

Site-Specific Recombination of Temperate *Myxococcus xanthus* Phage Mx8: Genetic Elements Required for Integration†

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Like most temperate bacteriophages, phage Mx8 integrates into a preferred locus on the genome of its host, *Myxococcus xanthus*, by a mechanism of site-specific recombination. The Mx8 *int-attP* genes required for integration map within a 2.2-kilobase-pair (kb) fragment of the phage genome. When this fragment is subcloned into a plasmid vector, it facilitates the site-specific integration of the plasmid into the 3' ends of either of two tandem tRNA^{ASP} genes, *trnD1* and *trnD2*, located within the *attB* locus of the *M. xanthus* genome. Although Int-mediated site-specific recombination occurs between *attP* and either *attB1* (within *trnD1*) or *attB2* (within *trnD2*), the *attP* × *attB1* reaction is highly favored and often is accompanied by a deletion between *attB1* and *attB2*. The *int* gene is the only Mx8 gene required in *trans* for *attP* × *attB* recombination. The *int* promoter lies within the 106-bp region immediately upstream of one of two alternate GTG start codons, GTG-5208 (GTG at bp 5208) and GTG-5085, for integrase and likely is repressed in the prophage state. All but the C-terminal 30 amino acid residues of the Int protein are required for its ability to mediate *attP* × *attB* recombination efficiently. The *attP* core lies within the *int* coding sequence, and the product of integration is a prophage in which the 3' end of *int* is replaced by host sequences. The prophage *intX* gene is predicted to encode an integrase with a different C terminus.

Temperate phage Mx8 infects *Myxococcus xanthus*, a social gram-negative bacterium that lives in soil. The myxobacteria undergo a complex, multicellular developmental cycle in response to starvation. Development involves the morphogenesis of fruiting structures comprised of hundreds of thousands of cells that support the differentiation of a minority of cells into spores. Mature spores are resistant to UV light, desiccation, and heat and can germinate to develop into vegetative cells when nutrients become available. Mx8 has coadapted with its host to respond to this complex developmental cycle. The Mx8 prophage is stable upon the passage of an *M. xanthus* lysogen through cycles of development and germination (22). How the prophage maintains lysogeny throughout the dramatic changes in host gene expression during both sporulation and germination remains a mystery.

In a previous report, we described the initial genetic analysis of the 9.5-kilobase-pair (kb) immunity (*imm*) region of the Mx8 genome that includes the genes necessary for prophage integration and superinfection immunity (28). These two functions are required for the stability of the Mx8 prophage throughout development. The genes in the Mx8 *imm* region are densely packed and transcribed in a single direction. Three genes in the Mx8 *imm* region, *mox*, *uoi*, and *int*, encode products with known or suspected functions. The *mox* gene encodes a nonessential DNA adenine methylase (18), whereas *uoi* encodes a putative Mx8 excisionase and *int* encodes the Mx8 integrase (28, 37).

Plasmid vectors carrying the Mx8 *int* and *attP* genes integrate at the *attB* locus (16, 28, 34, 37). Such vectors have been used extensively to introduce second copies of *M. xanthus* genes at this ectopic locus, allowing for the construction of merodip-

loids that are more stable than those formed by homologous recombination (35). The ability of *int-attP*⁺ plasmids to integrate is our starting point for the dissection of the mechanism of Mx8 site-specific recombination in *M. xanthus*.

An independent study of the mechanism of Mx8 integration has shown that, unlike those of most other temperate phages and viruses, the Mx8 *attP* phage attachment site lies within the *int* coding sequence (37). In this paper, we show that the *int* gene is the only gene required in *trans* for integration and define the *int* promoter and the extent of the *int* coding sequence required for integration. We also describe the primary structure of the *M. xanthus attB* locus, confirm that the Mx8 *attP* site lies within the *int* gene, and characterize the changes in the structure of a bipartite *attB* locus that occur upon integration. In the accompanying paper (19), we show that, because the *attP* site lies within the *int* coding sequence, the integration event alters the primary sequence of the *int* gene to regulate the specific activity of its product.

MATERIALS AND METHODS

Bacterial strains. *M. xanthus* DK1622 (11) is the wild type. The multiple-mutant strain DZ1 (4) is the preferred host for the growth of phage Mx8 and was used to assay plasmid integration dependent on subcloned Mx8 site-specific recombination functions. *Escherichia coli* JM107 (39) was used for the construction of plasmids and the preparation of plasmid DNA. Electroporation (36) was used to introduce plasmids into JM107. Liquid CTPM medium (38) was used for the routine growth of *M. xanthus*. Derivatives of DZ1 with integrated plasmids were grown in CTPM medium with kanamycin (40 µg/ml) and/or the combination of spectinomycin (800 µg/ml) and streptomycin sulfate (1 mg/ml). Derivatives of JM107 with plasmids were grown in Luria-Bertani medium supplemented with ampicillin (100 µg/ml), kanamycin (40 µg/ml), or spectinomycin and streptomycin (50 µg/ml each). Antibiotics were from Sigma Chemical Co. Oligonucleotides used for plasmid construction and mutagenesis were made by Biosource Inc.

Integration-proficient parental plasmids. Plasmids with portions of the Mx8 genome were derived in one or more steps from DNA isolated from the wild-type strain of Mx8 (20) and are listed in Table 1. These include the kanamycin-resistant (Km^r) plasmid pAY50, a derivative of pACYC177 (6) which carries the Mx8 functions both necessary and sufficient for integration and superinfection immunity on an 8.1-kb *Sau3AI-PvuII* fragment of Mx8 DNA (18). The sequence of a larger region of the Mx8 genome including this 8.1-kb fragment has been assigned GenBank accession no. U64984 (28); coordinates (base pairs) used

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† This work is dedicated to the memory of Hatch Echols, teacher and friend.

TABLE 1. Plasmids

Plasmid	bp ^a	Vector ^b	Relevant genotype ^c	Source or reference
pACYC177		Ap ^r Km ^r		6
pBGS18		Km ^r		33
pGB2		Sp ^r Sm ^r		7
pLITMUS28		Ap ^r		New England Biolabs
pLITMUS29		Ap ^r		New England Biolabs
pRS552		Ap ^r Km ^r	'lacZ	32
pAY50	1–8072	pBGS18/Km ^r		18
pAY60	4585–8072	pBGS18/Km ^r		28
pAY62	4585–8072	pBGS18/Km ^r		This study
pAY721	4585–6809	pBGS18/Km ^r	<i>uoi</i> ⁺ <i>int</i> ⁺ <i>attP</i> ⁺	This study
pAY952	4585–6809	pGB2/Sp ^r Sm ^r	<i>uoi</i> ⁺ <i>int</i> ⁺ <i>attP</i> ⁺	This study
pAY979	4585–6809	pBGS18/Km ^r	<i>intVA42</i>	28
pAY754	4585–6809	pBGS18/Km ^r	<i>intVA1</i>	28
pAY990	4585–6809	pBGS18/Km ^r	<i>intVA1 intVA42</i>	28
pAY722	4808–6809	pBGS18/Km ^r	<i>uoi</i> ⁺ <i>int</i> ⁺ <i>attP</i> ⁺	This study
pAY759	4979–6809	pBGS18/Km ^r	<i>uoi</i> ⁺ <i>int</i> ⁺ <i>attP</i> ⁺	This study
pAY743	4585–5403	pLITMUS29/Ap ^r	<i>uoi-14</i> (Am) <i>P</i> _{int} -14	This study
pAY951	4585–6809	pBGS18/Km ^r	<i>uoi-14</i> (Am) <i>P</i> _{int} -14	This study
pAY995	4585–6809	pBGS18/Km ^r	<i>uoi-14</i> (Am) <i>P</i> _{int} -14	This study
pAY950	4585–6809	pBGS18/Km ^r	<i>uoi</i> ⁺ <i>int</i> ⁺ <i>attP</i> ⁺	This study
pAY735	4585–6809	pBGS18/Km ^r	<i>uoi</i> ⁺ <i>int-nd attP</i> ⁺	This study
pAY980	4585–6809	pBGS18/Km ^r	<i>uoi</i> ⁺ Δ <i>int-5403/5851 attP</i> ⁺	This study
pAY725	4585–6809	pBGS18/Km ^r	<i>int-1</i> (Am)	This study
pAY982	4585–6809	pBGS18/Km ^r	<i>int-1</i> (Am)	This study
pAY703		pBGS18/Km ^r	<i>mgIBA</i> ⁺	18
pAY971	5073–6809	pBGS18/Km ^r	<i>mgIBA</i> ⁺ <i>int</i> ⁺ <i>attP</i> ⁺	This study
pAY972	5208–6809	pBGS18/Km ^r	<i>mgIBA</i> ⁺ <i>int</i> ⁺ <i>attP</i> ⁺	This study
pAY987	5238–6809	pBGS18/Km ^r	<i>mgIBA</i> ⁺ Δ <i>int-5238</i>	This study
pAY992	5298–6809	pBGS18/Km ^r	<i>mgIBA</i> ⁺ Δ <i>int-5298</i>	This study
pAY993	5337–6809	pBGS18/Km ^r	<i>mgIBA</i> ⁺ Δ <i>int-5337</i>	This study
pAY733	4585–6504	pBGS18/Km ^r	Δ <i>int-6504</i>	This study
pAY734	4585–6711	pBGS18/Km ^r	Δ <i>int-6711</i>	This study
pAY146	4585–6716	pBGS18/Km ^r	Δ <i>int-6716</i>	This study
pAY997	1–8072	pACYC177/Km ^r	<i>uoi</i> ⁺ <i>int-NotI</i> ^r <i>attP</i> ⁺	This study
pAY738	4585–5210	pRS552/Ap ^r Km ^r	<i>uoi</i> ⁺ <i>int-lacZ</i> (fusion to GTG-5085)	This study
pAY739	4585–5087	pRS552/Ap ^r Km ^r	Δ <i>uoi int-lacZ</i> (fusion to GTG-5208)	This study

^a Coordinates of Mx8 DNA are those of GenBank accession no. U64984.

