bacteria possessing only one polar flagellum.

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15. The basal disks were isolated from a filamentless mutant that has an active motor. Spheroplasts of this mutant were prepared as described for the wild type (*4*) and sonicated for 3 min. They were lysed by 2% $C_{12}E_8$ in 10 mM tris-HCl (pH 8), and DNA was digested as described (*4*). We added EDTA to a concentration of 27.5 mM and KOH to a pH of 11. After 2 hours of incubation the basal disks, of which about 50 to 70% were without a hook–basal body complex, could be isolated and purified by repeated low- and high-spin centrifugations in the detergent buffer used above. This preparation was negatively stained with uranyl acetate (2%). Stained areas appear black in the micrographs, whereas biological material is bright. Micrographs were taken in a Philips CM12 electron microscope at minimal-dose conditions at a primary magnification of ×35,000. The micrographs were digitized by means of an Eikonix 1412 camera at an aperture that gave a pixel size of 0.43 nm on the specimen level. Image analysis as well as model calculations were done with the Semper 6.2 image processing system.
16. The disks, isolated from an exponentially growing culture, were photographed and analyzed for their diameters. While the largest diameter might reflect the natural maximum of size, the smallest disks may be a result of the isolation procedure. It cannot be excluded that disks having an even smaller diameter may exist but were not observed because of their low number.
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25. H.E. and S.C.S. contributed equally to this study. Supported by a research grant to E.B. from the Deutsche Forschungsgemeinschaft.

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Replication-Specific Inactivation of the pT181 Plasmid Initiator Protein

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Replication of the *Staphylococcus aureus* plasmid pT181, which occurs by the rolling circle mechanism, is accompanied by the covalent attachment of a ≈12-residue oligodeoxynucleotide to one subunit of the dimeric plasmid-coded initiator protein, RepC. This oligonucleotide represents the plasmid sequence immediately 3′ to the initiating nick site. The resulting heterodimeric protein lacks the topoisomerase and replication activities of unmodified RepC, suggesting that the regulation of plasmid DNA replication requires postreplicational inactivation of the initiator protein as well as control of its synthesis.

Although the initiation of DNA replication is closely regulated in all organisms, the control of replication is not fully understood. We have used the *S. aureus* pT181 plasmids to study the regulation of DNA synthesis because these plasmids are maintained by precise control systems (at about 22 copies per cell). The pT181 plasmids replicate by the rolling circle mechanism, and initiation of their replication is regulated primarily by antisense-mediated control of synthesis of the 38-kD initiator protein, RepC (*1*). Leading strand replication begins with a site-specific nick introduced by RepC at the origin of replication (*ori*) (*2*). The protein is attached to the 5′ nick terminus by a phosphotyrosine bond (*3*). Although the synthesis of RepC is regulated precisely, we consider this regulation to be insufficient to ensure stable maintenance, as reutilization of the protein would probably lead to uncontrolled replication. Therefore, the Rep protein is likely to be degraded or inactivated after use.

To examine the fate of RepC, we incubated *S. aureus* containing a pT181 copy number mutant, *cop-608*, in the presence of chloramphenicol (Cm). Chloramphenicol blocks pT181 replication by inhibiting the synthesis of RepC (*4*). After addition of the drug, new plasmid initiation continues at a very low rate, for 5 to 10 min, during which the presumably small intracellular pool of active RepC is used up (*4*). In the presence of Cm, we did not find degradation of RepC; rather, we found two proteins (38 and 42 kD) that reacted with antibody to RepC (anti-RepC) and that remained present at constant amounts (Fig. 1) over a 30-min incubation. Both protein bands were detected also in the absence of Cm, but only the previously identified (*5*) 38-kD protein was seen in a preparation of RepC from *Escherichia coli*, in which pT181 does not replicate. Neither protein was seen in preparations from *S. aureus* strains that lacked plasmids. This result indicates that RepC is not degraded after its use and suggests that the 38-kD RepC molecule may be converted during replication to a different, slower migrating form. Residual replication in the presence of Cm is completed in less than 10 min, despite the continuing presence of a substantial amount of 38-kD material, suggesting that the 38-kD form is incapable of participating in replication under these conditions. There is no major change in the relative amounts of the 38- and 42-kD forms in the presence of Cm.

