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Characterization of the Genetic Elements Required for Site-Specific Integration of Plasmid pSE211 in *Saccharopolyspora erythraea*

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The 18.1-kilobase plasmid pSE211 integrates into the chromosome of *Saccharopolyspora erythraea* at a specific *attB* site. Restriction analysis of the integrated plasmid, pSE211^{int}, and adjacent chromosomal sequences allowed identification of *attP*, the plasmid attachment site. Nucleotide sequencing of *attP*, *attB*, *attL*, and *attR* revealed a 57-base-pair sequence common to all sites with no duplications of adjacent plasmid or chromosomal sequences in the integrated state, indicating that integration takes place through conservative, reciprocal strand exchange. An analysis of the sequences indicated the presence of a putative gene for Phe-tRNA at *attB* which is preserved at *attL* after integration has occurred. A comparison of the *attB* sites for a number of actinomycete plasmids is presented. Integration at *attB* was also observed when a 2.4-kilobase segment of pSE211 containing *attP* and the adjacent plasmid sequence was used to transform a pSE211$^-$ host. Nucleotide sequencing of this segment revealed the presence of two complete open reading frames (ORFs) and a segment of a third ORF. The ORF adjacent to *attP* encodes a putative polypeptide 437 amino acids in length that shows similarity, at its C-terminal domain, to sequences of site-specific recombinases of the integrase family. The adjacent ORF encodes a putative 98-amino-acid basic polypeptide that contains a helix-turn-helix motif at its N terminus which corresponds to domains in the Xis proteins of a number of bacteriophages. A proposal for the function of this polypeptide is presented. The deduced amino acid sequence of the third ORF did not reveal similarites to polypeptide sequences in the current data banks.

Numerous bacterial genetic elements undergo site-specific integration into their host chromosomes. Examples include the genomes of bacteriophages (reviewed by Weisberg and Landy [51]), the e14 element from *Escherichia coli* K-12 (10), the viruslike particle SSV1 of the archebacterium *Sulfolobus* strain B12 (45), and several plasmids from actinomycetes, the gram-positive bacteria that form mycelia and produce antibiotics (4, 6, 11–13, 21, 22, 34, 37, 43, 48). In four well-studied instances of actinomycete plasmid integration—pMEA100 from *Nocardia mediterranei*, SLP1 from *Streptomyces coelicolor*, pSAM2 from *Streptomyces ambofaciens*, and pIJ408 from *Streptomyces glaucescens*—it was shown that integration resulted from nonduplicative recombination between cognate plasmid (*attP*) and chromosomal (*attB*) sites that were found to share a sequence of perfect homology ranging in length from 43 to 58 base pairs (bp) (7, 26, 28, 30, 38, 48). An analysis of the secondary structure of the *attB* sites of several plasmids and bacteriophages has led to the proposal that they are contained within structural tRNA genes (44, 45). For pSAM2, a 2.5-kilobase (kb) segment that consists of *attP* and two open reading frames (ORFs) whose translated sequences show structural similarity to the integrases and excisionases of bacteriophages was found to be sufficient to promote site-specific integration (8, 26).

Saccharopolyspora erythraea (until recently known as *Streptomyces erythraeus* [27]) produces the clinically important macrolide antibiotic erythromycin. In previous work, we showed that *S. erythraea* contains two distinct elements, pSE101 (11.3 kb) and pSE211 (18.3 kb), that are normally present as integrated sequences but which could be detected in the free form under certain conditions (11, 12). When introduced into *S. erythraea* strains devoid of the particular sequence, each element was observed to undergo site-specific integration into its cognate *attB* site (11, 12). A 47-bp segment of pSE101 (*attP* site) was found as a direct repeat at the left (*attL*) and right (*attR*) pSE101^{int}-chromosome junctions (12). When introduced into *Streptomyces lividans*, pSE101 was observed to either undergo stable autonomous replication or integrate into one of several chromosomal sites employing the *attP* site for crossover (11). The other *S. erythraea* element, pSE211, is self-transmissible, produces pocks (lethal zygosis phenotype) after conjugal transfer to pSE211$^-$ *S. erythraea* strains (12), and probably corresponds to the *S. erythraea* sex factor designated SEP1 by Dewitt (15). pSE211 is maintained exclusively in the autonomous state in *Streptomyces lividans* and does not appear to undergo integration in this host (D. P. Brown, unpublished results). As a first step in our efforts to gain a better understanding of the processes involved in plasmid integration, we sought to identify the plasmid-encoded sequences that determine or regulate the recombination events. In this report we demonstrate that a 2.4-kb segment of pSE211 is required and sufficient for site-specific integration. A detailed analysis of the nucleotide sequence of this segment is presented, and the potential roles that the corresponding polypeptides may play in the process are discussed. Comparisons of the deduced amino acid sequences with recombination enzymes are presented. We also describe the sequences of the plasmid and chromosomal attachment sites and show that the *attB* site for pSE211 corresponds to a phenylalanine-tRNA gene.

MATERIALS AND METHODS

Bacteria, plasmids, growth, and transformation. *S. erythraea* ER720 is Ltz^s (15) and is devoid of pSE211 sequences (12). *Escherichia coli* HB101 (9) and DH5α (F$^-$ *endA1 hsdR17 supE44 thi-1* λ$^-$ *recA1 gyrA96 relA1* φ80d*lacZ*ΔM15) and plasmids pUC9 (50) and pUC19 (33) were obtained from commercial sources. pIJ704 (23) was obtained from E. Katz.

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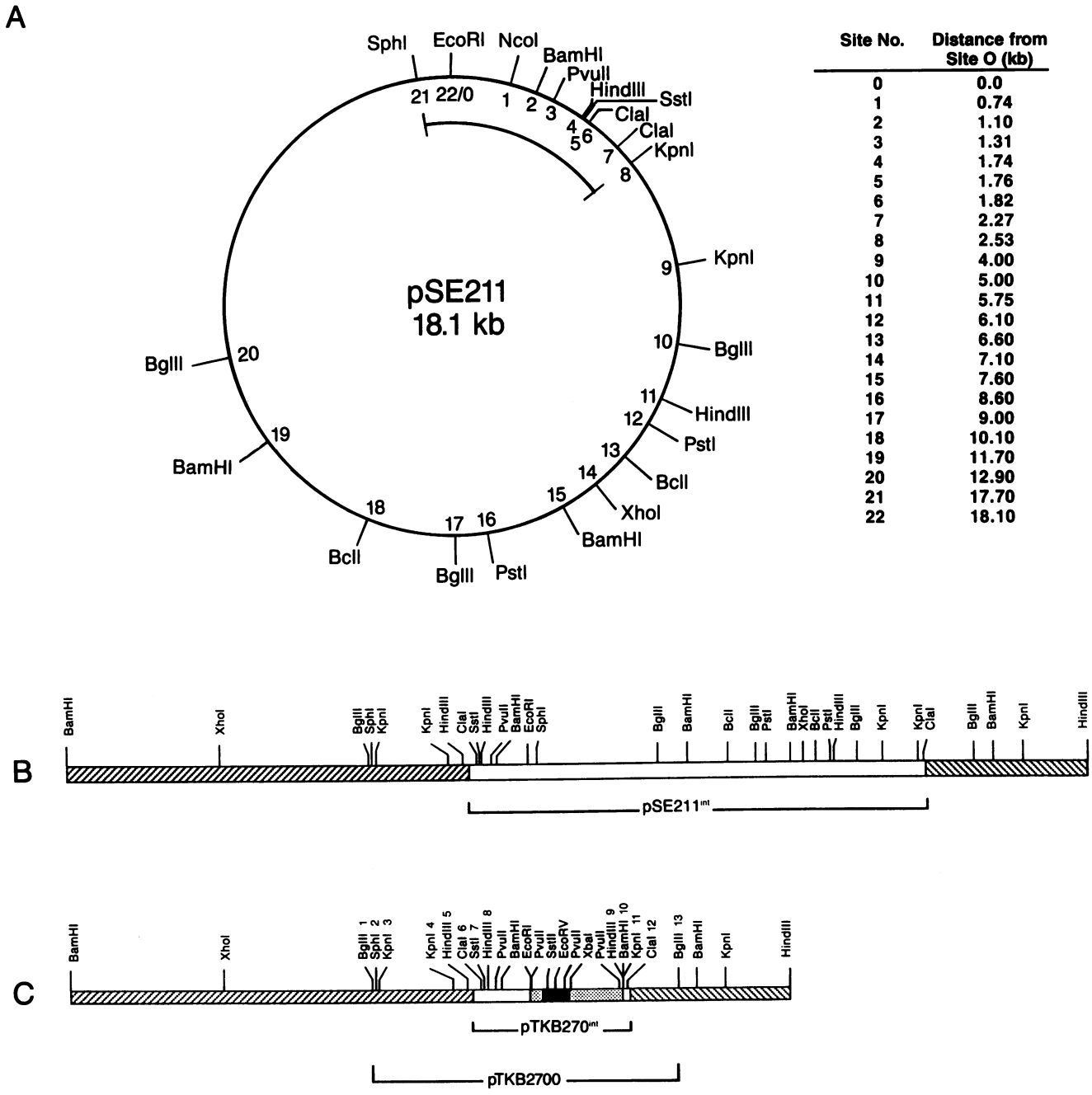


