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A Site-Specific Recombination Function in *Staphylococcus aureus* Plasmids

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All known small staphylococcal plasmids possess one or two recombination sites at which site-specific cointegrate formation occurs. One of these sites, RS_A, is present on two small multicopy plasmids, pT181 and pE194; it consists of 24 base pairs of identity in the two plasmids, the "core," flanked by some 50 base pairs of decreasing homology. Here we show that recombination at RS_A is *recA* independent and is mediated by a plasmid-encoded, *trans*-acting protein, Pre (plasmid recombination). Pre-mediated recombination is site specific in that it occurs within the core sequence of RS_A in a *recA1* host. Recombination also occurs between two intramolecular RS_A sites. Unlike site-specific recombination systems encoded by other plasmids, Pre-RS_A is not involved in plasmid maintenance.

Site-specific recombination is generally used to effect stable or semistable structural rearrangements of nonhomologous elements (integration of phage genomes and transposons; formation and resolution of cointegrates) or of segments within the same element (expression-related inversions) (1, 13, 15, 32, 40, 43). Site-specific recombination also mediates structural rearrangements involving homologous elements such as circularization of the terminally redundant linear P1 phage genome (36) and resolution of plasmid multimers. It has been suggested that the latter type of activity is involved in stable plasmid maintenance because certain plasmid mutants defective in site-specific recombination activity accumulate multimers and are hereditarily unstable (9, 38). A unit-copy replicon, such as P1, utilizes its site-specific recombination system to mediate dimer resolution into monomeric substrates for proper partitioning (2).

Site-specific recombination in *Staphylococcus aureus* plasmids is responsible for the formation of stable cointegrates (14). We have observed previously that most of the known small *S. aureus* plasmids contain one or two specific recombination sites, RS_A and RS_B (26). RS_B, about 30 base pairs (bp) in length, is present on all of the six plasmids then analyzed, namely, pT181, pE194, pC194, pC221, pS194, and pSN2. RS_B cointegrates were obtained only after cotransduction, and crossovers were later shown to map within a perfectly conserved 18-bp sequence (5'-AAGTTTTCTC GGCATAAA-3') (28). It was therefore suggested that RS_B recombination was mediated by a site-specific phage recombination function. The RS_A sequence was found in only two of the six plasmids, pT181, and pE194. In contrast to RS_B, plasmid transduction was not required for the formation of RS_A cointegrates. These were readily obtained with established heteroplasmid strains by selecting for the rescue of a temperature-sensitive mutant plasmid. In a Rec^+ host strain, recombination involving RS_A occurred at different locations within a 70-bp region consisting of a 24-bp fully conserved "core" sequence and flanking regions marked by a considerable number of mismatched nucleotides. No recombination occurred utilizing adjacent regions of extensive DNA homology in the two plasmids. These results suggested that RS_A recombination involved an unusual mechanism that recognized a specific short region but catalyzed strand exchange at different sites within that region.

In this communication, we identify a new plasmid-encoded recombination protein, Pre (plasmid recombination), that mediates RS_A, but not RS_B, recombination. Both of the plasmids that contain RS_A encode homologous Pre proteins, whereas plasmids lacking RS_A do not. We show that Pre-mediated recombination is site specific as it always occurs within the RS_A core sequence in a *recA1* background; this suggests that the previously observed site variability may reflect synergism between Pre and the host *rec* system.

MATERIALS AND METHODS

Bacterial strains and plasmids. The plasmids used in this study are listed in Table 1. The Rec^+ host strain was *S. aureus* RN450, a derivative of NTCC 8325 cured of all known prophages. *S. aureus* RN1030 (*recA1*) (42) is lysogenic for phage φ11. *Escherichia coli* BL21 (F^- *hsdS gal*) (37) was the host strain for plasmid pGEM1 (Promega Biotec) and its derivatives.

Nucleotide sequence coordinates are according to the published sequences of pT181 (16) and pE194 (11). Cloned fragments retain their original nucleotide sequence coordinates, which are suffixed (T) or (E) to indicate the plasmid of origin.

Media and culture conditions. Liquid and solid culture media for *S. aureus* were used as described (4). Tetracycline, chloramphenicol, and erythromycin were used at 5 μg/ml. Plasmid transfers were by transduction with phage φ11 (23) or by protoplast transformation (5).

For the growth of *E. coli* we used 2× YT broth, B agar, and M9 salts minimal medium (22); where appropriate, ampicillin (100 μg/ml) was added to the medium. Plasmid transformation of calcium chloride-treated cells was as described by Maniatis et al. (19).

Assay of plasmid stability. *S. aureus* cells from a selective plate were suspended in antibiotic-free liquid medium to a turbidity of approximately 20 Klett units (~2×10^8 CFU/ml) and grown to exponential phase. Dilutions (100 fold) of these cultures were grown nonselectively in liquid medium overnight. The cycle of dilution and growth in antibiotic-free broth was performed four times. Each cycle constituted approximately 20 generations. From each cycle, samples were diluted and spread onto antibiotic-free plates. The

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TABLE 1. Plasmids

Plasmid	Size (kilobases)	Description	Reference or source
pT181	4.4	Tc^r Inc3	(14b)
pE194	3.7	Em^r Inc11	(14a)
pRN5101	3.7	pE194 Tsr^a	A. Gruss
pC194	2.9	Cm^r Inc8	(14a)
pRN6321	2.8	pRN5101 Δ*Taq*I-C	A. Gruss
pRN8157	3.2	pT181 Δ*Dde*I-B + D	S. Projan
pRN6378	5.0	pC194::pT181 *Mbo*I-A	This work
pRN6450	3.1	pC194::pT181 RS_A (1931-2174)	This work
pRN6404	5.0	pRN6378 *Eco*RI linker at *Fnu*DII-1687	This work
pRN6010	8.1	pT181::pE194 *Xba*I cointegrate	(25)
pRN6019	5.9	2.2-kilobase deletion of pRN6010 Tc^s	(25)
pRN8110	4.4	pT181 *cop-633*	(4)
pGEM1	2.9	SP6-T7 cloning vector, Ap^r	Promega Biotech, Inc.
pRN8246	4.3	pGEM1::pT181 *Hgi*AI-*Rsa*I (T7 orient.)	This work
pRN8247	4.3	pGEM1::pT181 *Hgi*AI-*Rsa*I (SP6 orient.)	This work

[a] Tsr, Temperature sensitive for replication.

resulting colonies were replica plated on the appropriate antibiotic-containing plates and scored for resistance. The same procedure was used to detect cointegrate resolution. Colonies grown on tetracycline plus erythromycin plates at 32°C after 60 generations of growth in antibiotic-free liquid medium were replica plated on erythromycin plates at 43°C.