To confirm that the 42-kD material repre-

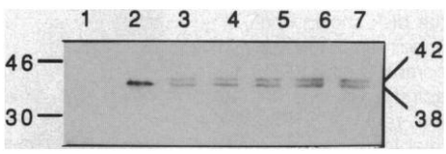

Fig. 1. Effect of chloramphenicol on RepC. A culture of *S. aureus* RN27 containing the high copy number mutant pT181*cop-608* was grown to a cell density of 7 × 10^8 bacteria per milliliter. Chloramphenicol was then added to a concentration of 150 μg/ml, and the culture was serially sampled. Whole-cell lysostaphin lysates from these samples were analyzed by SDS-PAGE followed by protein immunoblotting with rabbit anti-RepC. Blots were developed by treatment with goat antiserum to rabbit alkaline phosphatase conjugate and then stained for alkaline phosphatase. Lane 1, RN27 (plasmid$^-$); lane 2, RepC purified from MB2(pSK184) (*5*), an *E. coli* strain containing a cloned *repC* gene; lane 3, RN27(pT181*cop-608*) before Cm treatment; lanes 4 to 7, samples taken after 5, 10, 20, and 30 min of incubation in the presence of Cm. Numbers 46 and 30 represent molecular sizes (in kilodaltons) of protein standards; numbers 42 and 38 represent apparent molecular sizes of the two forms of RepC.

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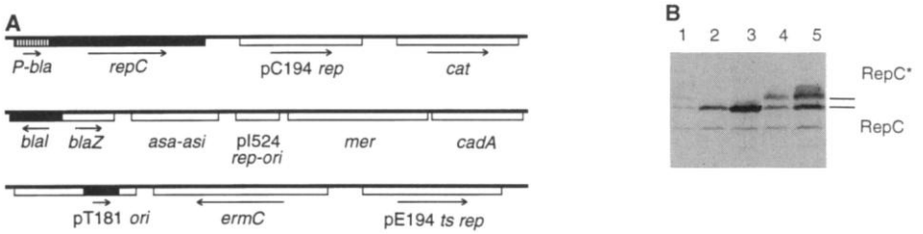


Fig. 2. Association between RepC* and the pT181 origin of replication. (**A**) Plasmid system for trans-activation of pT181 origin by induced RepC. Key elements of the interacting plasmids are shown in black. The plasmid pRN6759 (5.5 kb) is a construct in which RepC (*ori*$^-$) is under the control of the inducible promoter for staphylococcal β-lactamase (*P-bla*). The β-lactamase repressor (*blaI*) was provided in trans by the plasmid pI524 (30 kb). The plasmid pRN6366 (4.3 kb) contains the pT181 origin (*ori*) cloned into pRN5101, and requires RepC in trans for replication at 42°C. Other elements, shown by open boxes, are resistance genes (*asa-asi*, arsenate-arsenite; *mer*, mercury; *cadA*, cadmium; *cat*, chloramphenicol; *ermC*, erythromycin) and non-pT181 replicons that maintain these plasmids (pI524 *rep-ori*; pC194 *rep*; pE194 *ts rep*). Plasmid maps are opened at an arbitrary point and are not drawn to scale. (**B**) Lysates of exponentially growing *S. aureus* cultures were prepared as in Fig. 1 and analyzed by protein immunoblotting with rabbit anti-RepC. Lane 1, strain pT181*cop-608*; lanes 2 and 3, strain RN27 containing pRN6759 plus pI524 (RN7805); lanes 4 and 5, strain RN27 containing those two plasmids plus pRN6366 (RN7807); lanes 3 and 5, cultures induced with CBAP (5 μg/ml) for 2 hours before harvesting. Some RepC is present in the uninduced cultures (lanes 2 and 4) owing to the basal activity of the *bla* promoter. Lines at right indicate the 42-kD RepC* and 38-kD RepC bands.

