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Purification and Characterization of the PcrA Helicase of *Bacillus anthracis*

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PcrA is an essential helicase in gram-positive bacteria, and a gene encoding this helicase has been identified in all such organisms whose genomes have been sequenced so far. The precise role of PcrA that makes it essential for cell growth is not known; however, PcrA does not appear to be necessary for chromosome replication. The *pcrA* gene was identified in the genome of *Bacillus anthracis* on the basis of its sequence homology to the corresponding genes of *Bacillus subtilis* and *Staphylococcus aureus*, with which it shares 76 and 72% similarity, respectively. The *pcrA* gene of *B. anthracis* was isolated by PCR amplification and cloning into *Escherichia coli*. The PcrA protein was overexpressed with a His_6 fusion at its amino-terminal end. The purified His-PcrA protein showed ATPase activity that was stimulated in the presence of single-stranded (ss) DNA (ssDNA). Interestingly, PcrA showed robust 3′→5′ as well as 5′→3′ helicase activities, with substrates containing a duplex region and a 3′ or 5′ ss poly(dT) tail. PcrA also efficiently unwound oligonucleotides containing a duplex region and a 5′ or 3′ ss tail with the potential to form a secondary structure. DNA binding experiments showed that PcrA bound much more efficiently to oligonucleotides containing a duplex region and a 5′ or 3′ ss tail with a potential to form a secondary structure than to those with ssDNAs or duplex DNAs with ss poly(dT) tails. Our results suggest that specialized DNA structures and/or sequences represent natural substrates of PcrA in biochemical processes that are essential for the growth and survival of gram-positive organisms, including *B. anthracis*.

Bacillus anthracis is a gram-positive, spore-forming bacteria that is the etiological agent of anthrax in humans (reviewed in references 13, 20, and 27). *B. anthracis* is a potential biological weapon, and an in-depth understanding of the cellular processes that are important for its growth and survival is critical to combat bioterror agents created on the basis of this and related organisms. DNA helicases are required for critical cellular processes such as DNA replication, transcription, recombination, and repair (3, 9, 12, 22–24). Most bacterial species contain several DNA helicases. The DnaB helicase of gram-negative bacteria is necessary for cell survival and is known to be involved in the theta-type replication of the chromosome as well as of several plasmids (6, 9, 26, 33). Gram-positive organisms such as *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cereus*, and *B. anthracis* contain a homolog of the replicative DnaB helicase of *Escherichia coli* termed DnaC (www.tigr.org). It has been shown that DnaC is required for chromosome replication in *B. subtilis* and *S. aureus* (5, 28, 29), and it is highly likely that this is also the case for other gram-positive organisms, including *B. anthracis*.

In addition to DnaC, gram-positive bacteria also contain another helicase, PcrA, which is essential for cell survival in *S. aureus* and *B. subtilis* (14, 28). PcrA belongs to superfamily I of DNA helicases that share seven conserved motifs (3, 12). Helicases of this family include the UvrD (helicase II) and Rep helicases of *E. coli* and UL5 of herpes simplex virus type 1 (3, 12, 24, 36). The chromosome of *B. anthracis* contains a sequence encoding a putative 747-amino-acid PcrA helicase that shares 62% identity and 76% similarity with the PcrA of *B. subtilis* and 58% identity and 72% similarity with the PcrA of *S. aureus*, as revealed by a BLAST search (1, 31) (http://www.tigr.org). The PcrA helicases of gram-positive bacteria also share approximately 40% homology with the UvrD and Rep helicases of *E. coli* that are involved in DNA repair and rolling-circle (RC) replication of single-stranded (ss) DNA (ssDNA) phages such as M13 and ϕX174, respectively (4, 8, 12, 22–25). Although likely, the essentiality of PcrA for the viability of *B. anthracis* and closely related organisms such as *B. cereus* and *Bacillus thuringiensis* has not yet been demonstrated.

In *B. subtilis* and *S. aureus*, PcrA has been shown to be required for both DNA repair and the replication of small drug resistance plasmids that replicate by an RC mechanism (4, 7, 10, 18, 19, 34). The PcrA helicases of two gram-positive organisms, *Bacillus stearothermophilus* and *S. aureus*, have been characterized, and the crystal structure of the *B. stearothermophilus* PcrA has been determined (7, 34–36, 38). PcrA acts as a monomer, in contrast to the more common replicative helicases which act as hexamers (3, 22–25, 34–36, 38). In this paper, we describe the isolation of the *pcrA* gene of *B. anthracis* and the purification and characterization of this helicase. The PcrA protein contained an ATPase activity that was stimulated by ssDNA. Interestingly, PcrA was equally active as a 5′→3′ helicase and a 3′→5′ helicase. Electrophoretic mobility-shift assays showed that the interaction of PcrA with duplex DNA substrates containing an ss region with potential to form a secondary structure was much stronger than that with ssDNA or with duplex substrates with a linear ss region. Our results are consistent with the possibility that PcrA plays a role in the resolution of blocked intermediates in various DNA transac-

