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Characterization of the Integrase Gene and Attachment Site for the *Myxococcus xanthus* Bacteriophage Mx9

Bryan Julien*

Kosan Biosciences, Inc., Hayward, California 94545

Received 9 September 2002/Accepted 5 August 2003

Bacteriophage Mx9 is a temperate phage that infects *Myxococcus xanthus*. It lysogenizes the bacteria by integrating into the bacterial chromosome by site-specific recombination at one of two sites, *attB1* or *attB2*. Integration at *attB1* results in deletion of DNA between the two *attB* sites. The *attB2* site lies within the 5′ region of the *M. xanthus* tRNA^{Gly} gene. Mx9 integration requires a single protein, Int. Analysis of integration revealed that the phage attachment site (*attP*) is contained in the *int* gene and that upon integration, the 3′ end of the *int* gene is altered. Plasmids containing fusions of the *pilA* or *mgl* promoter to *lacZ* integrated at either Mx9 *attB* site have higher levels of transcription than the same fusions integrated at the Mx8 *attB* site.

Mx9 is a general transducing phage that infects the gram-negative bacterium *Myxococcus xanthus* (9). The phage particle has a polyhedral head and a very short tail. Structurally, this phage resembles Mx8, which also infects *M. xanthus*.

The integrase gene and attachment site for Mx8 have been characterized (7, 8, 11). Integration of Mx8 by site-specific recombination requires a single phage protein, Int, and the phage attachment site, *attP*. Unlike the situation in most temperate bacteriophages, the Mx8 *attP* site is contained in the *int* gene, and upon insertion into the *M. xanthus* chromosome, the 3′ end of the *int* gene is altered. This modified *int* gene produces a protein, IntX, with lower integrase specific activity (8).

Because no natural replicating plasmids have been identified for *M. xanthus* or for any other myxobacterium, phage attachment sites provide an efficient and stable alternative way to introduce new genes or add additional copies of existing genes to the cell. With the recent heterologous expression of the epothilone biosynthetic gene cluster in *M. xanthus*, the ability to engineer the host should prove to be valuable for further optimization of polyketide production (4). The Mx8 *int* gene and the attachment site can be used to integrate DNA into the chromosome, but expression of many genes is affected by insertion into the Mx8 *attB* sites; many developmental promoters, as well as two constitutive promoters, *mgl* and *pilA*, have reduced activity at the Mx8 sites (2, 6). Therefore, I set out to find another attachment site. Here I describe characterization of the *int* gene and *attP* from Mx9, as well as the sites of insertion in the *M. xanthus* chromosome.

MATERIALS AND METHODS

Bacteria, phage, and plasmids. DZ1 is a nonmotile strain of *M. xanthus* and was used for plating Mx9 and for characterization of the Mx9 attachment sites (12). DK816 is a natural *M. xanthus* isolate lysogenic for Mx9 (9). *M. xanthus* strains were grown in CYE medium (1) or 1% CTS (1% Casitone, 0.2% $MgSO_4 \cdot 7H_2O$, 50 mM HEPES; pH 7.6). Phleomycin (Cayla) was used at a concentration of 30 μg/ml. The Mx9 phage was reisolated from DK816 by growing a culture to the stationary phase, pelleting the cells, and plating dilutions of the supernatant onto DZ1. High-titer stocks of Mx9 were made by coring a plaque and placing it in phage buffer (10 mM morpholinepropanesulfonic acid [MOPS] [pH 7.6], 4 mM $MgCl_2$, 2 mM $CaCl_2$). The eluted phage were diluted and mixed with 0.5 ml of DZ1 in the early stationary phase. After the cells and phage were incubated at room temperature for 20 min, 2.5 ml of top agar was added, and the suspension was poured onto phage plates (1% BBL Trypticase, 0.1% $MgSO_4 \cdot 7H_2O$, 1% agar, 10 mM MOPS; pH 7.6). The plates that exhibited confluent lysis after 2 days of incubation at 30°C were overlaid with 5 ml of phage buffer and incubated at 4°C overnight. The eluted phage were stored at 4°C. Phage stocks with concentrations greater than 1×10^9 PFU/ml were obtained by this method. The plasmids used are described in Table 1.