Isolation and analysis of plasmid DNA. Plasmid DNA was isolated by ethidium bromide-cesium chloride centrifugation of cleared lysates prepared as described (27). Sheared whole-cell minilysates were prepared as described by Projan et al. (31) and analyzed by 1.0% agarose gel electrophoresis.

Restriction mapping and cloning. Restriction enzymes were purchased from New England Biolabs, Bethesda Research Laboratories, and International Biotechnologies, Inc. and used as recommended. Restriction mapping was performed with ethidium bromide-cesium chloride–purified plasmid DNA samples.

Cointegrates between pT181 and pE194 and their deletion derivatives were analyzed by single and double digestions with *Taq*I, *Mbo*I, *Hin*fI, and *Dde*I restriction endonucleases. The digestion products were separated by agarose or acrylamide gel electrophoresis. Junctions were identified as fragments that did not comigrate with any fragment from either parental plasmid, and, conversely, fragments of the parental plasmids containing the crossover point were identified by their absence from the cointegrate digests.

For molecular cloning, standard procedures were used as described (4).

DNA sequencing. Nucleotide sequences were determined by the dideoxy chain termination method (34) using linear plasmid DNA as a template and a synthetic oligonucleotide (position 1860–1880 in the pT181 map) as a primer. The DNA sequence of the *pre* coding region was determined from isolated restriction fragments cloned into either mp10 or mp11 M13 vectors (22), using the M13 universal primer.

Analysis of cointegrate formation. Heteroplasmid strains of *S. aureus* RN1030 (*recA1*) were obtained by ϕ11 transduction. For each heteroplasmid analyzed, 6 to 12 single colonies grown on doubly selective plates at the permissive temperature for the thermosensitive replication (Tsr) mutant of pE194 used (32°C) were picked and suspended in CY broth (4). Appropriate dilutions were plated on doubly selective plates and incubated at the permissive (32°C) and nonpermissive (43°C) temperatures for 48 h.

Cointegrate formation frequencies were calculated as the ratio of the number of colonies at 43°C divided by the viable counts at 32°C. Approximately 1/4 to 1/3 of the colonies obtained at 43°C were lysed and screened by 1% agarose gel electrophoresis for the presence of cointegrate molecules, and the colony counts at 43°C were corrected for noncointegrates.

RNA isolation. Lysostaphin protoplasts were lysed with 5M guanidine thiocyanate (Fluka Chemicals). The crude lysate was layered on a 3-ml CsCl cushion ($\rho = 1.76$) and spun at 35,000 rpm for 16 h. The RNA pellet was precipitated from 8 M guanidine hydrochloride (6, 8), followed by two precipitations from distilled water and then four rinses with 80% ethanol. The final RNA pellet was suspended in TE (10 mM Tris, pH 7.6, 1 mM EDTA) and stored in aliquots at −70°C. When necessary, RNA was treated with DNase I (Pharmacia) (8 U/100 μg of RNA) in the presence of RNase inhibitor (Pharmacia) at 15 U/U of DNase I for 30 min at 37°C.

Runoff transcription. In vitro transcripts were obtained using a modification of the procedure of Fisher et al. (7). DNA templates consisted of specific restriction fragments of pT181 isolated from 5% acrylamide gels (20) and labeled with [α-^{32}P]dATP by nick translation (33). From 500 to 750 ng of template was used in each reaction. Transcriptions were carried out in a volume of 50 μl. DNA, *E. coli* RNA polymerase (1 U; Boehringer Mannheim), and binding buffer (40 mM Tris, pH 7.6, 80 mM KCl, 8 mM $MgCl_2$, 1 mM dithiothreitol, and 40 μg of bovine serum albumin per ml) were combined and incubated for 5 min at 37°C. Heparin was then added (600 μg/ml, final), followed by [α-^{32}P]UTP. The reaction was started by adding cold nucleotide triphosphates (200 μM ATP, CTP, and GTP; 20 μM UTP) and incubated for a further 40 min at 37°C. Reactions were terminated by adding sodium acetate to 0.3 M, 20 μg of carrier tRNA, and 3 volumes of 95% ethanol. After alcohol precipitation, the dried pellets were suspended in 8 M urea, denatured at 65°C for 10 min, and loaded on a thin 6% acrylamide–8 M urea sequencing gel. Electrophoresis was carried out at 1,200 V until the xylene cyanol was approximately 10 cm from the bottom. The gel was fixed in 5% acetic acid-methanol, dried, and autoradiographed at −70°C, using Fuji AR film and a DuPont intensifying screen.

S1 nuclease protection. The procedure outlined by Maniatis et al. (19) was followed for nuclease protection. Samples of 20 μg of RNA were used in these experiments. The DNA probe fragments were the same as in the runoff experiments. These fragments were treated with 1 U of calf intestinal alkaline phosphatase (Boehringer Mannheim) for 60 min at 37°C and then end labeled with [γ-^{32}P]ATP and 20 U of kinase (Pharmacia) for 60 min at 37°C. The labeled fragments were gel purified from 5% acrylamide and used for hybridization. Approximately 5×10^4 to 7×10^4 cpm was used for each hybridization. Hybridizations were carried out at 21, 30, and 39°C for 16 h. Then 200 U of S1 was added, and the samples were incubated for 30 min at 37°C, precipitated, and vacuum dried. Pellets were suspended and analyzed as described for runoff transcripts.

Lac induction. A single colony of the test strain was inoculated into 10 ml of ampicillin-containing M9 medium and grown at 37°C with shaking to 5×10^8 to 10×10^8

CFU/ml. Induction was with 0.4 mM isopropyl-β-D-thiogalactopyranoside. Cells were harvested 3 h after induction, and pellets were frozen (37). Proteins were extracted by acetone-sodium dodecyl sulfate treatment of the pellets (3). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 12.5% gels was by the method of Laemmli (17).

RESULTS

A plasmid product, Pre, is required for recombination at RS_A. To determine whether a plasmid product is involved in recombination at RS_A, we directed attention to the region between RS_A and RS_B, which contains a homologous open reading frame (ORF) on pT181 and pE194 to which no function had been assigned. Delections affecting this region were constructed by removal of restriction fragments using pT181 wild type and pRN5101, a temperature-sensitive derivative of pE194. A schematic map of the two plasmids indicating the deleted regions is presented in Fig. 1.