FIG. 1. Restriction maps of free (A) and integrated (B) pSE211 and integrated pTKB270 (C). Sites are numbered as shown. The inner arc corresponds to the segment sequenced in Fig. 7. Maps B and C: ▨, chromosomal DNA; □, pSE211 DNA; ■, *tsr* gene; ▩, pUC9 DNA. Plasmid pTKB2700 shown in map C is the *Bgl*II(1) through *Bgl*II(13) segment of chromosomal DNA excised from the chromosome of ER720(pTKB270^{int}) and circularized as described in Table 1, footnote *a*, and Materials and Methods.

Other plasmids constructed in this work are described below. The growth of *S. erythraea* and transformation of protoplasts employing selection for thiostrepton resistance (Thio^r) on regeneration medium has been described previously (11). Transformation of *E. coli* was performed as described previously (19), employing selection for ampicillin resistance and, where applicable, X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside; Bethesda Research Laboratories, Inc., Gaithersburg, Md.) to screen for transformants with a putative insertion at the multiple cloning site of pAL7015 (see below).

DNA isolations and Southern hybridizations. pSE211 (Fig. 1A) was isolated as described previously (12). Other plasmids were prepared from *E. coli* by the method of Birnboim and Doly (5) and purified by centrifugation through cesium chloride-ethidium bromide density gradients. Total cellular DNA prepared from *S. erythraea* employed density gradient purification as described by Hopwood et al. (20). Southern hybridizations were performed as described by Brown et al. (11).

Recovery of DNA from agarose gels. After electrophoresis of restriction endonuclease-digested DNA through agarose

TABLE 1. Plasmid constructions and summary of properties

Plasmid designation	Summary of construction and relevant properties[a]	Site-speciifc integration[b]
pAL7001	Inserton of *tsr* gene from pIJ704 in pUC9 at *Bam*HI site	ND[c]
pTKB12	Insertion of pAL7001 in pSE211 at *Eco*RI[0] site	+
pTKB22	Deletion of 1.5-kb *Kpn*I[8]–*Kpn*I[9] segment of pTKB12	+
pTKB1410	Deletion of 8-kb *Bgl*II[10]–*Bgl*II[20] segment of pTKB12	+
pAL7015	Insertion of *tsr* gene at *Ssp*I site of pUC19	ND
pTKB26	Insertion of 0.26-kb *Cla*I[7]–*Kpn*I[8] segment of pSE211 in pAL7015	−
pTKB130	Insertion of 1.3-kb *Bam*HI[2]–*Kpn*I[8] segment of pSE211 in pAL7015	−
pTKB170	Deletion of 0.73-kb *Eco*RI[0]–*Nco*I[1] segment of pTKB270	−[d]
pTKB270	Insertion of 2.5-kb *Eco*RI[0]–*Kpn*I[8] segment of pSE211 in pAL7015	+
pTKB300	Insertion of 3.0-kb *Sph*I[21]–*Kpn*I[8] segment of pSE211 in pAL7015	+
pTKB420	Insertion of 4.2-kb *Hin*dIII[4]–*Hin*dIII[11] segment of pSE211 in pAL7015	−
pTKB520	Insertion of 5.2-kb *Eco*RI[0]–*Bgl*II[10] segment of pSE211 in pAL7015	+
pTKB640	Insertion of 6.4-kb *Bam*HI[2]–BamHI[15] segment of pSE211 in pAL7015	−
pTKB2700	12-kb *Bgl*II(1)–*Bgl*II(13) chromosomal segment of ER720(pTKB270^{int}) (Fig. 1C) rescued in *E. coli*; contains pTKB270^{int}, *attL*, and *attR*	ND
pTKB2900	Insertion of 2.9-kb *Hin*dIII-*Bgl*II pTKB2700-hybridizing chromosomal fragment of *S. erythraea* ER720 in pUC19; contains *attB*	ND

[a] Details of the constructions are given in Materials and Methods. Numbers in brackets denote the position of restriction sites of pSE211 shown in Fig. 1A; numbers in parentheses denote the position of restriction sites of pTKB270^{int} (pTKB2700) shown in Fig. 1C.

[b] Determined by demonstration of plasmid sequence in *S. erythraea* ER720 at *attB* by Southern hybridization.

[c] ND, Not done.

[d] Thio^r colonies observed but plasmid not observed at *attB* by Southern hybridization.

gels, slices containing the bands to be recovered were removed from the gel and dissolved in ca. 2× volumes of a saturated solution of KI and then diluted two- to fourfold with TTE buffer (0.1 M Tris hydrochloride [pH 7.7]–1 mM EDTA–10 mM triethylamine). The preparation was applied to a 3-ml C_{18} solid phase extraction column (Baker Chemical Co., Philipsburg, N.J.) that had been prewashed with methanol and equilibrated with TTE buffer. The column was washed with 2 ml of TTE buffer and then with 2 ml of H_2O, and the residual moisture was removed. DNA was eluted with 300 μl of a 50% methanol solution and concentrated by precipitation in ethanol. The DNA recovered was found to be a fully competent substrate for restriction endonuclease digestion, ligation, or nick translation.

Plasmids. DNA was subjected to restriction endonuclease digestion, ligation with T4 DNA ligase, or other manipulations, employing standard methods (31). pAL7001 and pAL7009 were constructed by ligation of the 1.1-kb *Bcl*I fragment of pIJ704 containing the *tsr* gene, conferring the Thio^r phenotype in *Streptomyces* species (49) and *S. erythraea* (11), to the *Bam*HI site of pUC9 and pUC19, respectively. The *tsr*-containing *Eco*RI-*Xho*I fragment of pAL7009 was treated with DNA polymerase I to produce blunt ends (31) and ligated to the *Ssp*I site of pUC19 to create pAL7015. Plasmid constructions employing insertion of all or part of plasmid pSE211 in pAL7001 or pAL7015 are summarized in Table 1. pTKB2700 was constructed as follows. Chromosomal DNA was isolated from a *S. erythraea* ER720-(pTKB270) transformant which carried the plasmid as an integrated sequence (Fig. 1C), digested with *Bgl*II, treated with T4 DNA ligase, and then used to transform *E. coli*. Since pTKB270 does not contain a site for cleavage with *Bgl*II, the resulting plasmid (pTKB2700) recovered from *E. coli* consisted of the segment of the *S. erythraea* chromosome containing integrated pTKB270 and left and right adjoining chromosomal DNA sequences to the nearest *Bgl*II sites (Fig. 1C). pTKB2900 was constructed as follows. Approximately 10 μg of *Hin*dIII plus *Bgl*II-digested chromosomal DNA of *S. erythraea* ER720 was subjected to agarose gel electrophoresis. The section of the gel that contained DNA fragments approximately 3 kb in size was removed and then used for DNA extraction as described above. The DNA recovered was ligated to *Hin*dIII plus *Bam*HI-digested pUC19, and the mixture was used to transform *E. coli*. Transformants carrying a putative insertion in pUC19 were subjected to colony hybridization (31), employing the 2.6-kb *Kpn*I(11) through *Bgl*II(13) (see Table 1, footnote *a* for explanation of restriction sites of plasmids) *attR* and adjacent chromosomal DNA-containing segment of pTKB2700 (Fig. 1C) as probe. pTKB2900, recovered from one of the clones, contained a 2.9-kb insert.