Isolation of phage DNA. The phage from a high-titer stock were pelleted by centrifugation in an SS-34 rotor at 28,000 rpm for 3 h and then resuspended in TE (10 mM Tris [pH 7.6], 1 mM EDTA). The phage proteins were removed by extraction twice with phenol and twice with phenol-chloroform-isoamyl alcohol. The DNA was precipitated and resuspended in TE.

Isolation and sequence of the phage attachment site. To isolate the phage attachment site, phage DNA was partially cleaved with *Hin*PI, and the fragments were ligated into pKOS35-93 cleaved with *Acc*I. Plasmid pKOS35-93 is pBluescriptII SK+ with the kanamycin resistance from Tn*5* ligated into the *Sma*I and *Eco*RI sites. One plasmid, pKOS35-117.9.7, integrated efficiently into the chromosome. The insert from this plasmid was sequenced.

Isolation of the bacterial attachment site. The bacterial attachment site (*attB*) was isolated by electroporating pKOS35-117.9.7 into DZ1, making chromosomal DNA, and then recovering the plasmid with flanking chromosomal DNA. Six kanamycin-resistant colonies were picked, and chromosomal DNA was prepared from each colony. The DNA was cleaved with either *Pst*I or *Xho*I, ligated, and then transformed into *Escherichia coli*. Three colonies from each of the electroporations were picked, and the plasmids recovered were cleaved with *Pst*I or *Xho*I. One plasmid from each preparation was sequenced by using either primer 183-66.3 (GAAGGAGGCACCATGCACGG) or primer 183-66.4 (CTCACTG AGAGTGAAGCCGC).

PCR amplification of Mx9 *attB*. Primers were designed to PCR amplify *attB1* and *attB2*. Primers 183-99.4 (CGAGGTCCGGGACGCGCGCA) and 183-99.6 (TGCCAGGGCTTACGGCTTC) were used to amplify a 285-bp *attB1* fragment, and primers 183-99.5 (TATCCCAGCAACCGCCGGAG) and 183-99.4 were used to amplify a 373-bp *attB2* fragment. To amplify the native *attB1* site, primers 183-99.6 and 249-179.7 (CAGCACGGGTGCAGCAAC) were used to amplify a 250-bp fragment. PCRs were performed by using chromosomal DNA from DZ1 and the FailSafe PCR system from Epicentre. The amplification conditions were 96°C for 2 min and then 30 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 2 min.

Construction of a minimal integration plasmid. The *int* gene was PCR amplified from pKOS35-117.9.7 by using primers 111-74.4 (CCCAATTGGCTCA GGGCAGCGGCTCATT) and 111-82.5 (CCCCATGGCGCTCAGGGGTGCG TCGGACGCC). The PCR amplification conditions were those described above. The amplified fragment was ligated into the *Eco*RV site of pLitmus 28 (New England Biolabs) to create pKOS249-12. The *int* gene was removed from this plasmid by cleavage with *Eco*RI, and the DNA ends were made blunt with the Klenow fragment of DNA polymerase, followed by cleavage with *Nco*I. The fragment was ligated with pUHE24-2B (3) that was cleaved with *Pst*I, and the

* Mailing address: Kosan Biosciences, Inc., 3832 Bay Center Place, Hayward, CA 94545. Phone: (510) 731-5209. Fax: (510) 732-8401. E-mail: julien@kosan.com.

TABLE 1. Plasmids

Plasmid	Characteristics
pKOS35-117.9.9	Amp^r Kan^r ColE1, 4.6-kb fragment from Mx9
pKOS139-29	Amp^r Co1E1, P_{T7A1} Mx8 *int attP*$^-$
pKOS139-47	Tc^r , p15A, P_{mgl} *lacZ*, Mx8 *attP*
pKOS178-86	Tc^r, p15A, P_{pilA} *lacZ*, Mx8 *attP*
pKOS178-177	Tc^r, p15A, P_{pilA} *lacZ*, Mx9 *int attP*
pKOS178-188	Tc^r, p15A, P_{mgl} *lacZ*, Mx9 *int attP*
pKOS249-31	Amp^r $Bleo^r$ ColE1, P_{T7A1} Mx9 *int attP*

DNA ends were made blunt with the Klenow fragment of DNA polymerase I and cleaved with *Nco*I. The resulting plasmid, pKOS249-23, contained the *int* gene under control of the *E. coli* phage T7 A1 promoter that was engineered to contain two LacI binding sites to repress transcription. The bleomycin resistance gene was added to this plasmid by isolating the bleomycin resistance gene from pKOS183-112 as a *Bam*HI-to-*Hin*dIII fragment, and the DNA ends were made blunt with the Klenow fragment of DNA polymerase I; then the fragment was ligated with pKOS249-23, which was cleaved with *Xho*I, and the DNA ends were made blunt with the Klenow fragment of DNA polymerase I. This plasmid was designated pKOS249-31.

