Biochemical Characterization of the *Staphylococcus aureus* PcrA Helicase and Its Role in Plasmid Rolling Circle Replication*

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Previous genetic studies have suggested that a putative chromosome-encoded helicase, PcrA, is required for the rolling circle replication of plasmid pT181 in *Staphylococcus aureus*. We have overexpressed and purified the staphylococcal PcrA protein and studied its biochemical properties in vitro. Purified PcrA helicase supported the in vitro replication of plasmid pT181. It had ATPase activity that was stimulated in the presence of single-stranded DNA. Unlike many replicative helicases, PcrA was highly active as a 5′ → 3′ helicase and had a weaker 3′ → 5′ helicase activity. The RepC initiator protein encoded by pT181 nicks at the origin of replication and becomes covalently attached to the 5′ end of the DNA. The 3′ OH end at the nick then serves as a primer for displacement synthesis. PcrA helicase showed an origin-specific unwinding activity with supercoiled plasmid pT181 DNA that had been nicked at the origin by RepC. We also provide direct evidence for a protein-protein interaction between PcrA and RepC proteins. Our results are consistent with a model in which the PcrA helicase is targeted to the pT181 origin through a protein-protein interaction with RepC and facilitates the movement of the replisome by initiating unwinding from the RepC-generated nick.

DNA helicases play critical roles in DNA transactions such as replication, transcription, recombination, and repair (1, 2). Most bacterial species contain several DNA helicases that are involved in one or more processes of DNA metabolism. *Escherichia coli* cells contain a number of helicases, of which DnaB, UvrD, and Rep are the best studied (1). The DnaB helicase is necessary for cell survival and is known to be involved in the theta-type replication of the *E. coli* chromosome and several plasmids. The UvrD helicase (DNA helicase (II) is involved in DNA repair in *E. coli*, whereas the Rep helicase is required for rolling circle (RC) replication of single-stranded (ss) DNA phages such as M13 and φX174 (1, 3–5). The *Staphylococcus aureus* pcrA gene was identified several years ago and found to be required for the RC replication of plasmid pT181 (6, 7). Subsequently, the pcrA gene was also identified in *Bacillus subtilis*, and genetic studies have shown that PcrA is required for both DNA repair and RC plasmid replication and appears to incorporate the function of both the Rep and UvrD proteins of *E. coli* (8). The *S. aureus* and *B. subtilis* PcrA helicases share 56% identity and 74% similarity, are required for cell viability, and may also play a role in replication of the chromosomal DNA (7, 8). The *S. aureus* PcrA helicase shares 39% homology with the UvrD and Rep helicases of *E. coli* (7). The pcrA gene has also been identified in several other Gram-positive bacteria such as *Bacillus steaerotherophilus*, *Lactococcus lactis*, *Streptococcus pyogenes*, and *S. pyogenes* and is likely that they have similar functions in their respective hosts. The PcrA helicases of Gram-positive bacteria belong to superfamily I of DNA helicases (1, 2).

Plasmid pT181 of *S. aureus* replicates by a rolling circle (RC) mechanism (9, 10). The RepC initiator protein encoded by pT181 nicks at the origin of replication and becomes covalently attached to the 5′ end of the DNA (11, 12). The 3′ OH end at the nick site then serves as a primer for displacement synthesis, which presumably involves unwinding of the DNA by the PcrA helicase ahead of the replication fork. During the termination of plasmid DNA replication, the RepC protein covalently attached to the DNA is expected to catalyze additional trans-esterification reactions leading to the release of the parental circular leading strand of the DNA and a supercoiled (SC) DNA containing a newly synthesized leading strand (13). An *S. aureus* mutant carrying the pcrA3 mutation was shown to be defective in the RC replication of plasmid pT181, but this mutation did not affect chromosome replication, replication of other RC plasmids, or cell survival (7). Additional *in vivo* studies showed that the pcrA3 mutants of *S. aureus* and *B. subtilis* accumulated nicked plasmid pT181 DNA, and further studies have suggested that PcrA may be required for unwinding of the plasmid DNA from the initiator protein-generated nick, an event that is required for plasmid RC replication (8, 14). Mutants in the replication initiator protein of pT181, RepC, have been isolated that allow plasmid replication in the pcrA3 strain, suggesting an interaction between PcrA and RepC proteins (15). The PcrA helicase of *B. steaerotherophilus* has been purified, and its crystal structure has been determined (16, 17). It shows a strong helicase activity with double-stranded (ds) substrates containing a 3′ ss tail and a limited activity with substrates containing a 5′ ss tail (16, 19, 20). This PcrA is one of the few helicases that act as monomers in contrast to the more common replicative helicases, which act as hexamers (1, 2, 4, 17, 18). It has been shown to bind to both ss as well as ds DNA (19, 20). The PcrA helicases of *B. steaerotherophilus* and *S. aureus* have 60% identity. The *B. steaerotherophilus* PcrA