DNA sequencing. M13mp18 or M13mp19 phage DNA carrying inserts and single-stranded M13 DNA sequencing templates were prepared as described by Messing (33). M13 clones were sequenced by the method of Sanger et al. (47) (employing universal M13 primers) and by extending the sequence with the use of synthetic oligonucleotides. Double-stranded plasmid sequencing was done by the method of Zhang et al. (52), employing sequencing kits obtained from US Biochemical Corp., Cleveland, Ohio, containing the enzyme Sequenase and 7-deaza-dGTP in place of dGTP. The sequencing of end-labeled DNA fragments was performed as described previously (32).

Computer-assisted sequence analysis. Sequence analyses were performed, using Intelligenetics Bionet (Mountain View, Calif.) or the University of Wisconsin Genetics Computer Group (UWGCG) (Madison, Wisc.) software packages on a VaxII/RC, Vax100, or Macintosh II computer. Comparisons of nucleotide or polypeptide sequences were performed, using the UWGCG programs COMPARE and DOTPLOT (14). COMPARE takes a segment of a sequence, specified by a window, and compares it with each window of the second polypeptide in every register permitting conservative amino acid substitutions to recognize similarities. If the number of similarities within the window is above the specified level (stringency), the region is noted. DOTPLOT graphically represents the information generated from COMPARE such that a dot is placed where the similarity was observed. CODONPREFERENCE (UWGCG) graphically displays the G+C composition of each of the base

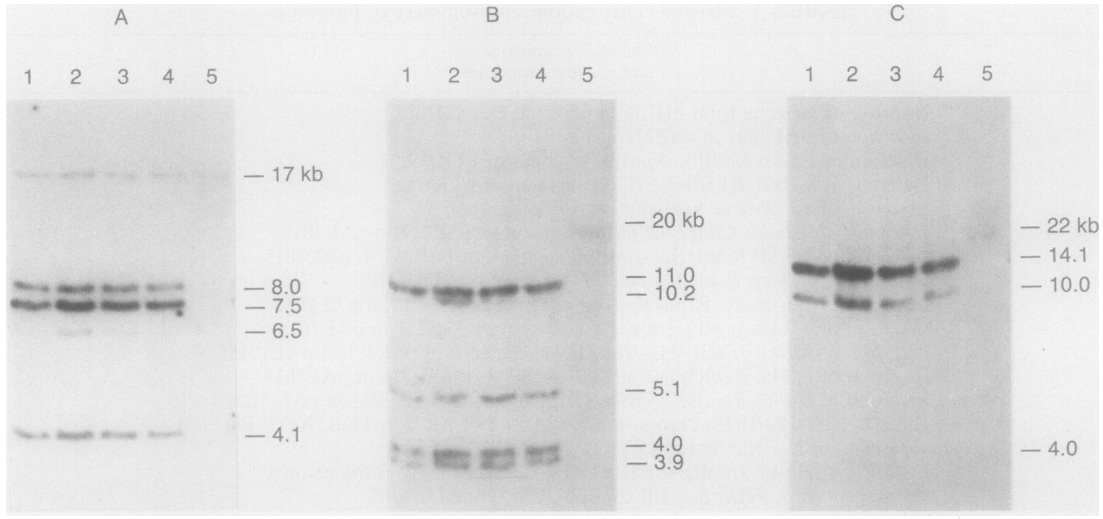


FIG. 2. Southern blot of integrated pSE211 from four *S. erythraea* ER720/pSE211 transformants. Total DNA was cut with *Bam*HI (A), *Bgl*II (B), and *Hin*dIII (C). In each panel, lanes 1 to 4 contain total DNA from corresponding transformants and lane 5 contains DNA from ER720. Probe, pSE211. Positions corresponding to lengths (in kilobase pairs [kb]) of selected fragments are shown.

positions in codons over a window (set here at 25 codons) and a codon usage table for high G or C bias in the third codon position compiled from several *Streptomyces* genes (3). Searches of the Protein Identification Resource, Swiss Prot, NBRF, and GenBank data bases for sequence homologies employed the programs WORDSEARCH, TFASTA (UWGCG), or FASTDB (Intelligenetics).

RESULTS

Location of the *attP* site in pSE211. Southern blots of total DNA from four *S. erythraea* ER720(pSE211) transformants (spores picked from the center of a pock on a regeneration plate) digested with various enzymes and probed with pSE211 are shown in Fig. 2. In the samples treated with *Bam*HI, pSE211-hybridizing fragments of 4.1, 7.5, 8.0, and 17.0 kb can be seen in each preparation (Fig. 2A). The 4.1- and 7.5-kb bands represent the *Bam*HI[15] through *Bam*HI[19] and *Bam*HI[19] through *Bam*HI[2] segments of the pSE211 sequence (Fig. 1A). The absence of a 6.5-kb pSE211-hybridizing band in lanes 1, 3, and 4, representing the *Bam*HI[2] through *Bam*HI[15] segment of the plasmid, indicates that integration utilizes a site somewhere within this segment; the 8.0- and 17.0-kb bands are plasmid-chromosome junction fragments. Similarly, the 3.9- and 4.0-kb pSE211-hybridizing bands in the *Bgl*II-digested preparations represent plasmid segments, and the 5.1- and 11.0-kb bands correspond to the plasmid-chromosome junctures (Fig. 2B). (The small amounts of the 6.5- and 10.2-kb bands seen in track 2 of Fig. 2A and C, respectively, indicate either tandem duplication of the integrated element in a small fraction of the chromosomes or a minor amount of free plasmid in the transformant population.) In the *Hin*dIII-digested samples, the 14.1-kb band represents a plasmid segment and the 10.0-kb band represents a plasmid-chromosome juncture (Fig. 2C). A second plasmid-chromosome junction fragment of 0.7 kb (not visible in Fig. 2C) was observed in subsequent Southern blots (not shown). In *Kpn*I-digested samples of the same total DNA preparations, plasmid bands of 1.5 kb and plasmid-chromosome juncture bands of 4.0 and 15.0 kb were observed (not shown). By examination of the blots and inspection of the restriction map in Fig. 1A, it could be concluded that the 0.79-kb *Hin*dIII[4] through *Kpn*I[8] segment of pSE211 is interrupted when the plasmid undergoes integration and thus contains the site at which crossover takes place. This site, whose length will be described below, is designated *attP*. By employing additional enzymes to analyze the integrated plasmid (designated pSE211^{int}) and adjacent chromosomal sequences, the restriction map shown in Fig. 1B was obtained. The chromosomal site at which pSE211 is integrated is designated *attB*.

