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Functional analysis of a palindromic sequence required for normal replication of several staphylococcal plasmids

(*palA* element/*Staphylococcus aureus*/*Bacillus subtilis*)

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ABSTRACT **Most small multicopy antibiotic-resistance plasmids of *Staphylococcus aureus* contain a major axis of hyphenated dyad symmetry (*palA*) that is required for normal replication and stability, although located outside of the minimal replicon. Rearrangements affecting *palA* cause plasmid instability, a marked reduction in copy number, and the accumulation of large quantities of strand-specific circular single-stranded plasmid DNA. In view of the recent observation that pT181 initiates replication by a nick and 3′-extension mechanism (S. Khan, personal communication), it is suggested that these plasmids replicate by an asymmetric rolling-circle mechanism in which the displaced plus strand remains single stranded until *palA* is exposed, forming a hairpin that serves as the lagging strand origin.**

Inverted duplications of nucleotide sequences (palindromes) have been increasingly recognized to play important roles in a variety of genetic activities such as control of gene function, initiation of replication, genetic transposition, transcription termination, and formation of deletions and inversions. Palindromes, which form hairpins when single-stranded, have been shown to function in the initiation and termination of replication of single-stranded DNA phages. In particular, in replicative form (RF)-to-RF rolling-circle replication, the displaced leading (plus) strand remains single-stranded until a palindromic element is exposed, forming a hairpin that serves as the lagging strand origin (1, 2). In duplex DNA, hairpins have yet to be demonstrated *in vivo*, so that palindromic elements are usually regarded as symmetry elements rather than as physical secondary structures. Palindromic elements also exist in the replication origins of many double-stranded DNA replicons such as the lambdoid phages (3), various plasmids, etc. (4, 5). With plasmid ColE1 and its relatives, palindromic elements in the origin region are involved in the formation of the RNA primer for replication (5); with R1, deletions affecting a large palindromic element near the origin cause instability and a decrease in copy number (6). We report here the identification of a major palindromic element, *palA*, widespread among plasmids from Gram-positive bacteria, outside of the minimal replicon but required for normal plasmid replication and stability. Analysis of this element suggests that these plasmids replicate by an asymmetric rolling-circle mechanism in which *palA* serves as the lagging strand initiation site.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids. The strains used are all derivatives of *Staphylococcus aureus* strain NCTC8325 (RN450) (7) or *Bacillus subtilis* strain BD170. Plasmids used are listed in Table 1. pT181, pC221, pC194, and pE194 are naturally occurring multicopy *S. aureus* antibiotic resistance plasmids. Copy number (*cop*) mutants were used in some experiments.

Media and Growth Conditions. Liquid and solid media for *S. aureus* and *B. subtilis* were used as described (11). Chloramphenicol and erythromycin were used at 5 μg/ml; tetracycline was used at 5 μg/ml for *Staphylococcus* and 25 μg/ml for *Bacillus*. Cells were grown at 37°C except where indicated. Plasmid stability was analyzed by plating after four generations of growth in nonselective broth and scoring for retention of plasmid markers. Protoplast transformation was performed as described (12).

Isolation and Analysis of Plasmid DNA. Preparation and analysis of plasmid DNA and determination of plasmid copy numbers were by published procedures (13–15). Enzymes were obtained commercially and used as recommended by the suppliers. Restriction fragment isolation (11), cloning (16), hybridization analysis (17), and DNA sequencing (18) were performed by standard procedures. For the identification of single-stranded DNA, agarose gels were blotted to nitrocellulose without alkali treatment (19).

Nucleotide coordinates for plasmid DNA segments are according to the published sequence for the wild-type plasmid (4, 8–10). Deletion derivatives were constructed by religation of plasmid DNA partially digested with the appropriate restriction enzyme or by digestion of linearized plasmid DNA with exonuclease BAL-31.

For the insertion of *Eco*RI linkers, plasmid linear forms were generated by partial digestion with *Rsa* I or *Hin*dIII and ligated to phosphorylated *Eco*RI octanucleotide (GGAATTCC) from New England Biolabs (16). DNA isolated from transformant colonies was redigested with *Eco*RI, religated, and used to transform other cells.