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The abbreviations used are: RC, rolling circle; SC, supercoiled; ss, single-stranded; ds, double-stranded; OC, open circular; nt, nucleotide(s); MBP, maltose binding protein.

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helicase activity is not very processive, but the RepD initiator protein encoded by the S. aureus plasmid pC221 enhances its processivity (21). This suggests that the B. stearothermophilus PcrA may interact with RC initiator proteins.

In an effort to understand the role of S. aureus PcrA in the RC replication of its native pT181 plasmid, we have overexpressed and purified this protein as a fusion with six histidine (His6) residues and studied its biochemical activities. Purified PcrA supported in vitro replication of pT181 in cell extracts made from the pcrA3 mutant of S. aureus, providing a direct biochemical evidence for its role in plasmid RC replication. PcrA was also found to have a robust ATPase activity that was stimulated in the presence of ssDNA. It had a strong 5′-3′ helicase activity. We also report that PcrA is able to unwind SC pT181 DNA nicked at the origin by the RepC initiator protein. This unwinding required both sequence-specific noncovalent binding of RepC to the origin, as well as the presence of covalently attached RepC at the nick site. Our data support a model in which PcrA is recruited to unwind the nascent leading strand.

Experimental Procedures

Cloning of His-PcrA—The pcrA gene was amplified by PCR from the chromosomal DNA of S. aureus S6 as the template. The sequences of the primers used were: 5′-CGGGATCCATGCGGTATATTACATCTATATGATTACAGGACAAAGTG-3′ for the forward primer and 5′-GGATCCGATGCCTGGTCTCTCCCAGGCTTATGATC-3′ for the reverse primer. The PCR primers contained BamHI linkers at the ends. The reaction mixtures contained 200 μM of each dNTP, 250 ng of S. aureus genomic DNA, 1 μM of each primer, and 5 units of the FpI polymerase (Stratagene, La Jolla, CA). The conditions of amplification were as follows: 94°C for 3 min; 94°C for 1 min, 60°C for 1 min, and 72°C for 4 min for 25 cycles; and 72°C for 10 min. The amplified product was digested with BamHI and then fused in-frame to the His6 epitope at the BamHI site of the pQE30 vector from Qiagen. This DNA was expected to encode a PcrA protein with His6 residues fused at its amino-terminal end. The ligation mixture was then introduced into E. coli M15 by electroporation, and the appropriate clones were isolated for protein overexpression.

Preparation of the His-PcrA Protein—A single colony of the E. coli strain expressing the His-PcrA protein was grown overnight in 10 ml of LB containing 50 μg/ml ampicillin and 25 μg/ml kanamycin at 37°C. This culture was diluted into 1 liter of prewarmed LB, and the cells were grown to the mid-exponential phase (A600 of ~0.5) at 37°C. Expression of the pcrA gene was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 0.25 mM, and the culture was further shaken at room temperature for 2 h. The cells were harvested by centrifugation and suspended in a final volume of 20 ml of the lysis buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 40 mM MgCl2, 3 mM Mg(II), 3 mM ATP, 5 mM dithiothreitol, 10% glycerol, 0.5% potassium phosphate (pH 7.5) buffer). The cells were then lysed by two quick freeze-thaw steps. Ultracentrifugation was performed in SW41 rotor at 33,000 rpm for 1 h at 4°C. The supernatant (about 10 ml) was collected and diluted with an equal volume of the lysis buffer lacking Mg2+-mercaptoethanol. The diluted supernatant (about 20 ml) was added to 1 ml of the nickel-coated resin and mixed by gentle inversion at 4°C for 1 h. The mixture was then packed onto a column that was washed with 10 column volumes of the lysis buffer containing 20 mM β-mercaptoethanol. The PcrA protein was then eluted with a buffer containing 50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 20 mM β-mercaptoethanol, 200 mM imidazole, and 10% ethylene glycol. Small aliquots (0.5 ml) were collected. The concentration of the His-PcrA preparation reached 1–2 mg/ml in the peak fractions, and the purity was about 95% based on SDS-PAGE and staining with Coomassie Brilliant Blue.

