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Genetics

Coding sequence for the pT181 *repC* product: A plasmid-coded protein uniquely required for replication

(complementation/initiation of replication/thermosensitive mutations/*Staphylococcus aureus* plasmids/tetracycline resistance)

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ABSTRACT **pT181 is a 4.4-kilobase plasmid from *Staphylococcus aureus* specifying tetracycline resistance and present in about 20 copies per cell. The existence of a diffusible pT181 product required for plasmid replication has been proposed on the basis of *trans*-complementable thermosensitive mutants defective in plasmid maintenance (phenotype Tsr). In this report, the Tsr mutants are shown to have primary replication defects, and the genetic complementation data are confirmed biochemically. All of five mutations are in a single cistron, the *repC* cistron; interruption of the plasmid DNA molecule at any of three neighboring restriction sites inactivates *repC* function. Analysis of the DNA sequence in this region reveals an open reading frame of 939 base pairs which encodes the *repC* product, a 313-amino acid protein. pT181 replication has been demonstrated in cell-free extracts to require specifically a pT181-coded protein of approximately the same size, and it is proposed that this protein is, indeed, the *repC* product. Preliminary evidence is discussed suggesting that the pT181 replication rate is controlled at the level of synthesis of the *repC* protein.**

As predicted by Jacob *et al.* (1), many plasmids specify one or more replicon-specific "*rep*" proteins that are required for replication (2–5). Such proteins presumably act as a precise functional link between the plasmid and the host by recognizing specifically the autologous plasmid origin and playing a critical role in the generation of a 3′ primer terminus. Therefore, a probable control point for replication rate is at the level of synthesis or action of *rep* proteins.

As part of a program intended to analyze the role of *rep* proteins in plasmid replication and its control, we have undertaken the study of pT181, a tetracycline (Tc)-resistant (Tc^r) plasmid from *Staphylococcus aureus*. pT181 is a 4.4-kilobase (kb) plasmid (6) with a copy number of 20–25 per cell (S. Projan, personal communication). Similar or identical plasmids are dispersed throughout the world and are probably responsible for the majority of Tc resistance encountered in veterinary and human clinical strains of *S. aureus* and *Staphylococcus epidermidis* (6, 7). pT181 was identified initially by Iordanescu, who isolated five mutants that were unable to be maintained at elevated temperature (phenotype Tsr) and could be complemented in *trans* by the wild-type plasmid (4). One of the mutants was unable to complement any of the others, suggesting that all five mutants are in the same cistron, designated *repC* (4).

Khan *et al.* (8) have identified a protein in extracts of pT181-containing cells that is required specifically for replication of pT181 DNA *in vitro*. More recently, this protein has been partially purified and found to have a M_r of 35,000–40,000 (unpublished data). It is overproduced by a control mutant, *cop-608*, which has a copy number approaching 1,000 per cell (unpublished data).

In this paper, we define the coding sequence for the *repC* protein of pT181 and present preliminary evidence suggesting that this protein is rate-limiting for replication of the plasmid *in vivo*.

A nearly identical Tc^r plasmid, pT127, has been analyzed by Ehrlich and co-workers (9, 10). The known replication functions of pT127 are clustered in a 1.5-kb region bounded by the *Mbo* I sites at 3.0 and 0.38 kb on our map (see Fig. 3 and ref. 9; B. Niaudet and S. D. Ehrlich, personal communication), and we assume provisionally that the same is true for pT181.

METHODS

Bacterial Strains and Culture Conditions. Strains of *S. aureus* and plasmids that were used are listed in Table 1. Strains were stored and cultured as described (14). Tc (gift of Chas. Pfizer & Sons), erythromycin (Em, gift of Eli Lilly), and chloramphenicol (Cm, gift of Parke Davis) were used at 10, 100, and 25 μg/ml, respectively.

Transduction was performed with phages φ11 and 80α as described (15). Transformation of protoplasts (16) was modified for *S. aureus* by the use of lysostaphin (30 μg/ml). Antibiotics for selection of transformants were at the following concentrations in μg/ml: Tc, 5; Em, 3; Cm, 10.