For reinsertion of pT181 *palA* or pC221 *palA* into pADG8201, the *Eco*RI–*Hin*fI fragment (bp 444–707) of pT181 or the *Rsa* I-B fragment (bp 391–871) of pC221 was isolated, treated with the Klenow fragment of DNA polymerase I, and ligated to pADG8201 linearized by cleavage at its unique *Fnu*DII site, position 1687.

RESULTS

***palA* Sequences and Structures.** Eight different small *S. aureus* plasmids, pT181 (20), pC221 (4), pE194 (9), pC194 (8), pS194, pE12/pIM13 [S. Projan and R.P.N., unpublished data (21)], and pSN2 (22), have an extensive region of hyphenated dyad symmetry, referred to as *palA* (Fig. 1), flanking the conserved recombination site RS_B (24). The three hairpins shown are approximately 70% base paired and have calculated free energies of formation of −64 to −84 kcal (1 kcal =

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Abbreviations: RF, replicative form; Tsr, temperature-sensitive replication.

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Table 1. Plasmids used

Plasmid	Size, kb	Copy no.*	*palA*	Description	Source or ref.
pC221	4.5	20	+	Cm^r Inc4 wild-type	Ref. 4
pADG5535	3.0	<10	−	pC221 *Mbo* I-A (bp 827–3836)	This work
pC194	2.9	40	+	Cm^r Inc8 wild-type	Ref. 8
pADG6407	2.7	40	+	pC194 Δ*Taq* I-D (bp 2283–2510)	This work
pADG6406	2.5	<10	−	pC194 Δ*Taq* I-D+E (bp 2110–2510)	This work
pE194	3.7	40	+	Em^r Incl1 wild-type	Ref. 9
pE194 *cop-6*	3.7	200	+	pE194 copy mutant	Ref. 9
pADG5111	3.6	<10	−	pE194*cop-6* Δ*Dde* I-B (bp 686–842)	This work
pT181	4.4	22	+	Tc^r Inc3	Ref. 10
pADG8053	4.4	22	+	pT181, new *Eco*RI site, bp 885	This work
pT181 *cop-620*	4.4	60	+	Copy mutant of pT181	Ref. 11
pADG8201	3.6	<10	−	pT181 *cop-620* Δ*Rsa* I-C+F (bp 444–1236)	This work
pSA0331	4.4	145†	+	pT181 *repC4 cop-634* Tsr	Ref. 11
pT181 *cop-619*	4.4	135	+	Copy mutant of pT181	Ref. 11

Cm^r, chloramphenicol resistance; Em^r, erythromycin resistance; Tc^r, tetracycline resistance; Inc, incompatibility group; bp, base pairs; Tsr, temperature-sensitive replication.
*In *S. aureus*.
†Measured at 32°C.

4.18 kJ) per mol (25). The base of the hairpin (capital letters) is virtually identical for all seven plasmids; the remainder is more variable and occurs in at least the two forms shown in Fig. 1. The pT181 type is shared by pSN2 and pE12/pIM13; the pC221 type, by pC194 and pE194. Within either type, many of the nucleotide differences occur in pairs, preserving the base pairing between the two stems. These conservative changes are illustrated in Fig. 1, for the pC221-pC194 pair, also as capital letters. This conservatism in the face of considerable sequence divergence suggests that *palA* is more likely to function as an actual hairpin than as a symmetry element in duplex DNA.