^b Antibiotic resistance determinants retained from the vector are indicated.

^c The *uoi-14* mutation inactivates the *int* promoter (*P*_{int}); *int-nd* is an uncharacterized PCR-induced mutation within bp 4585 to 6089 that inactivates the *int* gene. The *int-NotI* mutation is a 4-bp insertion within *int* made by filling in the *NotI* site and ligation.

throughout this paper are from this entry. The Km^r *int-attP* plasmids pAY60, pAY62, and pAY721 (28) are subclones of Mx8 DNA in plasmid pBGS18 (33). Plasmid pAY721 has a 2.2-kb insert of Mx8 DNA in pBGS18 with the *int* termination codon at its 3' end (28) and is used as a positive control in all electroporation experiments measuring the function of *int* and/or *attP*. Plasmid pAY952 is an Sp^r Sm^r, integration-proficient derivative of plasmid pGB2 (17). pAY952 has no sequence homology with pAY721, except within their common *EcoRI-HindIII* inserts of Mx8 DNA. None of the parental plasmids (pACYC177, pBGS18, or pGB2) used for subcloning of the Mx8 *int-attP* genes can integrate into the *M. xanthus* genome, as evidenced by their failure to give rise to antibiotic-resistant recombinants when electroporated into *M. xanthus*. Other plasmids were derived in one or more steps from these integration-proficient parental plasmids by standard cloning methods (29). Derivatives of pAY721 with the *intVA1* mutation (pAY979), the *intVA42* mutation (pAY754), and a combination of the *intVA1* and *intVA42* mutations (pAY990) have been described elsewhere (28).

Plasmids with deletions extending rightward into *uoi* and *int*. To map the *int* promoter, two derivatives of pAY721 were made with deletions extending rightward from a unique *MfeI* site at bp 4585 in the 8.1-kb sequence of the Mx8 immunity (*imm*) region to different end points upstream of *int*, bp 4808 and bp 4979. To construct pAY722, the PCR was used with 5' primers having the sequences CCAAGCTTCCTAGGTAGCGGAAGGGCTCTC and AAAAGA TCTACTGCGTGTGCATCG and template plasmid pAY60. The *BglII-HindIII* fragment from the product (bp 4808 to 6809) was ligated to the *BamHI* and *HindIII* sites of pBGS18. To make pAY759, the *Acc65I-StuI* fragment of pAY973 (bp 4979 to 6809) was ligated to the larger fragment of pAY721.

Plasmids with small substitutions or insertions in *uoi* or *int*. The open reading frame immediately upstream of *int*, *uoi*, is predicted to encode a putative excisionase and overlaps the 5' end of *int* (28). We replaced codon 14 (Ser) of *uoi*, AGC, with a TAG (amber) codon. Primer pairs with the sequences GGGGGA

AAAGATCTCGTGGACTGCAATTG and GGTGGATGTCTAGACACTTG CGAGGCTGCCGC and the sequences CACCAGTGCAGCAGCTGCCG ACAGCTCAGCAC and GCAAGTGTCTAGACATCCACCATTCCGCAAC were used to amplify Mx8 DNA. The products were annealed and then amplified in a second step with primers having the sequences GGGGAAAAGATCTC GTGGACTGCAATTG and CACCAGTGCAGCAGCTGCCGACGCGTCA GCAC. The product resulting from the second step was cleaved with *BglII* and *StuI* and ligated to pLITMUS29 to make pAY743. The 819-bp *MfeI-StuI* fragment with the *uoi-14*(Am) allele and the 5' end of *int* was joined together with the 1.4-kb *StuI-HindIII* fragment of pAY721 with the 3' of *int* to make pAY951. Cleavage of plasmid pAY743 with *XbaI* confirmed the presence of the *uoi-14*(Am) mutation. To confirm that the integration-defective phenotype of plasmid pAY951 is due solely to the *uoi-14*(Am) mutation, the largest *BglI* fragment of pAY951 was ligated to the smaller *BglI* fragments of pAY721 to make the isogenic integration-defective plasmid pAY995. The sequence of the region upstream of the *BglI* site was determined to show that the only change in this region is the desired one to amber. Plasmid pAY950, the integration-proficient, otherwise isogenic partner of pAY995 without the *uoi*(Am) mutation, was made by use of primers with the sequences GGGGAAAAGATCTCGTGGACTGC AATTG and CCCAAGCTTCCTAGGTAGCGGAAGGGCTCTC to amplify Mx8 DNA. The 2.2-kb product was cleaved with *BglII* and *HindIII* and then ligated to the *BamHI* and *HindIII* sites of pBGS18 to make pAY735. pAY735 and pAY721 were cleaved with *StuI* and *HindIII*, and the 1.4-kb fragment of pAY721 was ligated to the larger fragment of pAY735 to make pAY950. pAY950 differs from control plasmid pAY721 only in the extent of polylinker sequence that it retains from parental vector pBGS18. pAY980 is a derivative of pAY721 with a substitution of the 86-bp *StuI-PstI* fragment of pLITMUS28 for the *StuI-PstI* fragment internal to *int* (bp 5403 to 5851). A derivative of pAY721, pAY982, with the *int-1*(Am) mutation was constructed by two-step amplification of plasmid template pAY721 with primer pairs having the sequences CAGCA

CTGACCTTTTG and CTGGGCACCTAGGTGCGTCAAGAAGTCG and the sequences GCGACCTAGGTGCCAGCCGTCAGGAGT and AGCGGATAACAATTCACACAGGA. The amplified 1.7- and 0.5-kb products were annealed and amplified with primers having the sequences CAGCACTGACCC TTTTG and AGCGGATAACAATTCACACAGGA. The 2.2-kb product was cleaved with *EcoRI* and *HindIII* and ligated to pBGS18 to make pAY725. Plasmids pAY721 and pAY725 were cleaved with *BglI*, and the largest *BglI* fragment of plasmid pAY725 was ligated to the three smaller *BglI* fragments of plasmid pAY721 to make plasmid pAY982. Plasmids pAY725 and pAY982 were cleaved with *AvrII* to confirm the presence of the *int-1*(Am) mutation.

Plasmids that express *int* from the *mgl* promoter and their deletion derivatives. To express *int* from the constitutive *mglBA* promoter, several DNA fragments with different 5' ends were amplified and cloned downstream of the *mglBA* operon (9) in plasmid pAY703 (18). Primers with the sequences AAAGGTAC CCTGACGGTGGGCATCGTG and CCCGAATTCTCAGGTAGCGGAAG GGCTCT were used to amplify a 1.7-kb fragment from template pAY721 with the *int* coding sequence from bp 5073 to 6809. The product was cleaved with *EcoRI* and *Acc65I* and ligated to pAY703 to make plasmid pAY971. Primers with the sequences AAAGGTACCTGACGGTGGGCATCGTGGGTAAC GTCTATCGCAAGAAGGCCACT and CCCGAATTCTCAGGTAGCGGAAG GGGCTCT were used to amplify bp 5208 to 6809 from template pAY721. The 1.6-kb product was cleaved with *EcoRI* and *Acc65I* and ligated to pAY703 to make pAY972. Smaller fragments within *int*, bp 5238 to 5433, 5298 to 5433, and 5337 to 5433, were amplified with 5' primers having the sequences AAAGGTA CCCTGACGCTACTTGTGGATGTGGTGG, AAAGGTACCTGAGAAGA AGACGAAGTGAAGGCG, and AAAGGTACCTGAACCGAGAAGCGC GTGGCCGCG, respectively, the common 3' primer having the sequence ACG CGCCCTCCATCCACTTG, and template pAY721. DNA fragments were cleaved with *Acc65I* and *SmaI* to yield products of 167, 106, and 56 bp, which were ligated to pAY973 (19) to make plasmids pAY987, pAY992, and pAY993, respectively.

Plasmids with deletions extending leftward into *int*. To construct defined 3' deletions of *int*, we used the PCR to introduce premature termination codons within *int* in place of codons at bp 6504 (Leu474) and bp 6711 (Ala543). The common 5' primer with the sequence GGGGGAAAAGATCTCTGGTGAAGT CAATTG was used with a primer having the sequence CCCCAAGCTTGGC TACTGGCGCGGATTGGCGTAGAA or CCCCAAGCTTCTAAAGCCG CTCCAGGGCCATCAG to amplify Mx8 DNA. *BglII-HindIII* fragments made from the products (bp 4585 to 6504 and 4585-6711) were ligated to the *BamHI* and *HindIII* sites of pBGS18 to make plasmids pAY733 and pAY734, respectively. Plasmid pAY146 is a derivative of pAY62 made by cleavage with *NotI* and *HindIII*. Cleaved plasmid DNA was treated with the *E. coli* DNA polymerase I large fragment in the presence of deoxyribonucleoside triphosphates to fill in single-stranded ends and ligated. In plasmid pAY146, codon 543 of *int* is fused in frame to the 5' end of *lacZ* carried on pBGS18. The hybrid protein initiated at the GTG-5085 (GTG at bp 5085) start codon of *int* is predicted to have the first 543 amino acid residues of integrase and 56 residues of LacZ. Plasmid pAY997 is a derivative of pAY50 made by cleavage with *NotI*, filling in, and ligation. The expected sequence of the junction made by filling in was confirmed by cleavage of pAY997 with *EagI*, *NgoMI*, and *NotI*.