Isolation and Analysis of Plasmid DNA. Cultures were screened for the presence and the approximate sizes of plasmids by a modification of the method of Meyers *et al.* (17) in which lysostaphin was substituted for lysozyme. Lysates, prepared from about 2×10^9 cells, were analyzed by electrophoresis on horizontal agarose slab gels in Tris/borate/EDTA buffer (18) and were stained and photographed as described (19). Plasmid DNA in gel bands was quantitated by scanning the ethidium bromide-stained gel with a fluorescence-activated scanning densitometer (Shimadzu model CS910) (S. Projan, personal communication). The scanner gave a linear response from 5 to 1,500 ng of DNA; the intensity of fluorescence was stable during destaining for at least 2 hr in water, and the correction factor for differential binding of ethidium to supercoiled DNA in comparison to other forms (20) was determined empirically to be 1.36 under the conditions used.

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Abbreviations: kb, kilobase(s); Tc, tetracycline; Em, erythromycin; Cm, chloramphenicol; Em^r, Cm^r, and Tc^r, Em, Cm, and Tc resistant; Tsr, phenotype of thermosensitive mutants defective in plasmid maintenance; Cm^s, Em^s, and Tc^s, Cm, Em, and Tc sensitive.

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Table 1. Plasmids

Plasmid	Size, kb	Phenotype/genotype	Derivative*	Ref.
pT181	4.4	Tc^rInc3^+	Naturally occurring	7
pSA0301	4.4	Tc^rInc3^+ *repC1*	NNG mutagenesis of pT181	4
pSA0311	4.4	Tc^rInc3^+ *repC2*	NNG mutagenesis of pT181	4
pSA0321	4.4	Tc^rInc3^+ *repC3*	NNG mutagenesis of pT181	4
pSA0331	4.4	Tc^rInc3^+ *repC4*	NNG mutagenesis of pT181	4
pSA03451	4.4	Tc^rInc3^+ *repC5*	NNG mutagenesis of pT181	4
pSA5000	4.0	Cm^rInc3^+ $repC^+$	*In vivo* recombinant pSA5000–pC221	11
pSA5120	4.0	Cm^rInc3^+ *repC1*	*In vivo* recombinant pSA5000–pSA0301	11
pE194	3.6	Em^rInc11^+	Naturally occurring	12
pRN6010	8.0	$Tc^rEm^rInc11^+$	pT181::pE194 (*Xba* I clone)	—
pSA5500	8.0	$Tc^rEm^rInc11^+$	pT181::pE194 *in vivo* cointegrate	13
pRN6233 (pRN6010Δ141)	7.9	$Tc^rEm^rInc11\Delta141$	*In vitro* deletion of pRN6010	—
pRN6238 (pT181Δ141)	4.3	Tc^rInc3^+ $repC^-$ Δ141	pT181 moiety of pRN6233	—

* NNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

These data were used to calculate plasmid copies per cell, C_p, as $C_p = (D_p \cdot M_c)/(D_c \cdot M_p)$ in which D_p and D_c are the amounts of plasmid and chromosomal DNA in the gel, respectively, M_c is the chromosomal DNA per cell, and M_p is the M_r of the plasmid.

Plasmid DNA was isolated as described (19). Restriction endonucleases and other enzymes as noted were purchased from New England BioLabs and Bethesda Research Laboratories and were used in accordance with the instructions of the supplier. T4 DNA ligase was prepared by the method of Murray *et al.* (21). Analysis of restriction digests was by electrophoresis on horizontal agarose slab gels in Tris/borate/EDTA buffer.

Analysis of Plasmid Replication. Strains were pulse-labeled with [^{3}H]thymidine (10 μCi/ml; 1 Ci = 3.7×10^{10} becquerels) in vigorously aerated exponential broth cultures at the desired temperature. Sheared whole-cell lysates were prepared from the labeled cultures and analyzed by gel electrophoresis. DNA in gel bands was quantitated by fluorescence densitometry, and the bands were excised, melted at 100°C, and counted in Liquiscint.

Complementation Tests. Heteroplasmids (strains containing two or more different plasmids) were constructed by transformation, maintaining double selection for the two plasmids, and were analyzed for the ability of either plasmid to replicate by pulse-labeling with [^{3}H]thymidine after a shift to the restrictive temperature. Labeled cultures were analyzed as before.