It has been previously shown in this and other systems (18, 26) that cointegrates can be selected in heteroplasmid strains, where one plasmid of the pair is temperature sensitive, by plating at the nonpermissive temperature on medium selective for the temperature-sensitive replicon. We constructed heteroplasmid strains by transduction in both Rec^+ and *recA1* host strains containing either the two parental plasmids or one or both deletion derivatives. Therefore, four pairs were examined: pT181 and pRN5101; pRN8157 (pT181 Δ*Dde*I−B+D) and pRN5101; pT181 and pRN6321 (pRN5101 Δ*Taq*I-C); and pRN8157 (pT181 Δ*Dde*I−B+D) and pRN6321 (pRN5101 Δ*Taq*I–C). The heteroplasmid strains were plated on erythromycin (5 μg/ml) plates (see Materials and Methods) at 43°C, and colonies arising were subcultured. Of 454 colonies, 284 were screened for plasmid content; 95% of them contained a single plasmid species whose size corresponded to the sum of the two original plasmids. Frequency values (Table 2) are corrected accordingly. Separate frequencies were measured for several single-colony isolates (see Materials and Methods); frequencies obtained for each heteroplasmid strain are the mean of these independent determinations. With the pT181 *pre* gene intact, cointegrates were formed at frequencies of the order of 10^{-6} per CFU. When both plasmids had ORF deletions, the frequency of colony formation decreased by two orders of magnitude. If the pE194 ORF was intact but that of pT181 was deleted, intermediate frequencies of cointegrate formation were obtained. Although cointegrate formation in Rec^+ cells occurred at a slightly higher frequency than in the *recA1* host, the frequency was equally depressed by the double ORF deletion.

These results led to a twofold conclusion. First, the corresponding ORFs of pT181 and pE194 each encode a recombination-promoting function; the pE194 protein seems somewhat less effective than that of pT181 for the formation of heterologous cointegrates. Second, cointegrate formation between pT181 and pE194 is not promoted to a comparable extent by any host *rec* function.

The recombination function responsible for the formation of rare cointegrates in the *recA1* mutant host and in the absence of either plasmid protein is unknown; it is noted that the residual activity of the *recA1* host strain for homologous interplasmid recombination is about 10^{-4} of that of the Rec^+ host (41). The cointegrates were found to be stable during prolonged growth on nonselective medium. Single colonies from each type were grown for approximately 40 generations in broth and then tested by replica plating at the nonpermissive temperature. Loss of the temperature-sensitive marker was not detected. If it occurs, it does so at a frequency less than 10^{-4}.

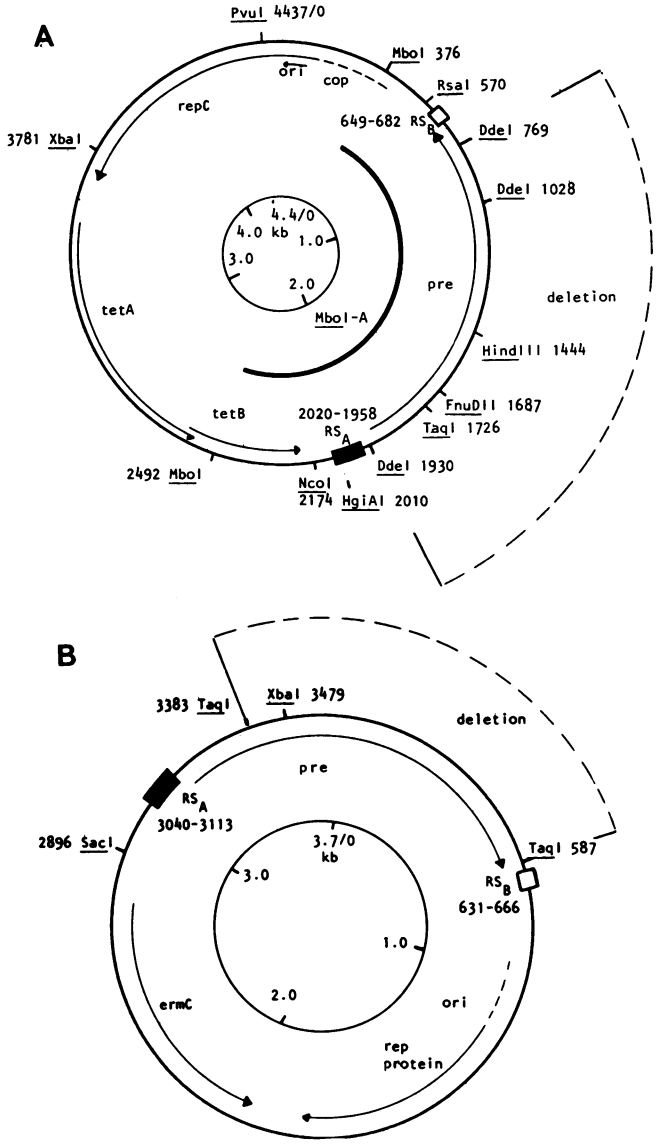

FIG. 1. Maps of *S. aureus* plasmids pT181 (A) and pE194 (B). Dark line inside the pT181 map represents the *Mbo*I-A fragment containing the *pre* gene. ori, Origin of replication; cop, copy control; repC, initiator gene; tetA, tetB, tetracycline resistance determinant. Numbers are nucleotide coordinates according to the published sequence (11, 16). Only relevant restriction sites are indicated.

The *Mbo*I-A fragment of pT181 encodes a *trans*-acting protein that mediates cointegrate formation. To test whether the ORF implicated in cointegrate formation acts in *trans*, we cloned the *Mbo*I-A fragment of pT181 (376–2492) into the unique *Hin*dIII site of the chloramphenicol resistance plasmid pC194 (12, 14). The cloned fragment contains the entire ORF and the two flanking recombination sites, RS_A and RS_B. The recombinant plasmid pRN6378 was introduced by transduction into the strain containing the deleted pT181 and pE194 replicons, and the frequency of cointegrate formation was determined by selecting for rescue of the temperature-sensitive pE194 plasmid. Since the three starting plasmids have different mobilities in agarose, the composition of the

TABLE 2. Frequencies of cointegrate formation

Plasmids present in heteroplasmid			Cointegrate formation frequencies[a] (10^{-6} CFU/ml)		*pre* genotype	
pT181	pE194	pC194::pT181 *Mbo*I-A	Wild type	*recA1*	pT181	pE194
pT181	pRN5101		3.8 ± 0.8	0.9 ± 0.4	+	+
pT181	pRN6321		NT	3.4 ± 1.9	+	−
pRN8157	pRN5101		NT	0.1 ± 0.09	−	+
pRN8157	pRN6321		0.05 ± 0.05	0.02 ± 0.04	−	−
pRN8157	pRN6321	pRN6378	NT	2.6 ± 2.3	+	−
					(in *trans*)	
pRN8157	pRN6321	pRN6404[b]	NT	0.04 ± 0.1	−	−
					(in *trans*)	

[a] Heteroplasmid strains were grown on selective plates at the permissive temperature (32°C). Single colonies were suspended in broth, diluted, and plated with double selection at 32 and 43°C. Frequencies represent the ratio of CFU at 43 and 32°C; values are the mean (±SD) of the frequency determinations on 6 to 12 colonies for each strain. NT, Not tested.