Plasmids that express *int-lacZ* translational fusions from the *int* promoter and β -galactosidase assays. Ap^r Km^r plasmid pRS552, used for constructing *int-lacZ* fusions, carries unique *EcoRI* and *BamHI* sites immediately upstream of the majority of the *lacZ* coding sequence (32). The 5' primer with the sequence AGCGGATAACAATTCACACAGGA and the 3' primers with the sequences GGGGGGATCCCCACGGCGCCCTGCCTT and GGGGGGATCC ACGATGCCAGCCGTCAGGA were used to amplify template pAY721. The products were cleaved with *EcoRI* and *BamHI* and then ligated to the same sites of pRS552 to make pAY738 and pAY739, which have in-frame translational fusions of the GTG-5085 and GTG-5208 start codons of *int*, respectively, to the seventh codon of *lacZ*.

For β -galactosidase assays, Km^r derivatives of DZ1(Mx8) carrying pAY738 or pAY739 integrated into the prophage genome by homologous recombination were constructed by electroporation of these plasmids into the Km^r lysogen and selection for Km^r recombinants. These recombinants and their parental strain were grown to a density of 4×10^8 /ml in CTPM medium at 32°C. Eight milliliters of each culture was pelleted by low-speed centrifugation, resuspended in 0.10 volume of TPM buffer (10 mM Tris, 8 mM MgSO₄, 1 mM potassium phosphate [pH 7.6]), and sonicated for three 15-s 5-W pulses with a Microson cell disrupter. After sonication, 0.25 volume of 0.1% sodium dodecyl sulfate was added, and assays for β -galactosidase activities and protein concentrations were performed as described previously (14). Activities are given in nanomoles of *o*-nitrophenol per minute per milligram produced by hydrolysis of the substrate *o*-nitrophenol- β -galactoside (Sigma).

PCR amplification of *M. xanthus* genomic DNA. Colonies of Km^r electroporants of host DZ1 were grown for 5 to 7 days on CTPM plates, patched on CTPM agar, and grown for an additional 2 to 3 days at 32°C. Approximately 0.1 g of cell paste was scraped from each plate and suspended in 200 μ l of TE buffer (10 mM Tris-HCl [pH 7.6], 0.1 mM EDTA) supplemented with 100 μ g of RNase A. Cells were incubated at 85°C for 5 min, extracted with an equal volume of distilled, buffered phenol, and centrifuged at $15,000 \times g$ for 15 min. The aqueous phase was extracted twice with an equal volume of chloroform, and 0.5 to 5.0 μ l of

supernatant was used as a template in 50- μ l reaction mixtures for PCR. Conditions for PCR amplification were as follows: 0.2 μ M each oligonucleotide primer, 1.5 mM MgCl₂, 0.25 mM each nucleotide triphosphate, and 0.5 to 2.0 U of *Taq* DNA polymerase (Gibco-BRL).

Amplification of *attP*, *attB*, *attL*, and *attR* sites with specific primer pairs. To determine where phages or *attP*-containing plasmids integrate within the *attB* locus (at site *attB1* or *attB2*), we performed two separate PCRs to amplify the *attL* and *attR* sites generated by phage or plasmid integration. These reactions yield products of different sizes, depending on whether integration occurs at the *attB1* or the *attB2* site. As controls for these PCR assays, we often tested whether strains carrying a prophage or integrated plasmid retained the *attB* sites or also had an *attP* site. *attB* primers with the sequences GGGGAATTCGTCGACT GCGCAGGTCCGCGGAGGA and AAAAAAGCTTCCGGGCGGCCTTGC GGAATGAT yield a 591-bp product when used to amplify template DK1622 or DZ1 genomic DNA (see below). *attP* primers with the sequences TCAGCGCT TCAGGTCCGGGACTGGGAC and CCCAAGCTTCTAGGTAGCGGAAG GGCTCTC yield a 969-bp product when used to amplify phage Mx8 DNA. *attL* primers with the sequences GGGGAATTCGTCGACTGCGCAGGTCCGCG GGAGGA and CCCAAGCTTCTAGGTAGCGGAAGGGCTCTC yield a product of 526 bp if integration occurs at *attB2* (or integration is accompanied by a deletion between *attB1* and *attB2*) or a product of 692 bp if integration occurs at *attB1*. *attR* primers with the sequences AAAAAAGCTTCCGGGCGGCCTT GCGGAATGAT and TCAGCGCTTCAGGTCCGGGACTGGGAC yield a 1,006-bp product if integration occurs at *attB2* and an 840-bp product if integration occurs at *attB1* (or integration is accompanied by a deletion between *attB1* and *attB2*).

To distinguish whether derivatives of plasmid pAY703 which contain the *mgl* operon have integrated at either the *mgl* or the *attB* loci, genomic DNAs isolated from Km^r electroporants were amplified with the *attR* primers. We confirmed that templates that yielded no product in these reactions had a plasmid integrated at the *mgl* locus by showing that they yielded a product when amplified with the *attP* primer pair.

Electroporation assays for plasmid integration. To quantify the efficiencies with which plasmids can integrate into the *M. xanthus* genome, 1 ml of DZ1 cells at an exponential density of 5×10^8 /ml was concentrated 25-fold and electroporated with plasmid DNA (100 to 200 ng) under conditions standard for the electroporation of *M. xanthus* (12). After electroporation, cells were suspended in 3 ml of CTPM medium to a density of 1.7×10^9 /ml and grown at 32°C for 16 h before being plated on CTPM agar plates with antibiotics. Coelectroporations were done in a similar way, with a mixture of 100 to 200 ng of each plasmid. Colonies formed by antibiotic-resistant electroporants were counted after 7 days of incubation at 32°C.

Nucleotide sequences of the *int* and *uoi* genes and the *attB* locus and their accession numbers. The sequence of a region of the Mx8 genome including *uoi* and *int* has been assigned GenBank accession no. U64984 (28). To determine the sequence of the *attB* locus, we amplified a 591-bp fragment of the *attB* locus (37) from both mutant strain DZ1 and wild-type strain DK1622 by using the *attB* primers. The products were sequenced by the dideoxy method (30). Sequencing runs were performed by Commonwealth Biotechnologies, Inc., Richlands, Va., and resolved on an ABI Prism model 377 automated sequencing apparatus. The sequence of the *attB* locus, determined to be identical for both DK1622 and DZ1, was the same as that determined by Tojo et al. (37), which has been assigned GenBank accession no. D26557.

RESULTS

The *int* promoter is located in a 106-bp region upstream of the *int* structural gene. The model for the site-specific integration of temperate phage genomes initially proposed by Campbell (3) has proven particularly robust. Almost all temperate phages integrate into their host chromosome by a mechanism of site-specific recombination, in which an integrase protein catalyzes a four-strand exchange event between an *attP* site on the circular phage chromosome and an *attB* site on the host chromosome. Integration generates a linear prophage chromosome with termini representing the *attL* and *attR* products of the integration reaction.

Like most temperate phages, bacteriophage Mx8 of *M. xanthus* integrates into the chromosome of its host by a mechanism similar to that for coliphage λ , as predicted by Campbell (3) over 25 years ago (Fig. 1). As for most temperate phages, the genetic elements necessary for the site-specific recombination of Mx8 are clustered on the phage genome. When subcloned onto a circular plasmid, these genes confer upon the plasmid the ability to integrate into the host chromosome.

Previously, we have shown that the addition of a 2.2-kb

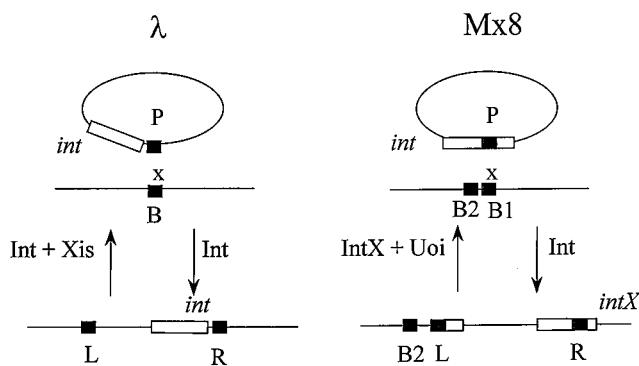


FIG. 1. Model for Mx8 prophage integration and excision. The integration of phage λ into its preferred site on the *E. coli* chromosome, *attB* (B), is catalyzed by the phage-encoded integrase (Int) enzyme, the product of the *int* gene. The *attB* \times *attP* (P) recombination event generates the *attL* (L) and *attR* (R) prophage junctions (3). Excision of the λ prophage involves the reciprocal *attL* \times *attR* event, in which the phage-encoded Xis (excisionase) protein controls the directionality of Int-catalyzed recombination. The integration of phage Mx8 into the *attB* locus on the *M. xanthus* chromosome is a more complex event. Integration can occur at either of two tandem sites (*attB1* and *attB2*) within the *attB* locus, catalyzed by the phage-encoded integrase (Int). The preferred product of integration, formed by recombination between the *attP* and *attB1* sites, is shown. Unlike the case for phage λ , the Mx8 *attP* site lies within the *int* gene, and the integration event changes the 3' end of *int* and the C terminus of its predicted product. We presume that excision of the Mx8 prophage is catalyzed by the modified, prophage-encoded integrase (IntX) and may be stimulated by the Mx8 Uoi protein, the predicted sequence of which is similar to those of other excisionases (28).

fragment of Mx8 DNA to Km^r plasmid pBGS18 enables the recombinant plasmid, pAY721, to integrate into the *attB* locus of *M. xanthus* (28). The Mx8 DNA fragment subcloned in pAY721 begins about 500 bp upstream of the first of two *int* start codons and ends at the *int* stop codon (Fig. 2). The *int* gene has two alternate translation start codons, GTG-5085 and GTG-5208. Derivatives of plasmid pAY721 in which only one of these GTG codons has been changed to GCG can form Km^r recombinants after electroporation into a sensitive *M. xanthus* host. In contrast, an otherwise isogenic plasmid in which both GTG codons have been changed to GCG cannot form Km^r recombinants (28).