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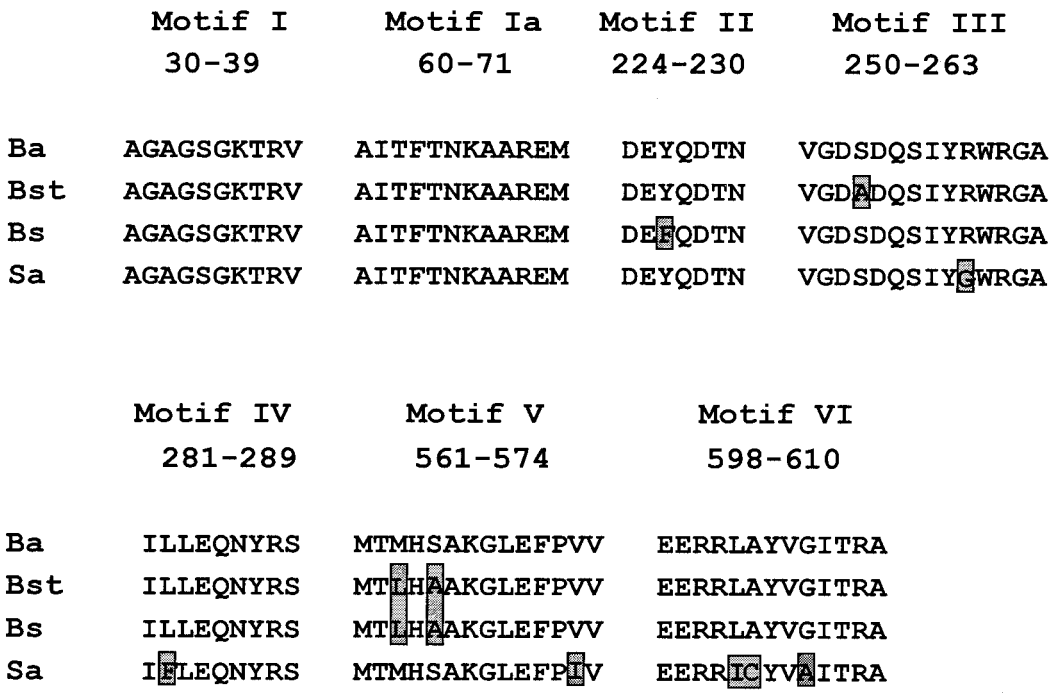

FIG. 1. Alignment of PcrA of *B. anthracis* (Ba), *B. stearothermophilus* (Bst), *B. subtilis* (Bs), and *S. aureus* (Sa) in the seven conserved helicase motifs. Amino acids that differ from those of *B. anthracis* PcrA are shaded and boxed. Numbers correspond to the amino acid positions of the *B. anthracis* PcrA.

tions such as recombination and/or replication, making it an essential helicase required for the growth and viability of gram-positive organisms.

MATERIALS AND METHODS

Isolation of genomic DNA from *B. anthracis*. Chromosomal DNA from the *B. anthracis* strain Sterne was isolated by incubating the cells in a cetyltrimethylammonium bromide solution at 65°C followed by chloroform extraction and isopropanol precipitation as previously described (2).

Cloning of the *pcrA* gene of *B. anthracis*. Preliminary sequence data for the *B. anthracis* genome were obtained from the website of The Institute for Genomic Research (http://www.tigr.org). The genomic DNA of *B. anthracis* isolated by the above-described procedure was used as a template for the amplification of the *pcrA* gene (2.2 kb). The sequences of the primers used were 5′ CCGGATCCA CAGATAGGTTATTAAATGGTTTAAACCCGCAACAAC 3′ for the forward primer and 5′ CCGGATCCCGTTTTTTTGCTATCTCTTTTGACATATCCTC ATTCC 3′ for the reverse primer. The PCR primers contained *Bam*HI linkers at their ends. The reaction mixtures contained 200 μM of each deoxynucleoside triphosphate, 50 ng of *B. anthracis* genomic DNA, 1 μM concentrations of each primer, and 5 U of *Pfu* polymerase (Stratagene, La Jolla, Calif.). 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 6 min for 25 cycles; and 72°C for 10 min. The amplified product was gel purified and digested with *Bam*HI. The *pcrA* gene was then fused in frame to the His_6 epitope at the *Bam*HI site of a pQE30 vector from Qiagen. This DNA was expected to encode a PcrA protein with His_6 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. The His-PcrA protein of *B. anthracis* was overexpressed by induction with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at 37°C for 2 h and purified by nickel affinity chromatography as described previously for the *S. aureus* PcrA helicase (7). The concentration of the His-PcrA preparation reached about 0.5 mg/ml in the peak fractions, and the purity was tested by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and staining with Coomassie brilliant blue.