A 2.4-kb segment of pSE211 is sufficient and required for site-specific integration. Plasmids that contained pUC9 or pUC19, a segment containing the *tsr* gene, the 0.79-kb *Hin*dIII[4] through *Kpn*[8] *attP*-containing segment of pSE211, and varying lengths of adjacent plasmid sequence (see Materials and Methods) (Table 1) were constructed and used for transformation of *S. erythraea* ER720 protoplasts, employing selection for resistance to thiostrepton. Stable Thio^r transformants (maintenance of Thio^r after spore-to-spore passage without selection) were obtained only with plasmids pTKB12, pTKB22, pTKB270, pTKB300, pTKB520, and pTKB1410 (Table 1). Transformation of ER720 with pTKB170 consistently resulted in the appearance of at least as many Thio^r colonies on regeneration plates as were obtained when the host was transformed with an equivalent amount of pTKB270 DNA, but the thiostrepton-resistant phenotype was always lost upon subsequent passage of the transformants. The use of plasmids pTKB26, pTKB130, pTKB420, and pTKB640 failed to yield Thio^r transformants of ER720. In addition, all other plasmids constructed that did not contain the intact *Eco*RI[0] through *Kpn*I[8] segment of pSE211 were also unable to yield Thio^r transformants of ER720 regardless of which other segments of pSE211 they carried.

Southern blots of *Eco*RI plus *Bgl*II-digested total DNA from seven *S. erythraea* ER720(pTKB270) transformants employing pTKB12 as probe are shown in Fig. 3. Three preparations displayed hybridizing bands of 5.7 and 6.4 kb (lanes 4, 6, and 7); the other four preparations displayed the 5.7- and 6.4-kb bands as well as a 6.2-kb band (lanes 1, 2, 3, and 5). An inspection of the restriction map of the chromosomal region adjacent to pSE211^{int} (Fig. 1B) shows that the

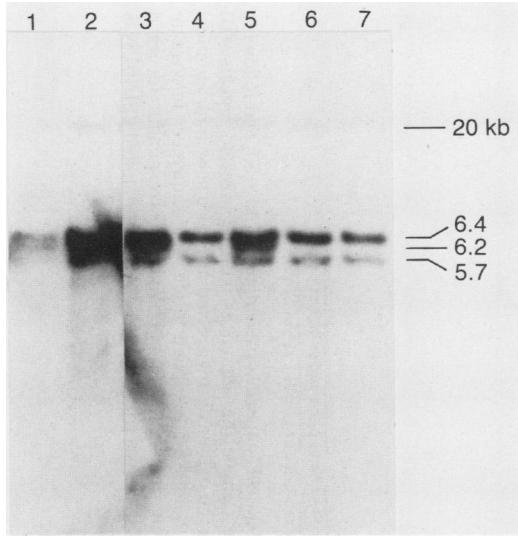


FIG. 3. Southern blot of integrated pTKB270 from seven *S. erythraea* ER720(pTKB270) transformants (lanes 1 to 7). In each lane, total DNA was digested with *Eco*RI and *Bgl*II. Probe, pTKB12 (Table 1). Positions corresponding to lengths (in kilobase pairs [kb]) of selected fragments are shown.

presence of the 5.7- and 6.4-kb bands in Fig. 3 are consistent with the interpretation that pTKB270 had integrated at the *attB* site and that it employed the *attP* site in the crossover event. Since pTKB270 is 6.2 kb and contains a single *Eco*RI site and free pTKB270 DNA was not detected in the transformants (data not shown), the simplest explanation consistent with the appearance of the 5.7-, 6.4-, and 6.2-kb bands in the other transformants is the presence of a tandemly duplicated copy of pTKB270 integrated at *attB*. Analyses of Southern hybridizations of total DNA from transformants carrying pTKB270 digested with other restriction enzymes appeared to confirm this hypotheses (data not shown). A restriction map of pTKB270^{int} and adjacent chromosomal DNA is shown in Fig. 1C. Southern blots of total DNA from several *S. erythraea* ER720(pTKB1410) and *S. erythraea* ER720(pTKB520) transformants also revealed pTKB12-hybridizing fragments with sizes that corresponded to those expected for integration of the respective plasmids at *attB* (not shown). These findings indicate that stable integration at *attB* can occur if the plasmid contains the *attP* site and adjacent 2.4-kb DNA segment to one side that corresponds to the *Eco*RI[0] through *Kpn*I[8] segment of pSE211 (Fig. 1A).

Nucleotide sequence and analysis of the *att* sites. The nucleotide sequences of segments carrying *attB*, *attL*, and *attR* were determined by employing double-stranded sequencing of pTKB2900 and pTKB2700 or other plasmids that contained the putative *att* sites. *attP* was obtained from the sequence of the 2.9-kb *Sph*I[21] through *Kpn*I[8] segment of pSE211 described below. Sequences of the four sites are shown in Fig. 4A. A 57-bp sequence common to all sites was observed. It can be seen (Fig. 4A) that the sequences preceding and following the common sequences in *attP* and *attB* are fully conserved in *attL* and *attR*, respectively, indicating that integration of the plasmid takes place through crossover at some point within the common 57-bp sequence in *attP* and *attB* without duplication of either site. A 38-bp inverted repeat is present at one end of *attP* (and *attL*). A small inverted repeat in the chromosomal sequence adjacent to the common sequence in *attB* (and *attR*) is also seen.

In previous work, tRNA genes were shown to be located at the *attB* sites of integrating plasmids and bacteriophages (44). The deduced RNA sequence of the 74-base segment of pSE211 *attB* from bases −31′ to 43′ (Fig. 4A) was found to conform perfectly to established rules for tRNA structure (45, 46). When searches of data bases were performed (as described in Materials and Methods), more than 800 sequences that either encode tRNA genes or rRNA regions were found to be homologous to this sequence. A cloverleaf representation of the putative 74-base RNA transcript from pSE211 *attB* (and *attL*) is shown in Fig. 5. The bases found to be invariant (Fig. 5, shaded letters) or highly conserved (Fig. 5, circled letters) in tRNA sequences (16) are shown. The sequence GAA in the anticodon loop indicates that *attB* (*attL*) encodes a putative tRNA^{Phe}. A cloverleaf structure could not be fashioned for the corresponding sequence in *attP* (or *attR*).

Nucleotide sequence of the plasmid segment involved in integration. The strategy employed for the sequencing of the 2,867-bp *Sph*I[21] through *Kpn*I[8] segment of pSE211 is shown in Fig. 6, and the complete nucleotide sequence of the segment is shown in Fig. 7. A modified representation of the output from the CODONPREFERENCE analysis is shown in Fig. 6. The sequence contains three likely ORFs, designated *orf1*, *orf2*, and *int* (Fig. 6, thick arrows). Each contains an average third codon position G or C bias of greater than 0.85 and generally follows the codon usage tables for *Streptomyces* genes (3). The deduced amino acid sequences of these ORFs is displayed in Fig. 7. *orf1* encodes a polypeptide of at least 263 residues, designated pSE211 Orf1, and terminates with the TGA stop sequence at position 794 of the sequence. It is not known whether the Met residue at the beginning of the amino acid sequence shown represents the amino terminus of the proposed polypeptide or whether *orf1* begins at a yet-to-be-determined site in the *Bgl*II[20] through *Eco*RI[0] segment of pSE211 (Fig. 1A). *orf2* is postulated to begin at ATG-791, to terminate at the TGA-1087, and to encode a basic polypeptide, designated pSE211 Orf2 (calculated pI, 10.68) of 98 amino acid residues with a predicted molecular weight of 10,503. *orf1* and *orf2* share a 2-bp overlap. *Streptomyces* ORFs are usually preceded by short sequences bearing homology to the 3′ end of 16S rRNA of *Streptomyces lividans* that are thought to serve as ribosome-binding sites (2). The putative start site of *orf2* is preceded by the sequence AGGGG, which could potentially fill this role. The third ORF, designated *int*, is contained within the segment ATG-1089 to TGA-2412 and encodes a postulated basic polypeptide (calculated pI, 10.57) of 437 amino acids with a predicted molecular weight of 50,327, designated pSE211 Int. The potential ribosome-binding site sequence AGGGA precedes the putative translation start site. Other potential in-frame translation start sites are located at ATG-1183 (not preceded by a recognizable ribosome-binding site) and GTG-1250 (preceded by the sequence AGGAG). *int* is followed by a 38-bp inverted repeat and the sequence previously designated *attP* (shown in reverse orientation in Fig. 4). Several putative ORFs encoded by the complement of the sequence were also observed. The longest, shown in Fig. 6, is contained on the complement of *orf1* from bp 394 (GTG start) past 1 and encodes a polypeptide of greater than 118 amino acids.