To map the *int* promoter, we determined the extent of sequences within this 500-bp upstream region required for Int-mediated site-specific recombination and made two derivatives of pAY721 with shorter upstream regions. These derivatives, pAY722 and pAY759, contain 277 and 106 bp, respectively, upstream of GTG-5085. Both of these deletion derivatives give rise to Km^r recombinants when electroporated into host DZ1 and retain the ability to integrate (see Fig. 2). These results show that the natural Mx8 promoter for *int* must lie within 106 bp upstream of its translation start codon GTG-5085.

A critical element of the *int* promoter lies within the upstream *uoi* gene. Both of these deletion derivatives, pAY722 and pAY759, also retain the *uoi* gene, which is predicted to encode a product of 74 amino acids. The sequence of the Uoi protein is similar to those of other phage and plasmid excisionases (28), site-specific DNA-binding proteins that control the directionality of the Int-mediated site-specific recombination reaction. pAY759 has only 12 bp of Mx8 DNA upstream of the start codon of *uoi* and is likely missing the *uoi* promoter. However, we considered the possibility that *uoi* might be required for integration and fortuitously expressed from this plasmid, because some temperate phages require the products of several genes for site-specific integration. To exclude this possibility, we made a derivative of pAY721, pAY995, with the

change of an ACG codon (Ser14) of *uoi* to TAG (amber). To our surprise, we found that this plasmid lost the ability to integrate (Fig. 2).

The *uoi-14*(Am) mutation could result in a loss of function for one of two possible reasons. Either both *uoi* and *int* are required in *trans* for integration or the *uoi-14* mutation inactivates the *int* promoter. To distinguish between these possibilities, we examined whether a plasmid with the *uoi-14* mutation and a plasmid with an *int* mutation could complement one another for integration. In this complementation test, as for its controls, we assayed plasmid integration after the coelectroporation of two different plasmids.

As shown in Table 2, Sp^r Sm^r plasmid pAY952, with the same subcloned region of Mx8 DNA as that in Km^r integration-proficient plasmid pAY721, gives rise to Sp^r Sm^r electroporants efficiently after electroporation into sensitive host DZ1. Km^r plasmid pAY980 is a derivative of pAY721 with a substitution of a short polylinker sequence for a large deletion (bp 5403 to 5851) internal to *int* (*int* Δ 5403/5851) that interrupts conserved domain I of integrase (Fig. 2). As expected, pAY980 (*int* Δ 5403/5851) cannot integrate (Table 2). However, when plasmids pAY952 (*int*⁺ Sp^r Sm^r) and pAY980 (*int* Δ 5403/5851 Km^r) are coelectroporated into DZ1, Km^r recombinants arise, and a significant fraction of these are Sp^r Sm^r . These results show that pAY980 (*int* Δ 5403/5851) has a functional *attP* site and that *int* function supplied by pAY952 can act in *trans* to mediate the integration of pAY980. Although pAY995 (*uoi-14* Km^r) cannot integrate, it too can be complemented for integration by pAY952 upon coelectroporation. However, when pAY980 (*int* Δ 5403/5851 Km^r) is coelectroporated with pAY995 (*uoi-14* Km^r), Km^r electroporants arise only at a low efficiency. This result suggests that the *uoi-14* mutation acts in *cis* to impair integration and inactivates the *int* promoter. It is likely that the defect caused by the *uoi-14* mutation is not due to a polar effect of this amber mutation. pAY982, an otherwise isogenic derivative of pAY721 with the *int-1*(Am) mutation, which replaces the first alternate start codon of *int*, GTG-5085, with TAG, can integrate as efficiently as its pAY721 parent (Fig. 2).

The *int* gene is the only Mx8 gene required in *trans* for integration. If the *uoi-14* mutation inactivates the *int* promoter, then the expression of *int* alone (in the absence of *uoi*) from a heterologous promoter should be sufficient to promote plasmid integration. We have shown that when the phage Mx8 *mox* gene (18), the *M. xanthus* *sglK* gene (38), or the *E. coli* *glk* gene (unpublished results) is subcloned into pAY703 and the subclones are integrated by homologous recombination into the *M. xanthus* *mgl* locus, the genes are expressed as part of the *mglBA* operon. Therefore, we made a series of plasmids in which the *int* gene or versions of the *int* gene truncated at the 5' end are expressed from the constitutive *mglBA* promoter. Portions of the *int* region in plasmid pAY721 were amplified and subcloned into plasmid pAY703 immediately downstream of *mglA*, the more distal gene in the *mgl* operon.

Because pAY703 has the *mglBA* genes, it can integrate into the *mgl* locus of DZ1 by homologous recombination and can give rise to Km^r recombinants after electroporation of this host (18). When we add the *int-attP* genes to pAY703, we give this plasmid the option of integrating either at the *mgl* locus by homologous recombination or at the *attB* locus by site-specific recombination. Site-specific recombination prevails over homologous recombination. Stephens and Kaiser (35) have shown that a plasmid with both the *mglBA* and the *int-attP* genes integrates preferentially at the *attB* locus.

Figure 3 shows that when *int* alone is expressed from the *mgl* promoter on pAY703, starting with either GTG-5085

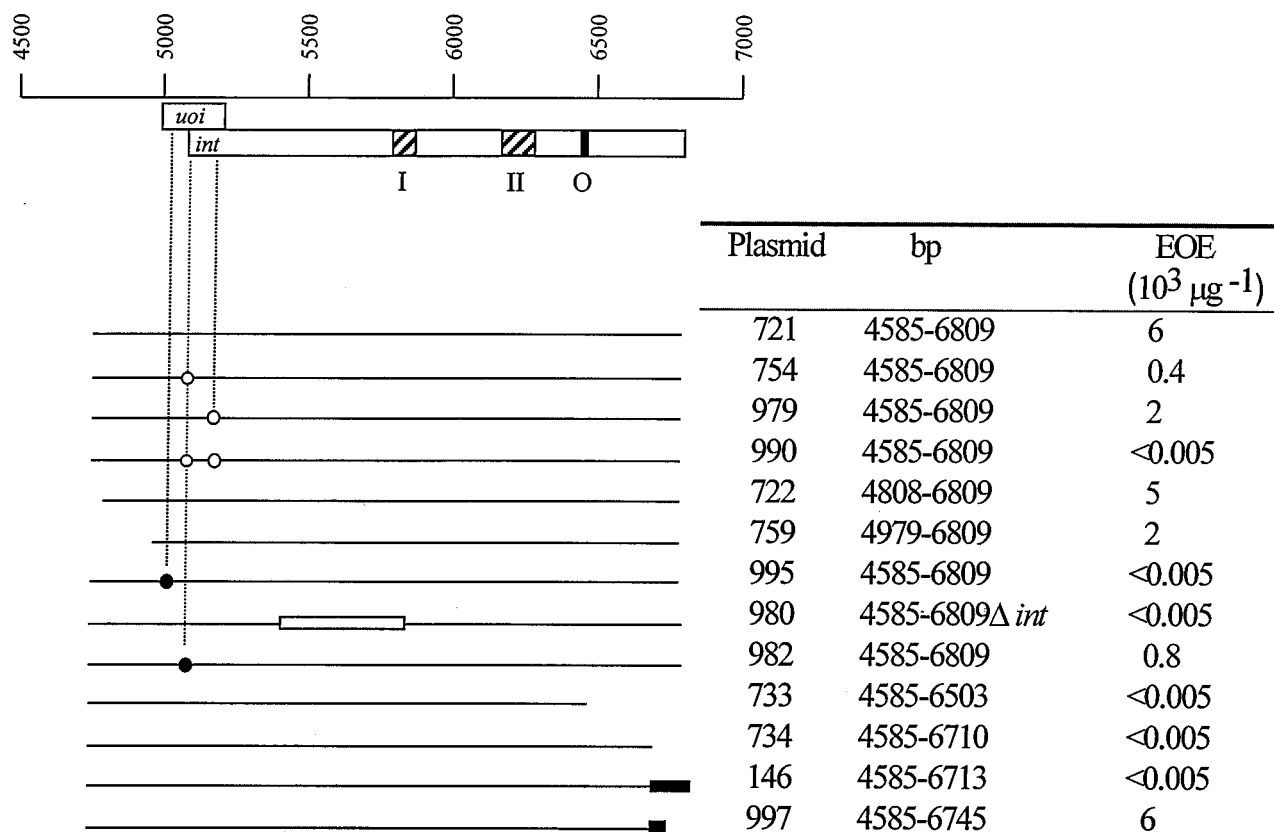


FIG. 2. Genetic elements required for the integration of plasmids with Mx8 DNA inserts. Coordinates of Mx8 DNA inserts (base pairs), based on GenBank accession no. U64984 (28), are shown above the open boxes representing the *uoi* and *int* coding sequences (top) which, like the coliphage λ *xis* and *int* genes (10), overlap. The hatched regions in the *int* gene correspond to conserved domains I and II of integrases (1, 15); conserved domain II includes the active-site tyrosine residue (23). The filled region within *int*, O, is the *attP* common core. The extents of Mx8 DNA inserts present in each plasmid are shown as horizontal thin lines (bottom left). Mutant plasmids are derived from pAY721, which contains a 2.2-kb region of the Mx8 genome with the functional *int-attP* genes. pAY754, pAY979, and pAY990 have missense GTG-to-GCG changes (open circles) of one or both of the two alternate *int* start codons (28). pAY722 and pAY759 have deletions extending into the 3' end of the 2.2-kb region. pAY980 is a derivative of pAY721 with a deletion, Δ *int-5403/5851* (open box), that removes a portion of domain I. pAY995 and pAY982 have amber codons (filled circles) in place of codon 14 of *uoi* and the first start codon (GTG-5085) of *int*, respectively. pAY734 and pAY733 are deletion derivatives with *int* coding sequences that terminate prematurely at engineered stop codons. pAY146 is a deletion derivative of pAY721 that makes a mutant integrase with an altered C terminus (small black box) encoded by adjacent vector sequences. The new C terminus of the active integrase made by pAY997 is produced from a shifted reading frame within *int*, translated due to the presence of a 4-bp insertion introduced into the 3' end of *int* by filling in of its internal, unique *NotI* site. The efficiencies of electroporation (EOE) of the plasmids are the numbers of Km^r recombinants arising per microgram of DNA electroporated into host DZ1 and are the averages of at least three independent determinations (see Materials and Methods).