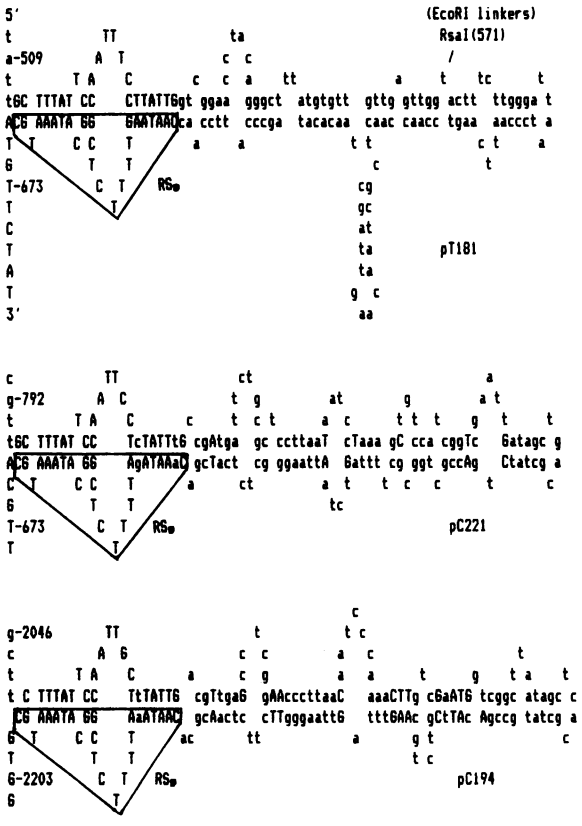

FIG. 1. Predicted *palA* secondary structures. Structures were predicted by computer analysis (23). Boxed region indicates the highly conserved RS$_B$ sequence (24), which is matched by a similarly conserved region at the opposite end. Nucleosides outside of this conserved region represented by capital letters indicate differences between the pC221 and pC194 *palA* elements that preserve or increase Watson–Crick base pairing between the two arms.

palA is asymmetric and is in the same orientation with respect to the replication (*rep*) gene of pT181 (10), pC221 (4), pE194 (9), pC194 (8), and pS194 (S. Projan and R.P.N., unpublished data). In pT181, pC221, pE194, and pS194, *palA* is located just upstream from *rep*; in pC194 it is about 1.4 kilobases (kb) upstream ("upstream" and "downstream" refer to the direction of *rep* gene transcription).

Deletion Mapping. Deletions and insertions affecting pT181 *palA* and the flanking regions are shown in Fig. 2. In *S. aureus*, we were unable to recover pT181 derivatives with *palA* defects. In *B. subtilis*, however, Gryczan *et al.* (27) had previously isolated deletion derivatives of pE194 lacking *palA* and we obtained deletions affecting all or part of the palindrome from pT181 *cop-620* (e.g., pADG8201; see Fig. 2), pE194 *cop-6* (pADG5111; see Table 1), pC221 (pADG5535; see Table 1), and pC194 (pADG6406; see Table 1) by partial digestion with restriction enzymes. Additionally, we generated a 120-base deletion (pADG8241), internal to pT181 *palA*, by BAL-31 digestion starting from a synthetic *Eco*RI site at position 571 (pADG8238). All of these *palA*$^-$ derivatives had copy numbers in *B. subtilis* that were equivalent to those of their *palA*$^+$ parents (Fig. 3, lanes 10 and 11). Once having obtained *palA*$^-$ derivatives in *B. subtilis*, we found it possible to transfer them, at very low frequency, to *S. aureus* by protoplast transformation. In *S. aureus*, all *palA*$^-$ derivatives showed a marked decrease in copy number (see Fig. 3 and Table 1), reaching approximately the same low level regardless of the copy number of the *palA*$^+$ plasmids from which they were derived. They were also highly unstable—e.g., pADG8201 (pT181 *cop-620* Δ444-1236; see Fig. 2) showed 10% loss after growth on nonselective medium for four generations.

Deletions or insertions involving sequences flanking *palA* in pT181, pC194 (Fig. 2, lane 7), or pE194 *cop-6* (not shown) were without effect. In pADG8181 (see Fig. 2) the deletion extends to position 675, the first paired base (see Fig. 1). The lack of effect of insertions (8 bp to 2 kb) at pT181 position 444 (see Fig. 2) suggests further that the distance between *palA* and the replication region need not be constant.

Reinsertion of *palA*. *palA*-containing restriction fragments of plasmids pT181 (bp 444–707) and pC221 (bp 391–871) were isolated and inserted into the unique *Fnu*DII (1687) site of

