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The IntP C-Terminal Segment Is Not Required for Excision of Bacteriophage Mx8 from the *Myxococcus xanthus* Chromosome

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During lysogenization of myxophage Mx8, phage DNA can be integrated into the *attB* site of the *Myxococcus xanthus* chromosome through site-specific recombination. We previously demonstrated that the Mx8 *attP* site is located within the coding sequence of the Mx8 *intP* gene. Hence, the integration of Mx8 into the *M. xanthus* chromosome results in the conversion of the 112-amino-acid C-terminal segment of the IntP protein into a 13-amino-acid C-terminal segment of a new protein, IntR. To examine whether IntR is active for Mx8 excision, we have constructed a series of plasmids carrying various lengths of the *intP-attP* or *intR-attR* regions as well as the *lacZ* gene. The integrated Mx8 was excised at a high frequency, indicating that IntR is active for the excision. For Mx8 excision, a gene designated *xis* was shown to be required in addition to *intR*.

During lysogenization of most temperate phages, their genomic DNA is integrated into the specific attachment site of the host chromosome by a mechanism originally proposed by Campbell (2). For example, phage λ is integrated into the *attB* site of the *Escherichia coli* chromosome by conservative site-specific recombination between the λ *attP* site and host *attB* site (1). The product of the λ *int* gene and the host integration host factor protein are required for integration. Upon induction of the SOS response λ phage is excised from the host chromosome. In this case the product of the λ *xis* gene is also required in addition to λ Int and integration host factor. On the λ genome, the *xis* and *int* genes overlap by 23 nucleotides and the *attP* site follows the *int* gene.

While the various site-specific recombinases of the integrase family have evolved diversely, all carry the conserved box I and box II motifs (13, 18) (see Fig. 5). An arginine residue in box I and histidine, arginine, and tyrosine residues in box II are completely conserved, and they form an active center for phosphotransfer reaction. The conserved tyrosine residue in box II forms a phosphodiester bond with the 3′ end of the recombining DNA. The processes of site-specific recombination were analyzed by X-ray crystallography for the Cre-*loxP* system (6–8). The *loxP* Holliday junction intermediate bound to four Cre molecules has been demonstrated.

Myxococcus xanthus is a unique gram-negative bacterium that can undergo multicellular development involving cell-to-cell interactions (for a review, see reference 4). When depleted of nutrients, cells on a solid medium aggregate to form mounds, which then convert to fruiting bodies. Rod-shaped vegetative cells change to round or ovoid myxospores. Myxophage Mx8 is a generalized transducing phage of *M. xanthus* (17, 23). Mx8 can be integrated into the *M. xanthus* chromosome by site-specific recombination during lysogeny (19). This recombination system has been used to introduce recombinant plasmids into the *M. xanthus* chromosome (22).

In a previous study (25), we have investigated the mechanism of Mx8 integration into the *M. xanthus* chromosome. The Mx8 *attP* site, *M. xanthus* chromosome *attB* site, and *attL* and *attR* phage-host junctions were cloned and sequenced. Based on sequence alignments of the *attP*, *attB*, *attL*, and *attR* sites, a strictly conserved 29-bp sequence was identified among the four sequences. The *intP* gene of Mx8 encodes an integrase family site-specific recombinase of 533 amino acid residues. Later it was found that the translation of the Mx8 *intP* gene can also be initiated by an alternative initiation codon located 123 bp upstream of the initiation codon that we have identified, yielding a 574-amino-acid protein (15, 16). The site-specific recombination system of Mx8 is unique, since the *attP* site is located within the *intP* coding sequence, as schematically illustrated in Fig. 1. Therefore, the integration of phage Mx8 into the *M. xanthus* chromosome *attB* site results in the conversion of the *intP* gene to the *intR* gene. As a result, the 112-residue C-terminal segment of the IntP protein is replaced by a 13-residue sequence of the IntR protein after Mx8 integration. The entire region of the *intP* gene, including the convertible C-terminal segment, was shown to be essential for the integration of Mx8 into the *M. xanthus* chromosome (25).

It is of great interest to examine whether the *intR* gene is active for Mx8 excision. In the present work, we analyzed the excision of Mx8 phage from the *M. xanthus* chromosome. It was found that the *intR* gene is sufficient for excision and that another gene, designated *xis*, is required for Mx8 excision.

MATERIALS AND METHODS

Bacteria, phage, and plasmids. *M. xanthus* DZF1 *sglA1* (12) was used. *E. coli* JM83 Δ(*lac-proAB*) *rpsL thi ara* φ80d *lacZΔM15* and JM109 *recA1* Δ(*lac-proAB*) *endA1 gyrA96 thi hsdR17 supE44 relA1*/F′ *traD36 proAB lacI*^{q}*ZΔM15* (26) were used for the construction of various plasmids. P1*clr100* Cm was used for the introduction of various plasmids from *E. coli* into *M. xanthus* cells (22). The Mx8 TM1 strain was from laboratory stock (25). pMC1403 and pSI1403 (3, 11) were used as vectors. Various segments of *intP-attP* and *intR-attR* were obtained from

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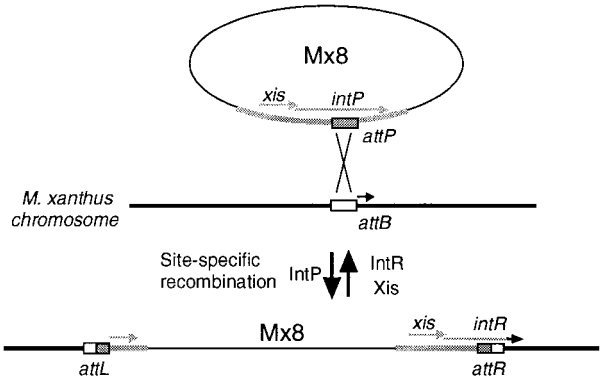

FIG. 1. Schematic illustration of the integration and excision of Mx8 by site-specific recombination. In the Mx8 integration system, the *attP* site is located within the *intP* coding sequence (25). The integration of Mx8 into the *M. xanthus* chromosome results in the conversion of the *intP* gene to a new gene, *intR*. As a result of this conversion, the 112-amino-acid C-terminal segment of the IntP protein is replaced by a 13-amino-acid segment of the IntR protein. The IntP protein, including the variable C-terminal segment, is required for Mx8 integration, while the IntR and Xis proteins are required for excision.

pMP001 and pMP004, respectively (25). pP1inc and pMXL101 (24) were also used.