Preparation of Plasmid DNA—Plasmid pT181cop608 was prepared by CaCl2/ethidium bromide density gradient centrifugation (22). Other plasmid DNAs were isolated by using the Maxi Prep kit from Qiagen. Chromosomal DNA from S. aureus S6 was prepared by phenol-chloroform extraction.

Preparation of Cell-free Extracts and in Vitro Replication—Cell-free replication extracts were prepared from the S. aureus strain RN4220 and the pcrA3 mutant as described (23, 24). Replication reactions (30 μl) contained 600 μg of protein extract, 500 ng of pT181cop608 DNA, 200 ng of RepC protein, and the indicated amounts of PcrA protein. Replication products were labeled with [α-32P]dATP. The reactions were incubated at 32°C for 1 h, treated with proteinase K, extracted with phenol/chloroform, and DNA-isolated by alcohol precipitation (23). The reaction products were subjected to electrophoresis on 1% agarose gels using Tris-borate-EDTA buffer containing 1 μg/ml of ethidium bromide (24). The gels were dried and subjected to autoradiography.

ATPase Assays—ATPase activity of PcrA was measured by hydrolysis of [α-32P]ATP or dATP. The reactions (30 μl) were carried out in 1× TEKEM buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 100 mM KCl, 10 mM Mg(II), and 10% ethylene glycol (v/v)) containing 1 μCi of [α-32P]ATP and the indicated amounts of PcrA. The reaction mixtures also contained 500 ng of SC pT181cop608 DNA, 100 ng of ss oligonucleotide, and 200 ng RepC where indicated. The reaction mixtures were incubated at 37°C for 1 h. To stop the reaction, EDTA was added to a final concentration of 83 mM, and 1-μl aliquots were subjected to thin layer chromatography on cellulose polyethyleneimine sheets using the 0.5 × 5 KH2PO4 (pH 3.5) buffer. The TLC sheets were dried and subjected to autoradiography.

Helicase Assays—Double-stranded oligonucleotide substrates containing 3′ or 5′ tails or blunt ends were prepared by labeling one strand with 32P at the 5′ end using T4 polynucleotide kinase (22) and annealing to the cold complementary strand. Oligonucleotide sequences used in this study are listed in Table 1. Helicase reactions were performed at 37°C for 30 min in a buffer containing 20 mM Tris-HCl (pH 7.5), 100 mM KCl, 3 mM MgCl2, 3 mM ATP, 5 mM dithiothreitol, 10% glycerol, ~1.75 ng of the DNA substrates, and the indicated amounts of PcrA helicase. The reactions were stopped by the addition of SDS dye, and the products were analyzed by 10% native polyacrylamide gel electrophoresis (22). The gels were subsequently dried and exposed to Kodak x-ray films.

DNA Relaxation and Unwinding Assays—DNA relaxation assays were performed in TEKEM buffer containing 5 mM ATP (11). One-half microgram of pT181cop608 DNA was incubated in the presence or absence of RepC (200 ng) and PcrA (200 ng) at 28°C for 30 min. RepC-nicked OC pT181cop608 DNA devoid of RepC was generated as follows. The plasmid DNA was nicked by RepC as described above, and the protein was removed by proteinase K digestion at 37°C for 30 min followed by phenol/chloroform extraction and alcohol precipitation. The nicked DNA was then used as a substrate for DNA unwinding assays with PcrA. The reaction products were subjected to electrophoresis on 1% agarose gels with Tris-borate-EDTA buffer containing 0.5 μg/ml ethidium bromide.

Table 1

Oligonucleotides used in this study

Numbers correspond to the nucleotide positions in the plasmid pT181 ori (1).

| Oligo 1 (5′ overhang) | 5′-ATAATCCACAGGATGCTGATC-3′ |
| Oligo 2 (3′ overhang) | 5′-CTGCATATATCTGCTGCTGCTG-3′ |
| Oligo 3 (5′ overhang) | 5′-GATCCTCATGACAGGATGATC-3′ |

S1 Nuclease Treatment of DNA—7.5 units of S1 nuclease in 300 μl of S1 buffer (0.28 mM NaCl, 0.05 mM sodium acetate, and 4.5 mM ZnSO4) were
PcrA is required for the in vitro replication of plasmid pT181. In vitro replication was carried out using the RepC protein and cell extracts from either wild-type or pcrA3 mutant S. aureus and the indicated amounts of the PcrA helicase. The positions of the supercoiled pT181cop608 DNA (SC), open circular DNA (OC), and replication intermediates (RI) are shown.