[b] Insertion of a synthetic octamer (*Eco*RI linker) at the *Fnu*DII site interrupts the *pre* gene.

cointegrates could be determined by screening the colonies by agarose gel electrophoresis for plasmid content. This screening showed that about 70% involved pE194 and pT181, whereas 30% involved pE194 and pC194. The overall frequency of cointegrates between pT181 and pE194 (Table 2) was approximately the same with the cloned *Mbo*I-A fragment in *trans* as with the native pT181 plasmid.

Additionally, we introduced a frameshift mutation into the ORF by inserting a synthetic octamer (commercial *Eco*RI linkers) at the unique *Fnu*DII site (position 1687 in pT181 sequence) and cloned the modified *Mbo*I-A fragment to pC194. The resulting plasmid, pRN6404, was introduced into the heteroplasmid strain carrying the pT181 and pE194 deletion derivatives used to demonstrate *trans* activity. In this case, no increase in cointegrate formation over background was observed (Table 2). We therefore suggest that the *Mbo*I-A fragment from pT181 encodes a *trans*-acting, recombination-promoting protein.

Nucleotide and amino acid sequence analysis. The published sequence of pT181 (16) shows two shorter, overlapping ORFs in comparison to the single 403-amino acid ORF of pE194 (11) encompassing the RS_A-RS_B region.

We resequenced the pT181 region between nucleotides 700 and 1800 and found six differences with the previously published sequence: an extra T at position 1520, a dinucleotide CA instead of TC at positions 1494–1495, and an extra A at position 1429 (Fig. 2). A was read instead of G at three different positions (795, 820, and 829). All changes refer to the bottom strand of pT181 as published.

The revised sequence contains a single ORF of 413 amino acids between positions 681 and 1917 of the plasmid map. The presence of only a single ORF is consistent with the protein analysis presented below.

A comparative analysis of the ORFs in pT181 and in pE194 shows a high degree of DNA homology (47%), which is reflected in the overall amino acid sequence (39%). The homology is much stronger in the N-terminal portion of the two polypeptides (approximately 60%) than in the C-terminal portion (approximately 20%) (Fig. 3). We refer to this ORF as Pre (plasmid recombination) henceforth, Pre(T) and Pre(E) being the pT181 and pE194 homologs.

The *pre* promoter. To map the *pre* promoter, we analyzed in vitro runoff transcripts by using two restriction fragments from pT181 as templates for *E. coli* RNA polymerase: *Mbo*I (2492)-*Taq*(1726) (766 bp) and *Mbo*I(2492)-*Hin*dIII(1444) (1,048 bp). These two fragments have the same *Mbo*I end but differ by 282 bp at the opposite *Taq*I and *Hin*dIII ends. Transcription products were run on a 6% acrylamide-urea sequencing gel next to a M13 dideoxy nucleotide sequencing ladder. The results (Fig. 4A) showed a unique band produced from each template. The band obtained from the shorter *Mbo*I-*Taq*I fragment was smaller than the band