Culture conditions. *M. xanthus* cells were grown in Casitone-yeast extract (CYE) medium (12) at 30°C. Solid medium contained 1.5% Bacto agar (Difco). Kanamycin sulfate (40 μg/ml) or oxytetracycline (6.25 μg/ml) was added for the selection of kanamycin-resistant (Km^r) or tetracycline-resistant (Tc^r) *M. xanthus* cells, respectively. Development of *M. xanthus* was induced on CF agar plates (10) as described previously (12).

E. coli cells were grown in Luria-Bertani medium (21) at 37°C. Ampicillin (100 μg/ml), tetracycline (12 μg/ml), and kanamycin sulfate (50 μg/ml) were used for the selection of plasmid-harboring cells.

Recombinant DNA techniques. Construction of plasmids, preparation of plasmid and chromosomal DNAs, transformation, Southern blot hybridization, and other methods for DNA manipulation were performed as described previously (21).

To construct plasmids pPFZ002 through pPFZ021 (Fig. 2A-1), a 1,368-bp *Aor*I-*Bam*HI internal segment of the *M. xanthus lonD* gene (encoding LonD residues 13 to 468) was first inserted into the *Sma*I-*Bam*HI site of pMC1403. Next, a 5.4-kb *Eco*RI-*Kpn*I fragment of the P1 phage incompatibility (P1*inc*) region was inserted. Finally, various lengths of the Mx8 *attP-intP* region (Fig. 2B) were excised from pMP001 DNA by using the indicated restriction enzymes and inserted into the resultant plasmid together with a 1.3-kb *Hin*dIII-*Sma*I Km^r fragment of Tn*5*. The Km^r fragment was inserted at both orientations in relation to the *intP* gene. pPFZ017, pPFZ018, pPFZ019, pPFZ020, and pPFZ021 carried a 4-bp deletion at the *Bgl*I site (position 1123 in the sequence under GenBank accession no. D86464), a 4-bp insertion at the *Nco*I site (position 2292), a 3-bp deletion at the *Bgl*I site (position 2486), replacement of AAC by TGCA at the *Bgl*I site (position 2486), and a 4-bp insertion at the *Not*I site (position 2657), respectively (25).

To construct plasmids pSZP002 through pSZP021 (Fig. 3A-1), a 4.5-kb *Dra*I-*Pvu*II segment of pSI1403 (a pMC1403 derivative carrying a translation initiation codon and the Shine-Dalgarno sequence for the *lacZ* gene) was first replaced by the P1*inc* fragment and the 1.4-kb *Eco*RI-*Bal*I Tc^r fragment of pBR322. Next, various lengths of the Mx8 *attP-intP* region with or without mutations (Fig. 3B) and the Tn*5* Km^r fragment were inserted into the resultant plasmid.

To construct plasmids pZLN002 through pZLN018 (Fig. 4A-1), the 4.5-kb *Dra*I-*Pvu*II segment of pSI1403 was first removed. Next, the 1.13-kb *Eco*RI-*Dra*I fragment of the resultant plasmid was replaced by the P1*inc* fragment, the pBR322 Tc^r fragment, an *M. xanthus lonD* internal segment, and a 0.72-kb *Sal*I fragment containing the Mx8 *attL* region. The *Not*I site within the *intP* C-terminal region of *attL* had been filled in. Finally, various lengths of the Mx8 *attR-intR* region with or without mutations (Fig. 4B) and the Tn*5* Km^r fragment were inserted into the resultant plasmid.

Assay for integration and excision activity of various lengths of *intP-attP* or *intR-attR* segments with or without mutations. The pPFZ series plasmids were transferred from *E. coli* into *M. xanthus* cells by P1 transduction to compare their frequencies of integration into the *lonD* locus or into the *attB* site of the chromosome. *M. xanthus* cells infected with P1 transducing phages were plated on CYE agar plates containing kanamycin and incubated for 5 to 7 days. When Km^r colonies appeared, the plates were overlaid with CYE soft agar containing 100 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) and incubated for 6 to 12 h. Yellow and green colonies were counted.

To determine the excision frequency, pSZP series plasmids were first introduced into *M. xanthus* cells by P1 transduction. Integration of pSZP series plasmids into the *attB* locus was confirmed by Southern blot analysis. *M. xanthus* cells carrying the pSZP series plasmids were grown overnight in CYE broth without kanamycin and plated on CYE agar without kanamycin. After colonies appeared, the plates were overlaid with CYE soft agar containing X-Gal as described above. Yellow and green colonies were counted.

The excision frequency was further determined by using pZLN series plasmids which carried artificial phage segments flanked by *attL* and *attR* sequences. pZLN series plasmids were introduced into *M. xanthus* cells by P1 transduction. Integration of pZLN series plasmids into the *lonD* locus was confirmed by Southern blot analysis. *M. xanthus* cells carrying pZLN series plasmids were grown overnight in CYE broth with oxytetracycline and plated on CYE agar with oxytetracycline. After colonies appeared, the plates were overlaid with CYE soft agar containing X-Gal. Yellow and green colonies were counted.