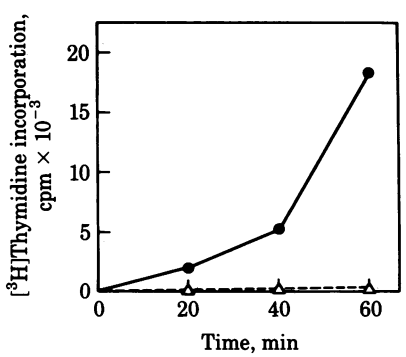

FIG. 1. Replication analysis of Tsr mutant plasmids. Strains were grown to early exponential phase at 32°C in CY broth and were then shifted to 43°C at $t = 0$. Aliquots were removed at various times and labeled for 20 min with [^{3}H]thymidine (10 μCi/ml) and then analyzed by agarose gel electrophoresis of sheared whole-cell lysates. The supercoiled plasmid bands were excised and assayed, and the resulting counts are plotted as a function of time after the temperature shift. ●, Incorporation for pT181; △, incorporation for the mutants. Data for all five mutants were indistinguishable.

RESULTS

Copy Number and Replication of *repC* Mutants. The exponential segregation of the Tsr plasmid mutants at 43°C suggested that they are defective in replication (4, 16). This was verified by direct measurements of replication as shown in Fig. 1. The mutants all stopped thymidine incorporation immediately after the temperature shift, indicating that each has a primary thermosensitive replication defect. These data, however, do not localize the defects to any point in the plasmid replication cycle.

The densitometric scans of the zero-time samples were used to calculate plasmid copy numbers at 32°C (Table 2). Copy numbers for 43°C were determined on cultures that had been grown on selective plates overnight at that temperature. Note that two of the Tsr mutants have higher copy numbers than the wild type does at the permissive temperature, whereas others are lower. Both of the former have independent mutations affecting replication and copy control.

Complementation Analysis. Incompatible heteroplasmids (14) were constructed by transformation of protoplasts prepared from strains containing each of the five Tsr mutants of pT181 with either pSA5000 ($repC^+$) or pSA5120 (*repC1*) DNA, maintaining Tc/Cm double selection. Note that pSA5000 and pSA5120 are derivatives of pT181 and pSA0301, respectively, in which the Tc^r determinant has been replaced by the Cm-resistant (Cm^r) determinant of pC221 (11). One strain from each cross, which was shown to lose the two resistant markers independently during growth on nonselective medium, was used

Table 2. Plasmid copy numbers

Plasmid	*repC* allele	Copies/cell 32°C	Copies/cell 43°C
pT181	$repC^+$	26	28
pSA0301	*repC1*	40	<2*
pSA0311	*repC2*	11	†
pSA0321	*repC3*	5	†
pSA0331	*repC4*	265	9*
pSA0341	*repC5*	21	≈2–3*
pSA5000	$repC^+$	25	20
pSA5120	*repC1*	40	≈2–3*

* Grew very poorly on selective media at 43°C. Plasmid bands for *repC1* and *repC5* mutants were barely detectable.
† Did not grow on selective media at 43°C.

to isolate a Cm-sensitive (Cm^s) Tc^r segregant. In each case, the Tc^rCm^s segregant was shown to have retained its Tsr phenotype. These heteroplasmid strains were subjected to successive 15-min pulses of [^{3}H]thymidine (10 Ci/ml), after a shift of exponential cultures from 32°C to 43°C, and the labeled cultures were analyzed. Both the *rep*$^+$ plasmid, pSA5000, and the *rep*$^-$ plasmid, pSA0331, replicated at the restrictive temperature, showing complementation of the latter by the former (Fig. 2*A*), whereas when both plasmids were *rep*$^-$ (Fig. 2*B*), neither replicated at the restrictive temperature, suggesting that the two *rep*$^-$ mutations are in the same cistron. Similar results were obtained for the other four mutants, pSA0301, pSA0311, pSA0321, and pSA0341 (not shown), confirming the genetic complementation data obtained by Iordanescu and Surdeanu (11).

Physical Map of pT181. The plasmid was analyzed to determine the physical location of the *repC* cistron. A restriction map of the pT181 plasmid molecule (Fig. 3) was determined by standard techniques involving single and multiple restriction enzyme digests, and full documentation is given by Adler (22).