These results showed that the PcrA helicase of S. aureus is the only replication protein defective in the pcrA3 mutant and that the purified PcrA protein is biologically active. Because the native unfused PcrA protein from S. aureus has not been purified, we have assumed in this study that the His-tagged PcrA is as active as the wild-type enzyme.

ATPase Activity of the PcrA Protein—The S. aureus PcrA had an NTase activity that efficiently hydrolyzed ATP (Fig. 2A) as well as dATP (Fig. 2B). PcrA also hydrolyzed other nucleotides such as dGTP, dCTP, and TTP (not shown). The ATPase activity was not affected in the presence of either the RepC initiator protein or SC pT181cop608 DNA and the RepC protein were included in the reactions (Fig. 2A). However, the ATPase activity was stimulated when both the pT181cop608 DNA and the RepC protein were included in the reactions (Fig. 2A). This is likely due to the “activation” of the DNA unwinding activity of PcrA in the presence of a RepC-generated nick at the pT181 origin of replication (see below). The ATPase activity of PcrA was also significantly stimulated in the presence of ssDNA (Fig. 2B).

DNA Helicase Activity of PcrA—As discussed earlier, PcrA is expected to act as a helicase during the RC replication of plasmids. We tested the helicase activity of PcrA as well as its directionalcy using several oligonucleotides representing various regions of the pT181 origin (Table I). Polyacrylamide gel electrophoresis demonstrated that PcrA unwound a ds oligonucleotide containing a 5′ ss tail (oligonucleotide 1) in a dose-dependent manner (Fig. 3A). However, it had an ~10-fold weaker helicase activity with oligonucleotide 2 that contained a 3′ ss tail (Fig. 3B). Results similar to those shown in Fig. 3 (A and B) were obtained when additional oligonucleotides with 5′ or 3′ tails, respectively, were used in these experiments (data not shown). As expected, no DNA unwinding was observed in the absence of ATP. A ds 30-mer oligonucleotide containing 4-nt-long 5′ overhangs at both ends (oligonucleotide 3) was as efficiently unwound as oligonucleotide 1 by PcrA (Fig. 3C), demonstrating that 4 nt are sufficient for the loading of PcrA onto the 5′ end of the DNA. PcrA failed to unwind a blunt-ended ds oligonucleotide (data not shown), demonstrating a requirement for an ss region for its helicase activity. To rule out the possibility of any contamination from E. coli helicases in the PcrA preparation, proteins from the host strain lacking the PcrA gene were subjected to mock purification through a nickel affinity column. The eluted fractions from this column did not contain any detectable helicase activity (data not shown).

PcrA Can Initiate Unwinding from RepC-nicked pT181 DNA—The observation that nicked OC form of pT181 DNA

![Fig. 1. PcrA is required for the in vitro replication of plasmid pT181. In vitro replication was carried out using the RepC protein and cell extracts from either wild-type or pcrA3 mutant S. aureus and the indicated amounts of the PcrA helicase. The positions of the supercoiled pT181cop608 DNA (SC), open circular DNA (OC), and replication intermediates (RI) are shown.](http://www.jbc.org/)

![Fig. 2. ATPase activity of PcrA. A, products of [α-32P]ATP hydrolysis by PcrA (100 ng) in the presence or absence of the RepC protein (100 ng) and/or pT181cop608 DNA. B, stimulation of the dATPase activity of PcrA by a 53-nt-long oligonucleotide (ssDNA). The products of [α-32P]ATP or [α-32P]dATP hydrolysis were analyzed by TLC.](http://www.jbc.org/)