ATPase assays. The ATPase activity of PcrA was measured by hydrolysis of [α-^{32}P]ATP, as described earlier (7). The products of the reaction were subjected to thin-layer chromatography followed by autoradiography, as previously described (7).

DNA helicase assays. Double-stranded (ds) oligonucleotides containing a 5′ or 3′ ss region at one end were prepared by labeling one strand with ^{32}P at the 5′ end with T4 polynucleotide kinase (32) and annealing the cold complementary strand at a threefold molar excess. 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 $MgCl_2$, 3 mM ATP, 5 mM dithiothreitol, 10% glycerol, ~1 ng of the DNA substrates, and the indicated amounts of the PcrA helicase. The reactions were stopped by the addition of SDS dye, and the products were analyzed by 10% native polyacrylamide gel electrophoresis (7). Gels were subsequently dried and exposed to Kodak X-ray films.

DNA binding assays. The binding of the PcrA helicase to various DNA substrates was studied by electrophoretic mobility-shift assays. Approximately 1 ng of probes labeled at the 5′ end with ^{32}P was incubated with the indicated amounts of PcrA in a reaction buffer consisting of 20 mM Tris-HCl (pH 8.0), 100 mM KCl, 1 mM EDTA, 100 ng of poly(dI-dC), 5 mM dithiothreitol, and 10% ethylene glycol. The reactions were incubated at room temperature for 15 min, and the DNA-protein complexes were resolved by electrophoresis on 6% native polyacrylamide gels. The gels were dried and subjected to autoradiography.

RESULTS

Overexpression and purification of the *B. anthracis* PcrA helicase. The *pcrA* gene of *B. anthracis* was identified by a homology search of the *B. anthracis* genome sequence with the *pcrA* genes of *S. aureus*, *B. stearothermophilus*, and *B. subtilis*. While various PcrA helicases share approximately 60% identity in their amino acid sequences, they are more than 95% identical in the seven conserved motifs found in helicases belonging to superfamily I (Fig. 1). The *pcrA* gene of *B. anthracis* includes an open reading frame (ORF) of 747 amino acids.

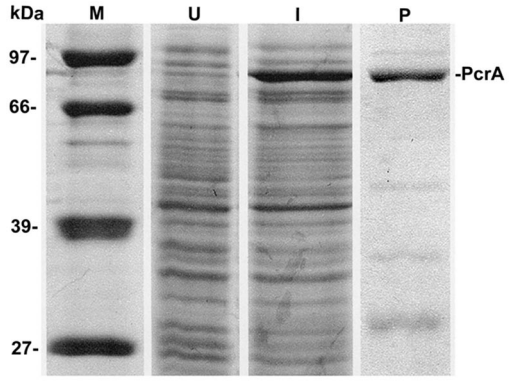

FIG. 2. SDS-PAGE analysis of the purified *B. anthracis* PcrA protein. U, lysates from uninduced cells; I, lysates from IPTG-induced cells overexpressing the His-PcrA protein; P, PcrA protein purified by nickel affinity column chromatography; M, protein molecular-weight standards (in kilodaltons). Smaller fragments likely correspond to breakdown products of PcrA.

The *pcrA* ORF contained the ATG sequence at the first and third codons. The *pcrA* ORF was amplified by PCR such that it included amino acids 4 to 745 and was fused in frame (using the vector pQE30) to His_6 residues at its amino-terminal end. The first three amino acids were not included to rule out the possibility of initiation from the ATG codons of *pcrA* instead of the ATG codon of the His_6 epitope. The sequence of the cloned *pcrA* gene was confirmed by automated DNA sequencing. The His-PcrA fusion protein was purified by nickel affinity chromatography, and protease inhibitors were included throughout the purification procedures. SDS-PAGE analysis (Fig. 2) showed that the full-length protein was approximately 80 to 85% pure. The impurities essentially consisted of breakdown products of PcrA. In spite of the use of protease inhibitors during the purification procedures and differing conditions for IPTG induction of the cloned *pcrA* gene, the breakdown products persisted.

ATPase activity of the PcrA helicase. The ATPase activity of PcrA was tested by incubating different amounts of PcrA with α-^{32}P-labeled dATP. Thin-layer chromatography showed that PcrA had a robust level of ATPase activity (Fig. 3A). Furthermore, its ATPase activity was stimulated in the presence of ssDNA (Fig. 3B).