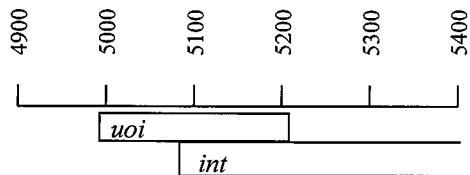
(pAY971) or GTG-5208 (pAY972), Km^r electroporants are obtained at high efficiencies. These recombinants are formed by site-specific recombination. When chromosomal DNA is isolated from these recombinants and amplified with the *attL* and *attR* primer pairs, products of the expected sizes are ob-

served, indicating the presence of recombinant *attL* and *attR* sites. This result confirms that *uoi* is not required for integration. When otherwise isogenic derivatives of pAY703 that express 5' truncated versions of *int* beginning at potential start codons ATG-5250 (pAY987), GTG-5310 (pAY992), and

TABLE 2. The *uoi-14* mutation reduces the activity of the *int* promoter^a

Plasmid(s) [relevant genotype(s)]	EOE (10 ³ μg ⁻¹) for the following recombinants:		% Sm ^s Sp ^s electroporants
	Sm ^r Sp ^r	Km ^r	
pAY952 (<i>int</i> ⁺ <i>attP</i> ⁺)	4.3 × 10 ⁵		100
pAY980 (Δ <i>int-5403/5851</i>)		<0.005	
pAY995 (<i>uoi-14</i>)		<0.005	
pAY952 (<i>int</i> ⁺ <i>attP</i> ⁺) + pAY980 (Δ <i>int-5403/5851</i>)	7.0 × 10 ⁵	0.94	50
pAY952 (<i>int</i> ⁺ <i>attP</i> ⁺) + pAY995 (<i>uoi-14</i>)	5.0 × 10 ⁵	0.81	58
pAY980 (Δ <i>int-5403/5851</i>) + pAY995 (<i>uoi-14</i>)		0.008	

^a The efficiency of electroporation (EOE) of each plasmid alone or in coelectroporations was measured as the numbers of either Sp^r Sm^r or Km^r recombinants of host DZ1 as described in Materials and Methods. To determine the percentage of Sp^s Sm^s electroporants, 100 independent Km^r electroporants were plated on CTPM medium with spectinomycin and streptomycin, and growth was scored after 72 h of incubation at 32°C.



Plasmid	Genotype	Start Codon <i>int</i>	EOE (10 ³ μg ⁻¹)	Fraction in <i>attB1</i>
pAY971	<i>int</i> ⁺	GTG-5085	5	8/8
pAY972	<i>int</i> ⁺	GTG-5208	8	8/8
pAY987	Δ <i>int</i>	ATG-5250	6	0/8
pAY992	Δ <i>int</i>	GTG-5310	16	0/8
pAY993	Δ <i>int</i>	GTG-5349	25	0/8

FIG. 3. The *int* gene is the only Mx8 gene required in *trans* for site-specific integration. Amplified regions of the *int* gene beginning with its first five potential translation start codons and ending with its stop codon were subcloned downstream of the *mglBA* genes on plasmid pAY703 (18). The 5' ends of the portions of Mx8 DNA subcloned into these derivatives of pAY703 are shown as horizontal lines below the map of *uoi* and the 5' end of *int*. The relative positions of these genes with respect to the Mx8 *imm* sequence are shown (see Fig. 5). Each of the plasmids expresses *int* or a 5' truncated version of *int* as the third gene in the constitutive *mgl* operon (arrows). All five plasmids give rise to Km^r recombinants when electroporated into host DZ1. These can arise due to either Int-mediated site-specific recombination into the *attB* locus or homologous recombination into the *mgl* locus (35). When DNAs isolated from eight independent recombinants carrying integrated plasmids pAY971 and pAY972 are amplified with the *attL* primer pair, all of these recombinants yield an *attL* junction site fragment, indicating that they have integrated at *attB* (see Fig. 6). In contrast, none of eight independent recombinants carrying plasmids pAY987, pAY992, and pAY993 yield an *attL* junction fragment in the same assay. EOE, efficiency of electroporation.

GTG-5349 (pAY993) are electroporated into host DZ1, a different result is obtained. All of these Km^r recombinants carry plasmids integrated at the *mgl* locus. When chromosomal DNA is isolated from these recombinants and amplified, a primer pair specific for *attL* or *attR* yields no amplification products, whereas an *attP*-specific primer pair yields a product of the expected size (data not shown). These results indicate that the 5' coding sequence between GTG-5208 and ATG-5250 is essential for *int* function and are consistent with our finding that *int* has two alternate start codons, GTG-5085 and GTG-5208 (28).

The C-terminal 30 amino acids of Int are dispensable for integrase activity. To define the extent of the *int* coding sequence distal to the *attP* core required in *trans* for the *attP* × *attB* reaction, we made additional derivatives of plasmid

pAY721. Deletion derivatives pAY733 and pAY734, in which the *int* coding sequence is terminated prematurely by amber stop codons in place of codons Leu474 (bp 6504) and Ala543 (bp 6711), respectively, do not integrate after electroporation into host DZ1 (Fig. 2). Neither does plasmid pAY146, in which the coding sequence for 56 amino acids in the N-terminal region of LacZ is added to the 3' end of *int*, terminating with Ala543.

In contrast, plasmid pAY997, made by filling in the unique *NotI* site within *int*, does integrate. This plasmid is predicted to encode a version of the Int protein that diverges in primary sequence from the wild type after Ala544 and has 10 amino acids added after this residue before translation termination (Fig. 4). A derivative of pAY50 with a large insert of DNA present at the filled-in *NotI* site also is able to integrate (data

Plasmid	C-terminal sequence	Integration
pAY721	<u>‡GLEP</u> VLLGSFYANPRL-60-SLMALERLAAALHRTHAADDRWSDCPACRAVADGESPSAT	+
pAY997	<u>‡GLEP</u> VLLGSFYANPRL-60-SLMALERLAA <u>GR</u> PAPHP <u>CRR</u>	+
pAY146	<u>‡GLEP</u> VLLGSFYANPRL-60-SLMALERL <u>ASLALAVVLQRRD</u> WENPGVTQLNRLAAHPPFA... * * * * *	-
pAY734	<u>‡GLEP</u> VLLGSFYANPRL-60-SLMALERL	-
pAY733	<u>‡GLEP</u> VLLGSFYANPRL	-
pAY999	<u>‡GLEP</u> VASGVTGRRSNQLN	+

FIG. 4. The last 30 amino acid residues of the Int C terminus are not required for integration. The predicted amino acid sequences of the C termini of the mutant integrases made by plasmids with changes in the 3' end of *int* (see Fig. 2) are shown; 60 refers to a stretch of 60 residues in the wild-type sequence that is not shown. Residues corresponding to the common core are shown in bold, and those encoded by vector sequences are underlined. Residues conserved between new C termini and the wild-type C terminus are indicated by asterisks. Integration-proficient plasmids pAY721 and pAY997 diverge in sequence after residue Ala544. pAY146, pAY734, and pAY733, which either change or remove Ala544, cannot integrate. In the accompanying paper (19), we show that the IntX integrase encoded by plasmid pAY999 is active.

not shown). Taken together, these results suggest that the most C-terminal 30 amino acid residues of Int are not essential for *attP* × *attB* recombination but that C-terminal alterations of Int that are predicted to remove 31 or more amino acids from the C terminus of Int abolish integration (Fig. 4).

Tojo et al. (37) reported that a similar plasmid with the filled-in *NotI* site lost the ability to integrate. However, they based their conclusion on their failure to find recombinants and on the sequence determination of the mutational change. To draw our conclusions, we have quantified the average efficiency of electroporation from at least three independent determinations for each plasmid and have measured the efficiency of electroporation of pAY721 as an internal, positive control for each preparation of electrocompetent host cells. In addition, we confirmed the genotype of the mutation carried by this plasmid by a method that does not rely solely on DNA sequence (see Materials and Methods). The dideoxy method (30) yields a highly compressed sequence ladder across this region of *int* with this mutation and is difficult to interpret.

Structure of the *attB* locus: two *M. xanthus* *attB* sites for phage Mx8 integration lie within a cluster of three tandem tRNA genes. Tojo et al. (37) determined the sequences of the prophage junction sites, *attL* and *attR*, formed upon the integration of phage Mx8 into its preferred locus on the *M. xanthus* genome, *attB*. Their analysis revealed the surprising result that the Mx8 *attP* site lies within the *int* coding sequence. This result predicts that, upon integration of the Mx8 prophage, the sequence of the *int* gene will be modified by the integration reaction catalyzed by its product. If this is the case, the integration will result in the formation of a lysogen in which the recombinant derivative of the *int* gene, *intX*, encodes a product with a different C terminus. The C terminus of the prophage-encoded IntX protein is predicted to have 13 amino acid residues in place of the 112 residues that terminate the phage-encoded Int protein. However, our 3' deletion analysis of the phage *int* gene shows that substitution mutations that are predicted to alter more than 30 C-terminal residues of the Int protein result in a loss of function. From these results, we might predict that the product of the prophage *intX* gene should be inactive.