RESULTS

Site-specific integration of *intP-attP* segments into the *M. xanthus* chromosome. In a previous study (25), we qualitatively determined the integration activities of various *intP-attP* segments by examining the formation of Km^r colonies when test plasmids carrying the *intP-attP* region were introduced into *M. xanthus* cells. To quantitatively estimate the frequency of site-specific integration, pPFZ series plasmids were constructed (Fig. 2A-1). These plasmids carried a 1,368-bp *Aor*I-*Bam*HI internal segment (corresponding to codons 13 to 468) of the *M. xanthus lonD* gene (24), to which the *E. coli lacZ* gene was fused in frame. They also carried various *intP-attP* segments with or without the Km^r promoter. pPFZ series plasmids can be integrated into the *M. xanthus* chromosome in two ways. First, they can be integrated into the *lonD* locus of the *M. xanthus* chromosome by homologous recombination (Fig. 2A-3). In the recombinant *M. xanthus* cells, the active *lonD-lacZ* fusion gene, which was previously shown to be expressed during vegetative growth (24), is constructed, whereas the cells are deficient in development. Second, when the *intP-attP* segment is active for integration, the pPFZ series plasmids can be integrated into the *attB* site of the *M. xanthus* chromosome by site-specific recombination (Fig. 2A-4). In this case, the recombinant cells are deficient in β-galactosidase activity but proficient in development. Thus, the frequency of site-specific recombination can be estimated as a relative ratio to that of homologous recombination.

pPFZ series plasmids were introduced into *M. xanthus* cells from *E. coli* cells by P1 transduction, and Km^r transductants were selected. Since the pPFZ series plasmids cannot replicate in *M. xanthus* cells, Km^r recombinants carry pPFZ series plasmids that have integrated into the *M. xanthus* chromosome by either homologous recombination or site-specific recombination as described above. To determine whether each transductant carries a pPFZ series plasmid at the *lonD* locus or at the *attB* site, the β-galactosidase activity of Km^r colonies was determined by pouring soft agar containing X-Gal onto the plate. After a few hours, yellow and green colonies were found. Southern blot analysis indicated that pPFZ002 integrated into