Helicase activity of PcrA. The helicase activity of PcrA was evaluated using partially ds oligonucleotides (Table 1). One set of probes consisted of an 18-bp duplex region with an oligo$(dT)_{20}$ ss tail at the 5′ (oligonucleotide a) or 3′ (oligonucleotide b) end. Both oligonucleotides a and b were unwound by PcrA to similar extents (Fig. 4), suggesting that the *B. anthracis* PcrA helicase has both 3′→5′ and 5′→3′ helicase activities. To rule out the possibility that this dual activity was due to a contaminating enzyme in the PcrA preparations, protein lysates from the host *E. coli* M15 cells lacking the PcrA overexpressing plasmid were fractionated on a nickel affinity resin under conditions identical to those used for the purification of PcrA. Fractions corresponding to the sites at which PcrA eluted were then used in the helicase assays. No 3′→5′ or 5′→3′ helicase activity was detectable at concentrations at which the PcrA preparations showed robust helicase activity (data not shown). We wished to determine whether PcrA is able to unwind oligonucleotides in which the 5′ or 3′ ss region can assume a secondary structure. For this, we utilized oligonucleotide probes containing a 25-bp duplex region and a 28- or 29-nucleotide (nt) ss region at their 5′ or 3′ ends (oligonucleotides c and d) (Table 1). These probes represent the inverted repeat II region from the plasmid pT181 origin in which the sequences present in the 5′ or 3′ ss regions can assume a hairpin structure (16, 17). The underlined regions in oligonucleotides c and d (Table 1) contain 5′CCGG3′ sequences which (upon intrastrand base pairing) generate a cleavage site for the restriction endonuclease *Hpa*II. Probes c and d were found to have been cleaved by *Hpa*II, confirming that the 5′ or 3′ ss regions were present in a folded structure (data not shown). Oligonucleotides c and d are still expected to have a 6- or 7-nt unpaired sequence at their 5′ and 3′ ends, respectively (Table 1). Both oligonucleotide c and oligonucleotide d were efficiently unwound by PcrA (Fig. 4). These results showed that PcrA has both 3′→5′ and 5′→3′ helicase activities and that it can unwind DNA in which the ss region can acquire a secondary structure. At higher PcrA concentrations, an additional slow-migrating complex that presumably corresponds to a PcrA-DNA complex was observed with oligonucleotides b, c, and d (Fig. 5).

DNA binding activity of the PcrA helicase. Experiments were carried out to determine whether PcrA can bind stably to the DNA substrates used in the helicase assays. PcrA bound weakly to oligonucleotide a and oligonucleotide b containing a duplex region along with 5′ or 3′ ss oligo(dT) tails (Fig. 5). Interestingly, PcrA showed much stronger binding to ds probes containing 5′ or 3′ ss tails that can potentially fold into hairpin structures (oligonucleotides c and d) (Fig. 5). Surprisingly, the interaction of PcrA with ssDNAs (representing the top and bottom strands of oligonucleotides d and c, respectively) with the potential to form secondary structures was very weak and detectable only upon overexposure of the gel (Fig. 5). These results suggested that PcrA binds most efficiently to DNAs containing ss-ds junctions along with folded ss regions. We further analyzed the binding of PcrA to oligonucleotides c and d representing similar substrates with either a 5′ or 3′ ss tail with the potential for secondary structure. PcrA bound efficiently to both of these probes in a dose-dependent manner, although binding to oligonucleotide d was more robust (Fig. 6). These data showed that dsDNA substrates containing a 5′ or 3′ ss region with a potential for secondary structure along with a short unpaired region at their ends were the preferred substrates for DNA binding by the PcrA helicase.

DISCUSSION

PcrA helicase is conserved in most gram-positive bacteria and has been shown to be essential for the viability of *S. aureus* and *B. subtilis* (14, 15, 29, 30). Although yet to be demonstrated, it is highly likely that *pcrA* is also an essential gene in *B. anthracis* and related organisms such as *B. cereus* and *B. thuringiensis*. PcrA has been shown to be required for UV DNA repair and the replication of small drug resistance plasmids that replicate by an RC mechanism in gram-positive organisms (4, 7, 10, 18, 19, 34). RCR plasmids (such as pC194 and pE194) from *S. aureus* can be established in *B. anthracis* (20). Since these plasmids require PcrA for replication in their