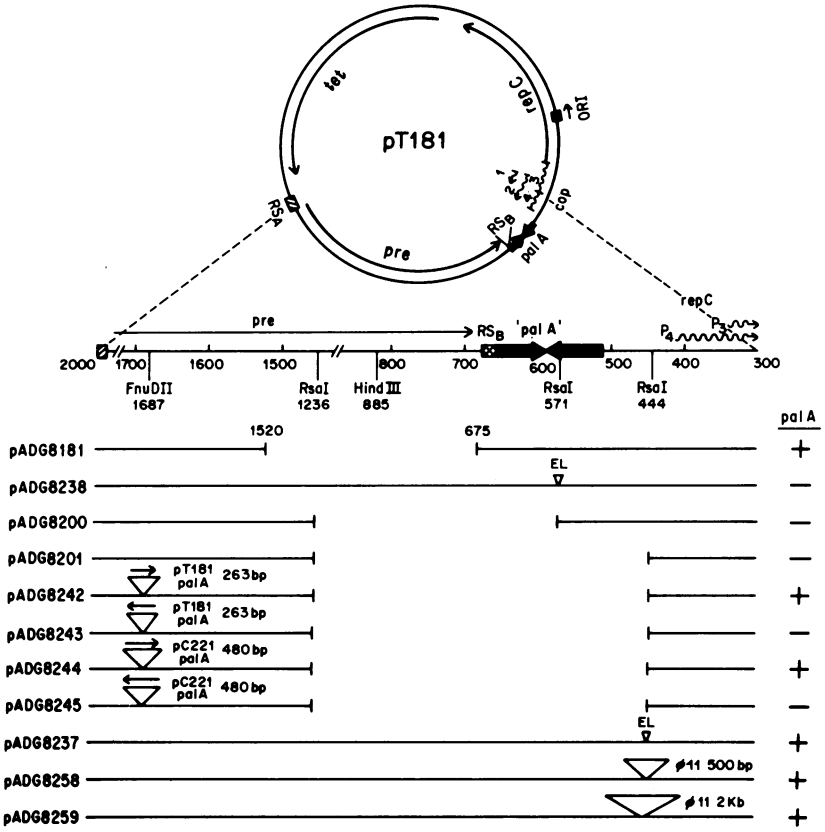

FIG. 2. Schematic representation of pT181 and mutations. RS_A and RS_B are short conserved recombination sites (24). Wavy arrows 1 and 2, and 3 and 4, on the circular map correspond to regulatory countertranscripts and *repC* mRNAs, respectively (26); P_3 and P_4 on the linear map are the *repC* promoters (26). Genetic symbols are *pre*, plasmid recombination (M. Gennaro and R.P.N., unpublished data); *cop*, copy number control; *repC*, replication initiator gene; *ori*, origin of replication; and *tet*, tetracycline resistance. Smooth arrows correspond to major open reading frames. Wavy arrows indicate transcripts. Relevant restriction sites are as indicated. Numbers refer to nucleotide positions according to Khan and Novick (10); derivatives of pT181 or pT181 *cop-620* are indicated below the plasmid map. Gaps indicate deletions, triangular loops indicate insertions, and arrows correspond to orientation of inserted *palA* DNA: →, native orientation; ←, reversed orientation. EL represents *Eco*RI linker insertion. With the exception of pADG8181, which is a BAL-31 deletion of pADG8053 (see Table 1), all the deletions were generated by the removal of *Rsa* I fragments. pADG8242–5 were constructed by inserting *palA*-containing fragments of pT181 (*Rsa* I-444–*Hin*fI-707) or pC221 (*Rsa* I-391–*Rsa* I-871) into the *Fnu*DII site of pADG8201 in either the native (pADG8242 and pADG8244) or reverse (pADG8243 and pADG8245) orientation. pADG8258 and pADG8259 were constructed by inserting 500-bp and 2-kb *Eco*RI fragments of phage φ11 DNA into the *Eco*RI-444 site of pADG8237.

pADG8201, a *palA*$^-$ derivative of pT181 *cop-620* (see Fig. 2). The cloning site is about 1 kb upstream of the original location of *palA*. Insertion of either fragment repaired the *palA*$^-$ defect but only when the palindrome was in its native orientation (see Fig. 4*A* and Table 1).