The *Pvu* I site at 12 o'clock has been chosen for the origin of map coordinates, which read in the clockwise direction. Determination of the primary nucleotide sequence of the entire plasmid (unpublished data) has confirmed the existence of the restriction sites indicated in Fig. 3. As has been shown for pT127 (9), the single *Kpn* I site at 2.83 kb was found to be within the Tc^r determinant of pT181; pRN6010 DNA was digested with *Kpn* I and incubated with *Escherichia coli* DNA polymerase I in the presence of the four dNTPs, followed by T4 DNA ligase. The resulting preparation was introduced into *S. aureus* cells by protoplast transformation with selection for Em resistance and was scored for Tc resistance. Several Em^r/Tc^s transformants were isolated, and all were found to lack the *Kpn* I site (experiment performed by Alexandra Gruss). The sequence studies (unpublished data) revealed that the *Kpn* I site is located within the coding sequence (from position 3,480 to 2,595) of a M_r 35,000 polypeptide that we conclude to be required for Tc resistance; the *Mbo* I site at 3.0 kb is also in this same coding sequence.

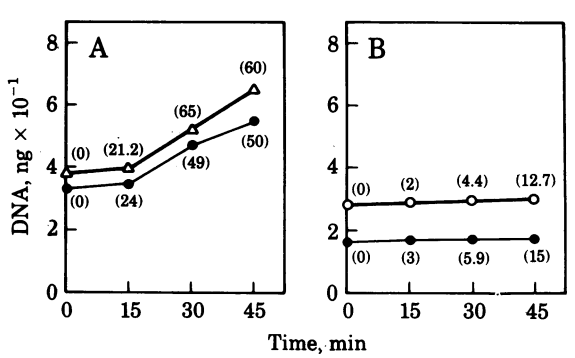

FIG. 2. Complementation of Tsr mutants. Heteroplasmid strains containing both a mutant plasmid, pSA0331, and either pSA5000 (*repC*$^+$) or pSA5120 (*repC1*) were constructed and grown to early exponential phase in CY broth at 32°C and were shifted to 43°C at t = 0, pulse-labeled, and analyzed by agarose gel electrophoresis of sheared whole-cell lysates. The resulting gels were scanned by fluorescence densitometry, and the amount of DNA in each supercoiled plasmid band was calculated by means of DNA standards run on the same gel. The supercoiled plasmid bands were excised and counted, and the resulting counts were divided by the amounts of DNA in the respective bands to give specific incorporation rates. Total plasmid DNA recovered is plotted for each plasmid as a function of time, and the specific incorporation rates (cpm/ng) are given as numbers on the graph. (*A*) Results with pSA5000 (△) and pSA0331 (●). (*B*) Results with pSA5120 (○) and pSA0331 (●).

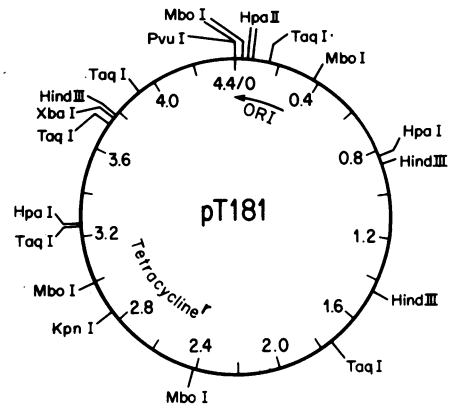

FIG. 3. Restriction map of pT181. Distances are given in kilobases with the coordinates originating at the *Pvu* I site. The precise size of the plasmid is actually 4,437 nucleotide pairs (unpublished data). The location of the Tc^r determinant was determined by filling in the *Kpn* I site (see text); the location of *ori* and the direction of replication were determined by *in vitro* analyses (unpublished data).

Identification of Sites Essential for pT181 Replication. Suggestive evidence for the essentiality of the *Hin*dIII site at 3.8 kb was obtained in an experiment involving religation of a complete *Hin*dIII digest of pT181 DNA and transformation with selection for Tc resistance. Ten Tc^r transformants contained all three *Hin*dIII fragments, and seven lacked the smallest fragment, C. Members of these two classes were indistinguishable from the wild-type plasmid with respect to copy number and stability, and so it is concluded that fragment C is dispensable. A similar finding has been reported for pT127 (10). All seven of the plasmids lacking fragment C were analyzed by digestion with *Hpa* I, which revealed that fragments A and B were always in the same relative orientation, suggesting that the A–B junction is essential. The function spanning this junction is not, however, that of Tc resistance, inasmuch as the A fragment by itself is sufficient for the expression of the Tc^r phenotype (9, 22); thus, it appears probable that the A–B junction is necessary for autonomous replication.