On the other hand, lysogens of *M. xanthus* carrying a single Mx8 prophage release infectious particles by spontaneous induction. When DNA is isolated from these phage particles and analyzed, the *int* gene is found to be identical in structure to that of the parental phage used to construct the lysogens (data not shown). For Mx8, as for other temperate phages, we expect that prophage excision as well as integration will depend on integrase. If the excision of Mx8 depends on the prophage *intX* gene, then the product of *intX* should be active.

Therefore, we examined whether the *attP* site lies within the *int* gene and whether the prophage *intX* gene encodes an active product. In this study, we confirm the findings of Tojo et al. (37) and show that, indeed, the *attP* site is within *int*. We also find that the integration of a plasmid with the *int-attP* genes into the *M. xanthus* genome can occur at two different *attB* sites, *attB1* and *attB2*. The integration of Mx8 causes changes in the primary structures of two transcription units, the phage *int* gene and either of two *M. xanthus* tRNA genes, within which the *attB* sites lie. In the accompanying paper (19), we show that, indeed, the *intX* gene encodes an active product, and we measure the relative abilities of the Int and IntX integrases to catalyze a variety of site-specific recombination reactions.

To confirm that *attP* lies within *int* and to characterize the *attP* and *attB* sites required in *cis* for integrative recombination, we first confirmed the sequence of the *attB* locus. From the

published sequences of the *attL* and *attR* prophage junction sites, we designed oligonucleotide primers and used PCR to amplify and clone the *attB* locus from both wild-type *M. xanthus* DK1622 and its mutant derivative DZ1. The sequence of the *attB* locus was found to be identical for both strains. Analysis of this sequence shows that Mx8 integrates into the second gene of a cluster of three tRNA genes, *trnD2*, *trnD1*, and *trnV1* (Fig. 5). Each of these genes encodes a class I or a class II tRNA (13, 40) with 4 bp in the stem of the dihydrouracil arm, 5 bases in the variable loop, and the 13 invariant bases shared by all tRNA molecules. Whereas *trnD1* has a CCA acceptor stem within its coding sequence, the coding sequence for *trnD2* terminates with CTA, and that for *trnV1* terminates with CTG. We presume that, as for other class II tRNAs, the CCA acceptor stem is added enzymatically to the products of the class II *trnD2* and *trnV1* genes during their maturation.

The *attB1* and *attB2* sites, located at the 3' ends of tandem tRNA^{ASP} genes, are both functional, but integration into the *attB1* site is highly favored. As diagrammed in Fig. 6, when pAY721 is electroporated into *M. xanthus*, Km^r recombinants can arise from site-specific recombination events involving the *attP* site on the plasmid and one or both of the *attB* sites. Alignment of the regions of homology between *attP* and the 3' ends of *trnD1* and *trnD2* shows that all three sites have a common core of 26 bp within which site-specific recombination occurs (Fig. 7). Overlapping this area of homology, the *attP* site has a 29-bp sequence with striking dyad symmetry consisting of 7 bp flanked by inverted repeats of 11 bp. A shorter, dyad symmetry element consisting of this 7-bp sequence flanked by 5-bp repeats defines the common core, designated O. In the 5' direction, the homology between *attP* and each of the tRNA genes extends beyond these 5-bp inverted repeats by 6 bp for *attB1* (*trnD1*) but by only 3 bp for *attB2* (*trnD2*). In the 3' direction, both *trnD* genes share the same 48-bp (bottom-strand) coding sequence but diverge in sequence immediately before the first nucleotide of the coding sequence.

The integration of a plasmid or a phage into either tRNA^{ASP} gene, *trnD1* or *trnD2*, results in the simultaneous alteration of two different transcription units, one on the plasmid (or phage) and one on the host genome (Fig. 7). This is because *attP* lies within *int* and an *attB* core lies at the 3' end of each *trnD* gene, before its transcription terminator. The change in the *int* gene results in a gene, *intX*, encoding an integrase with a new C terminus. This new C terminus of IntX is the same whether integration occurs at *attB1* or at *attB2*. The change in the *trnD1* and *trnD2* transcription units involves the replacement of the natural terminators for these tRNA genes with an Mx8-specified terminator, t_{Mx8}. The t_{Mx8} sequence is embedded within the antisense strand of the *int* gene, immediately distal to the *attP* core.

The majority of Km^r recombinants are formed by the simple integration of pAY721 into the *attB1* site within the 3' end of *trnD1*. A minority of Km^r recombinants are formed by the integration of pAY721 into either the *attB1* or the *attB2* site accompanied by a deletion between *attB1* and *attB2*. Extremely rarely, Km^r recombinants are formed by the simple integration of pAY721 (or related *int-attP* plasmids) into *attB2*. As shown in Fig. 8, when DNA is isolated from eight independent Km^r electroporants of pAY721 and amplified with primer pairs for the *attL* and *attR* sites, five of eight templates (lanes a, d, e, f, and g) give rise to a 692-bp *attL* product and an 840-bp *attR* product. This is the result expected for strains carrying plasmids that have integrated into the (preferred) *attB1* site. All of the DNA templates from these five strains give rise to an *attP* product of 969 bp, the size of the *attP* product obtained with circular plasmid pAY721 DNA as a template. This observation

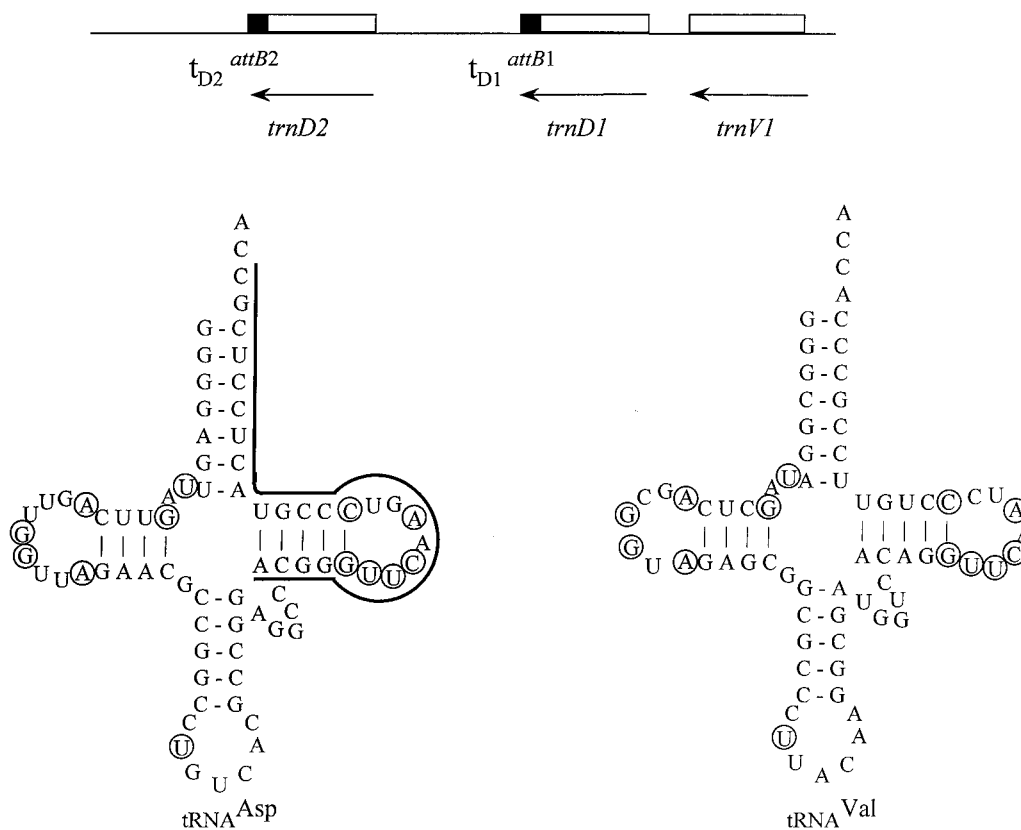


FIG. 5. Structure of the *M. xanthus* *attB* locus and its tRNA products. (Top) The *attB* locus contains three tandem tRNA genes, *trnD2*, *trnD1*, and *trnV1*, represented as open boxes. All three genes are transcribed from right to left, as indicated by the arrows. The two filled portions at the 3' ends of the *trnD1* and *trnD2* genes indicate the positions of the 26-bp core sequence shared by the *attB2*, *attB1*, and phage Mx8 *attP* sites, the region of homology in which *attP* × *attB* recombination occurs. Two terminators, *t*_{D1} and *t*_{D2}, follow the tRNA^{Asp} genes that overlap the two *attB* sites within the *attB* locus. The *trnV1* gene, which encodes tRNA^{Val}, is located immediately upstream of *trnD1*, and these two genes may be cotranscribed. (Bottom) Predicted cloverleaf secondary structures of the tRNA^{Asp} and tRNA^{Val} products of these two genes. Circled nucleotides are the invariant tRNA bases (13, 40).

shows either that multiple copies of pAY721 can integrate into the same *attB1* site or that, in some cells, pAY721 has excised from the *M. xanthus* chromosome.

The latter argument is sufficient to explain this observation, because it is known that integrated plasmid pAY721 can undergo excision in these recombinants to generate free, circular forms of plasmid DNA. Furthermore, when total chromosomal DNA is prepared from these Km^r recombinants and electroporated into *E. coli*, Km^r electroporants with circular plasmid pAY721 are recovered at low, but reproducible, efficiencies (ca. 10 to 100 μg⁻¹). Thus, Km^r *M. xanthus* strains with pAY721 carry the plasmid in a dynamic equilibrium between integrated and excised forms.