sents a form of RepC and that the modification is replication-dependent, we used a combination of plasmids (Fig. 2A) in which one plasmid, pRN6759 (6), encodes RepC, and a second compatible plasmid, pRN6366 (7), contains the pT181 *ori* in the vector, pRN5101 (8). The endogenous origin of the vector pRN5101 does not function at 42°C. The RepC gene in pRN6759 is controlled by the inducible pI258 β-lactamase (*bla*) promoter. In this construct the pT181 *ori* located within *rep*C has been inactivated. Using a strain containing pRN6759 plus pI258, which supplies the *bla* repressor, with or without the *ori*-containing plasmid, pRN6366, we tested for co-induction of the 38- and 42-kD RepC forms by the β-lactamase inducer, carboxyphenyl benzoyl-6-aminopenicillanic acid (CBAP). The cultures were grown at 42°C to inhibit the pRN5101 origin. RepC antigen produced by cells containing only pRN6759 was exclusively the 38-kD form and was inducible by CBAP (Fig. 2B). In the strain containing both plasmids, RepC antigen was found in both 38- and 42-kD forms, both of which were equally inducible by CBAP. This induction was accompanied by a twofold increase in the copy number of pRN6366. These results suggest that the 42-kD antigen is a form of RepC modified during pT181 replication; we have designated it RepC*. Preparations of RepC antigen from strains containing an active pT181 *ori* are equal mixtures of RepC and RepC*.

Approximately one molecule of RepC is synthesized with each replication of a single full-length plasmid (9), and the RepC molecules are stable (Fig. 1). Thus, if RepC is inactivated during replication, most of the RepC from pT181-containing bacteria should be inactive; a small fraction of active protein would be expected, representing material that has not yet been used for replication.

To test this hypothesis, we compared the activity of RepC antigen from a pT181*cop-623* (8) containing strain with that from a strain containing pRN6759 (RepC$^+$*ori*$^-$) for the three known enzymatic activities of RepC, namely, *ori*-specific topoisomerase-like (relaxation) activity, nicking activity (2), and initiation of replication in cell-free extracts (10). The *cop-623* preparation, a 50-50 mixture of RepC and RepC* (Fig. 3A), had no detectable topoisomerase or nicking activity in comparison with an equivalent quantity of RepC purified from the *ori*$^-$ strain of *S. aureus* (Fig. 3A). In other experiments of this type, preparations containing RepC-RepC* often had the slight activity expected, always less than 10% of the activity seen with RepC (Fig. 3B). RepC-RepC* mixtures also had less than 10% of the replication activity of native RepC in an in vitro replication assay (10) (Fig. 3C).

The addition of RepC-RepC* to RepC caused no inhibition of the relaxation activity of RepC (Fig. 3B), suggesting that RepC-RepC* preparations contain no independent inhibitory activity and confirming that the apparent inhibition of RepC by RepC* is stoichiometric.

The fact that RepC* migrates more slowly than RepC on SDS–polyacrylamide gel electrophoresis (SDS-PAGE) suggested that the protein was inactivated by a covalent adduct. Deoxyribonuclease I (DNaseI), pancreatic ribonuclease (RNase), and calf intestinal phosphatase had no detectable effect on the mobility of RepC*. Nuclease S1, however, converted the modified protein to a new form of intermediate mobility (Fig. 4A). Thus, the difference between RepC and RepC* is the presence of an oligodeoxynucleotide. Terminal transferase labeled the RepC* band but not the RepC band (Fig. 4B). This result suggests that the 3′ end of the bound oligonucleotide is accessible to terminal transferase and that the 5′ end is bound covalently to form RepC*. Because the difference in mobility between RepC and RepC* is about 4 kD, we estimate that the oligomer would be about 12 nucleotides in length. Slight heterogeneity in mobility of RepC* suggests that there may be some variation in the length of the