β-Galactosidase assays. Seed cultures of two isolates for each integration site were grown in 1% CTS (5 ml) to the mid-log to late log phase. To start an assay culture, 35 ml of 1% CTS was inoculated with 1 ml of a seed culture at an optical density at 600 nm (OD_{600}) of 0.073. β-Galactosidase assays were performed by removing an aliquot of cells and adding them to Z buffer to obtain a combined volume of 1 ml. The cells were lysed by adding 1 drop of 0.1% sodium dodecyl sulfate and 2 drops of chloroform and vortexing the sample for 5 s. The assay was initiated by adding 0.1 ml of *o*-nitrophenyl β-D-galactopyranoside (8 mg/ml) and mixing. The reactions were stopped by adding 0.5 ml of 1 M Na_2CO_3. The OD_{600} of the cell culture and the OD_{420} of the enzyme reaction mixtures were determined with a SpetraMax 250 plate reader. Miller units were determined as previously described (10).

Nucleotide sequence accession numbers. The Mx9 sequence has been deposited under GenBank accession number AY247757. The accession numbers for *attB1* and *attB2* are AY297770 and AY297771, respectively.

RESULTS

Identification of the Mx9 *int* gene and attachment site. To identify the *int* gene and attachment site, a library of 5- to 8-kb fragments of Mx9 was made, and a clone that was able to integrate into the *M. xanthus* chromosome was identified. The insert in this plasmid, pKOS35-117.9.7, was sequenced. Five complete open reading frames (ORFs) and one partial ORF were identified in the 4.6-kb fragment (Fig. 1). ORF 1 was the only reading frame whose product exhibited amino acid similarity with the products of other known integrase genes and therefore was designated *int*. The other ORFs resembled ORFs from Mx8; ORF 2, ORF 3, ORF 4, ORF 5, and ORF 6 showed similarity to P15, P14, P16, P17, and P18, respectively, from Mx8. Based on the degrees of similarity of these ORFs in Mx8 and Mx9, it appears that Mx8 and Mx9 are very similar phages.

Because the Mx8 attachment site is located within the Mx8 *int* gene, the Mx9 *int* gene was examined for sequences which indicate that there is an attachment site. This analysis revealed a DNA segment within the *int* gene (nucleotides 1397 to 1428 [Fig. 2]) that exhibited sequence similarity to $tRNA^{Gly}$ from various organisms. Since Mx8 integrates into the $tRNA^{Asp}$ gene of *M. xanthus*, the sequence that showed similarity to $tRNA^{Gly}$ was predicted to serve as the site of integration for Mx9.

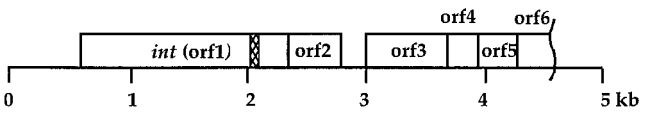

FIG. 1. Physical map of the *int* region of Mx9. The boxes represent putative ORFs. The cross-hatched box in *int* indicates the position of *attP*.

To test this prediction, chromosomal DNA from six integrants containing pKOS35-117.9.7 were cleaved with restriction enzymes, ligated, and transformed into *E. coli* to recover the plasmid along with flanking chromosomal DNA. Sequencing performed with primers adjacent to the proposed attachment site revealed that the point of recombination was indeed that of the putative $tRNA^{Gly.}$ Furthermore, the sequence of flanking chromosomal DNA showed that there were two *attB* sites. It appeared from the number of integrants at each site (three for *attB1* and three for *attB2*) that the two sites served equally well as the insertion site (Fig. 3).