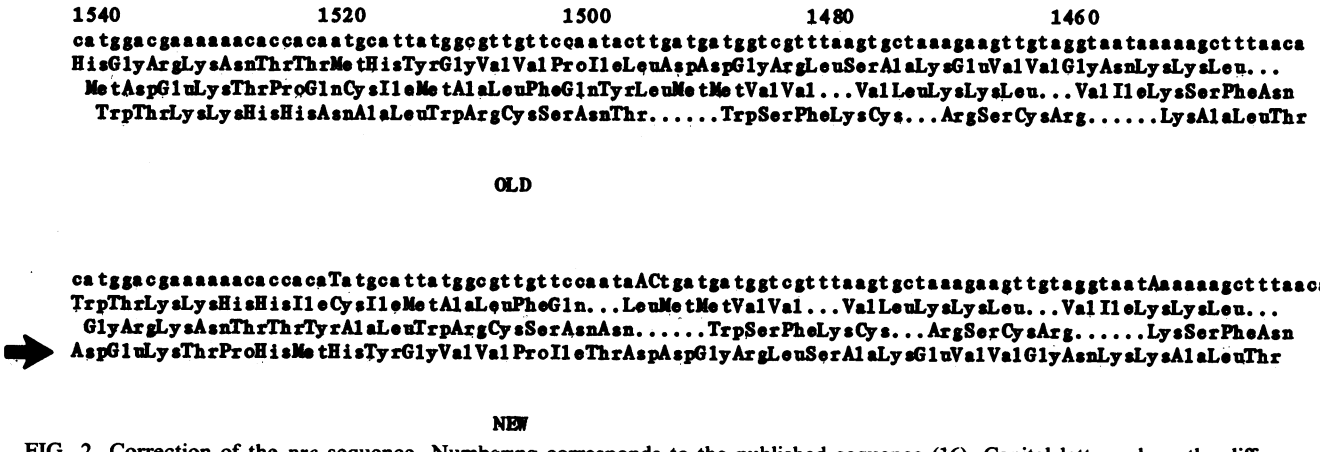

FIG. 2. Correction of the *pre* sequence. Numbering corresponds to the published sequence (16). Capital letters show the differences between the published sequence and that determined in this work. An arrow indicates the putative ORF for *pre*.

```
pE194   MSHSILRVARVKGSSNTNGIQRHNQRENKNYNNKDINHEETYKNYDLINAQNIKYKDKID (60)
        ::.::.::..::...:: :::.: :::: :: : ::.:  :: ::::.::.   . . ::
pT181   MSYSIVRVSKVKSGTNTTGIQKHVQRENNNYENEDIDHSKTYLNYDLVNANKQNFNNLID (60)

pE194   ETIDENYSGKRKIRSDAIRHVDGLVTSDKDFFDDLSGEEIERFFKDSLEFLENEYGKENM (120)
        : :..::.::::::.:::.:.:::.::: ::::. . :.  .:: . ::::.::::.:.
pT181   EKIEQNYTGKRKIRTDAIKHIDGLITSDNDFFDNQTPEDTKQFFEYAKEFLEQEYGKDNL (120)

pE194   LYATVHLDERVPHMHFGFVPLTEDGRLSAKEQLGNKKDFTQLQDRFNEYVNEKGYELERG (180)
        :::::.::. ::::.:.::.:.::::::: .:::: .: .:::::.: ..:: ::::
pT181   LYATVHMDEKTPHMHYGVVPITDDGRLSAKEVVGNKKALTAFQDRFNEHVKQRGYGLERG (180)

pE194   TSKEVTEREHKAMDQYKKDTVFHKQEL-------QEVKDELQKANKQLQSGIEHM--RST (231)
         :..::   : ..::::. : .:::::          .:    : .. : ..  .
pT181   QSRQVTNAKHEQISQYKQKTEYHKQEYERESQKTDHIKQKNDKLMQEYQKSLNILKKPIN (240)

pE194   KPFDYENER-TGLFS--GREETGRKILTADEFERLQETISSAERIVDDYENIKSTDYYTEN (289)
         :.. : :.  :::::   .:.:  ...  .:  .:  : .:. : .::: :::     .
pT181   VPYEQETEKVGGLFSKEIQEAGNVVISQKDFNEFQKQIKAAQDISEDYEYIKSGRALDDK (300)

pE194   QELKKRRESLKEVVNIWKEGYHEKSKEVNKLKRENDSLNEQLNWSEKFQASTV-TLYRA- (347)
          . .. :          :          : :        : :.. .. :.     .  : :.
pT181   DKEIREKDDLLNKAVERIE---NADDNFNQLYENAKPLKENIEIALKLLKILLKELERVL (357)

pE194   ARANFPGFEKGFNKLKEKF--FNDSKFERVGQFMDVVQDNVQKVDRKREKQRTDDLEM   (403)
        .:  :.     .     :.  .  .  ...  .   :.. :   .   . :   :
pT181   GRNTFAERVNKLTEDEPKLNGLAGNLDKKMNPELYSEQEQQQEQQKNQKRDRGMHL     (413)
```

FIG. 3. Comparison of the Pre proteins encoded by pT181 and pE194. Proteins are aligned to obtain maxima homology, with dashes (—) replacing missing amino acids. Colon (:) indicates residue identity between the two proteins; dot (.) indicates conservative changes. Figures in parenthesis at the end of each line indicate position number of last residue in the row.

obtained from the longer *Mbo*I-*Hin*dIII fragment. This indicated that the direction of transcription is from the *Mbo*I end toward the *Taq*I end. By comparison with the sequencing ladder, the *Mbo*I-*Taq*I runoff transcript measured approximately 240 nucleotides, which maps the transcription start site at around position 1968 in pT181 sequence. The *Mbo*I-*Hin*dIII runoff, which would be about twice as long, was too large to measure accurately.

To confirm the in vitro runoff results, we performed S1 nuclease protection studies. Whole-cell RNA isolated from an *S. aureus* strain carrying a pT181 copy mutant was hybridized to the *Mbo*I-*Taq*I and *Mbo*I-*Hin*dIII fragments end-labeled with kinase and [γ-^{32}P]ATP. S1-protected hybrids were run on a 6% sequencing gel next to an M13 dideoxy sequencing ladder. The results (Fig. 4B) showed that a protected band was obtained from both fragments. Consistent with the runoffs, the band protected by the *Mbo*I-*Taq*I fragment was shorter than the band protected by the *Mbo*I-*Hin*dIII fragment. Measurement of the S1-resistant band from the *Mbo*I-*Taq*I fragment gave a length of 232 nucleotides. This is in good agreement with the in vitro transcription results and maps the site of transcription initiation to position 1960 in pT181 sequence. The presence of a second, higher-molecular-weight protected band is noted here. This band, present with both probes, was seen preferentially at lower hybridization temperatures, suggesting a lower degree of homology. The origin and nature of this species are unknown. Figure 5 shows the sequences containing the promoter and initiation site of the *pre* gene.

A protein is made from the 413-amino acid ORF. To test whether the *Mbo*I-A transcript was translated, we cloned the *Hgi*AI(2010)-*Rsa*I(570) fragment, internal to the *Mbo*I-A region but containing the entire *pre* coding sequence, into *E. coli* expression vector pGEM1, a 2.9-kilobase ampicillin resistance plasmid containing a polylinker region between T7 and SP6 promoters; depending on the orientation of the insert, transcription will be driven by either promoter.

We chose the orientation in which the insert was cloned under control of the T7 promoter and transformed the recombinant plasmid to *E. coli* BL21 (lambda DE3 lysogen) (37), which contains the T7 RNA polymerase gene in the chromosome under control of the inducible *lacUV5* promoter. Addition of isopropyl-β-D-thiogalactopyranoside induces T7 RNA polymerase, which in turn results in high-level expression of the target gene cloned in plasmid pGEM1 (see Materials and Methods).

Figure 4C shows the result of a sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the protein content of crude cell lysates prepared from isopropyl-β-D-thiogalactopyranoside-induced cultures containing either the recombinant plasmid or the vector plasmid. The gel reveals a single prominent protein band in the recombinant plasmid-containing cells that is absent in the controls. The apparent molecular weight of this protein is approximately 50,000,