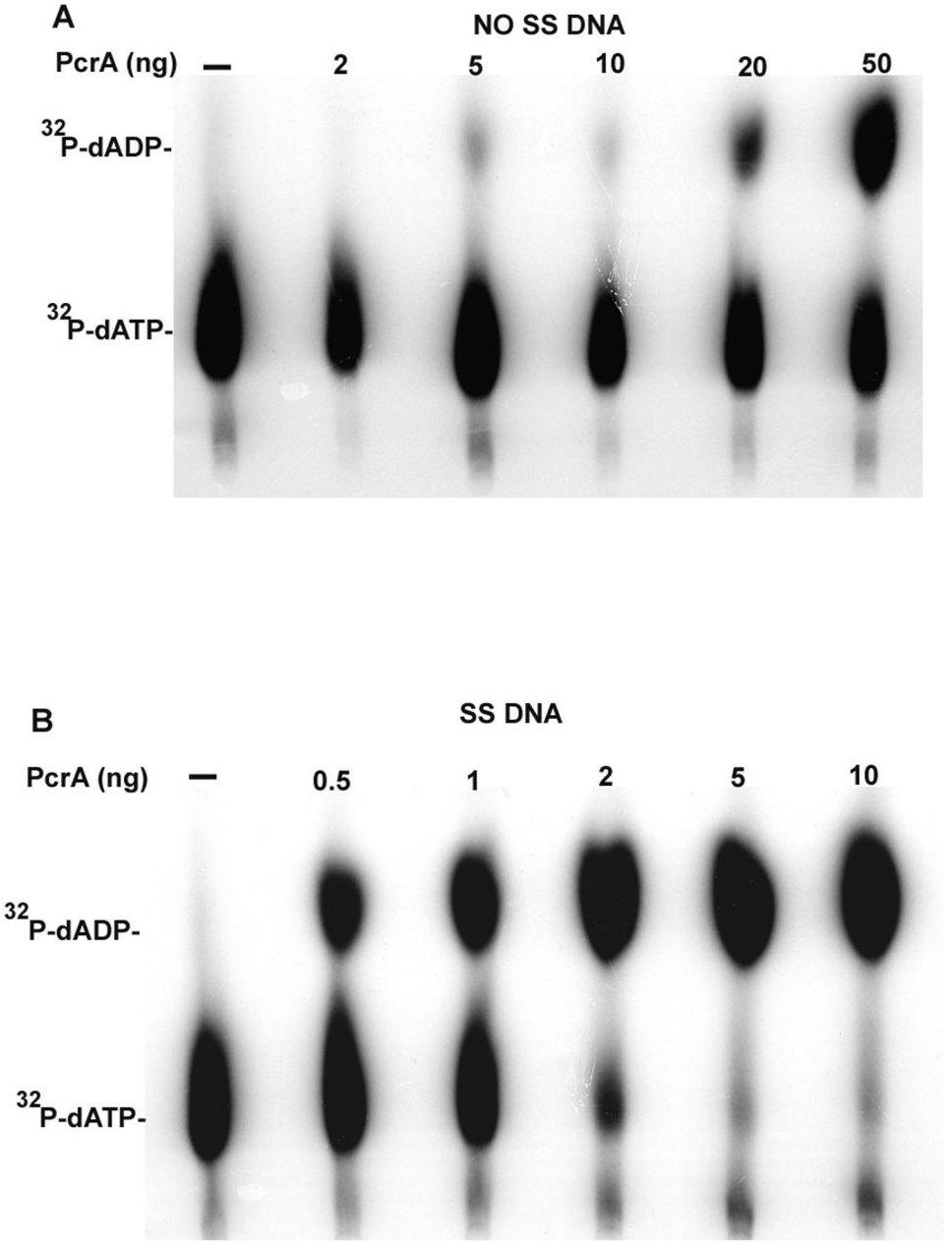

FIG. 3. ATPase activity of PcrA. (A) Products of [α-^{32}P]dATP hydrolysis in the presence of increasing amounts of PcrA. (B) Stimulation of the dATPase activity of PcrA by a 53mer ss oligonucleotide (ssDNA). The products of [α-^{32}P]dATP hydrolysis were analyzed by thin-layer chromatography.

native host, it is likely that the heterologous PcrA helicase of *B. anthracis* can support their replication. Conditional knockouts of *pcrA* in *B. subtilis* have been used to show that chromosome replication is affected only slightly in such strains (29). It is unclear what function of PcrA is essential for cell viability given that it is not the major replicative helicase. Recently, suppressor mutants have been identified in *B. subtilis* that allow cell growth in the absence of the PcrA helicase (30). These suppressors are located in the *recF*, *recO*, and *recR* genes that are involved in promoting *recA*-dependent homologous recombination (30). These studies suggest that the resolution of blocked recombination structures generated by the RecFOR proteins that can interfere with DNA replication and/or resolution of stalled replication forks is a critical role for the PcrA helicase in gram-positive bacteria (30). In the absence of PcrA, these DNA intermediates may be toxic to the cell.