Structure of the two *attB* sites. Figure 3 shows 360 bp from each of the *attB* sites. The two sites have a common 42-bp core sequence that is also found in the Mx9 *int* gene. In addition, there are 22 bp 5′ to both *attB* sites that are identical at 21 positions. There is a putative inverted repeat that may play a role in integrase protein binding at the *attB* and *attP* sites (Fig. 3B). The site of integration within *attB2* lies in the 5′ end of $tRNA^{Gly}$ gene, as shown in Fig. 3B. However, the sequence of *attB1* does not contain a complete $tRNA^{Gly}$ gene. Figure 4 shows the predicted folding of this segment of *attB2* into a corresponding tRNA.

Analysis of the *attR* and *attL* half-sequences for both *attB* sites revealed that the two *attR* sequences are identical, whereas the *attL* sequences differ. This is also the case with the two Mx8 *attB* sites (7). Plasmids containing the Mx8 *int* gene preferentially integrate at *attB1*, and this integration often is accompanied by a deletion between *attB1* and *attB2* (8).

To determine if the identical *attR* sites are due to the presence of two *attB* sites containing identical *attR* sites or due to deletion of the DNA between the two *attB* sites after integration into one of them, PCR analysis was performed either with primers 183-99.4 and 183-99.6 for *attB1* or with primers 183-99.4 and 183-99.5 for *attB2*. A PCR fragment was detected by using the primers specific for *attB2*, but no fragment was detected by using the primers specific for *attB1* (data not shown). This suggests that a deletion may occur upon integration of *attB1*, but to be certain that the lack of a PCR product was not due to a failure to PCR amplify the DNA fragment, further experiments were performed.

Next, the genomic sequence of *M. xanthus* strain DK1622, generated by Monsanto and available at The Institute for Genome Research web site, was examined for the two *attB* sites (www.TIGR.org). The *attB2* sequence was almost identical to the sequence identified previously (Fig. 3B), but only the first 178 bp of the *attB1* site shown in Fig. 3A was present before the sequence diverged. By using this sequence information for *attB1*, a primer was designed that was approximately 100 bp downstream from the point at which the sequence diverged (primer 249-179.7). By using this primer along with 183-99.6, the primer 5′ to the *attB1* site, and DZ1 genomic DNA, a PCR product that was approximately 250 bp long was isolated and sequenced. This PCR product was identical to that obtained