Analysis of pSE211 Int. A number of site-specific recombinases belong to a family of integrases that share significant sequence homology in their C-terminal regions (1). An alignment of a 42-amino-acid sequence encompassing the homologous domain of several integrases is shown in Fig. 8.

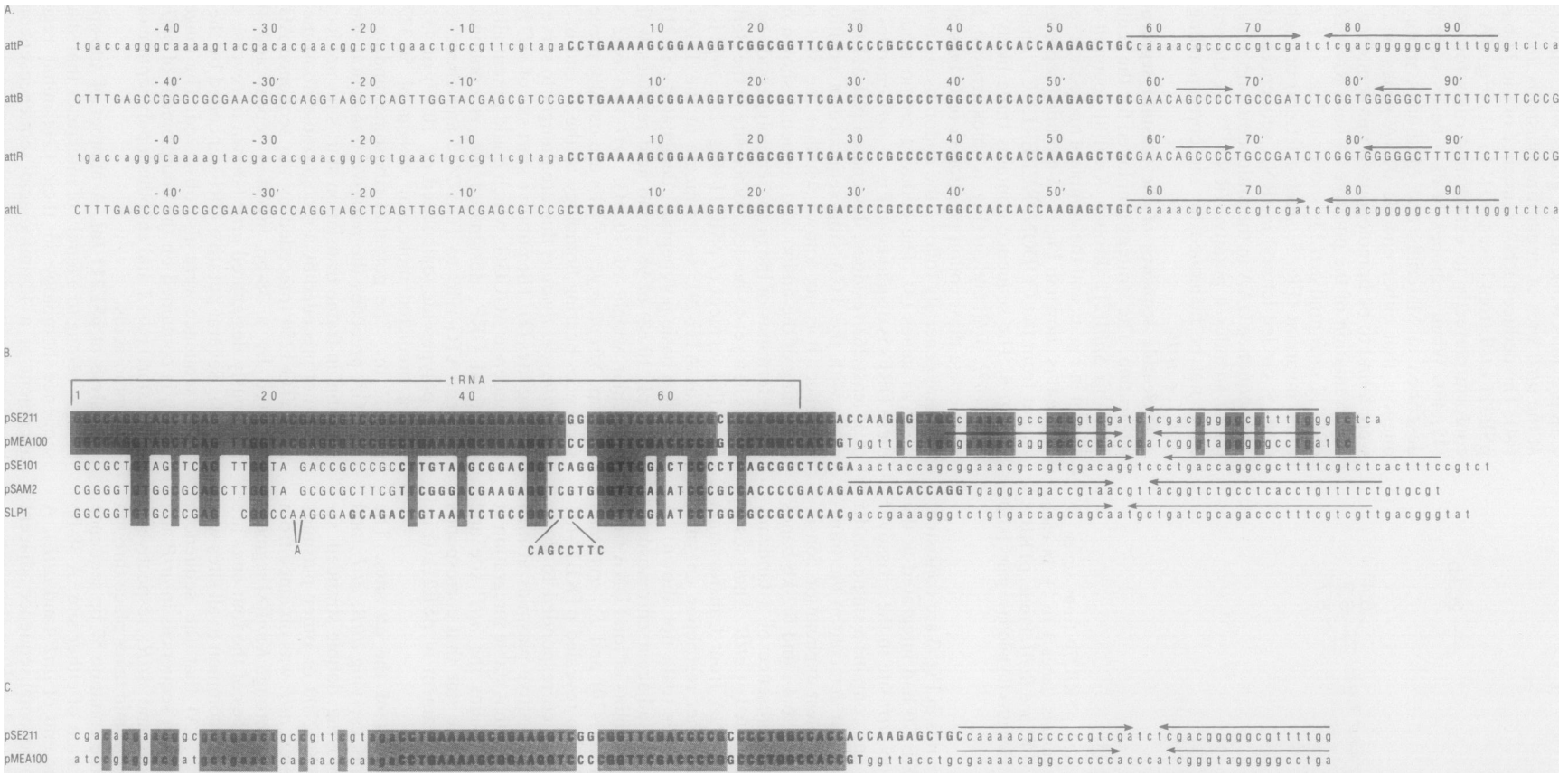


FIG. 4. (A) Nucleotide sequence of pSE211 *att* sites. Identical 57-bp sequence 1(1′) through 57(57′) is shown in boldface. Identical sequences preceding (−1 to −50) and following (59 to 100) the 57-bp conserved sequence are shown in lowercase or uppercase letters for *attP* and *attB*, respectively. Arrows above the sequences indicate inverted repeats. (B) Comparison of *attL* sequences for various actinomycete plasmids. Sequences are aligned so that the shaded bases correspond to each other as shown. Shading is also shown for identical bases in pSE211 and pMEA100. The sequences of bases 1 to 73 correspond to putative structural genes for tRNAs. Letters in boldface correspond to identical sequences in cognate *attP* sites. (C) Comparison of pSE211 and pMEA100 *attP* sequences. Sequences are aligned to allow maximum matches shown by shading. Letters in boldface correspond to identical sequences in cognate *attB* sites. pMEA100 *attP* and *attL* were from Madon et al. (30); pSAM2 *attL* was from Boccard et al. (7); SLP1 was from Omer and Cohen (37); and pSE101 was from D. P. Brown, D. Backer, K. I. Idler, and L. Katz (unpublished results).

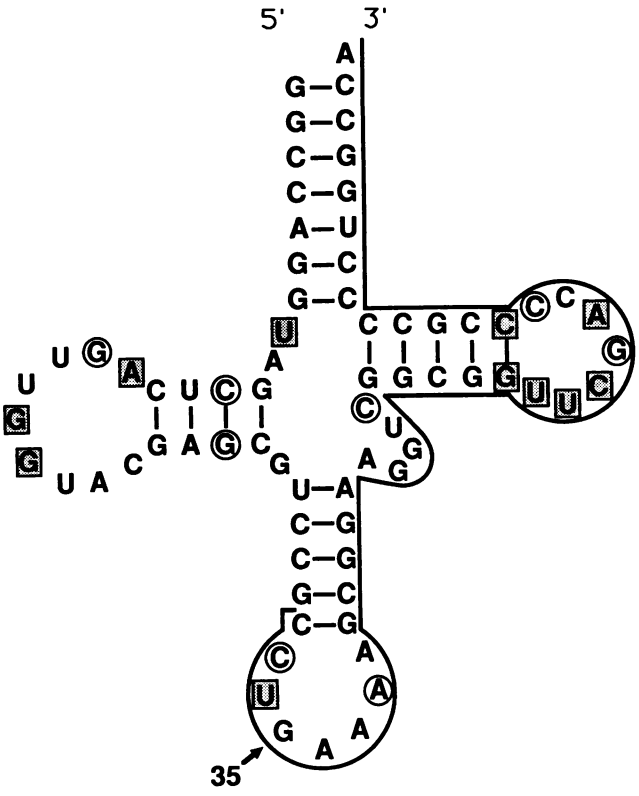


FIG. 5. Cloverleaf structure alignment of the putative transcript from pSE211 *attB* (*attL*) site. Sequence corresponds to bases 1 to 73 of pSE211 *attL* in Fig. 4C. Shaded or circled bases are invariant or highly conserved, respectively, in tRNAs (16). Sequence outlined by solid line corresponds to matching sequence in *attP* DNA. Anticodon begins at base 35 and corresponds to TTC-Phe.