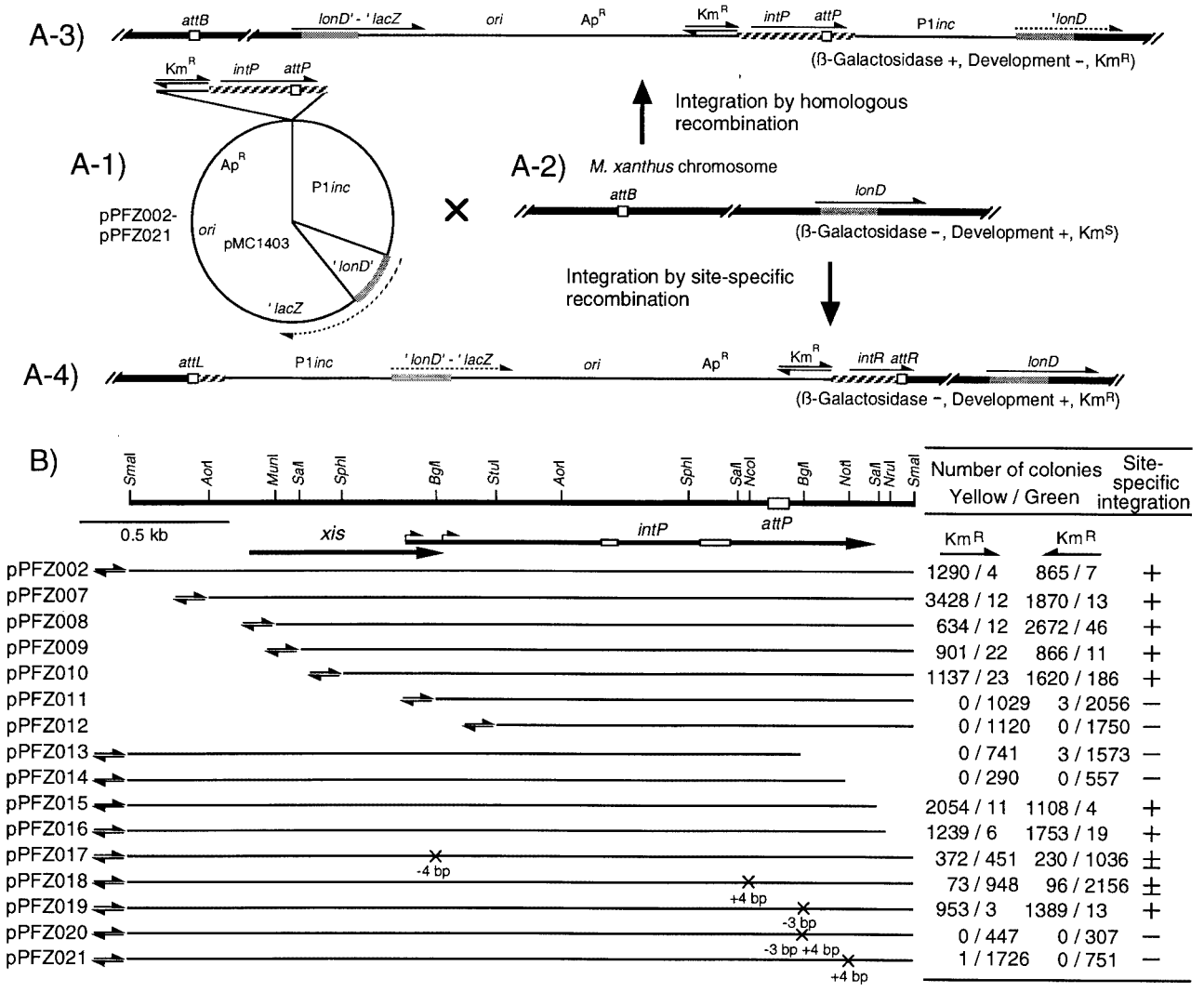

FIG. 2. Integration of pPFZ series plasmids into the *M. xanthus* chromosome by site-specific or homologous recombination. (A-1) Structure of the pPFZ series plasmids containing various lengths of Mx8 *intP-attP* segments and the *'lonD-lacZ* fusion gene without the N-terminal region. (A-2) Chromosomal structures of *M. xanthus* recipient cells around the *attB* site and the *lonD* locus. (A-3 and A-4) *M. xanthus* chromosomal structure in which the pPFZ series plasmids were integrated into the *lonD* locus by homologous recombination (A-3) or into the *attB* site by site-specific recombination (A-4). Hatched bars, Mx8 DNA fragment; open boxes, four *att* sites; solid bars, *M. xanthus* chromosome; stippled bars, a portion of *lonD* coding sequence; solid lines, vector and P1*inc* sequences; solid arrows, locations and orientations of active genes; dotted arrows, truncated genes. Integration of the pPFZ series plasmids into *M. xanthus lonD* by homologous recombination (A-3) results in reconstruction of an active *lonD-lacZ* fusion gene and inactivation of the *lonD* gene, while integration into *attB* by site-specific recombination (A-4) keeps the truncated *'lonD-lacZ* fusion gene inactive and the *lonD* gene active. (B) Integration of the pPFZ series plasmids into *attB* or *lonD* of the *M. xanthus* chromosome. At the top, a restriction map of the 2.9-kb *Sma*I fragment of Mx8 is shown together with the *attP* core sequence (open box). Short and long arrows represent the coding sequences and orientations of the *xis* and *intP* genes, respectively. Two alternative initiation codons for *intP* are indicated by bent arrows. Open bars on the *intP* coding sequence represent box I and box II motifs. Solid lines below the map indicate DNA portions present in various pPFZ series plasmids. pPFZ013, pPFZ014, pPFZ018, pPFZ020, and pPFZ021 encode truncated IntP proteins lacking the C-terminal 87, 30, 152, 87, and 30 amino acids, respectively. pPFZ019 encodes modified IntP with conversion of the 446th lysine and 447th leucine (from the second initiation codon) by methionine. The orientations of the Km^r genes are indicated. Crosses on the lines indicate the mutations constructed by modifying various restriction sites. pPFZ series plasmids were introduced into *M. xanthus* cells by P1 transduction. The numbers of yellow and green Km^r colonies were counted after X-Gal treatment. Ability for site-specific integration was inferred, as follows: +, high frequency; ±, low frequency; −, deficient.

the *attB* site in *M. xanthus* cells from the yellow colonies, whereas it integrated into the *lonD* locus in cells from the green colonies. In addition, the cells from the yellow and green colonies were found to be proficient and deficient for development, respectively. The numbers of the yellow and green colonies were counted (Fig. 2B). In pPFZ002 to pPFZ010, pPFZ015, and pPFZ016, yellow colonies were 300- to 9-fold more numerous than green colonies, indicating that these plasmids integrated predominantly into the *attB* site of the *M. xanthus* chromosome by site-specific recombination. Plasmids pPFZ011 through pPFZ014 produced practically no yellow colonies, indicating that these plasmids integrated only into the