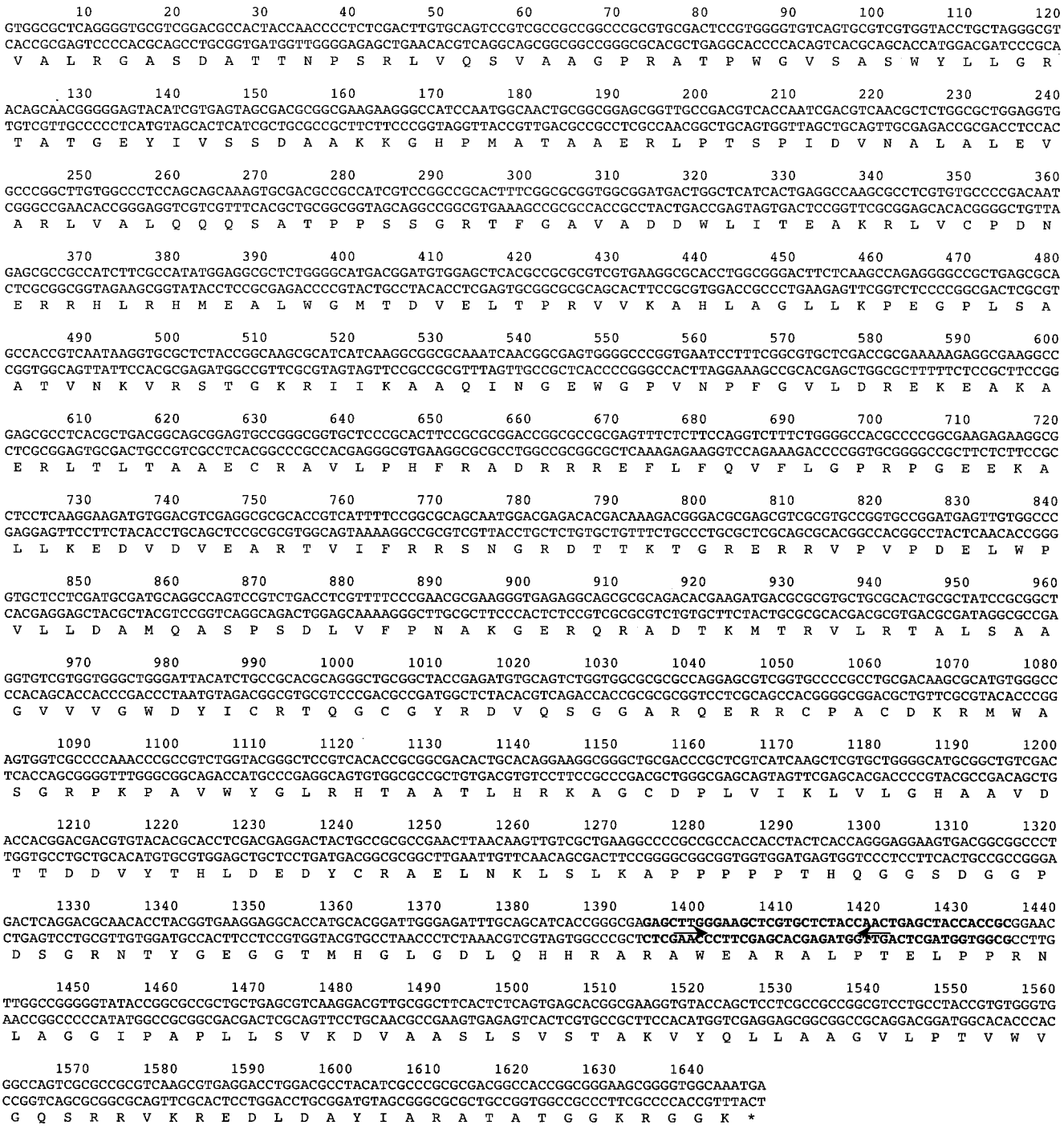

FIG. 2. Nucleotide sequence of the Mx9 *int* gene and the deduced amino acid sequence. The amino acids are indicated by single letters under the DNA sequence. Stop codons are indicated by an asterisk. The sequence in boldface type is the Mx9 *attP* sequence. The arrows indicate inverted repeats.

from the DK1622 genomic sequence (Fig. 3C). Analysis of the sequence revealed that only 16 bp of the 42-bp core *att* site was present in the native *attB1* site.

Final proof that a deletion does occur between *attB1* and *attB2* is shown in Fig. 5. By using primers 183-99.4 and 183-99.5, the primers that amplify the *attB2* site, PCR amplification was performed with genomic DNA from the wild-type strain or strains harboring a plasmid integrated at either *attB1* or *attB2*. By using chromosomal DNA from DZ1, a strain with no plasmid integrated at either *attB* site, a 372-bp PCR product containing the *attB2* site was detected (Fig. 5, lane 2). Two strains that had insertions at *attB2* (Fig. 5, lanes 5 and 6) did not