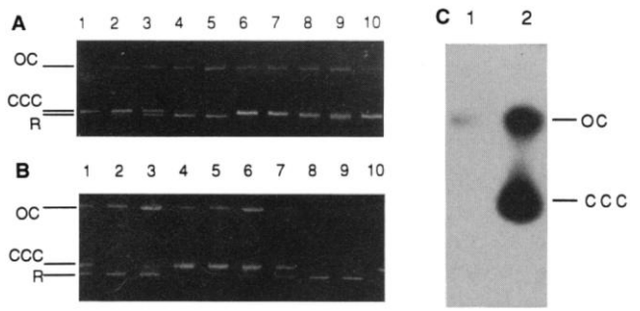


Fig. 3. Activity of RepC-RepC* in vitro. RepC was prepared from strain RN27 harboring plasmid pRN6759 (RN7805). RepC-RepC* was prepared from strain RN27 containing the high copy number mutant pT181*cop-623* (RN4111). (**A**) A constant amount of supercoiled pT181*cop-608* DNA (0.8 μg per sample) was incubated with different amounts of partially purified RepC or RepC-RepC* (12) for 30 min and then analyzed by agarose gel electrophoresis in the presence of ethidium bromide (0.25 μg/ml). R, relaxed covalently closed circular DNA; CCC, covalently closed supercoiled circular monomers; OC, open circular (nicked) DNA. Lanes 1 to 5, RepC antigen from RN7805 (inactive *ori*); lane 1, 6 ng; lane 2, 12 ng; lane 3, 25 ng; lane 4, 50 ng; and lane 5, 100 ng. Lanes 6 to 9, RepC-RepC* preparation from RN4111 (active *ori*); lane 6, 17 ng; lane 7, 35 ng; lane 8, 75 ng; lane 9, 150 ng; and lane 10, no RepC antigen. (**B**) Effect of RepC-RepC* on RepC relaxation activity. Reactions were prepared as in (A). Lanes 1 to 3 contained an equal mixture of RepC and RepC-RepC*; lane 1, 20 ng; lane 2, 35 ng; and lane 3, 100 ng. Lanes 4 to 6 contained RepC-RepC* alone; lane 4, 10 ng; lane 5, 35 ng; and lane 6, 100 ng. Lanes 7 to 9 contained RepC alone; lane 7, 10 ng; lane 8, 35 ng; lane 9, 100 ng; and lane 10, no RepC antigen. (**C**) Replication activity in cell-free extracts. Extracts of a plasmid-negative strain were prepared and assayed as described (10). Reaction mixtures contained 25-ng samples of RepC (lane 2) or RepC-RepC* (lane 1), 2 μg of supercoiled pT181*cop-608* DNA, 1 μCi of [α-^{32}P]deoxyadenosine triphosphate (dATP), and 50 μl of extract. Mixtures were incubated at 30°C for 30 min and then electrophoresed on a 1% agarose gel and autoradiographed. Radioactivity was quantified with the use of a Betagen beta scanner.

oligonucleotide. The relative mobility of the nuclease S1–treated material is consistent with this expectation, because the bond between the last nucleotide and the protein would be resistant to S1.

As RepC is covalently attached to plasmid DNA by a phosphotyrosine bond during replication (*3*), it seems likely that the oligonucleotide adduct (containing the origin) is generated through this attachment and therefore is at the active site tyrosine (Fig. 5). This model predicts that RepC* would be generated at the end of replication by the modification of one subunit of the RepC dimer, giving rise to a RepC-RepC* heterodimer. We have tested this prediction using streptavidin-coated paramagnetic beads (Dynal, Inc.) to which was bound a synthetic oligonucleotide, 5′-biotin-AAA-ACCAACCGGCTA-TT-3′ (Fig. 5D, top strand, nucleotides 58 to 70), complementary to the sequence immediately 3′ to the nick site (Fig. 5D, bottom strand, nucleotides 58 to 70). These beads selectively bound the RepC-RepC* complex, but did not bind the RepC homodimer (Fig. 4C). Treatment with NaOH eluted the bound RepC-RepC* complex quantitatively, both subunits being present in equimolar amounts in the eluate (*11*). This result confirms both the heterodimeric nature of the complex and the sequence of the bound oligonucleotide. Further evidence supporting the heterodimeric nature of RepC-RepC* was obtained by gel filtration analysis, in which the RepC-RepC* complex migrated with an apparent molecular size of 80 kD (*11*).

We conclude that the pT181 initiator protein RepC is inactivated as a direct consequence of its use for replication. This result contrasts with the behavior of the analogous ϕX174 CisA protein, which is repeatedly recycled until enough progeny phage are produced to lyse the cell. The use-specific inactivation of RepC is consistent with the hypothesis that stable regulation of DNA replication requires control of the fate as well as the synthesis of the initiator protein.

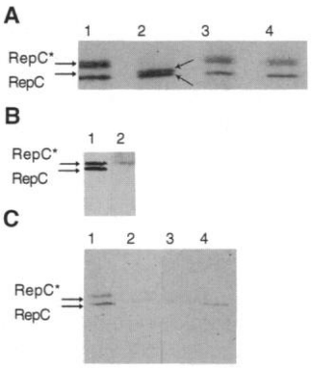
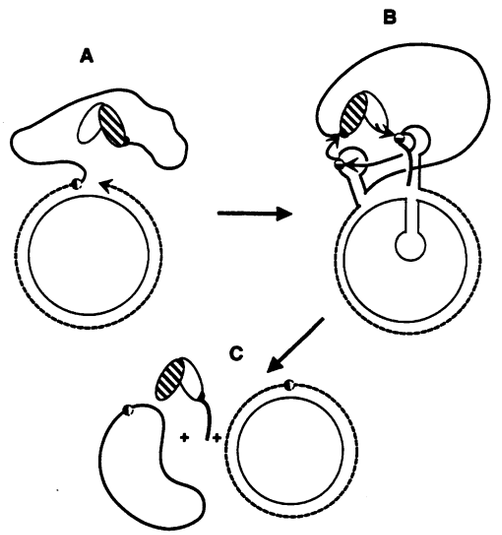