Single-Stranded Plasmid DNA. *S. aureus* and *B. subtilis* strains carrying various staphylococcal plasmids contain circular, strand-specific, single-stranded plasmid monomers (19); moreover, the pT181 initiator protein, RepC, introduces a site-specific nick at the origin that is utilized for the initiation of replication by 3′ extension and strand displacement (ref. 28; S. Khan, personal communication); we have noted that the displaced strand is the same as that detected in the single-stranded form by te Riele *et al.* (19). These observations suggested that *palA* might be involved in lagging strand initiation and that the PalA$^-$ phenotype might be due to a defect in this process. As shown in Fig. 4, *S. aureus* strains carrying *palA*$^-$ plasmids produced large amounts of single-stranded plasmid DNA, whereas those carrying *palA*$^+$ plasmids produced only trace amounts, in proportion to plasmid copy number (lanes 3 and 4). In Fig. 4*A* is shown an ethidium bromide-stained agarose gel separation of whole cell minilysates, in 4*B* and 4*C*, hybridization patterns of identical gels transferred to nitrocellulose with (4*B*) or without (4*C*) denaturation (19). Signals that are present in both autoradiograms correspond to single-stranded DNA (lower band in Fig. 4*B*), whereas those present in 4*B* but not 4*C* correspond to double-stranded DNA. Recloning of pT181 *palA* or pC221 *palA* in *palA*$^-$ pT181 *cop-620* in the original orientation reduced the accumulation of single-stranded DNA to wild-type levels (lanes 8 and 10). Any other rearrangement affecting *palA*, including linker insertion (lane 5), deletion (lanes 6 and 7), or inversion (lanes 9 and 11) caused the same accumulation of single-stranded DNA as did deletion of the entire palindrome (lane 7). Note, however, that pADG8241 (lane 6), which has a 120-bp deletion within *palA*, shows a less severe defect than pADG8238 (lane 5), which has only an EcoRI linker insertion within the palindrome.

We confirmed the strand specificity of the pT181 single-stranded material by blot hybridization with strand-specific probes (hybridization performed by S. Majumder; data not shown). We assume that this material is circular.

Effect of Replication. If single-stranded material is generated during replication it should decrease when replication initiation is blocked. Accordingly, a strain carrying pSA0331, a Cop$^-$ RepC temperature-sensitive double mutant of pT181 (11), was analyzed for single-stranded DNA at permissive (32°C) and nonpermissive (42°C) temperatures in comparison with pT181 *cop-620*. As shown in Fig. 5, the single-stranded material disappeared during a 2-hr incubation at 42°C of the Tsr strain (lanes 1–3) but persisted in the RepC$^+$ strain (lanes

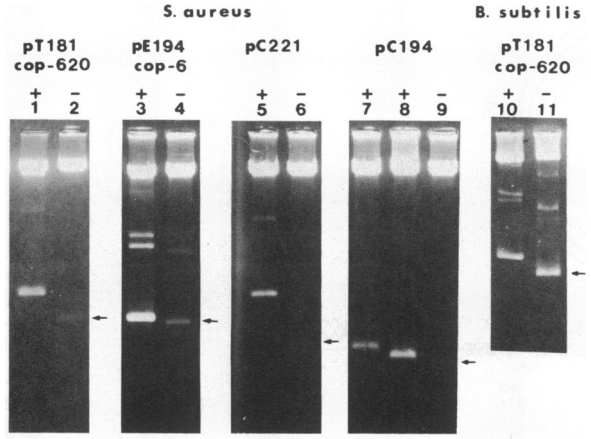

FIG. 3. Effect of *palA* deletions on plasmid copy number. Whole-cell sheared minilysates of *S. aureus* and *B. subtilis* strains containing the indicated plasmids were separated on 1% agarose in Tris borate buffer for 2.5 V·hr/cm, stained with ethidium bromide, and photographed. Each sample contained material derived from approximately 10^8 cells; lanes 1–9, *S. aureus* strains; lanes 10 and 11, *B. subtilis*; + and − indicate the presence or absence of *palA* sequences. The *palA*$^-$ derivatives used were pADG8201 (lanes 2 and 11), pADG5111 (lane 4), pADG5535 (lane 6); and pADG6406 (lane 9). In lane 8 is pADG6407, the immediate precursor pADG6406. The positions of *palA*$^-$ monomers are indicated by arrows. Intermediate plasmid bands correspond to topoisomers and multimers.