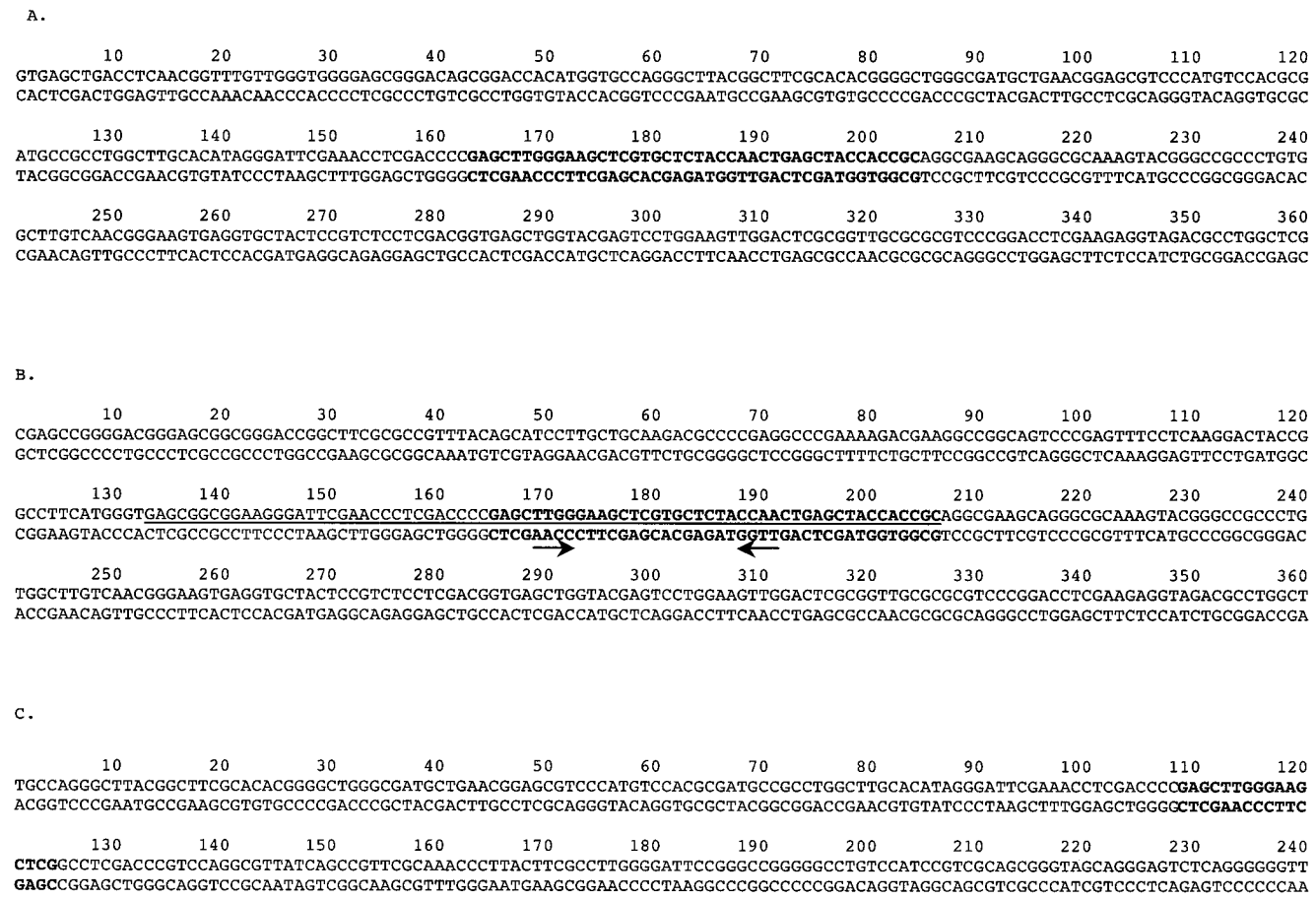

FIG. 3. (A) Nucleotide sequence of the reconstituted Mx9 *attB1* site. (B) Nucleotide sequence of the Mx9 *attB2* site. The arrows indicate an inverted repeat in *attB2*. (C) Nucleotide sequence of the native Mx9 *attB1*. Boldface type indicates the core *att* site. The underlined nucleotides encode $tRNA^{Gly}$.

produce the 372-bp band and should not have amplified *attB2* due to the presence of a plasmid integrated at that site. If a deletion did occur between *attB1* and *attB2*, there should have been no detectable amplification of *attB2* when a plasmid integrated at *attB1*. The results showed that no *attB2* PCR product was detected, indicating that there was a deletion of DNA between *attB1* and *attB2* when integration occurred at *attB1* (Fig. 5, lanes 3 and 4).

Integration results in alteration of the carboxy terminus of the Mx9 Int protein. Because *attP* lies within the *int* gene, integration into the chromosome alters the 3′ end of the *int* gene. In the 1,160 bp of *attR* that has been sequenced, no stop codon has been identified (data not shown). Thus, 70 amino acids should be removed from Int, and more than 389 amino acids should be added to the Int protein that is synthesized after integration into the chromosome. These additional amino acids presumably reduce the enzymatic activity of Int because the IntX protein of Mx8 has lost 112 residues and contains 13 added amino acids and is less active in site-specific recombination (8).

Mx9 Int is the only phage protein required for integration. To determine whether *int* is necessary and sufficient for integration, the *int* gene was PCR amplified and ligated into an *E. coli* expression vector that uses an engineered phage T7 A1 promoter. When plasmid pKOS249-31 was electroporated into DZ1, it integrated efficiently into the chromosome; approximately 1×10^4 colonies per μg of DNA were obtained. Thus, the Mx9 *int* gene is the only phage-encoded protein required for integrative recombination into the bacterial chromosome.