Integration of a plasmid with the functional *int-attP* genes often is accompanied by a deletion within the *attB* locus. Three of the electroporants analyzed in Fig. 8 (lanes b, c, and h) give rise to a mixture of amplified *attL* products but yield a single *attR* product of 840 bp, indicating that the right arm of the right prophage attachment site is derived from *attB1*. None of these recombinants gives rise to an amplified *attP* product of 1,135 bp, indicating that they do not have two copies of pAY721, one integrated at *attB1* and another integrated at *attB2* (Fig. 6). When segregants of these colonies are analyzed, many of them yield an *attL* fragment of the predicted size for a plasmid that has integrated into the *attB2* site and an *attR* fragment of the predicted size for a plasmid that has integrated into the *attB1* site. These segregants yield an *attP* product of 969 bp, not 1,135

bp. Together, these data show that a deletion between the *attB2* and *attB1* sites has occurred in these strains upon integration. Among 100 independent Km^r strains arising from the integration of pAY721 (and other, related *int-attP* plasmids) that we have analyzed by using the PCR with the *attL* and *attR* primers, only 3 have been found to have plasmids integrated only at *attB2* (data not shown).

The *int* promoter is repressed in the prophage state. When plasmids with the Mx8 *attP-int* genes are integrated at the *attB* locus, the recombinant strains maintain the integrated plasmids stably. This result suggests that, like the expression of other phage *int* genes, such as the phage λ *int* gene (31), the expression of the Mx8 *intX* gene may be repressed during the maintenance of the prophage state. Furthermore, we and others have found that when fusions of *M. xanthus* genes to the *lacZ* gene are subcloned onto a plasmid with the Mx8 *int-attP* genes and then integrated at the *attB* locus, these fusions are expressed at lower levels from the *attB* locus than from merodiploids with these fusions integrated at their normal chromosomal loci (8, 16). These results suggest that when *M. xanthus* genes are integrated ectopically at the *attB* locus, their expression is silenced.

Surprisingly, Tojo et al. (37) have reported that β-galactosidase activity is expressed from *int-lacZ* translational fusions on integrated plasmids at relatively high levels. To determine whether this is the case in our study as well, we constructed plasmids pAY738 and pAY739 with the *int* promoter and in-

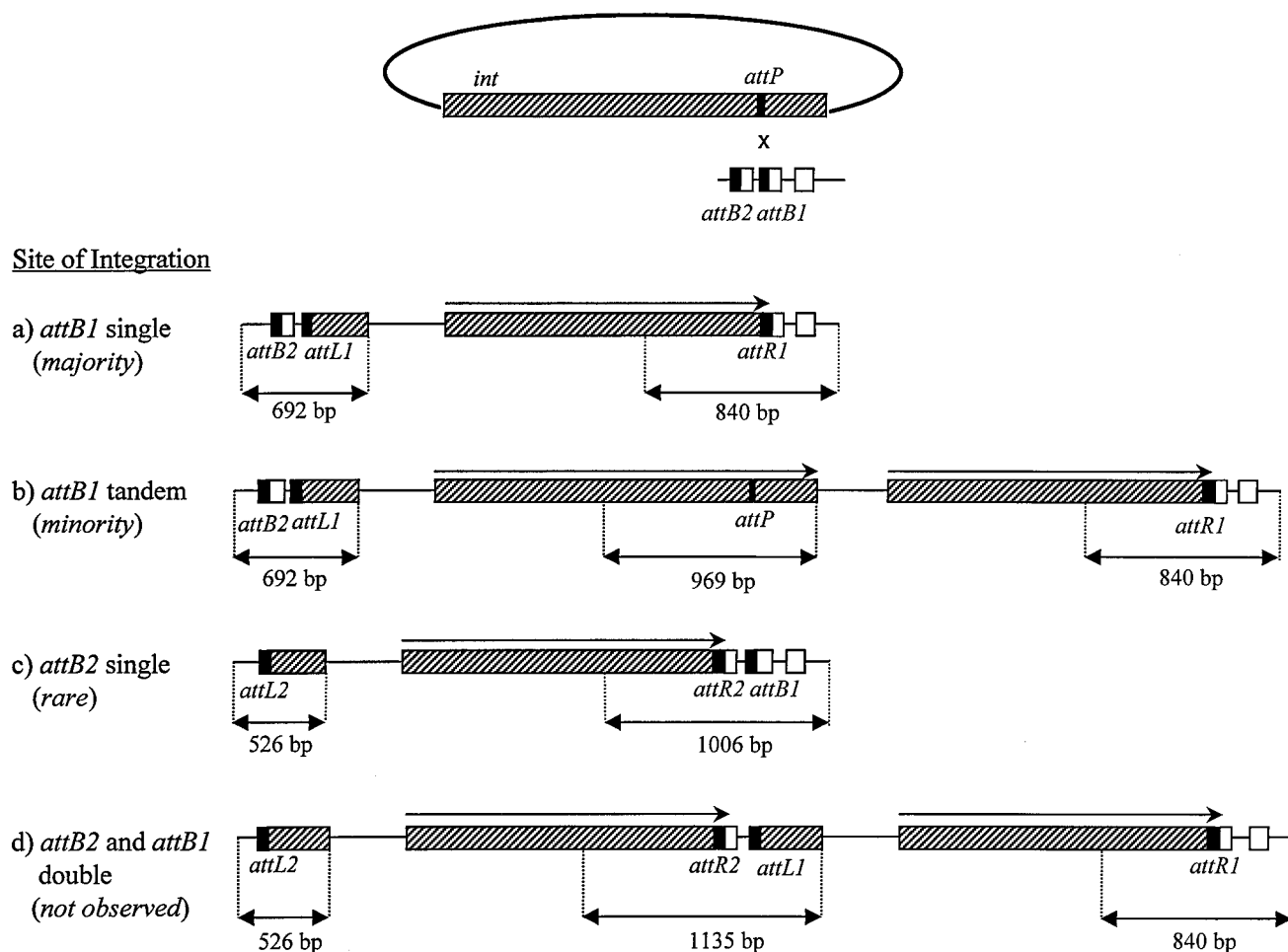


FIG. 6. PCR can be used to distinguish the possible products of plasmid integration. Integration of a plasmid at the *attB1* site generates prophage junctions that, when amplified with the *attL* and *attR* primer pairs, yield fragments of 692 and 840 bp, respectively. If integration at *attB1* (or *attB2*) is accompanied by a deletion between the tandem *attB* sites, then we observe *attL* and *attR* products of 562 and 840 bp. Integration at *attB2* generates junctions that yield amplified fragments of 526 and 1,006 bp. If two copies of a plasmid were to integrate, one at *attB1* and the other at *attB2*, then amplification of the *attP* junction site would yield a product of 1,135 bp.

frame translational fusions of each of the two GTG start codons of *int* to the *lacZ* structural gene on Km^r plasmid pRS552 (32). These recombinant plasmids were integrated by homologous recombination into a lysogen of host DZ1 carrying wild-type Mx8 as a prophage. The β -galactosidase activity produced from the expression of the *int-lacZ* fusion genes in the recombinants was assayed. Each of the strains was found to produce <1 nmol of β -galactosidase activity per min per mg, a specific activity indistinguishable from the background level of activity made by the lysogen alone or present in control assays with no added source of enzyme. This result shows that the *int* promoter is expressed at an extremely low level from prophage Mx8.

We can account for the discrepancy between our results and those reported by Tojo et al. (37) because they did not control for the possibility that their *int-lacZ* fusions were expressed from a fortuitous upstream promoter within their plasmid vector sequence. Because we integrated our plasmids carrying *int-lacZ* fusion constructs by homologous recombination into an Mx8 prophage, our fusions could be expressed only from an upstream Mx8 promoter. Therefore, we can conclude that the expression of the *int* gene and likely of the *int* promoter is repressed in the prophage state.

DISCUSSION

Mx8 integrates into a preferred target locus, *attB*, on the *M. xanthus* genome. The only phage-encoded function required for the integration reaction in *trans* is that of integrase, the product of the Mx8 *int* gene, which has two alternate translation start codons. The *int* promoter is located immediately upstream of the *int* structural gene in a small, 106-bp region, and at least one sequence element required for promoter function lies within the overlapping *uoi* gene, which may encode an excisionase function.