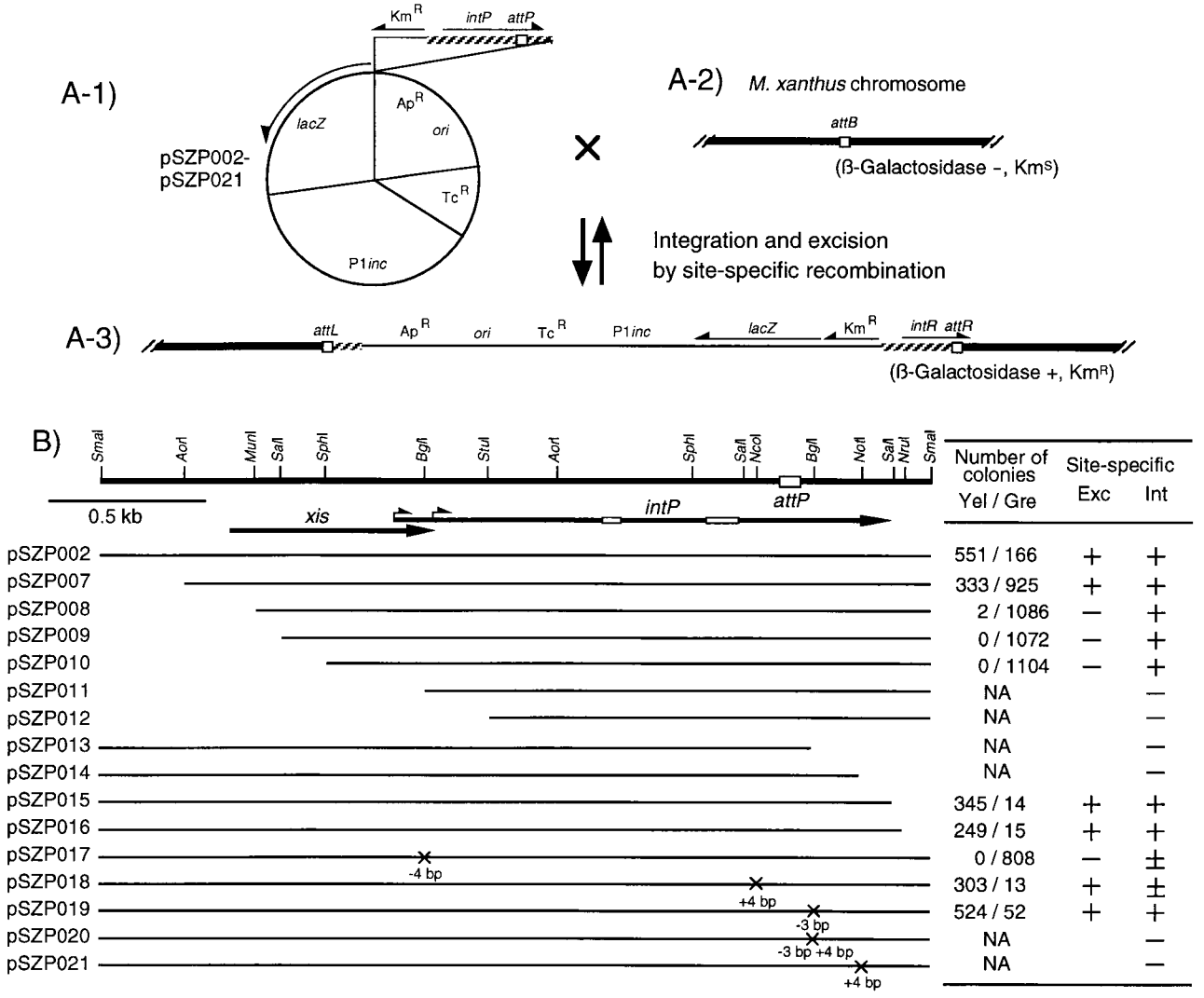

FIG. 3. Excision of integrated pSZP series plasmids from the *M. xanthus* chromosome. (A-1) Structure of the pSZP series plasmids containing a promoterless *E. coli lacZ* gene and various lengths of the Mx8 *intP-attP* segments. The *lacZ* gene is expressed by transcription from the Km^r gene. (A-2) The *attB* site of the *M. xanthus* chromosome without integration of the pSZP series plasmids. (A-3) Structure of the *M. xanthus* chromosome where the pSZP series plasmids were integrated into the *attB* site by site-specific recombination. (B) Excision of the pSZP series plasmids from the *attB* site of the *M. xanthus* chromosome. At the top, a restriction map of the 2.9-kb *Sma*I fragment of Mx8 is shown together with the *attP* core sequence (open box). Short and long arrows represent the coding sequences and orientations of the *xis* and *intP* genes, respectively. Open bars on the *intP* coding sequence represent box I and box II motifs. Solid lines below the map indicate DNA portions present in the pSZP series plasmids. Crosses on the line indicate the mutations constructed by modifying various restriction sites. After the pSZP series plasmids integrated into the *attB* site of the *M. xanthus* chromosome, their excision from *attB* was examined on CYE plates without kanamycin. Numbers of yellow (Yel) and green (Gre) colonies were counted after X-Gal treatment. NA, not applicable. Ability for site-specific excision (Exc) was inferred, as follows: +, high frequency; ±, low frequency; −, deficient. Ability for site-specific integration (Int) is also indicated.

lonD locus by homologous recombination. Frameshift mutations within the *intP* coding sequence weakly or strongly reduced the activity for site-specific recombination, while pPFZ019, encoding IntP with one amino acid deleted, integrated predominantly into the *attB* site. The direction of the external Km^r promoter did not affect the integration frequencies. These results are consistent with our previous observation that the entire IntP protein, including the convertible C-terminal segment, is required for site-specific integration of Mx8 (25).

Site-specific excision of the integrated plasmids from the *M. xanthus* chromosome. To examine the excision activity of Mx8, pSZP series plasmids were constructed (Fig. 3A-1). These plasmids carried various *intP-attP* segments as well as an intact *lacZ* gene transcribed from the Km^r promoter. Hence, *M. xanthus* cells carrying integrated pSZP series plasmids produce β-galactosidase (Fig. 3A-3). If pSZP series plasmids are excised from the chromosome of *M. xanthus* cells, however, *M. xanthus* cells become deficient in β-galactosidase activity.

pSZP series plasmids were introduced into *M. xanthus* cells by P1 transduction, and Km^r transductants were selected. Plasmids pSZP002 through pSZP010 as well as pSZP015 and