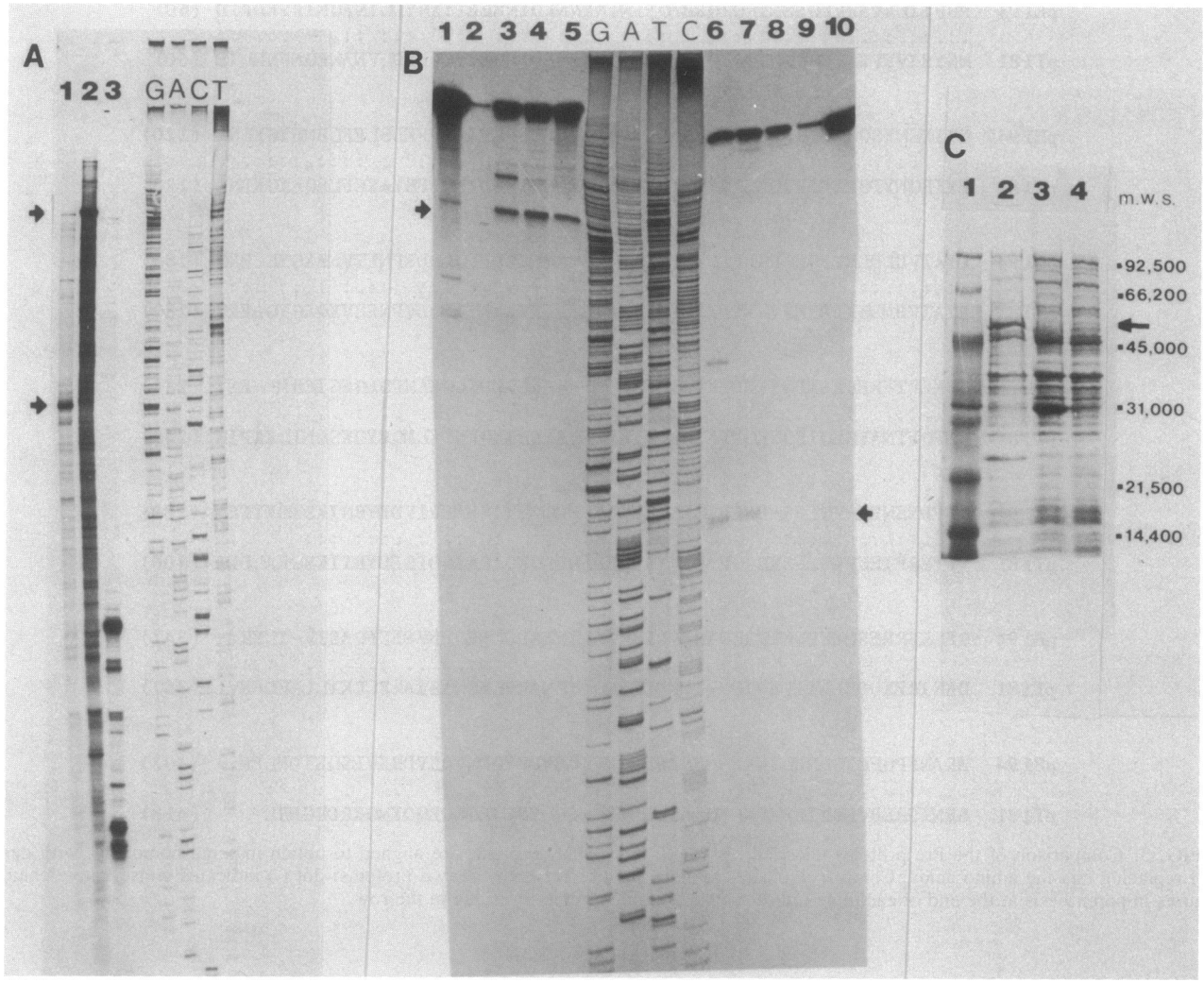

FIG. 4. Pre expression. (A) Runoff transcription experiments were carried out as described in Materials and Methods, and the reaction products were visualized by electrophoresis on a 6% polyacrylamide gel containing 8 M urea. Two fragments were used as templates for *E. coli* RNA polymerase-directed in vitro transcription: pT181 *Mbo*I(2492)-*Taq*I(1726) (lane 1) and *Mbo*I(2492)-*Hin*dIII(1444) (lane 2). pT181 *Mbo*I-D fragment (31–376) was used a template for the control reaction (lane 3), since it gives rise to transcripts of known length. GACT is an M13 ladder used as a size marker. Arrows indicate bands corresponding to a transcript of approximately 240 bases (lane 1) and a larger transcript (lane 2) whose size is not measurable under these experimental conditions. The results indicate the direction of transcription of the *pre* gene (see text). (B) The same pT181 fragments as in runoff transcription were used for S1 protection experiments, and the products were electrophoresed on a 6% polyacrylamide–8 M urea gel: pT181 *Mbo*I(2492)-*Hin*dIII(1444) (lanes 1 through 5) and pT181 *Mbo*I(2492)-*Taq*I(1726) (lanes 6 through 10). GATC is an M13 ladder used as a size marker. Lanes: 1 and 10, purified fragment, no RNA and no S1; 2 and 9, purified fragment + S1, no RNA; 3 to 5 and 6 to 8, purified fragment + RNA + S1 (DNA-RNA hybridization was carried out at different temperatures: 3 and 6, 21°C; 4 and 7, 30°C; 5 and 8, 39°C). Arrows indicate protected bands obtained with the two templates, corresponding to the 5′ end of the *pre* mRNA. (C) Expression of *pre* gene product in *E. coli*. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of whole-cell lysates upon *lac* induction, as described in Materials and Methods, visualized by Coomassie staining. Lanes: 1, molecular weight standards (m.w.s.) (BioRad Laboratories); 2, pGEM1::pT181 *Hgi*AI-*Rsa*I (T7 orientation); 3, pGEM1; 4, pGEM1::pT181 *Hgi*AI-*Rsa*I (SP6 orientation). The arrow indicates a protein band of approximately 50,000 molecular weight produced by the recombinant plasmid pGEM1::pT181 *Hgi*AI-*Rsa*I (T7 orientation) (Lane 2), which is absent in the other lanes.

which is in agreement with the predicted molecular weight (55,700). Therefore, these results confirm the sequence data indicating a single ORF in the *pre* region.

Pre-mediated cointegrate formation occurs at RS_A. Restriction analysis of 41 cointegrates derived from the first three classes of heteroplasmid strains listed in Table 2 (in which at least one of the parental plasmids carried *pre*) showed that (i) as previously observed, the two plasmids are always in the same orientation relative to each other in the cointegrate molecule, and (ii) the crossover point was always located in the RS_A region and never in the flanking *pre* homologous regions (data not shown). Additionally, 8 cointegrates of the 48 obtained in the presence of the cloned pT181 *pre* gene were analyzed by restriction enzyme digestion; they were all RS_A cointegrates. These observations confirmed previous findings (28) that cointegrates form primarily at RS_A, despite the presence of adjacent homologous sequences, and indicated that the *pre* gene product promotes these events. We determined the DNA sequence of one crossover junction of seven of the RS_A cointegrates formed in the presence of Pre

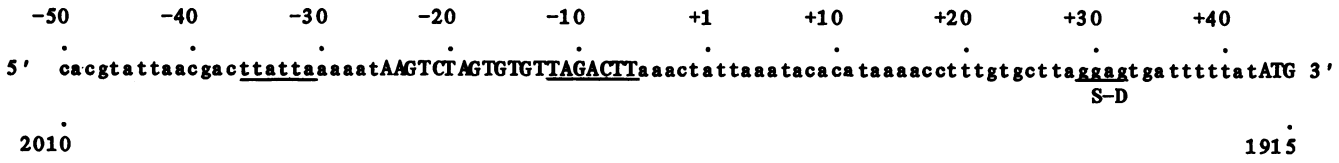

FIG. 5. Promoter region of pT181 *pre* gene. The nucleotide sequence of pT181 from position 2010 to position 1915 is shown, indicating the start site (+1) of the *pre* mRNA. Putative −10 and −35 regions of the *pre* promoter are underlined. RS_A core sequence is shown in capitals. The putative Shine-Dalgarno sequence (S-D) and the ATG initiation codon are also indicated.

in a recombination-deficient host. In each case, the crossover site was contained within a 24-bp sequence of identity, the RS_A core (Fig. 6A), suggesting that Pre-mediated cointegrate formation is site specific. In contrast, of five cointegrates from a heteroplasmid strain containing the two Pre^- plasmids, three were identified as RS_A and two as RS_B cointegrates, suggesting the existence of additional low-activity *rec* functions capable of recognizing RS_A and RS_B, possibly on the basis of homology.