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accumulates in pcrA3 mutants suggests that PcrA is required for the unwinding of SC pT181 DNA that has been nicked at the origin by RepC (14). This postulate was tested by incubating SC pT181cop608 DNA with PcrA in the presence and absence of RepC. As expected, PcrA by itself did not unwind SC pT181 DNA (Fig. 4). Incubation of pT181cop608 DNA with RepC resulted in the generation of relaxed, covalently closed circular DNA as well as nicked OC DNA. The covalently closed circular DNA migrates faster than the SC DNA in agarose gels in the presence of ethidium bromide (Fig. 4). The DNA migrating slower than the OC form presumably corresponds to dimers of OC DNA. When pT181cop608 DNA was incubated with both PcrA and RepC, a new band migrating faster than the relaxed covalently closed circular DNA was observed (Fig. 4). This form presumably corresponds to the unwound "U" form of the DNA (25). In addition, a diffused band corresponding to OC DNA possibly with various extent of unwinding was obtained (Fig. 4). Under the same conditions of S1 treatment, the SC and OC forms of pT181cop608 DNA were mostly unaffected, whereas the ss M13 DNA was totally digested (Fig. 4). These data showed that the conversion of pT181 DNA to the more quickly migrating U form as well as more slowly migrating unwound forms is dependent upon both PcrA and RepC.

Both DNA Binding and Nicking Activities of RepC Are Required for pT181 DNA Unwinding by PcrA—During the initiation of pT181 RC replication, RepC nicks at the origin, the replisome presumably assembles at the nick, and replication proceeds upon unwinding of the DNA by PcrA. We wished to determine whether unwinding of the nicked pT181 DNA by PcrA requires the presence of RepC covalently attached to the nick site. For this, pT181 DNA was first nicked and relaxed by RepC, and the protein was removed by treatment with proteinase K and phenol extraction. Incubation of PcrA with the OC pT181 DNA did not result in the generation of any unwound DNA (Fig. 5A), suggesting that unwinding by PcrA from the RepC-generated nick requires the presence of RepC covalently attached to the nicked DNA.

In addition to nicking the pT181 origin, the RepC protein binds noncovalently to the origin through a sequence-specific interaction (26). We wished to determine whether this interaction is important for the recruitment of PcrA helicase to the pT181 origin. The RepC protein contains separate DNA binding and nicking-closing domains, and we have previously shown that these two activities of RepC can be mutationally uncoupled (27). Two RepC mutants, nickbind/H11001 and nickbind/H11002 (27) were used in these experiments. Incubation of the DNA-binding mutant of RepC (nickbind/H11001) with SC pT181 DNA generated relaxed covalently closed circular DNA because this mutant has nicking-closing activities (Fig. 5B). However, addition of PcrA helicase to the reaction in the presence of this mutant did not generate any unwound U form of the DNA that was observed in the presence of wild-type RepC (Fig. 5B). Incubation of PcrA with pT181 DNA in the presence of the nickbind RepC mutant, which is able to bind noncovalently...
to the pT181 origin but is defective in nicking-closing, did not change the migration pattern of SC pT181 DNA (Fig. 5B). These results showed that both nicking and stable noncovalent interaction between RepC and the pT181 origin is required for PcrA to be targeted to the nick site and to initiate DNA unwinding.

Protein-Protein Interaction between the PcrA Helicase and RepC—Previous genetic studies as well as indirect in vitro studies have suggested an interaction between PcrA and RepC. Our experiments (Figs. 4 and 5) also suggested an interaction between these proteins in the presence of the pT181 origin. We wished to determine whether there is a direct physical interaction between the PcrA and RepC proteins in vitro. For this, we made use of the different epitope tags present on the PcrA and RepC proteins. E. coli lysates containing the overexpressed MBP-RepC protein were mixed with amylase resin and unbound proteins washed with a buffer containing 1% bovine serum albumin. This was followed by addition of His-PcrA to the resin. The wash was followed by buffer, and the bound proteins were eluted from the resin by the addition of SDS-PAGE sample buffer. SDS-PAGE analysis of these samples followed by Western blot analysis using either anti-MBP or anti-His6 monoclonal antibody showed that MBP-RepC was bound to the amylase resin as expected (Fig. 6). Furthermore, although His-PcrA did not bind to the amylase resin, it was retained on the resin to which MBP-RepC was bound (Fig. 6). These results suggested that PcrA and RepC can physically interact.