Fig. 4. (**A**) Effect of nucleases on RepC*. Partially purified RepC-RepC* (25 ng) was incubated with 150 U of S1 (lane 2), 0.5 μg of DNase I (lane 3), 0.5 μg of pancreatic RNase (lane 4), or no enzyme (lane 1) for 5 min at 37°C. Samples were analyzed by SDS-PAGE and protein immunoblotting as in Fig. 1. The thin arrows in lane 2 indicate RepC (lower) and RepC with at least one S1-resistant nucleotide attached (upper). (**B**) Labeling of the 3′ end of RepC* oligonucleotide with [α-^{32}P]dATP, using terminal transferase. RepC-RepC* was incubated with 160 U of terminal transferase and 2.5 μCi of [α-^{32}P]dATP for 60 min at 37°C. Samples were analyzed by SDS-PAGE and protein immunoblotting as in Fig. 1. Lane 1, RepC-RepC* immunoblot; lane 2, autoradiogram. (**C**) Specific adsorption of RepC-RepC* to magnetic beads. Two nanograms of a biotinylated oligonucleotide, synthesized at the Public Health Research Institute, were adsorbed to 100 μl of streptavidin-coated paramagnetic beads (Dynal), and 50-μl samples of the resulting preparation were incubated with RepC or RepC-RepC* in 50 mM NaCl, 10 mM tris (pH 8), 1 mM EDTA for 10 min at 15°C. The supernatant was then removed, and the beads were washed three times with 100-μl portions of the same buffer and then treated with 40 μl of 0.1 M NaOH for 5 min at room temperature. The beads were removed, and the eluate was neutralized with 10 μl of 0.4 M HCl plus 0.2 M tris. The resulting samples were then separated on SDS-PAGE and analyzed by protein immunoblotting as in previous figures. Lane 1, RepC-RepC* complex, NaOH eluate; lane 2, RepC-RepC*, bead supernatant; lane 3, RepC, NaOH eluate; and lane 4, RepC, bead supernatant.



D

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    replication  <-----      70 71
pT181 53  5′ TGCGTCCAACCGGCTATT-AGAGTAGCCGGTTTTAGAAAAA  3′ 92
             ACGCAGGTTGGCCGATAA TCTCATCGGCCAAAATCTTTTT
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Fig. 5. Possible mechanism for the generation of RepC*. (**A**) Diagram of the expected configuration of a plasmid DNA molecule nearing completion of a replication cycle. One subunit (crosshatched) of the RepC dimer is attached to the 5′ end of the displaced plus strand, and the growing end of the nascent strand (arrow) is approaching the *ori* nick site (*2*). In (**B**), the nick site has been replicated, the leading strand extended a short distance further, and the *ori* hairpin (*13*) has formed in both the new and displaced leading strands, as well as in the old template strand. The second subunit of RepC (clear) initiates a concerted strand transfer reaction (arrows) by nicking the newly synthesized *ori* and thus acquiring the observed oligonucleotide, which therefore consists of the ≈12-nucleotide 3′ half of the *ori* hairpin. The newly generated 3′-OH group then attacks the old *ori*, and the displaced 3′-OH then attacks the old RepC-DNA bond, displacing RepC and restoring continuity of the displaced plus strand, which is released as a single-stranded circle. In (**C**), the final products are shown, a single-stranded circular monomer representing the displaced leading strand, a RepC heterodimer with the oligonucleotide attached to one subunit, and a double-stranded plasmid monomer containing the new leading strand. The single-stranded monomer then replicates by an independent mechanism, not involving RepC, to complete the cycle. In (**D**), the *ori* sequences surrounding the nick site are shown. The opposite-facing dashed arrows indicate the stem of the *ori* hairpin.

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