We have cloned and overexpressed the *B. anthracis pcrA* helicase gene in *E. coli*. The amino-terminal end of the PcrA helicase was fused to six histidine residues, and the fusion protein was purified by affinity chromatography. Previous studies with *S. aureus* PcrA had shown that His-PcrA was biologically active (7). The *B. anthracis* His-PcrA helicase was used to

TABLE 1. Oligonucleotides used in this study

Oligonucleotide	Sequence[a]
a [5′ oligo(dT) overhang]	5′ GCCTCGCTGCCGTCGCCA 3′ 3′ CGGAGCGACGGCAGCGGTTTTTTTTTTTTTTTTTTT 5′
b [3′ oligo(dT) overhang]	5′ GCCTCGCTGCCGTCGCCATTTTTTTTTTTTTTTTTTT 3′ 3′ CGGAGCGACGGCAGCGGT 5′
c (5′ overhang)	5′ CATATGCACACAGTATGTGCGTCCA 3′ 3′ GTATACGTGTGTCATACACGCAGGTTGGCCGATAATCTCATCGGCCAACCTAG 5′
d (3′ overhang)	5′ GATCCATATGCACACAGTATGTGCGTCCAACCGGCTATTAGAGTAGCCGGTTGGATCC 3′ 3′ CTAGGTATACGTGTGTCATACACGCAGGT 5′

[a] Underlined sequences indicate complementary regions that can pair to generate a hairpin structure. Oligonucleotides c and d are expected to contain six or seven unpaired nucleotides at the 5′ or 3′ end, respectively.

study its biochemical activities. Our experiments showed that PcrA had a robust ATPase activity that was stimulated in the presence of ssDNA (Fig. 3A and B). The ATPase activity of different helicases is generally stimulated in the presence of ssDNA. This is consistent with the observation that helicases use ss regions as entry points to translocate on the DNA and utilize the energy of ATP hydrolysis for their movement (3, 8, 11, 12).