Examination of pRN6010, a pT181::pE194 cointegrate generated by cloning the two plasmids at their unique *Xba* I sites (22), showed that interruption of the *Xba* I site (at 3.78 kb) also inactivates the replicon. This composite plasmid had a copy number of about 100, which is close to that of pE194 (about 120) (22). Because pE194 is slightly thermosensitive for replication relative to pT181 (13), it was possible to test for the ability of the pT181 moiety to drive the replication of the cointegrate by testing for replication at a temperature that is restrictive for pE194 but permissive for pT181. Because the temperature differential is small, this test was performed by growth on a temperature gradient. The constructed cointegrate pRN6010 had the same thermosensitivity as its pE194 component, whereas pSA5500 [an *in vivo* cointegrate between these same two plas-

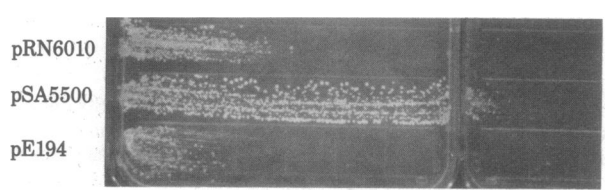

FIG. 4. Temperature gradient analysis. Strains to be tested were streaked on square Petri dishes containing GL agar plus Em (1 μg/ml) and incubated on a temperature gradient. The portion of the gradient illustrated was from 41°C to 44°C.

mids in which the pT181 replicon is intact (13)] can replicate, as can pT181, at temperatures at which pE194 cannot (Fig. 4).

Resection Analysis of the Plasmid Replicon. Plasmid deletions were prepared by digestion of *Pvu* I-linearized DNA of pRN6010 with exonuclease III (22). One of these derivatives—pRN6233, which lacks about 60 base pairs surrounding the *Pvu* I site (Δ141) (22)—was found to have a *trans*-complementable defect in replication. Digestion of pRN6233 DNA with *Xba* I, followed by religation and transformation for Tc^r, gave rise only to transformants containing Tc^rEm^r plasmids indistinguishable from pRN6233. The same experiment with pRN6010 DNA gave approximately equal numbers of Tc^rEm^s transformants (which contained pT181 alone) and doubly resistant transformants (which contained either a pRN6010-sized plasmid or two plasmids corresponding to pT181 and pE194). In a second transformation experiment with the same pRN6233 ligation mixture, an aliquot of intact pSA5000 DNA was added to the ligated DNA sample. In this case, selection for Tc resistance yielded 33 Tc^rEm^s transformants in addition to 34 Tc^rEm^r ones, and all of the former were also Cm^r. Examination of 20 of these by gel electrophoresis revealed that 19 contained two plasmids, one the size of pT181Δ141 and the other the size of pSA5000. The 20th transformant contained a single large plasmid that was presumably an *in vivo* recombinant. The pT181Δ141/pSA5000 heteroplasmids were unstable, as would be expected on the basis of autonomous Inc3 incompatibility, but gave rise to only one class of segregants, namely Cm^rTc^s. Examination of several of these by gel electrophoresis revealed that pT181Δ141 (pRN6238) had been lost and that pSA5000 had been retained. These results together suggest that the 60-base deletion Δ141 affects a function that is essential for replication and can be supplied in *trans* by pSA5000.