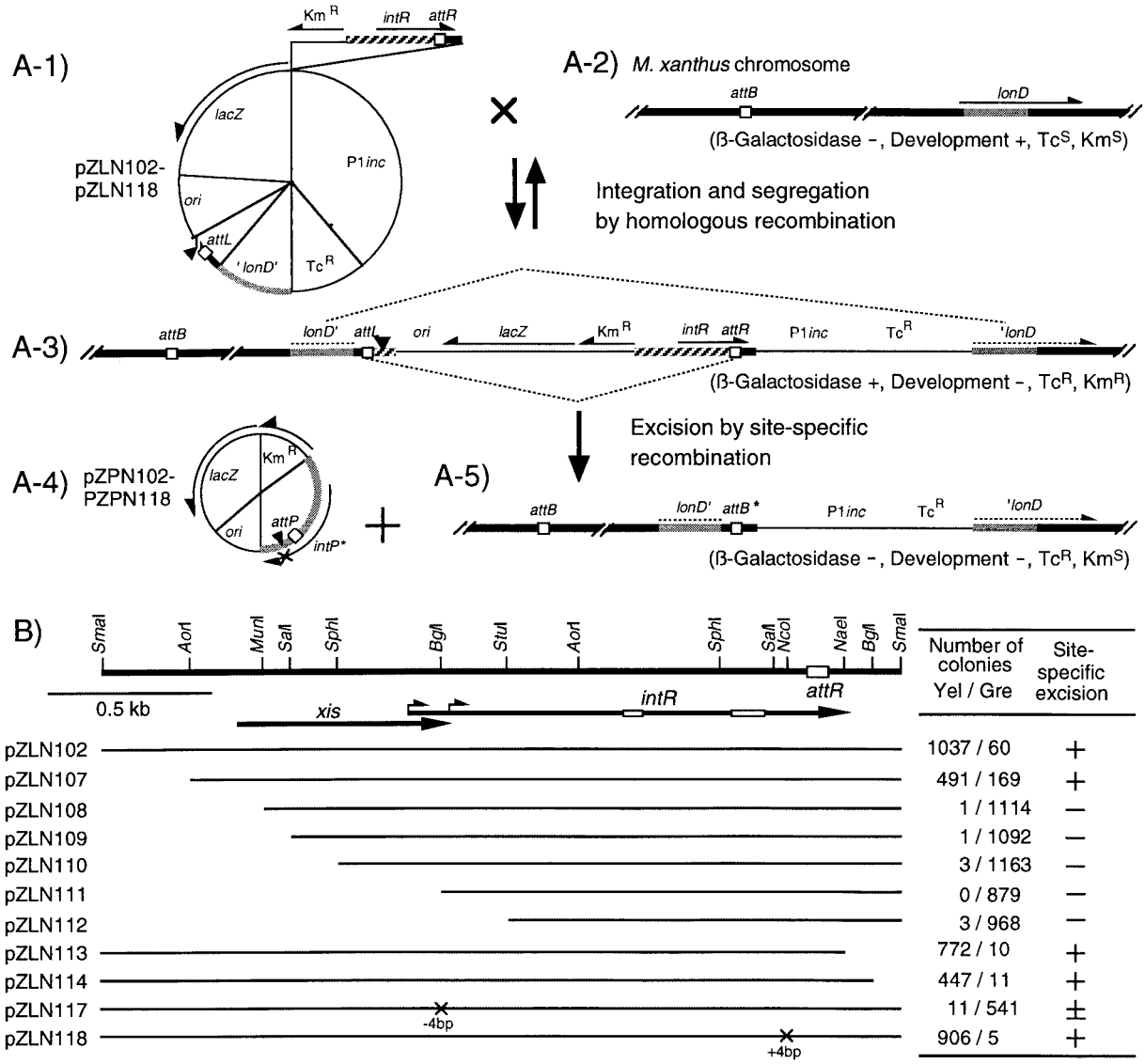

FIG. 4. Excision of pZPN series plasmids from pZLN series plasmids integrated in the *M. xanthus* chromosome. (A-1) Structure of the pZLN series plasmids containing a promoterless *lacZ* gene, the Mx8 *attL* segment with a 4-bp insertion at the *Not*I site (solid triangle), a truncated *M. xanthus lonD* gene, and various lengths of Mx8 *intR-attR* segments. The *lacZ* gene is expressed by transcription from the Km^r gene. (A-2) Chromosomal structures of *M. xanthus* recipient cells around the *attB* site and the *lonD* gene. (A-3) Structure of the *M. xanthus* chromosome where pZLN series plasmids were integrated into the *lonD* gene by homologous recombination. (A-4) Structure of the pZPN series plasmids produced by excision through site-specific recombination between the *attL* and *attR* sites. pZPN series plasmids are deficient in site-specific integration into the *M. xanthus* chromosome, since they carry a 4-bp insertion at the *Not*I site which leads to the inactivation of the *intP* gene (see Fig. 2B). (A-5) Chromosome structure after excision of pZPN series plasmids. *attB** represents a newly generated *attB* site. (B) Excision of pZPN series plasmids from the artificial *attB* site of the *M. xanthus* chromosome. At the top, a restriction map of the 2.66-kb *Sma*I fragment of the Mx8 *attR* junction is shown with the *attR* core sequence (open box). Short and long arrows represent the coding sequences and orientations of the *xis* and *intR* genes, respectively. Open bars on the *intR* coding sequence represent box I and box II motifs. Solid lines below the map indicate DNA portions present in various plasmids. Crosses on the line indicate the mutations constructed by modifying various restriction sites. After the pZLN series plasmids integrated into the *lonD* gene of the *M. xanthus* chromosome, excision of pZPN series plasmids was examined on CYE plates without kanamycin. Numbers of yellow (Yel) and green (Gre) colonies were counted after X-Gal treatment. Ability of site-specific excision was inferred, as follows: +, high frequency; ±, low frequency; −, deficient.

pSZP016 were integrated into the *attB* site of the *M. xanthus* chromosome at high frequencies, while pSZP017 and pSZP018 were integrated at low frequencies (Fig. 3B). In contrast, the remaining pSZP series plasmids could not be integrated into the *M. xanthus* chromosome. These results for integration activity are consistent with the results in Fig. 2B. *M. xanthus* cells carrying integrated pSZP series plasmids were grown overnight in CYE medium without kanamycin and plated on a CYE plate. After colonies formed, β-galactosidase activity was determined as described above (Fig. 3B). From *M. xanthus* cells carrying integrated pSZP002, pSZP007, pSZP015, pSZP016, pSZP018, and pSZP019 plasmids, yellow colonies appeared at

high frequencies. In contrast, from *M. xanthus* cells carrying integrated pSZP008, pSZP009, pSZP010, and pSZP017 plasmids, practically no yellow colonies appeared. These results indicate that some pSZP series plasmids were excised from the *M. xanthus* chromosome at a high frequency, while the other pSZP series plasmids were not. pSZP008, pSZP009, pSZP010, and pSZP017 integrated into the *attB* site of the *M. xanthus* chromosome but could not be excised, suggesting that a gene designated *xis* (Fig. 3B) is required for the excision of Mx8. The Mx8 *xis* gene may start from a GTG initiation codon located at the nucleotide 436 of the previously described sequence (GenBank accession no. D86464), and it encodes a 241-amino-acid basic protein with a calculated molecular weight of 25,324.

To further examine Mx8 excision, pZLN series plasmids were constructed (Fig. 4A-1). These plasmids contained artificial Mx8 segments flanked by the *attL* and *attR* sites, in which the intact *lacZ* gene transcribed from the Km^r promoter was inserted. To exclude the possibility of reintegration of the excised DNA element, the *Not*I site within the coding sequence for the IntP C-terminal segment was filled in by DNA polymerase I Klenow fragment. pZLN series plasmids carried various lengths of *intR-attR* segments (Fig. 4B). They can be integrated into the *lonD* locus of the *M. xanthus* chromosome by homologous recombination (Fig. 4A-3). The entire plasmid or a portion of the integrated pZLN series plasmids can be excised from the *M. xanthus* chromosome in two ways. First, the entire pZLN series plasmids can be excised from the *M. xanthus* chromosome by homologous recombination (Fig. 4A-1). Second, since the DNA segment flanked by the *attL* and *attR* sites constitutes an artificial Mx8 phage, the pZPN series plasmids (Fig. 4A-4) can be excised from the *M. xanthus* chromosome by the Mx8 site-specific recombination system when the *intR* segment is active for excision. In this case, a portion of the pZLN series plasmids, including the Tc^r gene, remained on the *M. xanthus* chromosome (Fig. 4A-5). The excised pZPN series plasmids cannot be reintegrated into the *attB* site of the *M. xanthus* chromosome by site-specific recombination, since the reconstituted *intP* gene carries a frameshift mutation at the *Not*I site.