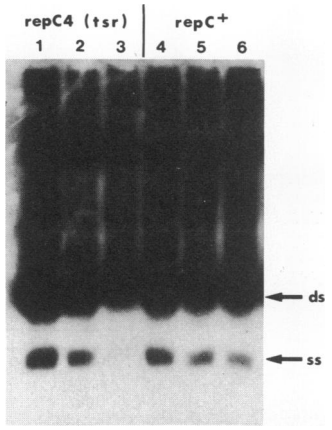

FIG. 5. Effect of replication. *S. aureus* cultures of strains containing either pSA0331 (Tsr) or pT181 *cop-619* were transferred to 42°C and maintained in exponential growth by dilution. Samples containing equivalent number of cells were used to prepare whole-cell sheared minilysates, which were treated as for Fig. 4. The gel was denatured and blot-hybridized with a radiolabeled pT181 probe. Lanes 1–3 contain pSA0331 samples; lanes 4–6, pT181 *cop-619*. Lanes 1 and 4 contain 32°C cultures; lanes 2 and 5, 1 hr at 42°C; lanes 3 and 6, 2 hr at 42°C. ds, Double-stranded DNA; ss, single-stranded DNA.

4–6). Disappearance of single-stranded DNA in the Tsr strain seemed not to be due to degradation; in a similar experiment using a Tsr *palA*$^-$ plasmid, the large accumulation of single-stranded plasmid DNA persisted with little or no decrease during a 5-hr incubation at 42°C (data not shown). These results suggest that single-stranded DNA is generated only during replication and is converted to the double-stranded form rather than degraded, and that efficient conversion requires an intact correctly oriented *palA* element.

Effect of *palA* in *B. subtilis*. As shown in Fig. 4 (lanes 1 and 2), *B. subtilis* carrying pT181 *cop-620* accumulates substantial amounts of single-stranded plasmid DNA irrespective of the presence of *palA*. An as-yet-uncharacterized slower-moving band of single-stranded DNA appears uniquely with *palA*$^-$ plasmids in both species (Fig. 4*C*). The amount of single-stranded DNA seen with *palA*$^+$ pT181 *cop-620* is much greater in *B. subtilis* than in *S. aureus*. Notable also is the large quantity of multimeric double-stranded plasmid DNA in *B. subtilis* compared to *S. aureus* strains. Evidently, *S. aureus* and *B. subtilis* differ sharply in functions involved in the accumulation, processing, or both, of single-stranded and multimeric double-stranded plasmid DNA.

DISCUSSION

As noted, pT181 evidently utilizes a rolling-circle mode of replication in which the Rep protein functions similarly to the CisA protein of phage φX174 (29). Our data are consistent with an asymmetric mechanism, similar to that utilized for RF-to-RF replication of the single-stranded DNA phages (2, 30) in which the initiation of replication on the displaced strand occurs, *de novo*, only after the palindromic lagging strand initiation site is exposed. As pT181 *palA* is close to the