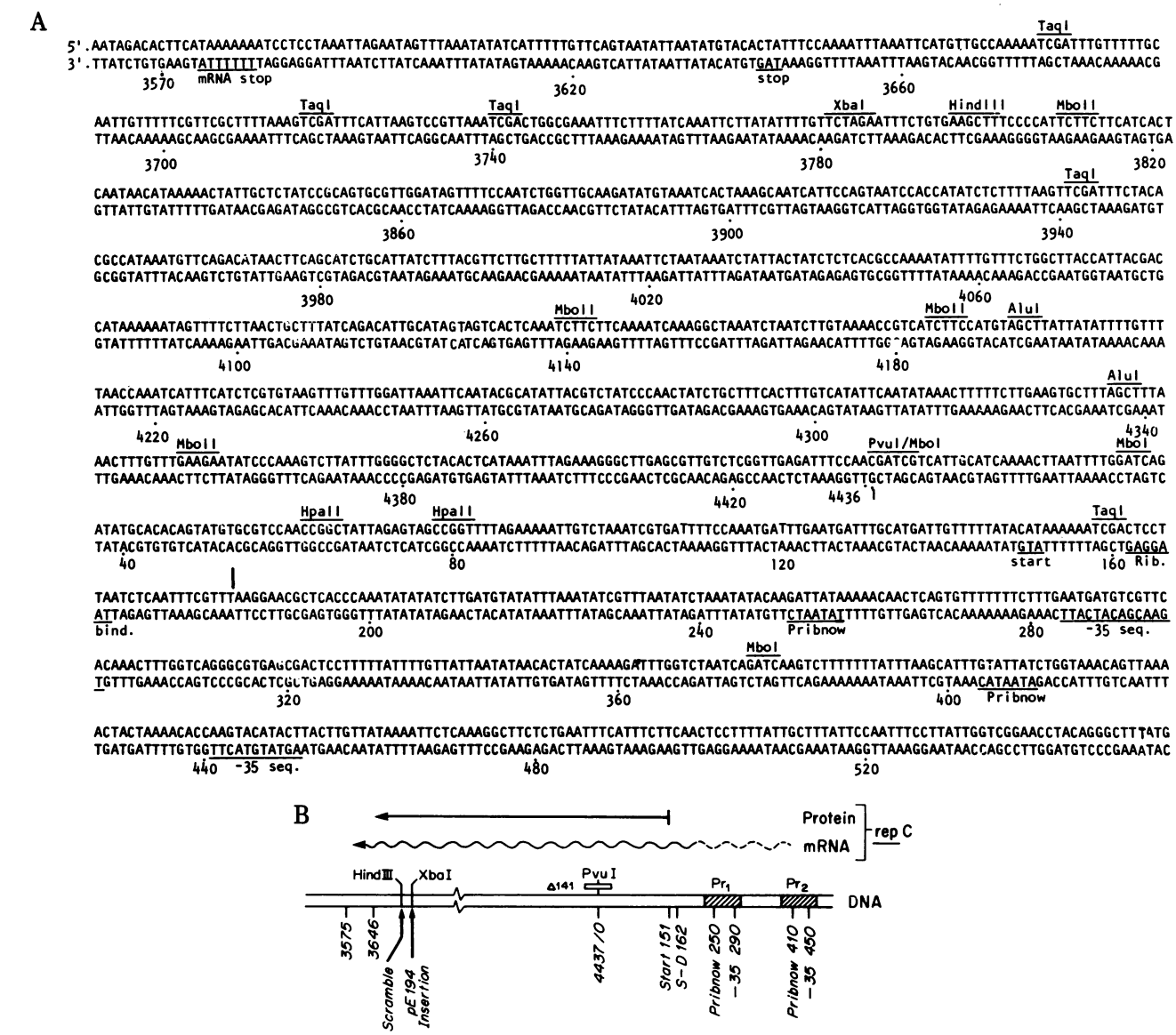

FIG. 5. The *repC* region. (*A*) Nucleotide sequence of the region of pT181 DNA from nucleotide position 3,563 to 556, determined by the Maxam–Gilbert procedure (23). A methionine codon at position 151 marks the start of a 939-base-pair open reading frame, which terminates at a stop codon at position 3,646. Putative mRNA start and stop positions are at positions 245 and 3,575, respectively. Possible promoter sequences are as indicated. Because little is known about the distances between Pribnow and −35 sequences in *S. aureus*, we have arbitrarily assigned the latter to sequences that most closely match the canonical −35 sequences in *E. coli* (24) rather than to matching nucleotide positions. (*B*) Diagrammatic representation of the proposed features of the *repC* region. The mRNA transcript has two possible starts (Pr_1 and Pr_2, respectively) inferred from the sequence; the upstream end is shown partially dashed to reflect this. The indicated restriction sites are those whose interruption inactivates the *repC* function. "Scramble" signifies unsuccessful attempts to rearrange the indicated *Hin*dIII fragments; "S–D" signifies the putative Shine–Dalgarno sequence for ribosome binding at the *repC* start.