pZLN series plasmids were introduced into *M. xanthus* cells by P1 transduction, and Km^r transductants were selected. When *M. xanthus* cells carrying the integrated pZLN series plasmids were grown in CYE medium without antibiotics, very few segregants exhibiting tetracycline sensitivity were produced, indicating that the frequency of segregation of pZLN series plasmids by homologous recombination is very low. *M. xanthus* cells carrying integrated pZLN series plasmids were grown overnight in CYE medium with tetracycline and plated on a CYE plate with tetracycline. After colonies appeared, their β-galactosidase activities were determined as described above (Fig. 4B). From *M. xanthus* cells carrying integrated pZLN102, pZLN107, pZLN113, pZLNP114, and pZLN118 plasmids, yellow colonies appeared at high frequencies, whereas from those carrying pZLN117, yellow colonies appeared at a very low frequency. The plasmid DNA fraction was extracted from *M. xanthus* cells carrying integrated pZLN102, pZLN107, pZLN113, pZLNP114, and pZLN118 plasmids and was used to transform *E. coli* cells. From transformed *E. coli* cells, pZPN series plasmids (Fig. 4A-4) were recovered, indicating that DNA recombination between the *attL* and *attR* sites of the integrated pZLN series plasmids had occurred. In contrast, from *M. xanthus* cells carrying integrated pZLN108, pZLN109, pZLN110, pZLN111, and pZLN112, practically no yellow colonies appeared. These results confirm that the pZPN series plasmids are excised from the *M. xanthus* chromosome by site-specific recombination at a high frequency and that the *xis* gene is required for Mx8 excision.

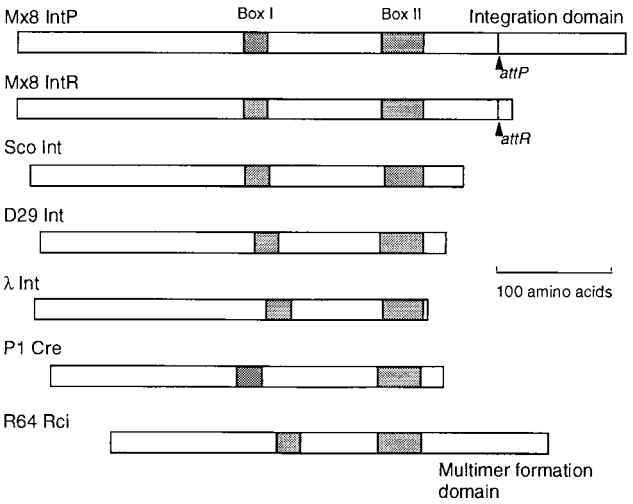

FIG. 5. Specific functions of the C-terminal domains of Mx8 IntP and R64 Rci recombinases. Structures of seven site-specific recombinases of the integrase family are compared. Sco, *S. coelicolor*. Only smaller forms of Mx8 IntP and IntR are illustrated. Locations of the box I and box II motifs in each recombinase are indicated by stippled bars. Locations of the *attP* and *attR* sites in Mx8 IntP and IntR, respectively, are also indicated. Only Mx8 IntP and R64 Rci carry additional C-terminal domains with specific functions.

DISCUSSION

In the present study, we have determined the frequencies of site-specific integration and excision of myxophage Mx8 into and from the *attB* site of the *M. xanthus* chromosome. Construction of pPFZ series plasmids enabled us to estimate the ability for site-specific integration of various lengths of *intP-attP* segments as a frequency relative to that of homologous recombination. The frequency of Mx8 integration was found to be up to 300-fold higher than that of homologous recombination. Higher frequencies of Mx8 integration have been reported previously (5, 22). With one exception, the results of the present study are in accord with those of our previous study (25), in which site-specific integration was determined by the formation of *M. xanthus* Km^r colonies after P1 transduction.

The result for pPFZ018 was unexpected: pPFZ018 exhibited a low level of site-specific integration, while mutants producing longer IntP fragments (pPFZ013, pPFZ014, pPFZ020, and pPFZ021) exhibited no site-specific integration. Low site-specific integration of pPFZ018 may be explained as follows. The Mx8 IntP protein consists of two segments, the N-terminal integrase segment and the C-terminal integration accessory domain, which are linked around the *Nco*I site in which the frameshift mutation was introduced in pPFZ018 (Fig. 5). The

Mx8 IntP N-terminal integrase segment corresponds to the entire sequence of most integrases of other phages. The 381-amino-acid N-terminal segment (from the second initiation codon up to the *Nco*I site) of Mx8 IntP shares 18 to 20% sequence identity with the entire integrases of *Streptomyces coelicolor*, *Bacillus subtilis*, and phage D29 and contains the box I and box II motifs conserved within the integrase family of site-specific recombinases (Fig. 5). The IntP C-terminal domain is specific for Mx8 among phage integrases and was shown to be required for integration but not for excision in the present study. In pPFZ018, the translation of the truncated IntP protein stops with eight additional amino acids following the *Nco*I site. This protein may exhibit full excision activity and low integration activity. In pPFZ013, pPFZ014, pPFZ020, and pPFZ021, however, the truncated IntP proteins carry fragments of the C-terminal domain, which may disturb Mx8 integration but not excision.

While the presence of an additional C-terminal domain is specific for Mx8 IntP among the various phage integrases, an additional C-terminal domain has also been identified in the shufflon-specific Rci recombinase, which belongs to the integrase family of site-specific recombinases (13, 14). Plasmid R64 bears a multiple inversion system designated shufflon, in which four DNA segments invert independently and in groups by the function of Rci. Shufflon-specific recombination sites (*sfx*), consisting of a central 7-bp spacer sequence and left and right 12-bp arms, were shown to be asymmetric: only the spacer sequence and right arm sequence are conserved among various R64 *sfx*s, whereas the left arm sequences are not conserved and are not related to the right arm sequence (9). Two Rci molecules are thought to bind to the *sfx* right and left arms in sequence-specific and non-sequence-specific manners, respectively. The Rci C-terminal domain is suggested to play a key role in non-sequence-specific Rci binding to the *sfx* left arm (9).

