific excisive recombination (12); both have closely related structures and similar mechanisms of DNA bending (18, 35). The *Anabaena* HU-like protein (17), encoded by *hanA* (24), is essential for sensitivity to cyanophage A-4(L) and heterocyst differentiation (19). It is synthesized in the vegetative cells, but not in the heterocysts, and is degraded during differentiation (24). Integrating this information leads to the possibility that the *Anabaena* HanA might substitute for the functions of IHF and Xis. Alternatively, the site-specific recombinase XisF of *Anabaena* sp. strain PCC 7120 (3), which is involved in regulating *fdxN* during heterocyst differentiation, requires two additional accessory proteins (XisH and XisI) whose functions are yet to be disclosed (28).

The independence of HK022 Int activity on any imported accessory protein in *Anabaena* and its extraordinary high efficiency can be useful as a novel effective tool for DNA manipulations in *Anabaena* and other cyanobacteria, for example, to remove genes for antibiotic resistance from transgenic strains designed for release in nature (e.g., *Agmenellum quadruplicatum* PR-6, *Synechocystis* sp. strain PCC 6803, *Synechococcus* sp. strain PCC 7942, and *Anabaena* sp. strain PCC 7120) that express one or a combination of genes for mosquito larvicidal proteins from *Bacillus thuringiensis* subsp. *israelensis* (26).

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