Identity of the Essential Coding Sequence and *repC*. A test of whether the region of the plasmid defined as essential by the effect of insertions and deletions is the same as the *repC* determinant defined genetically was performed by testing for the ability of pT181Δ141 to be complemented in *trans* by pSA5120, the *repC1*-bearing derivative of pSA5000 (11). In this test, pT181Δ141 was transduced into strain 8325 (pSA5120) with selection for Cm and Tc resistances, and the presence of both plasmids was verified by gel electrophoresis of whole-cell lysates. Being incompatible, these plasmids required double selection for comaintenance and, at the permissive temperature, showed the same instability characteristics as the pT181Δ141/pSA5000 heteroplasmid strain. At 43°C, the nonpermissive temperature, both plasmids were lost together at the same high frequency as pSA5120 alone, indicating that pT181Δ141 is unable to supply in *trans* the product whose thermosensitivity is responsible for the inability of pSA5120 to replicate—namely, the *repC* product.

The *repC* Coding Sequence. The pT181 nucleotide sequence from position 3,563 to 556 (Fig. 5*A*) was determined by the Maxam–Gilbert procedures (23) and will be described in detail elsewhere. The sequence shows a single open reading frame of 939 base pairs extending from position 151 to 3,656 (counterclockwise) and preceeded by appropriate transcription and translation signals as indicated and summarized in Fig. 5*B*. Within this reading frame are located all three of the restriction sites defined above as essential for integrity of the replicon—namely, the *Hin*dIII site at 3.80 kb and the single *Xba* I and *Pvu* I sites (at 3.78 and 1 kb, respectively).

DISCUSSION

Because complementation analysis was performed with the wild-type plasmid (which complemented all of the five available mutants) and with only one of the mutants (*repC1*, which complemented none of the others), these data do not formally exclude the possibility that two products are required and that "*repC1*" is actually mutant in both. However, the molecular analysis presented here strongly supports the inference of a single *rep* cistron. The connection between this cistron as defined genetically and the coding sequence from position 3,587 to 151 has been made by the finding that Δ141 is also in this cistron. These results suggest that pT181 encodes only one major protein required for its own replication, the *repC* protein, and that the coding sequence for this protein is as shown in Fig. 5*A*. Given cloning results that localized pT181 replication functions to the region between 0.38 and 3.0 kb, it is possible that a second *rep* function lies between 3.0 and 3.6 kb. The only discernible polypeptide that could be encoded in this region is the *tet* protein (unpublished data), which is clearly not involved in replication as its determinant straddles the *Mbo* I site at 3.0 kb. Consistent with these results is the finding that extracts of pT181-containing cells contain a protein of $M_r \approx 35{,}000$–$40{,}000$ that is essential for the activity of the extracts (8). Although it is likely that this protein is the one specified by the *repC* coding sequence, formal proof of this correspondence will require additional experiments.

Preliminary and circumstantial observations suggest that the concentration of the *repC* protein is rate-limiting for pT181 replication under normal circumstances and that the control of replication rate is exerted at the level of *repC* synthesis. Extracts prepared from strains containing a copy mutant of pT181, *cop-608*, have 5- to 10-fold more *repC* activity than do extracts prepared from strains containing the wild-type plasmid (8); the activity of mixed extracts is additive, suggesting that the difference is not due to an inhibitor and that, therefore, the mutant extracts have more *repC* protein. *In vivo*, this copy mutant acts in *trans* to elevate the copy number of a wild-type pT181 plasmid (unpublished data) consistent with the overproduction of a rate-limiting substance required for replication. Several of the Tsr mutants have lower than normal copy numbers, even at the permissive temperature. If these are, indeed, missense mutations in the structural *repC* gene, then they should not reduce the amount of *repC* protein. Therefore, it is argued, because a change in the efficiency of the protein causes a substantial reduction in replication rate, the protein is probably rate-limiting. Similar results have been obtained for Tsr mutants of *S. aureus* penicillinase plasmids (25), and there is also preliminary evidence to suggest that the replication rate of *E. coli* plasmid RI*drd19* may be controlled at the level of *rep* protein synthesis (26). Proof of this hypothesis will depend on further *in vitro* analysis.

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