Transcription from the *pilA* and the *mgl* promoters integrated at the two Mx9 *attB* sites. Because the interest in Mx9 integration was to find a phage attachment site on the *M. xanthus* chromosome that supports efficient expression of genes from a variety of promoters, fusions of *lacZ* to the *mgl* or *pilA* promoters were constructed, and transcription from these promoters at the two Mx9 *attB* locations, the Mx8 *attB* location, and the native chromosomal location was analyzed. Figure 6A shows the levels of expression of the *pilA* promoter (P_{pilA}) at the four different locations. Surprisingly, there was little transcription when the P_{pilA} plasmid was integrated by homologous recombination at the *pilA* location (pKOS178-86). This suggests that there may be a deletion in the *pilA* promoter region that eliminates activation of the *pilA* promoter in DZ1 since there was no expression in several isolates that were examined. As observed previously, little transcription from P_{pilA} is seen when it is integrated at the Mx8 *attB* site

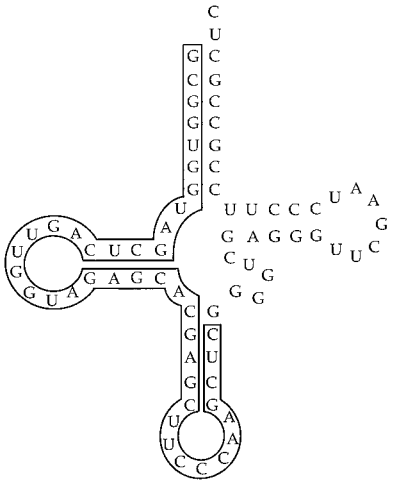

FIG. 4. Predicted cloverleaf secondary structure for $tRNA^{Gly}$ from *M. xanthus*. The bases that are within the core *attB* sequence are outlined.

(pKOS178-86 plus pKOS139-29). However, the Mx9 sites showed high levels of transcription from P_{pilA} (pKOS178-177), and the levels were fairly similar at the two sites, although *attB2* had high variability of expression in the two isolates examined. In addition, the regulation at the two sites was similar; transcription from P_{pilA} increased during the late log and stationary phases.

The results of transcription from the *mgl* promoter (P_{mgl}) are shown in Fig. 6B. Transcription from P_{mgl} at the two Mx9 *attB* (pKOS178-188) sites was better than transcription at the Mx8 site (pKOS139-47 plus pKOS139-29), but it was not as good when the promoter was integrated by homologous recombination at the chromosomal *mgl* location (pKOS139-47). However, the lower level of expression at the two Mx9 sites may have been vector dependent. When a plasmid that contained only the *attP* site was used and integrated by supplying

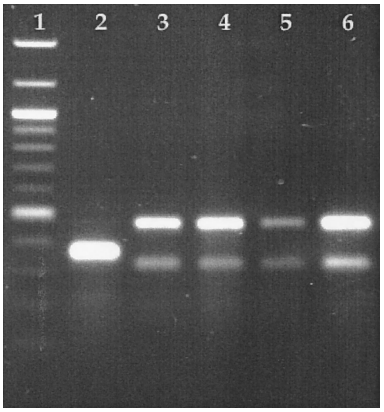

FIG. 5. Agarose gel containing PCR-amplified DNA fragments. Lane 1, 100-bp ladder from New England Biolabs; lane 2, PCR amplification for detection of *attB2* in wild-type strain DZ1; lanes 3 and 4, PCR amplification for detection of *attB2* in two independent isolates that contain a plasmid integrated at *attB1*; lanes 5 and 6, PCR amplification for detection of *attB2* in two independent isolates that contain a plasmid integrated at *attB2*.