**DISCUSSION**

The pcrA gene was originally identified in *S. aureus* as being required for the RC replication of staphylococcal plasmid pT181 as well as for the viability of this organism (6, 7). Subsequently, related pcrA genes were also identified in other Gram-positive bacteria such as *B. subtilis and B. stearothermophilus* and were found to have ~60% identity with the pcrA of *S. aureus*. Genetic studies have shown that the *B. subtilis* PcrA helicase is required for the replication of the heterologous pT181 plasmid in this organism and is also involved in UV repair (8). Because PcrA is essential for cell viability in both *S. aureus* and *B. subtilis*, it is likely to have additional roles such as in the replication of the chromosome or other critical cellular DNA metabolism. The *B. stearothermophilus* PcrA helicase has been purified, and its biochemical activities and crystal structure have been determined. Similar to the UvrD and Rep helicases of *E. coli*, this PcrA is a 3′→5′ helicase, although at higher concentrations it can also act in the 5′→3′ direction (16, 28). Mutational and crystal studies with the *B. stearothermophilus* PcrA helicase have identified the domains that are involved in its ATPase, helicase, and DNA binding activities (20, 29, 30). Based on the above studies as well as studies with 5′→3′ helicases, it has been suggested that a conserved motif known to be involved in the 3′→5′ helicase activity of PcrA may also be involved in 5′→3′ helicase activity (17). Unlike several other helicases involved in DNA replication, PcrA of *B. stearothermophilus* acts as a monomer, and its movement along the DNA appears to involve a “inchworm” rather than a more conventional “active rolling” mechanism (30). Despite extensive structural studies, very little is known about the biochemical role of the PcrA helicase in DNA metabolism. To evaluate the role of *S. aureus* PcrA in the RC replication of its native plasmids, we have overexpressed and purified this protein from *S. aureus* and studied its biochemical properties and role in plasmid pT181 RC replication in vitro.

The *S. aureus* PcrA helicase was purified as a His6 fusion at its amino-terminal end by affinity chromatography. We made use of the *pcrA*3 mutant of *S. aureus* to directly evaluate a role for PcrA in pT181 replication in vitro. Although cell-free extracts from wild-type *S. aureus* supported replication of pT181 DNA in the presence of RepC, extracts from the *pcrA*3 mutant were essentially inactive in replication (Fig. 1). The faint band seen at the OC position with the mutant extracts may represent the incorporation of a few nucleotides at the RepC-generated nick site. The IRII region of the pT181 origin (positions 60–83) is present as a hairpin, and the RepC nick site is located in the bottom strand of the loop of IRII (Table I and Refs. 11, 31, and 32). Based on sensitivity to bromoacetaldehyde and KMNO₄, it has been postulated that binding of RepC to the IRII region in SC pT181 DNA results in cruciform excision in which the IRII stem is melted to generate an ss region (32). The above observations are consistent with the finding that ~11 nt, including those contained in the downstream arm of IRII (pT181 positions 70–60), can be incorporated at the RepC nick site by DNA polymerase extension synthesis even in the absence of the helicase activity in the *pcrA*3 mutant (14, 32). However, for replication to proceed further, the helicase activity of PcrA is expected to be required for unwinding of the duplex DNA downstream of position 60 of pT181. Addition of the purified PcrA helicase to the *pcrA*3 mutant extracts restored pT181 replication (Fig. 1), demonstrating that this is the component missing in this mutant and is required for plasmid RC replication in vitro.

The *pcrA*3 mutant, which contains a threonine to isoleucine substitution at position 61 of the *S. aureus* PcrA helicase, is defective in plasmid pT181 replication (7). RepC mutants have been isolated that allow pT181 replication in the *pcrA*3 mutant,
suggesting an interaction between PcrA and RepC (15). Indirect evidence also suggests that the PcrA helicase of \textit{B. stearothermophilus} interacts with the pC221-encoded RepD protein at the plasmid origin (21). Our SC pT181 DNA unwinding experiments (Figs. 4 and 5) also suggest an interaction between PcrA and RepC in the presence of the pT181 origin. These data postulate that RepC may recruit PcrA to the pT181 origin during the initiation of plasmid replication through a specific protein-protein interaction. To directly test this possibility, pull-down assays were performed using affinity-tagged proteins (Fig. 6). These experiments showed that His-PcrA was retained on an affinity column containing MBP-RepC, and provide a direct evidence for PcrA-RepC interaction. Because the pcrA3 mutation does not affect cell growth or the replication of other RC plasmids, it is likely that the Thr-61 residue of PcrA lies in a domain that is involved in its specific interaction with RepC. This postulate is consistent with the conservation of the Thr-61 residue in the PcrA helicases of \textit{S. aureus}, \textit{B. subtilis}, and \textit{B. stearothermophilus} and in the related UvrD helicase of \textit{E. coli} (8). It is known that pT181 can replicate in \textit{S. aureus} and \textit{B. subtilis} that and UvrD can support replication of RC plasmids in \textit{E. coli} (8, 9, 33).