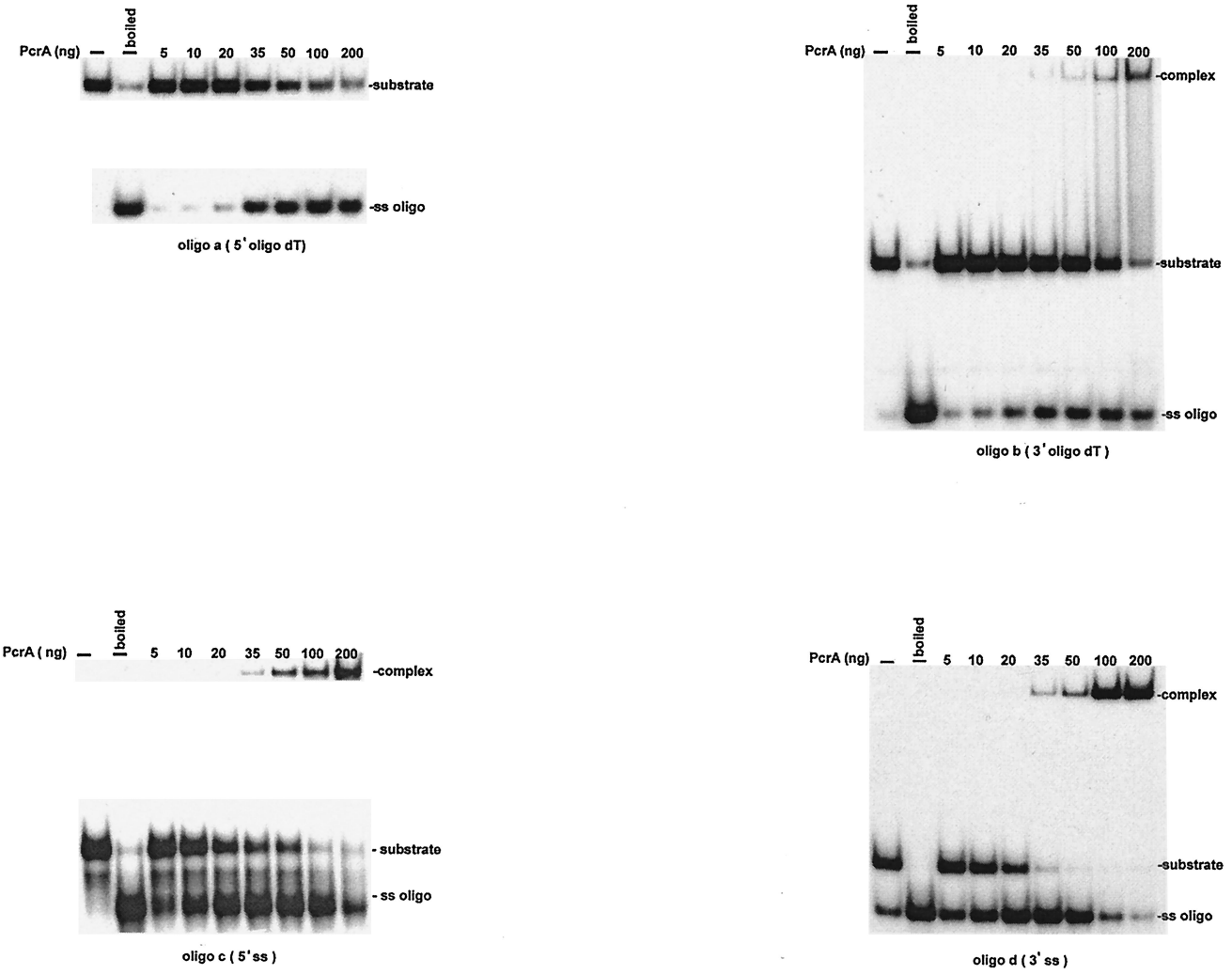

FIG. 4. Helicase activity of the PcrA protein. ^{32}P-labeled substrates were incubated with the indicated amounts of PcrA, and the products were resolved by native polyacrylamide gel electrophoresis. The probes used are listed in Table 1 and correspond to duplex oligonucleotides (oligo) containing either a 5′ or 3′ ss region. Only one strand of the probe was labeled. "Complex" corresponds to a PcrA-DNA complex.

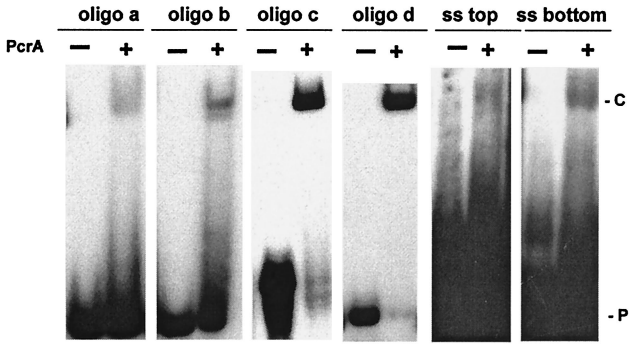

FIG. 5. Binding of the PcrA helicase to various DNA substrates. The PcrA helicase (200 ng) was incubated with probes labeled at the 5′ end, and the DNA-protein complexes were resolved by electrophoresis on native 6% polyacrylamide gels. The probes used are indicated. ss top, top strand of oligonucleotide d; ss bottom, bottom strand of oligonucleotide c. P, free probe; C, PcrA-DNA complex.

Our results demonstrate that PcrA can efficiently unwind duplex DNA containing a 3′ or 5′ oligo(dT) ss tail (Fig. 4). PcrA also unwinds duplex substrates containing 5′ or 3′ ss regions that can potentially assume a folded structure (Fig. 4). Thus, *B. anthracis* PcrA has robust 3′→5′ as well as 5′→3′ helicase activities. The *S. aureus* PcrA also has both 3′→5′ and 5′→3′ helicase activities, while the PcrA of *B. stearothermophilus* has a robust 3′→5′ and a weak 5′→3′ helicase activity (7, 34). Generally, most helicases preferentially unwind DNA containing either a 3′ or 5′ ss region (22, 23). Therefore, the PcrA helicases of gram-positive bacteria appear to be unusual in that they are effective both as 3′→5′ and 5′→3′ helicases. Interestingly, both 3′→5′ and 5′→3′ helicases belonging to superfamily I share the seven conserved motifs shown in Fig. 1 (3, 12, 36). Our results suggest that the *B. anthracis* and other PcrA helicases function in multiple DNA transactions that require either of these two activities (see below).

Interestingly, our DNA binding studies demonstrate that PcrA interacts weakly with ssDNA and duplex DNAs containing ss oligo(dT) tails at the 5′ or 3′ end (Fig. 5). On the other hand, PcrA bound with high affinity to dsDNAs containing 5′ or 3′ ss tails with a potential to form a secondary structure (Fig. 5 and 6). Most DNA helicases are known to require ss regions for loading onto the DNA and therefore bind to ssDNAs efficiently (3, 9, 12, 22–25). The *B. stearothermophilus* PcrA binds with greater affinity to ssDNA than to dsDNA (34–36, 38). Our results showed that the interaction of *B. anthracis* PcrA with oligonucleotides c and d containing ssDNA-dsDNA junctions is much stronger than that with ssDNA representing the top or bottom strand of these oligonucleotides (Fig. 5). Since PcrA is essential for cell growth and viability, one or more of its biochemical activities must be required for an essential cellular process. Our observations with *B. anthracis* PcrA suggest that DNAs with certain structural or sequence features represent its natural substrates in vivo. Such structures might be generated during DNA recombination and replication processes (9, 22–24, 28).