Intramolecular recombination occurs within the RS_A core sequence. Plasmid pRN6010 is the prototype of a series of cointegrates constructed in vitro by ligating pT181 (wild type or copy mutants) and pE194 at their unique *Xba*I sites (25). A schematic map of pRN6010 is shown in Fig. 7. The plasmid contains the intact pT181 *pre* gene, whereas the corresponding pE194 *pre* coding region is interrupted by the cloning procedure. The tetracycline resistance (Tc^r) gene of pT181 is flanked in this construct by homologous sequences in the direct orientation, including the RS_A sites of each parent. These cointegrates (but not pRN6050, in which pT181 and pE194 are in the opposite orientation) give rise to spontaneous deletions of the Tc^r marker at very low frequency ($<10^{-4}$). Tc^s spontaneous deletion derivatives could be obtained by exploiting the presence of a unique *Kpn*I site within the tetracycline resistance gene (G. K. Adler and R. P. Novick, unpublished data). Plasmid DNA from cultures carrying pRN6010 was digested with *Kpn*I before transformation; only *Kpn*I-resistant molecules (i.e., missing tetracycline resistance gene sequences) could give rise to Em^r transformants. As expected, these plasmids carried a spontaneous deletion in the tetracycline gene (25). We sequenced the deletion site in Tc^s derivatives of seven cointegrates of the pRN6010 family. The deletion occurred, in each case, by a site-specific crossover within the 24-nucleotide RS_A core; its sequence is identical to that shown in Fig. 6B. It is therefore likely that these events are Pre mediated.

Pre^- plasmids are not detectably defective in maintenance and do not accumulate multimers. Since other site-specific recombination systems act to resolve plasmid multimers, we tested Pre^- or RS_A^- plasmids (or both) for instability in *S. aureus* and for the accumulation of multimers. No loss of Pre^- plasmids from either Rec^+ or *recA1* host strains was detected after growth for approximately 40 generations in nonselective medium.

The presence of multimers was analyzed by 0.8% agarose gel electrophoresis of whole-cell lysates containing Pre^- or Pre^+ plasmids. Accumulation of multimers was greater with Pre^+ as opposed to Pre^- plasmids and in a Rec^+ as opposed to a *recA1* background (Fig. 8). Similar results were obtained with RS_A^- plasmids (not shown) which, due to the overlapping of RS_A sequences with the *pre* promoter, are also Pre^-.

DISCUSSION

We have shown that two *S. aureus* plasmids, pT181 and pE194, possess a site-specific recombination system. This system comprises a *cis*-acting element, the recombination site RS_A, and a *trans*-acting product which we have designated Pre. The RS_A sites in the two plasmids have identical 24-bp core sequences and are 80% homologous in the flanking sequences. A perfect 7-bp inverted repeat with 6-bp spacer region (5'-GTGTGT-3') is located within the core sequence (Fig. 6A). Some dyad symmetry is present in other sites involved in site-specific recombination, such as *res* (Tn*3*), *parB* (CloDF13), and *loxP* (P1), and is common to many other protein-binding sites.

Although the two Pre proteins are similar in size [413 amino acids for Pre(T) and 403 amino acids for Pre(E), respectively] and amino acid sequence (39% overall homology; 60% in the N-terminal region), Pre(T) seems considerably more active than Pre(E) in the formation of pT181-pE194 RS_A cointegrates. Indeed, Pre(E) may even interfere with Pre(T) in this situation; when both were present in the heteroplasmid the cointegration frequency was considerably lower than when Pre(T) alone was present. The Pre proteins

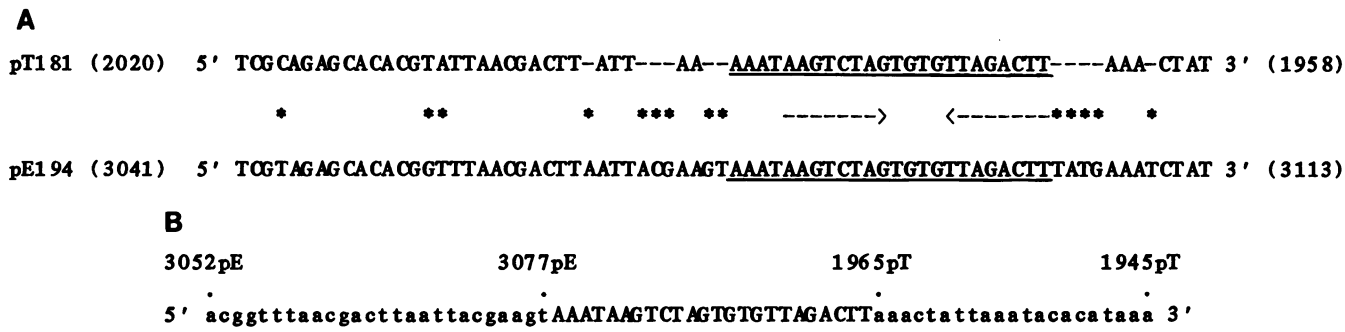

FIG. 6. Recombination site A (RS_A). (A) Sequence of the RS_A site in pT181 and pE194. Mismatched bases are starred; a 24-nucleotide sequence of identity, referred to as core sequence, is underlined (adapted from reference 28). (B) Sequence of the junction site of in vivo pT181::pE194 cointegrates. The crossover point maps within the RS_A core sequence, which is shown in capital letters.