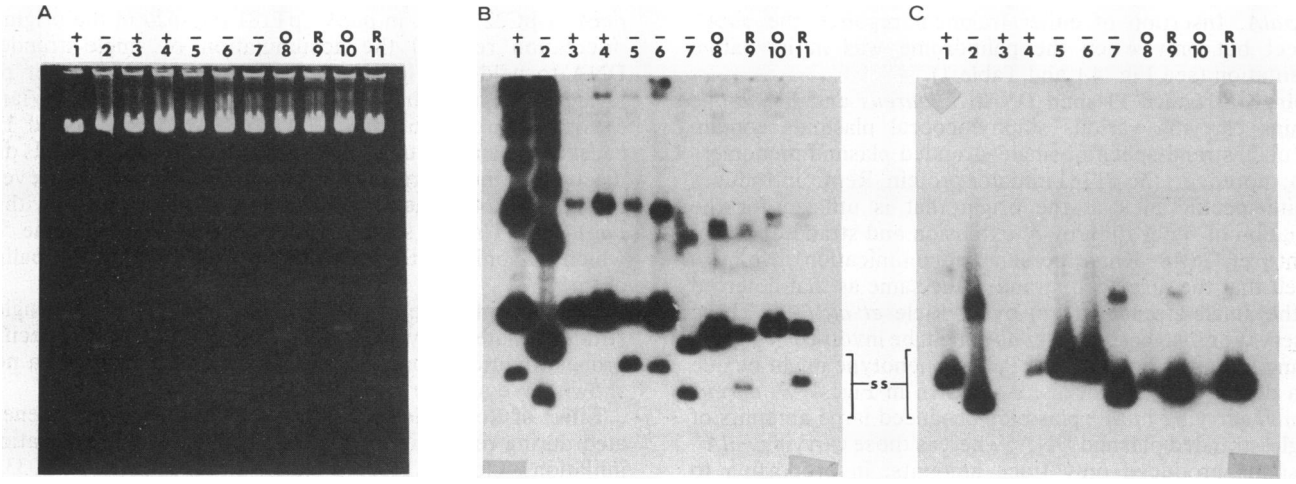

FIG. 4. Single-stranded plasmid DNA. Whole-cell sheared minilysates were separated on agarose gels containing ethidium bromide at 0.5 μg/ml. Duplicate gels (*A*) were transferred to nitrocellulose with (*B*) and without (*C*) prior denaturation. Nitrocellulose blots were hybridized with a radiolabeled sample of pT181 *Hin*fI-A (bp 755–160). Lanes 1 and 2 contained *B. subtilis* samples; lanes 3–11, *S. aureus* samples. Lanes 1 and 4, pT181 *cop-620*; lanes 2 and 7, pADG8201; lane 3, pT181; lane 5, pADG8238; lane 6, pADG8241; lane 8, pADG8242; lane 9, pADG8243; lane 10, pADG8244; lane 11, pADG8245. ss, Single-stranded material; + or −, *palA*$^+$ or *palA*$^-$; O, *palA* in original orientation; R, in reverse orientation.

leading strand termination site, the displaced plus strand would have to be nearly fully single-stranded before lagging strand synthesis could initiate, so that a short delay in lagging strand initiation would give rise to fully single-stranded molecules. In *palA*$^-$ derivatives, such molecules would accumulate because they would be defective for the initiation of replication. However, as deletion of *palA* is not lethal to the plasmid, an inefficient alternative lagging strand initiation site(s) or mechanism would have to exist.

As *palA* is far from a perfect palindrome, its orientation specificity is not surprising; indeed, all of the eight known *palA* sequences contain a highly conserved seven-base segment, 3′-CTCTTTT-5′, that is matched by a similarly conserved but noncomplementary six-base segment, 5′-AATT(T)C-3′, on the opposite stem. These segments may be sequence-specific functional elements of the palindrome.

On the basis of organizational similarities among pT181 and the other staphylococcal plasmids in their replication regions (S. Projan, personal communication), *palA* is likely always to be in the same orientation with respect to the direction of replication. Although pE194 has been reported to replicate in the opposite direction with respect to *palA* (31), it accumulates DNA of the strand predicted on the basis of its *palA* orientation (S. D. Ehrlich, personal communication); therefore, the direction of replication of this plasmid remains uncertain.

The only staphylococcal plasmid known to lack *palA* is the recently sequenced (32) pUB110, which does not accumulate single-stranded DNA (19). We have recently inserted into a *palA*$^-$ derivative of pT181 a pUB110 segment, distant from the replication region, that restored normal copy number and eliminated accumulation of single-stranded DNA in both *S. aureus* and *B. subtilis* (unpublished data). Thus pUB110 has an effective *palA* analog that functions in both species, and it may therefore replicate by the same asymmetric rolling-circle mechanism as the other staphylococcal plasmids.

The lack of any demonstrable requirement for *palA* in *B. subtilis* suggests that some other lagging strand initiation mechanism must exist in this species. A replication fork could be generated by a RecA-mediated interaction between single- and double-stranded molecules, similar to that observed for T4 (33); alternatively, nonspecific lagging-strand initiation sites could be utilized.

On the basis of the results presented here, one may envision at least two widely disparate plasmid replication strategies. Plasmids such as F and its relatives have low copy number, symmetrical θ replication, and specific partitioning, suggesting an evolutionary relationship to chromosomal replicons; pT181 and its relatives have high copy number and asymmetrical rolling-circle replication, suggesting an evolutionary relationship to the single-stranded DNA phages. This latter strategy is widespread among plasmids from Gram-positive bacteria, and it is predicted that it will shortly be identified in Gram-negative species.

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