Two recent reports have shown that the Srs2 helicase of *S. cerevisiae*, which has limited homology with PcrA, can inhibit DNA strand exchange mediated by the Rad51 protein (a ho-

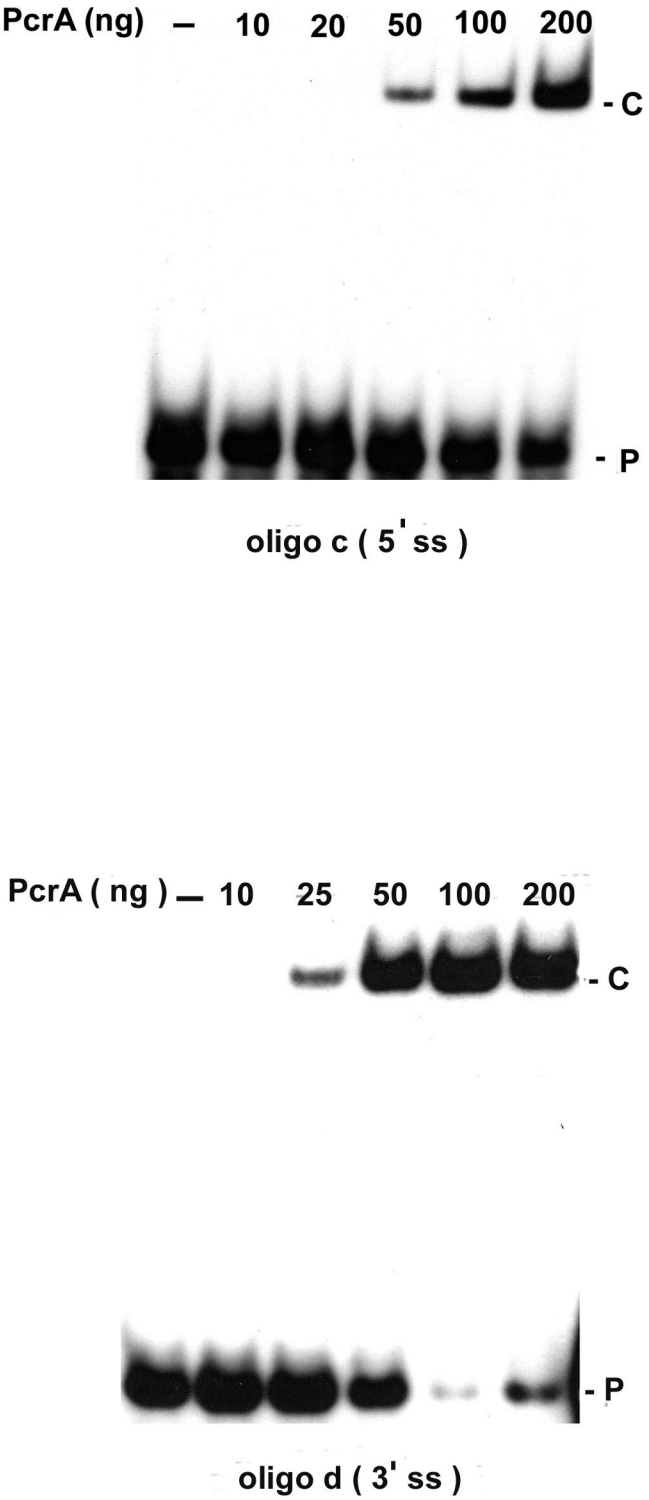

FIG. 6. Dose-dependent binding of PcrA to duplex DNA substrates containing 5′ or 3′ ss regions (oligonucleotide [oligo] c or oligonucleotide d, respectively). ^{32}P-labeled oligonucleotides were incubated with the indicated amounts of PcrA, and the products were resolved by polyacrylamide gel electrophoresis. P, free probe; C, PcrA-DNA complex.

molog of RecA) (21, 37). Srs2 was also shown to disrupt Rad51 filaments formed on ssDNA (21, 37). Our results are consistent with the possibility that PcrA plays a similar role in the resolution of blocked recombination intermediates generated by the RecFOR proteins of the RecA pathway that may interfere with DNA replication. In addition, PcrA may also inhibit strand exchange catalyzed by the RecA protein or be required for the resolution of replication forks stalled at different regions of the chromosome. It is possible that the functions of PcrA in the above-described biochemical pathways and in UV repair and plasmid RC replication involve the recognition of structurally different substrates and specifically require either its 5′→3′ or its 3′→5′ helicase activity. Future studies should reveal the specific cellular pathways that are dependent upon the function of this essential helicase.

The various biochemical activities of PcrA may represent important new targets for the future development of drugs to combat *B. anthracis* and other gram-positive pathogens. The crystal structure of the *B. stearothermophilus* PcrA has been determined, and future studies on the structure-function relationship of the PcrA helicases could be important for rational drug design. Furthermore, several assays that may be amenable to high-throughput analysis could be utilized for PcrA, including ATPase and helicase assays with fluorescently labeled nucleotides.

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