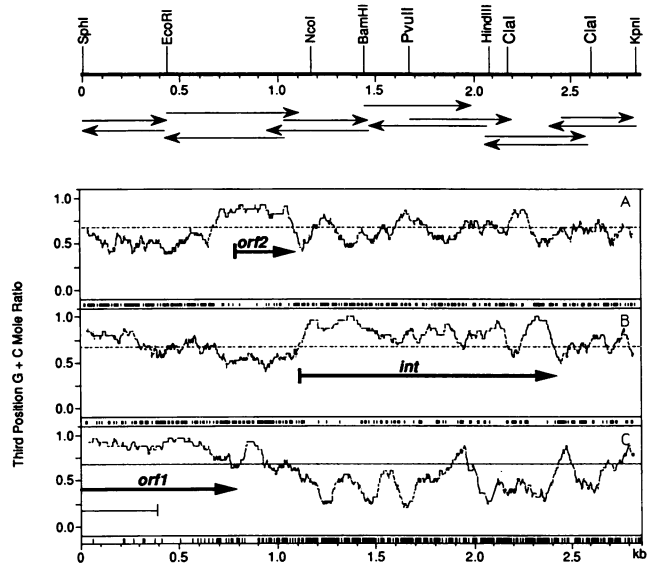
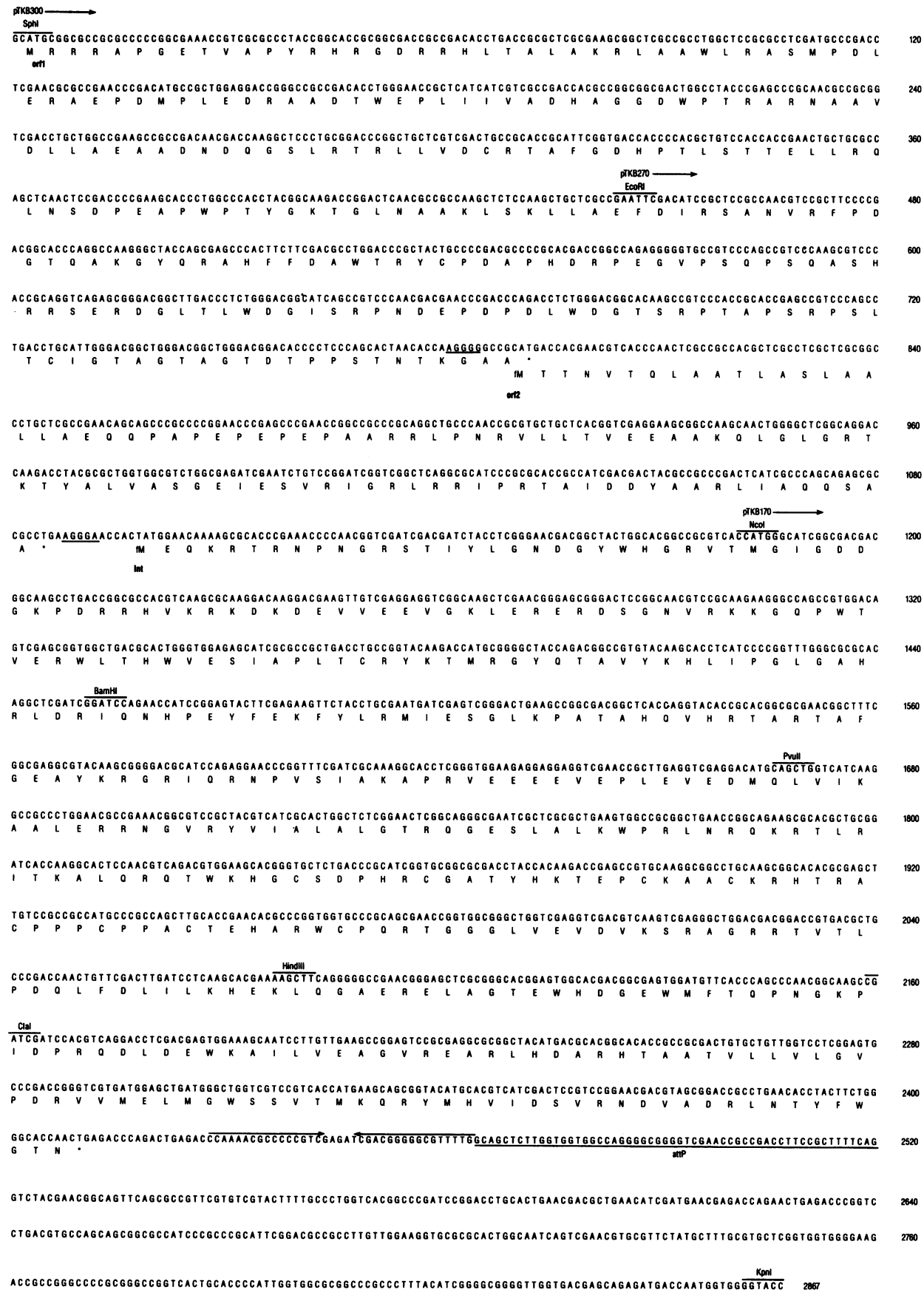


FIG. 6. Restriction map, sequencing strategy, and modified CODONPREFERENCE plot of pSE211 sequence corresponding to *Sph*I[21] through *Kpn*I[8] (Fig. 1A). The average G+C mole ratio over a 25-codon window is plotted for the second (A), first (B), and third (C) positions in each triplet of the sequence shown in Fig. 7. Corresponding restriction is shown above. Usage of a rare codon (3) is indicated by a mark at the position indicated as shown at the bottom of each panel. ORFs (AUG or GUG starts) are represented by arrows in panels A, B, and C. Dotted lines represent average G+C mole ratio of DNA sequence. Horizontal arrows under the restriction map represent direction of DNA fragments sequenced.

These sequences include the integrases of bacteriophages P2, P4, P22, 186, λ, and φ80; the Cre protein that resolves phage P1 dimers; the D protein of the F plasmid; the products of the *fimB* and *fimE* genes involved in inversion-controlled expression of fimbriae in *E. coli*; putative polypeptides of genes of transposons from *Staphylococcus aureus* (Tn*554*), *Bacillus thuringiensis* (Tn*4430*), and *E. coli* (Tn*2603*) that function in transposition; and the putative integrase of plasmid pSAM2 from *Streptomyces ambofaciens*. The corresponding segment of pSE211 Int from residues 376 to 416 is also shown. It can be seen that the residues His-396, Arg-399, and Tyr-433 (family positions), conserved in all sequences shown in Fig. 8 (with the exception of pSAM2 Int), are present at amino acid positions 379, 382, and 414, respectively, in the pSE211 Int sequence. Tyr-342 of λ Int (family position 433) has been shown to be the residue at which the integrase forms an *O*-phosphotyrosine Int-*att* covalent bond during the recombination event (42). It can also be seen that 12 of the 17 additional residues of the pSE211 Int sequence exhibit a significant degree of conservation within this domain. By using the programs COMPARE and DOTPLOT of the UWGCG software package, graphic representations of the extent of homology between pSE211 Int and various other integrases were determined. As expected, it was found that the greatest extent of homology was preserved only within the C-terminal regions of these polypeptides (data not shown), except for the comparison between pSE211 Int and pSAM2 Int (Fig. 9) in which additional segments of the polypeptides displayed homology to each other.