Deletion analysis of the 3' end of the *int* gene shows that, although the last 30 amino acid residues are not critical for Int function, deletions that are predicted to truncate the Int protein and alter the last 31 or more residues abolish function. Although this result predicts that the prophage-encoded IntX integrase should be inactive, the Mx8 prophage, as well as plasmids carrying functional *int-attP* genes that have integrated into the *M. xanthus* chromosome, can excise from the chromosome. The excision is site specific, because it regenerates a functional *int* gene with its internal *attP* site and occurs spontaneously at a measurable frequency. In the accompanying paper (19), we show that the prophage *intX* gene encodes an

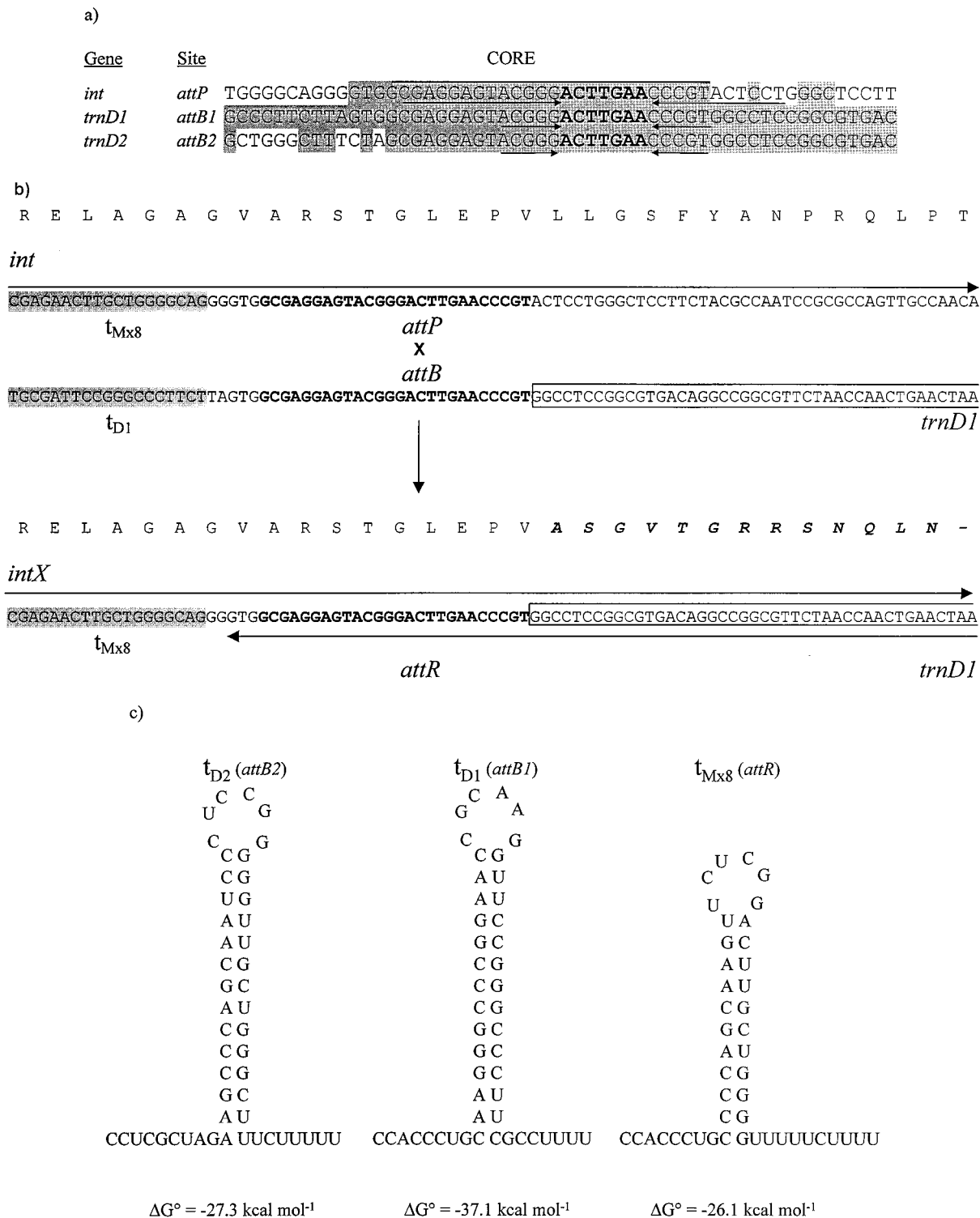


FIG. 7. The integration of Mx8 or plasmids carrying the Mx8 *int-attP* genes results in changes in two transcription units. (a) The top-strand sequences of portions of the *attP*, *attB1*, and *attB2* sites, located within the *int*, *trnD1*, and *trnD2* genes, respectively, are aligned to show the 26-bp core sequence shared by these sites. Dyad sequences (arrows) flank the likely 7-bp target of cleavage and joining (bold). Bases identical to the corresponding bases in the preferred *attB1* bacterial attachment site are shaded. (b) Embedded in the *int* coding sequence lies a terminator sequence, t_{Mx8} (shaded), and the common core of *attP* (bold). A portion of the amino acid sequence of integrase is shown above the *int* coding sequence. Arrows indicate the directions of *int* and *trnD1* transcription. The 3' end of *trnD1* contains the common core of *attB1* (bold) and a terminator sequence, t_{D1} (shaded), distal to *attB*. The bottom sequence shows the *attR* product of *attP* × *attB* recombination in which the *int* coding sequence has been changed to *intX* and t_{Mx8} replaces the t_{D1} terminator. The *intX* coding sequence, which terminates at a TAA stop codon, encodes a less active integrase with a new C terminus. (c) Predicted stem-loop secondary structures of the *trnD* gene terminators. The terminator sequences distal to both the *trnD2* and the *trnD1* genes, t_{D2} and t_{D1}, respectively, are replaced by the weaker Mx8 terminator, t_{Mx8}, upon integration. Free energies for hairpin formation were calculated with the RNAFOLD program (5).

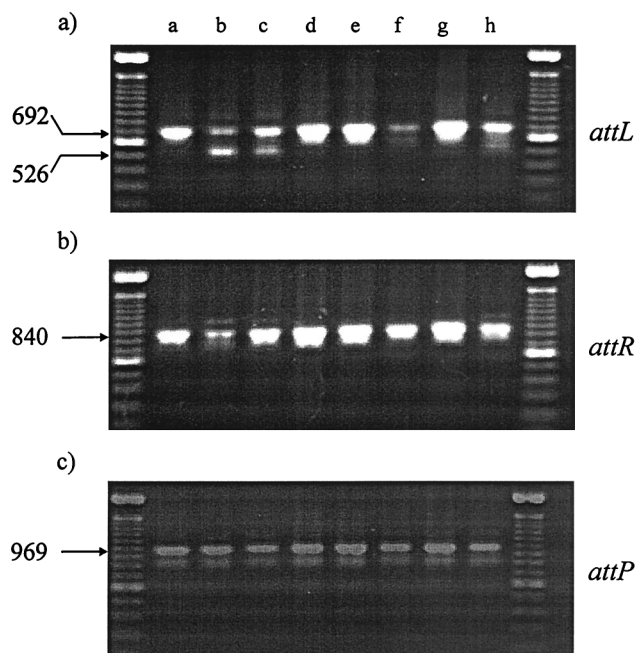


FIG. 8. Plasmid pAY721 prefers to integrate into the *attB1* site. Chromosomal DNA was isolated from eight independent Km^r electroporants of host strain DZ1 (lanes a to h) with plasmid pAY721 and amplified with the *attL* (a), *attR* (b), and *attP* (c) primer pairs as described in Materials and Methods. Amplified products were resolved by electrophoresis through a 1.5% agarose gel and visualized after staining with ethidium bromide. Size standards flanking the amplified products are the Gibco-BRL 100-bp ladder.

active integrase that can promote site-specific recombination events with a reduced efficiency relative to that of the phage-encoded *Int* protein.

Like many other temperate phages, as well as archaeobacterium virus SSV1 of *Sulfolobus shibatae* (27), the preferred site for the integration of Mx8 lies within the 3' end of a tRNA gene. The sequences of the three tRNA genes within the *attB* locus are the first reported sequences of tRNA genes for *M. xanthus* and offer few surprises. These three genes are predicted to encode tRNAs with all 13 invariant bases and the additional semivariant bases that contribute to tertiary tRNA structure (13, 40). In addition, these tRNAs are predicted to have a common secondary structure that includes a 7-bp amino acid acceptor stem, a 3- to 4-bp D stem with 7 to 10 bases in the D loop, a 5-bp anticodon stem, and a 7-base anticodon loop.

The common core within which site-specific recombination occurs between the Mx8 genome and its host genome is duplicated in close proximity on its host genome. Plasmids carrying the Mx8 *int* and *attP* functions required for site-specific recombination integrate frequently into the preferred *attB1* site within *trnD1* but only rarely into the *attB2* site within *trnD2*. For several temperate phages, including λ , the sequences within the *attB* locus necessary and sufficient for efficient site-specific recombination do not extend far beyond the core sequence. For λ , the core sequence is comprised of 7-bp imperfect inverted repeats flanking a unique 7-bp sequence, and only an additional flanking 2 bp is required for the full activity of *attB* (21); even less of the core homology is required for *attP* function (2).

If the same is true for Mx8, because there are two different *attB* core sequences within the Mx8 *attB* locus (Fig. 3), we might expect Mx8 to integrate into either site at the 3' end of either tRNA^{ASP} gene (into *attB1* or *attB2*) with approximately

equal efficiencies. This is the case for temperate corynephages β , ω , and γ , which can integrate into either of two different attachment sites on the *Corynebacterium diphtheriae* C7 chromosome with equal efficiencies (24, 25). Like the *M. xanthus* *attB1* and *attB2* sites, the *C. diphtheriae* *attB1* and *attB2* sites lie within the 3' ends of repeated tRNA genes close to one another on the host chromosome (26). When *tox*⁺ corynephages integrate at both sites, the lysogens are stable and produce higher levels of diphtheria toxin, perhaps conveying a selective advantage on their lysogenic hosts. In contrast, lysogens resulting from two corynephage genomes integrating at the same attachment site are unstable (24, 25).

The integration of Mx8 into the *M. xanthus* *attB1* and *attB2* sites follows different rules. Plasmids with the Mx8 *attP-int* genes prefer to integrate into the *attB1* site. Integration into the *attB1* site often results in a deletion between the *attB1* and *attB2* sites. Plasmids integrate only rarely into the *attB2* site. Differences in the sequences flanking the *attB1* and *attB2* core sequences likely contribute to this site preference for integration. The integration of Mx8 into the *M. xanthus* *attB* locus is unusual in another respect. Unlike the situation for most other integrative elements, Mx8 site-specific recombination involves an *attP* site located within the *int* gene, and the integration reaction changes the structure of the phage *int* gene by altering its 3' end to generate the prophage *intX* gene. In the accompanying paper (19), we show that and explain why Mx8 uses a mechanism of site-specific recombination in which the integration reaction serves to reduce the specific activity of the enzyme that catalyzes this reaction.

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