The PcrA helicase of \textit{S. aureus} has an ATPase activity that is not affected in the presence of either SC pT181 DNA or RepC alone. However, its ATPase activity is stimulated when both SC pT181 DNA and RepC are present together (Fig. 2). This is likely to be due to the activation of the helicase activity of PcrA upon generation of a nick at the pT181 origin by RepC (see below). The helicase activity of PcrA was also stimulated in the presence of ssDNA, consistent with the results obtained with several DNA helicases.

During plasmid RC replication, the PcrA helicase is expected to unwind the DNA and move ahead of the replication fork. Our experiments show that the \textit{S. aureus} PcrA helicase has a much stronger helicase activity with a DNA containing a 5' ss tail as compared with a substrate containing a 3' ss tail (Fig. 3). Based on the substrate preference of PcrA and its comparison with other well studied helicases (1, 3, 19, 20), our results suggest that the \textit{S. aureus} PcrA has a weak 3' → 5' and a much stronger 5' → 3' helicase activity. We used synthetic oligonucleotides containing either 3' or 5' tails or blunt ends in the helicase assays. PcrA efficiently unwound a ds substrate with a 23-nt 5' tail as well as ds oligonucleotides containing either 26- or 47-bp duplexes and 4-nt-long 5' tails at both ends (Fig. 3, A and C, and data not shown). These results showed that PcrA can efficiently unwind substrates with a 5' tail and that an ss region of 4 nt was sufficient for the helicase activity of PcrA. PcrA also unwind substrates containing 3' tails, but this activity was much weaker, and it failed to fully unwind the DNA even at the highest concentration tested (Fig. 3B). PcrA failed to detectably unwind blunt-ended oligonucleotides (data not shown). The PcrA of \textit{B. stearothermophilus} has a much stronger 3' → 5' helicase activity as compared with its 5' → 3' activity (16). This helicase was also inefficient in unwinding duplexes of greater than 20 bp (28). On the other hand, the \textit{S. aureus} helicase efficiently unwound oligonucleotides containing a duplex region of 47 bp (data not shown). \textit{S. aureus} PcrA also extensively unwind nicked OC pT181 DNA in the presence of RepC (Fig. 4). The differences in the helicase activities of the \textit{S. aureus} and \textit{B. stearothermophilus} PcrA proteins may reflect inherent differences in their activity/specificity, or it may be due to the nature of the substrates used in these studies (partially duplex oligonucleotides \textit{versus} oligonucleotides annealed to the M13 ssDNA, respectively). It is known that \textit{S. aureus} and \textit{B. subtilis} cells contain the replicative helicase DnaC (homolog of \textit{E. coli} DnaB) that is presumed to play an essential role in the theta-type replication of the chromosome. However, PcrA may also be required for chromosome replication and possibly in other DNA transactions such as DNA repair and recombination. Some of the above activities of PcrA may require its 5' → 3' helicase activity. Although the weak 3' → 5' helicase activity of the \textit{S. aureus} PcrA helicase may be involved in pT181 replication, it is possible that this helicase may translocate in a 5' → 3' direction on the displaced leading strand during plasmid RC replication. After nicking the pT181 origin, one monomer of the dimeric RepC becomes covalently attached to the 5' P of the DNA through its Tyr-191 residue (27). Because RepC also catalyzes DNA cleavage/relinkation events during the termination of plasmid RC replication, it is expected to be in close proximity to the replication fork as it reaches the termination site, \textit{i.e.}, the regenerated origin sequence (9). Because PcrA and RepC interact, it is possible that RepC (tethered to PcrA) moves along with PcrA just ahead of the replication fork. Because RepC is covalently attached to the displaced leading strand of the DNA, PcrA may either translocate on the template strand (in a 3' → 5' direction) or on the displaced strand (5' → 3' direction). Future studies should identify the directionality of the \textit{S. aureus} PcrA helicase during plasmid RC replication.