Analysis of pSE211 Orf2. A DOTPLOT comparison of pSE211 Orf2 with pSAM2 Xis (Fig. 9) illustrates that homology between the two polypeptides is preserved throughout the entire pSAM2 Xis sequence. Searches of the data bases for homology to pSE211 Orf2 did not reveal any other significant matches. An alignment of a 22-amino-acid sequence encompassing the conserved regions of the Xis proteins of various bacteriophages, the putative Xis protein of pSAM2, and pSE211 Orf2 (residues 42 through 63) is shown in Fig. 10A. pSE211 Orf2 has a high calculated pI (10.68) and contains a potential helix-turn-helix motif (41) encompassed within the 20-amino-acid sequence from Val-45 to Ala-64 (Fig. 7), suggesting that it functions as a DNA-binding protein. Figure 10B displays an alignment of the helix-turn-helix domain of pSE211 Orf2 (residues 42 through 63) to a number of DNA-binding proteins, including the following: the Tn*917* resolvase; transcriptional activators and repressors AraC, λ Cro, and CI; the deduced amino acid sequence of a segment of the *korA* gene from the *Streptomyces lividans* plasmid pIJ101 (25) that participates in regulating expression of the *kilA* and *kilB* genes of this plasmid (24); and the deduced amino acid sequence of a segment of the OrfC region of *imp* from SLP1, thought to repress expression of plasmid replication functions (18). In the Xis alignment (Fig. 10A), it can be seen that pSE211 Orf2 contains the highly conserved residues (41) at positions 1 to 6, 11, 13, 15, 16, and 22 but is missing the Arg- or Lys-12 residue found in the other proteins. Similarly, pSE211 Orf2 contains the highly conserved sequence Ala-8 Hydrophobic-11 Gly-12 Hydrophobic-13 Hydrophobic-21 (41), but the highly conserved Ile- or Leu- or Val-18 residue present in


pTKB300
SphI
orf1
pTKB270
EcoRI
orf2
pTKB170
NcoI
fM
Int
BamHI
PvuII
HindIII
ClaI
attP
KpnI

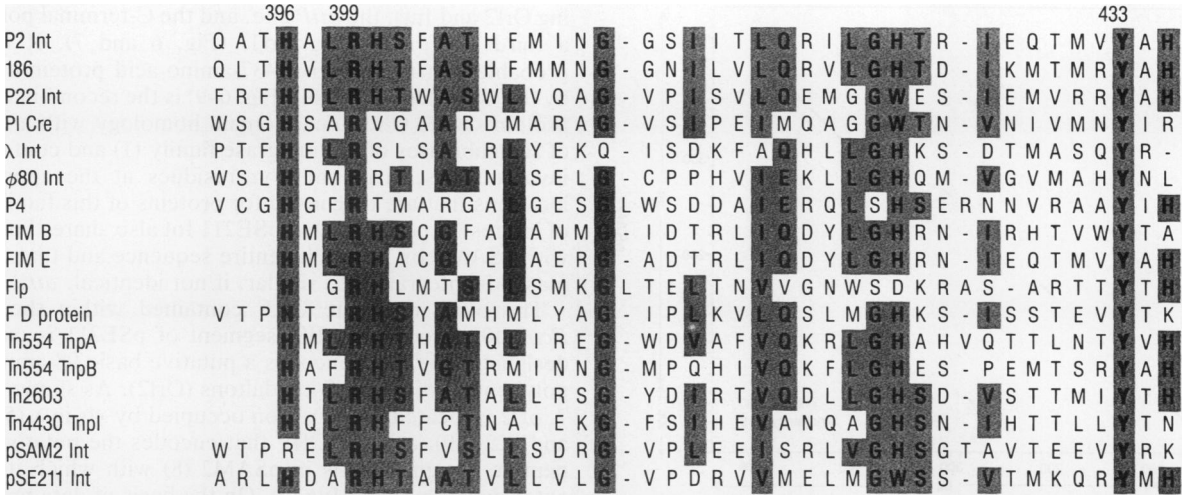


FIG. 8. Sequence comparisons of the C-terminal segments of the integrase (Int) family of site-specific recombinases. Alignments and numbering were made as described by Argos et al. (1). Shading indicates conserved or identical residues at a particular position. Sequences of P2, 186, P22, φ80, and P4 integrases, P1 Cre, λ Int, and FLP were from reference 1; FD protein, Tn*554* TnpA and TnpB, and Tn*2603* and Tn*4430* TnpI were from Murphy (35); pSAM2 Int was from Boccard et al. (8); FIM B and FIM E were from Glasgow et al. (17); and pSE211 Int was from Fig. 7.

many of the DNA-binding proteins (41) is replaced by Thr-18 in pSE211 Orf2 (Fig. 10B).

DISCUSSION

Nonduplicative recombination has been demonstrated here for integration of pSE211 by observing conservation of the plasmid and bacterial sequences at the left and right plasmid-chromosome junctions of pTKB270^{int} (Fig. 4A). That pSE211 shares common site-specific recombination mechanisms with other actinomycete plasmids (for a review, see reference 39) and with a variety of bacteriophages, transposons, and other genetic elements is suggested from examination of the nucleotide sequences of the *att* sites and the organization and sequences of the postulated polypeptides of the plasmid-encoded ORFs involved in recombination. *attL* (shown for five actinomycete plasmids in Fig. 4B) is composed of *attB* and an adjacent plasmid sequence that carries genes involved in site-specific recombination in the cases of pSE211 (Fig. 7), pSAM2 (7, 8), SLP1 (38), and pSE101 (D. P. Brown, unpublished results). The function of the plasmid sequence at the *attL* site of pMEA100 is not currently known. The first 75 bases of the sequences shown here for pSE211 correspond to a sequence for a putative tRNA gene for Phe (Fig. 5). The *attL* sequences of pMEA100 and SLP1 correspond to putative Phe-tRNA and Tyr-tRNA genes, respectively, as previously reported (44). Similarly, it can be seen that the *attL* (*attB*) sequences of pSE101 and pSAM2 also contain the 11 invariant bases (Fig. 4B and 5) and many of the other highly conserved bases found in tRNA genes and can also be configured in a cloverleaf arrangement (and thus correspond to Thr-tRNA and Pro-tRNA genes, respectively). The *attP* site of each plasmid contains a short sequence (43 to 58 bp in length) of perfect homology to the 3′ end of the cognate tRNA gene and adjacent downstream chromosomal sequence. In each case, after *attP* × *attB* recombination has taken place, the functional tRNA gene is preserved at *attL*. The mismatches previously reported between *attP* and *attB* of SLP1 (38) and between the *attP* site of pSAM2 and its *attB* site in *Streptomyces lividans* (8) lie, in each case, downstream of the sequence that corresponds to the tRNA. However, it remains to be determined how well mismatches within the putative tRNA structural genes would be tolerated.

A feature of *attL* common to all five actinomycete plasmids is the presence of long inverted repeats in the sequence immediately adjacent to the 3′ end of the putative tRNA genes. In each case, the inverted repeats were found in the corresponding *attP* site and thus were crossed in at integration. Since in pSE211 (Fig. 7), pSAM2 (8), and pSE101 (D. P. Brown et al., in preparation) the *attP* sites lie approximately 90 bases downstream of the 3′ end of the *int* genes, *attP* × *attB* recombination results in the placement of *int* adjacent to the tRNA gene at *attL* in the orientation such that their transcripts converge. It is likely that the inverted repeats that lie between the genes serve as transcription terminators for one or both of the genes. It is not yet known, however, whether *int* is expressed when the plasmid is present in the integrated state. This point is discussed in more detail below.