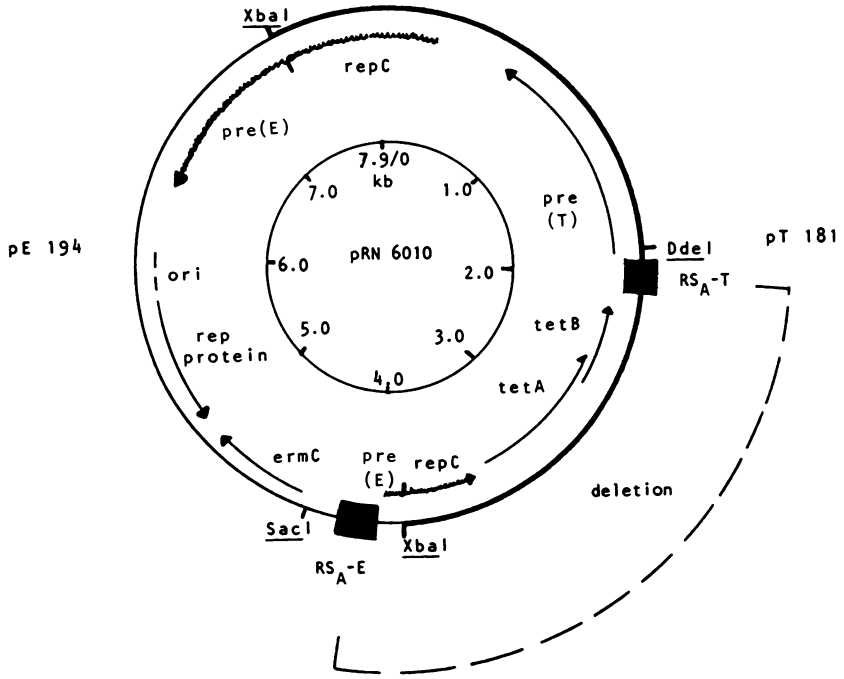

FIG. 7. Intramolecular recombination at the RS_A site. Map of pRN6010, a pT181::pE194 *Xba*I cointegrate. pT181 sequences are represented by a thick line; those from pE194 are indicated by a thin line. Coding sequences in the two plasmids interrupted by the cloning procedure are shown as wavy lines [*pre*(E) and pT181 *repC*]. The spontaneous deletion of a 2.2-kilobase fragment including the tetracycline resistance determinant is indicated by the dashed lines. The sequence of the crossover point is the same as in Fig. 6B.

are encoded by the largest ORF present in each plasmid. We have shown that Pre(T) is synthesized in vivo; Dubnau and co-workers (35) had previously shown that a 54-kilodalton protein, indicated as E1, was synthesized from the *pre* coding region in *Bacillus subtilis* minicells containing pE194.

We have focused our attention on the pT181-encoded system and shown that Pre-mediated crossover events always take place within the 24-bp core sequence of RS_A. This site seems to be used more efficiently for inter- than for intraplasmid recombination, since multimers accumulated with Pre^+-RS_A^+ but not with Pre^--RS_A^- plasmids.

We were able to detect Pre-mediated intraplasmid recombination only in a situation permitting molecular selection (for *Kpn*I resistance). Unfortunately, no estimate of frequency could be obtained in such experiments. We were also unable to measure resolution frequency directly, as it was below the detection limit ($<10^{-4}$ per cell per generation).

Since all the experiments involved heterologous RS_A sites, it was considered important to examine the behavior of homologous sites. Cointegrates were obtained between pSA0301 (a Tsr pT181 mutant) and pRN6450 (a pC194 derivative carrying a 240-bp RS_A-containing fragment from pT181) at a frequency of 10^{-5} per cell.

We do not know the function responsible for formation of these cointegrates, because the *recA1* host has residual homologous interplasmid recombination activity. However, the resolution frequency of these cointegrates was again below the limit of detectability ($<10^{-4}$). While these experiments do not permit any definite conclusion about the relative frequency of cointegrate formation versus resolution, they argue strongly against the possibility that the biological function of the RS_A-Pre system is multimer resolution. Any resolution function would have to operate at an efficiency many orders of magnitude greater than that observed for this system to play any meaningful role in the plasmid replication-cell division cycle. In contrast, the other plasmid-coded site-specific recombination systems, which do function in multimer resolution, have activity frequencies of the order of 10^0 to 10^{-1} per cell per generation (2, 9, 38).

The RS_A-Pre system may be widespread among plasmids in gram-positive bacteria. pUB110, a small multicopy plasmid originally isolated from *S. aureus* (30), has been recently

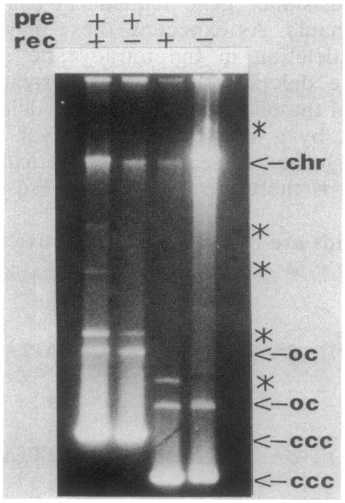

FIG. 8. Analysis of multimer formation. Whole-cell sheared lysates containing the indicated plasmids were separated on 0.8% agarose in Tris borate buffer for 18 h at 2.5 V/cm, stained with ethidium bromide, and photographed. Lanes: 1 and 2, pT181 (pre^+); 3 and 4, pRN8157 (pre^-). rec, Host recombination function; pre, plasmid Pre phenotype; chr, chromosomal DNA; oc, relaxed plasmid DNA; ccc, supercoiled plasmid DNA. Asterisks indicate multimeric forms.

sequenced (21). It contains an ORF of comparable size and with 34% amino acid sequence homology to that of *pre* in pT181 and pE194. Upstream of it there is a sequence that is almost identical to the RS_A core, with one mismatch in the inverted repeat and the same 6-bp spacer.

The RS_A sequence and the *pre* promoter overlap (as shown in Fig. 5). The promoter for the *tnpR* gene in Tn*3*-γδ and most of the promoters for the *din* genes (encoding expression-related invertases) overlap with their crossover sites, and the *tnpR* gene product has been shown to act as a repressor of its own synthesis (10, 29). Experiments in progress have shown that Pre(T) synthesis is autoregulated. Observations by D. Dubnau and co-workers (personal communication) also suggest that Pre(E) synthesis may be autoregulated.

Although the presence of Pre is a primary requirement for RS_A recombination, a host *rec* function may play an additive role by perhaps increasing the overall efficiency of the reaction and, more interestingly, by causing the initiation of strand exchange to take place at different points within the recombination site. Such synergism between Pre (site-specific) and host *rec* (homologous) functions operating on the same site has not heretofore been observed.

It is difficult to imagine how the role of RS_A-Pre in the formation (rather than resolution) of cointegrates and multimers is directly involved in plasmid maintenance. Two alternatives are as follows. (i) It could be involved in plasmid transfer by transduction, a process that requires the formation of concatemers (39; R. P. Novick, I. Edelman, and S. Lofdahl, in press); this seems unlikely, as we have observed that the transduction frequency for RS_A-Pre^- plasmids is the same as for RS_A-Pre^+ (unpublished data). Alternatively (ii), its primary function could be the formation of heterologous cointegrates, which would imply that these have selective value in *S. aureus* and other gram-positive bacteria. For example, they could be intermediates in the evolution of larger plasmids carrying multiple antiobiotic resistance determinants.

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