We also tested whether PcrA has pT181 origin-specific unwinding activity that is expected to be required for plasmid RC replication. Inclusion of PcrA in the relaxation reactions with pT181 DNA and RepC resulted in the generation of a more quickly migrating band that presumably corresponds to the unwound U form of the DNA (Figs. 4 and 5). No ss circular or linear DNA was detectable (data not shown), suggesting that PcrA is unable to fully unwind the pT181 DNA in the absence of other replisome components. However, this is not surprising because replisome proteins such as the single-stranded DNA-binding protein and others may promote unwinding of the DNA by PcrA during replication. The U form DNA contained extensive ss regions because it was sensitive to digestion by the S1 nuclease (Fig. 4). The single band for the U form may reflect either DNA unwinding by PcrA to a relatively fixed extent or a unique DNA conformation in which DNA has been unwound to different extent but migrates to the same position. For example, it is possible that the more quickly migrating U band reflects unwound DNA in which the RepC-bound displaced strand is held close to PcrA through a protein-protein interaction, a situation similar to that expected to occur during RC replication (9, 24). The U form is unlikely to resemble OC DNA with different extent of unwinding because such DNA is expected to migrate near the OC form. The above postulates are consistent with the sensitivity of the U form of the DNA and DNA species migrating near the OC position to cleavage by the S1 nuclease (Fig. 4) because they are expected to contain extended ss regions. Digestion of both types of DNA by S1 would presumably generate a smear of smaller dsDNA bands that may not be visible as specific bands.

\textit{In vitro}, nicking of the pT181 DNA by RepC is followed by religation that results in the generation of relaxed DNA. Therefore, only a small amount of OC DNA may be transiently available for PcrA-driven unwinding. This prediction is consistent with the limited amounts of the U form generated in the presence of both RepC and PcrA \textit{in vitro} (Fig. 4). During the initiation of RC replication, RepC is expected to interact stably with the origin through sequence-specific binding (26, 32). The generation of nicked OC DNA is likely to be coordinated with the recruitment of PcrA to the origin through protein-protein interactions. Following this, it is likely that origin nicking by RepC is followed by unwinding of the DNA by the PcrA helicase, binding of SSB to the ssDNA,
and synthesis of the leading strand of the DNA by Pol III involving a strand displacement mechanism (9, 24). To test whether stable binding of RepC to the origin (as compared with transient nicking-closing) is important for unwinding by PcrA, we utilized DNA-binding and nicking mutants of RepC. The bind- nick- RepC mutant is able to nick-close pT181 DNA through a transient interaction but does not bind stably to the pT181 origin (27). Although the pT181 DNA was relaxed by the bind- nick- mutant, PcrA was unable to unwind the DNA from the nick in the presence of this mutant (Fig. 5B). As expected, no unwinding of DNA by PcrA was observed in the presence of a bind- nick- mutant of RepC because this mutant is unable to nick the DNA (Fig. 5B). PcrA was also unable to unwind pT181 DNA nicked at the origin from which RepC had been removed (Fig. 5A). The above results are consistent with the postulate that stable binding of RepC to the pT181 origin facilitates recruitment of PcrA followed by DNA nicking and unwinding. As seen with the related RepD protein encoded by the pC221 plasmid of S. aureus (21), RepC may also increase the processivity of the PcrA helicase.

The PcrA helicase is known to be required for the replication of several different classes of RC plasmids (6–8). The RC plasmids of Gram-positive bacteria have been divided into four major families: the pT181, pE194/pMV158, pUB110/pC194, and pSN2 families (9, 10). Plasmids belonging to individual families have identical or very similar nicking domains in their Rep proteins and nick sites in their origins (9, 10). Thus, it is likely that the Rep proteins of the RC plasmids are capable of specific interaction with the PcrA helicases of Gram-positive bacteria. It is well established that although several plasmids of Gram-positive bacteria have a broad host range, others are stable only in their native hosts (9, 10). One determinant of narrow versus broad host range appears to be the single strand origin contained within the RC plasmids (9, 34, 35). It is possible that the ability of a particular RC plasmid to replicate in a wide range of Gram-positive organisms may also depend, in part, on the ability of its Rep protein to efficiently interact and recruit PcrA to the leading strand origin of replication. Future studies are expected to deal with this interesting possibility. The availability of purified PcrA helicase from S. aureus should allow an investigation of its possible roles in chromosome replication in Gram-positive organisms. It should also facilitate studies on the possible roles of the PcrA helicase in DNA repair, including whether it interacts with DNA mismatch repair proteins such as MutL, which is known to interact with the UvrD helicase in E. coli (3). Furthermore, because of its requirement for cell viability, PcrA may also represent an important target for the development of antibacterial agents against Gram-positive organisms.

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