Plasmids pMEA100 and pSE211 integrate in similar Phe-tRNA genes in *N. mediterranei* and *S. erythraea*, respectively (Fig. 4B). Furthermore, upon examination of the corresponding *attP* sequences (FIg. 4C), it can be seen that the same segments of the tRNA genes (bases 32 to 76) are

FIG. 7. Nucleotide sequence of pSE211 segment corresponding to *Sph*I[21] through *Kpn*I[8] (Fig. 1A). The deduced amino acid sequence is shown below the DNA sequence for segments in 5′ to 3′ direction corresponding to *orf1*, *orf2*, and *int* (Fig. 6) in standard one-letter amino acid designations. The regions upstream of the putative start sites for translation of *orf2* and *int* that could serve as ribosome-binding sites are underlined. The *attP* site (whose complement is shown in inverse orientation in Fig. 4A) is designated and underlined. Arrows above the sequence indicate inverted repeat sequences. Designations pTKB300, pTKB270, and pTKB170 followed by arrows indicate the beginning of the sequence shown to the *Kpn*I site present in plasmids designated (Table 1).

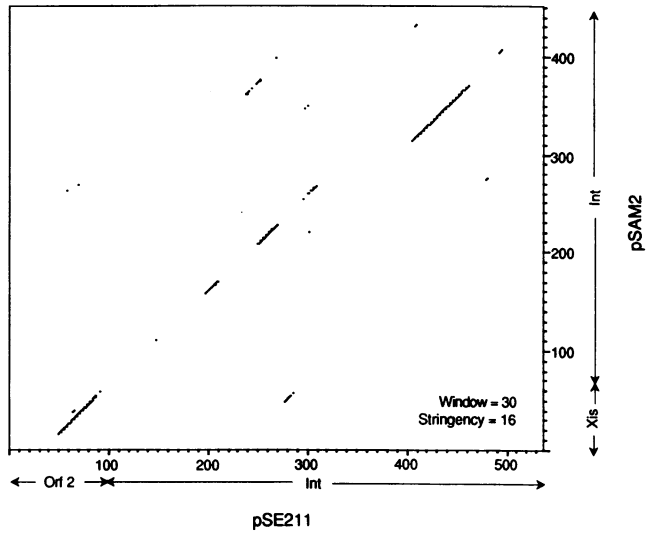


FIG. 9. DOTPLOT comparison of pSE211 orf2 plus Int proteins and pSAM2 Xis plus Int proteins (8). For the purposes of comparison, the Orf2 and Xis sequences were placed immediately in front of their cognate integrase (Int) sequence to correspond to their locations in the DNA sequence. Window and stringency explanations are given in Materials and Methods.

present in the two plasmids along with an additional nonmatching 3′ sequence. Further sequence analysis of pMEA100 and experiments to see whether one plasmid can integrate into the *attB* site of the other could potentially provide information into the nature of recombinase or *att* site specificity.

As shown in Fig. 3 and described in Results, the pSE211 sequence required and sufficient for integration is contained in the 2.4-kb *Eco*RI[0] through *Kpn*I[8] segment of the plasmid (Fig. 1A) that contains two complete ORFs (encoding Orf2 and Int), the *attP* site, and the C-terminal portion of a third ORF (encoding Orf1) (Fig. 6 and 7). It is most probable that the putative 437-amino-acid protein encoded by the ORF designated *int* (Fig. 6–9) is the recombinase. The protein shares C-terminal domain homology with a number of recombinases of the integrase family (1) and contains the invariant His, Arg, and Tyr residues at the appropriate locations that are diagnostic for proteins of this family. It is of interest to point out that pSE211 Int also shares homology with pSAM2 Int across its entire sequence and that the two plasmids integrate into similar, if not identical, *attB* sites.

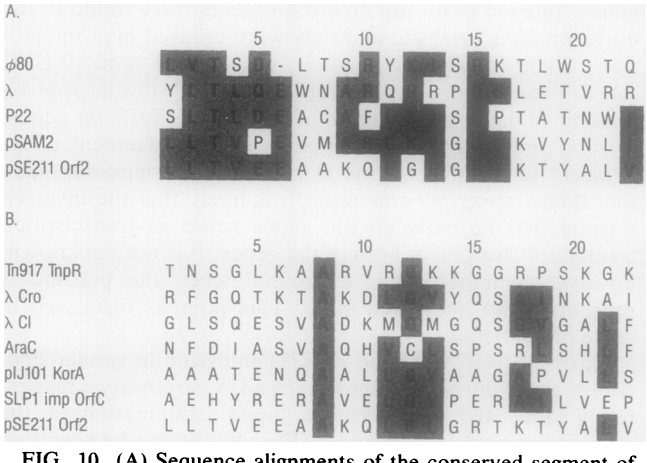


FIG. 10. (A) Sequence alignments of the conserved segment of various known or presumed Xis proteins. Sequences of λ, P22, and φ80 were from Leong et al. (29), and pSAM2 was from Boccard et al. (8). Shading indicates conserved or identical residues at a particular position. (B) Sequence alignments of the helix-turn-helix region of various DNA-binding proteins. Sequences of Tn*917* TnpR, λ Cro, λ CI, and AraC were from Pabo and Sauer (41); pIJ101 KorA was from Kendall and Cohen (24); SLP1 *imp* OrfC was from Grant et al. (18); and pSE211 Orf2 was from Fig. 7. Shading indicates conserved or identical residues at a particular position.

The other complete ORF contained within the 2.4-kb *Eco*RI[0] through *Kpn*I[8] segment of pSE211 is currently designated *orf2* and encodes a putative basic 98-amino-acid polypeptide of ca. 10.5 kilodaltons (Orf2). As shown in Fig. 7, *orf2* is located in the region occupied by *xis* in λ (50), φ80 and P22 (29), and the ORF that encodes the putative polypeptide designated Xis in pSAM2 (8) with which it shares extensive homology (Fig. 9). On the basis of data presented here, conclusions on the function of Orf2 cannot be drawn, but it is possible that Orf2 does not act as a classical excisionase. As described in Results, the introduction of pTKB170 (which was missing *orf2* [and *orf1*] and the 5′ segment of *int* corresponding to the first 28 N-terminal amino acids of Int) into a pSE211$^-$ strain consistently resulted in the appearance of unstable transformants that could not be propagated further when cultivated in the presence of thiostrepton. In contrast, plasmids devoid of functional Int, such as pTKB130 or pTKB640, yielded no transformants. A possible explanation for the unstable Thio^r phenotype is that pTKB170 is transiently maintained in the host through repeated cycles of integration and excision but is eventually lost from the transformants since it cannot replicate autonomously. One model provides that Orf2 normally binds to the *att* site(s) of the integrated plasmid but in contrast to Xis, its binding prevents the Int- or Int-plus-host-factor-mediated *attL* × *attR* recombination that would result in excision. In the pTKB170-containing transformants, therefore, the absence of Orf2 could not prevent the subsequent excision that would occur after an integration event had taken place. A second possibility is that the truncated Int protein produced from pTKB170 does not permit normal integration to take place. We are presently examining mutants of Orf2 to determine more precisely its role in the integration-excision process.

The sequence encoding the 116-amino-acid C-terminal end of a third ORF (*orf1*) is also contained within the 2.4-kb *Eco*RI[0] through *Kpn*I[8] segment of pSE211. We do not currently believe that this polypeptide plays a direct role in plasmid integration or excision. The upstream nucleotide sequence revealed that Orf1 contains at least 263 amino acid residues (Fig. 6 and 7). This protein does not bear resemblance to any polypeptide known or presumed to be involved in site-specific recombination. The C-terminal fragment of Orf1 produced from pTKB270 contains less than half the complete sequence and therefore would not be expected to function properly, yet pTKB270 appears to integrate normally. In addition, we have found that disruption of *orf1* (by integration of plasmid pAL7001 at the *Eco*RI site of pSE211 to yield plasmid pTKB12) does not affect integration or excision but does result in a 20-fold reduction in the frequency of conjugal transfer of pTKB12 and the disappearance of pocks (K. A. Boris, J. P. Dewitt, and L. Katz, unpublished results). Orf1 possibly plays a role in conjugal transfer, but the basis for the translational coupling of *orf1* and *orf2* (Fig. 7) is not presently understood.

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