The present experiments using the pSZP series and pZLN series plasmids revealed that the integrated Mx8 is excised from the *M. xanthus* chromosome at a high frequency. For the Mx8 excision, IntP was not required and IntR was sufficient. The *xis* gene was required for Mx8 excision, as in the case of other phage integration-excision systems (1). The high frequency of Mx8 excision was unexpected, since Mx8 integration into the *M. xanthus* chromosome has been reported to be stable (5, 22). One possible reason for this discrepancy is that the Mx8 TM1 strain used in the present experiments happened to carry a mutation. Salmi et al. (20) independently sequenced the Mx8 *int* region and found that the Mx8 TM1 strain carries a 89-bp deletion just upstream of the *xis* gene in comparison to wild-type Mx8. A good Shine-Dalgarno sequence, GAGGT, was formed 5 bp upstream of the putative initiation codon of the *xis* gene as a result of the 89-bp deletion in the Mx8 TM1 strain. In the wild-type Mx8 strain, the corresponding Shine-Dalgarno sequence, GGGGT, is poor. Higher expression of the *xis* gene may increase the excision frequency in Mx8 TM1.

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REFERENCES

1. **Azaro, M. A., and A. Landy.** 2002. λ integrase and λ Int family, p. 118–148. *In* N. L. Craig, R. Craigie, M. Gellert, and A. M. Lambowitz (ed.), Mobile DNA II. ASM Press, Washington, D.C.
2. **Campbell, A. M.** 1962. Episomes. Adv. Genet. **11:**101–145.
3. **Casadaban, M. J., J. Chou, and S. N. Cohen.** 1980. In vitro gene fusions that join an enzymatically active β-galactosidase segment to amino-terminal fragments of exogenous proteins: *Escherichia coli* plasmid vectors for the detection and cloning of translation initiation signals. J. Bacteriol. **143:**971–980.
4. **Dworkin, M., and D. Kaiser (ed.).** 1993. Myxobacteria II. American Society for Microbiology, Washington, D.C.
5. **Gill, R. E., and L. J. Shimkets.** 1993. Genetic approaches for analysis of myxobacterial behavior, p. 129–155. *In* M. Dworkin and D. Kaiser (ed.), Myxobacteria II. American Society for Microbiology, Washington, D.C.
6. **Gopaul, D. N., F. Guo, and G. D. van Duyne.** 1998. Structure of the Holliday junction intermediate in Cre-*loxP* site-specific recombination. EMBO J. **17:** 4175–4187.
7. **Guo, F., D. N. Gopaul, and G. D. van Duyne.** 1997. Structure of Cre recombinase complexed with DNA in a site-specific recombination synapse. Nature **389:**40–46.
8. **Guo, F., D. N. Gopaul, and G. D. van Duyne.** 1999. Asymmetric DNA bending in the Cre-*loxP* site-specific recombination synapse. Proc. Natl. Acad. Sci. USA **96:**7143–7148.
9. **Gyohda, A., N. Furuya, N. Kogure, and T. Komano.** 2002. Sequence-specific and nonspecific binding of the Rci protein to the asymmetric recombination sites of the R64 shufflon. J. Mol. Biol. **318:**975–983.
10. **Hagen, D. C., A. P. Bretscher, and D. Kaiser.** 1978. Synergism between morphogenetic mutants of *Myxococcus xanthus.* Dev. Biol. **64:**284–296.
11. **Horiuchi, T., M. Taoka, T. Isobe, T. Komano, and S. Inouye.** 2002. Role of *fruA* and *csgA* genes in gene expression during development of *Myxococcus xanthus:* analysis by two-dimensional gel electrophoresis. J. Biol. Chem. **277:**26753–26760.
12. **Inouye, M., S. Inouye, and D. R. Zusman.** 1979. Gene expression during development of *Myxococcus xanthus*: pattern of protein synthesis. Dev. Biol. **68:**579–591.
13. **Komano, T.** 1999. Shufflons: multiple inversion systems and integrons. Annu. Rev. Genet. **33:**171–191.
14. **Komano, T., A. Kubo, and T. Nisioka.** 1987. Shufflon: multi-inversion of four contiguous DNA segments of plasmid R64 creates seven different open reading frames. Nucleic Acids Res. **15:**1165–1172.
15. **Magrini, V., C. Creighton, and P. Youderian.** 1999. Site-specific recombination of temperate *Myxococcus xanthus* phage Mx8: genetic elements required for integration. J. Bacteriol. **181:**4050–4061.
16. **Magrini, V., M. L. Storms, and P. Youderian.** 1999. Site-specific recombination of temperate *Myxococcus xanthus* phage Mx8: regulation of integrase activity by reversible, covalent modification. J. Bacteriol. **181:**4062–4070.
17. **Martin, S., E. Sodergren, T. Masuda, and D. Kaiser.** 1978. Systematic isolation of transducing phages for *Myxococcus xanthus*. Virology **88:**44–53.
18. **Nunes-Düby, 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.
19. **Orndorff, P., E. Stellwag, T. Starich, M. Dworkin, and J. Zissler.** 1983. Genetic and physical characterization of lysogeny by bacteriophage MX8 in *Myxococcus xanthus.* J. Bacteriol. **154:**772–779.
20. **Salmi, D., V. Magrini, P. L. Hartzell, and P. Youderian.** 1998. Genetic determinants of immunity and integration of temperate *Myxococcus xanthus* phage Mx8. J. Bacteriol. **180:**614–621.
21. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. **Shimkets, L. J., and S. J. Asher.** 1988. Use of recombination techniques to examine the structure of *csg* locus of *Myxococcus xanthus*. Mol. Gen. Genet. **211:**63–71.
23. **Stellwag, E., J. M. Fink, and J. Zissler.** 1985. Physical characterization of the genome of the *Myxococcus xanthus* bacteriophage MX-8. Mol. Gen. Genet. **199:**123–132.
24. **Tojo, N., S. Inouye, and T. Komano.** 1993. The *lonD* gene is homologous to the *lon* gene encoding an ATP-dependent protease and is essential for the development of *Myxococcus xanthus*. J. Bacteriol. **175:**4545–4549.
25. **Tojo, N., K. Sanmiya, H. Sugawara, S. Inouye, and T. Komano.** 1996. Integration of bacteriophage Mx8 into the *Myxococcus xanthus* chromosome causes a structural alteration at the C-terminal region of the IntP protein. J. Bacteriol. **178:**4004–4011.
26. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.