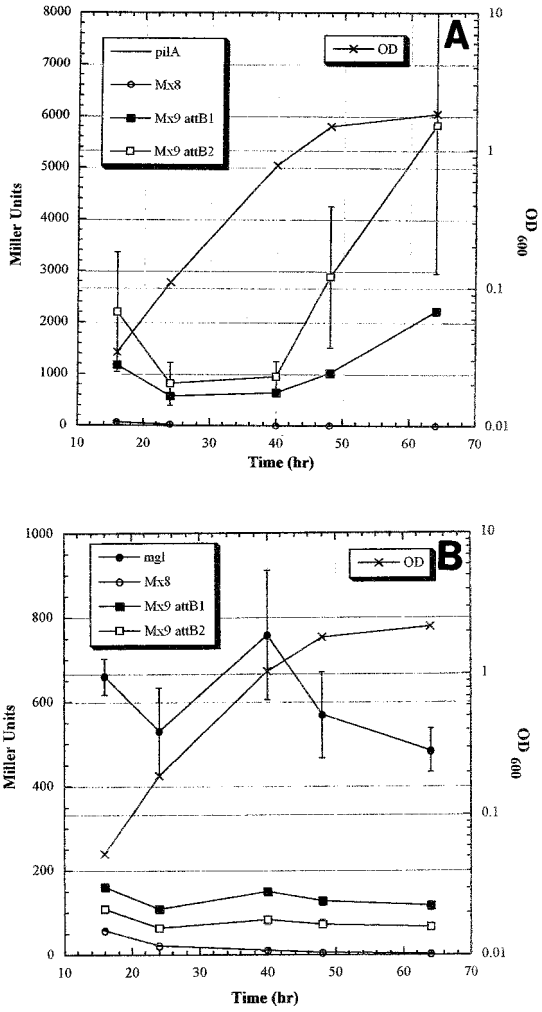

FIG. 6. (A) *lacZ* gene transcribed from the *pilA* promoter integrated at either the *pilA* chromosomal location, Mx9 *attB1* or *attB2*, or the Mx8 *attB* sites. (B) *lacZ* gene transcribed from the *mgl* promoter integrated at either the *mgl* chromosomal location, Mx9 *attB1* or *attB2*, or the Mx8 *attB* sites.

the *int* gene in *trans*, P_{mgl} functioned just as well at both Mx9 sites as it did at the chromosomal *mgl* location (data not shown). The data from these studies indicate that the Mx9 *attB* sites are good sites for expression of foreign or native genes.

DISCUSSION

The Mx9 *int* gene and attachment site were identified, along with the site of integration into the *M. xanthus* chromosome. The analysis revealed remarkable similarity to the *int* gene and attachment site in the myxophage Mx8 (7, 8, 11). Both phages contain *attP* within the *int* gene and integrate within a tRNA gene. They both have two *attB* sites, and it appears that adjacent chromosomal DNA is deleted when integration occurs at one of the sites. For both, Int is the only phage-encoded protein needed for integration.

One difference between the Mx8 and Mx9 phage integration systems is the length of the core sequences. The core sequence for Mx8 integration is smaller, composed of 29 bp. The *attB2*

site has two nucleotides that differ at one end, which may account for the preference of Mx8 for inserting at *attB1*. The *att* core region of Mx9 is 42 bp long, but only one of the two integration sites, *attB2*, contains all 42 bases. The *attB1* site contains only 16 bases of the core sequence. The lack of a complete core sequence in *attB1* may explain why there is always a deletion between *attB1* and *attB2* when integration occurs at *attB1*. The Int protein may bind to the inverted repeat within the 42-bp core. Binding of the λ Int protein to its *att* sites has been demonstrated (5). Since *attB1* contains one-half of the inverted repeat, only one-half of the necessary protein complex can form; however, once it has assembled, it may interact with the complementary half of proteins formed from *attB2* to allow integration. This should result in a looping out of the DNA between *attB1* and *attB2* and its subsequent loss upon integration of DNA.

In PCRs to detect *attB1* with primers 183-99.4 and 183-99.6, the conditions were such that if the distance between *attB1* and *attB2* was less than 2 kb, then a PCR product should have been detected. Since no product was observed, the results suggest that the distance between the two sites is greater than 2 kb. Analysis of the DK1622 sequence showed that the two *attB* sites are 6.7 kb apart. Analysis of this sequence revealed two ORFs that have sequence similarity to transposase genes, suggesting the presence of a transposon. The product of another ORF that was identified exhibited high levels of sequence similarity to proteins whose functions are unknown. Clearly, the ORFs between the two *attB* sites are not critical for growth under laboratory conditions since strains with integrations at *attB1* have no visible growth defects.

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