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Isolation of a Minireplicon of the Virulence Plasmid pXO2 of *Bacillus anthracis* and Characterization of the Plasmid-Encoded RepS Replication Protein

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A minireplicon of plasmid pXO2 of *Bacillus anthracis* was isolated by molecular cloning in *Escherichia coli* and shown to replicate in *B. anthracis*, *Bacillus cereus*, and *Bacillus subtilis*. The pXO2 replicon included (i) an open reading frame encoding the putative RepS replication initiation protein and (ii) the putative origin of replication. The RepS protein was expressed as a fusion with the maltose binding protein (MBP) at its amino-terminal end and purified by affinity chromatography. Electrophoretic mobility shift assays showed that the purified MBP-RepS protein bound specifically to a 60-bp region corresponding to the putative origin of replication of pXO2 located immediately downstream of the RepS open reading frame. Competition DNA binding experiments showed that the 5′ and central regions of the putative origin were important for RepS binding. MBP-RepS also bound nonspecifically to single-stranded DNA with a lower affinity.

Bacillus anthracis is a gram-positive bacterium that is the etiological agent of anthrax (reviewed in references 18, 24, and 31). There is a high degree of similarity between *B. anthracis* and members of the *Bacillus cereus* group (*B. cereus*, *Bacillus thuringiensis*, and *Bacillus mycoides*), with the major differences between these organisms being the presence or absence of two large virulence plasmids, pXO1 and pXO2 (18, 19, 23, 24, 31, 33, 36, 39, 40). Plasmid pXO1 (181.6 kb) encodes the anthrax toxin proteins termed protective antigen, lethal factor, and edema factor (16, 20, 23, 24, 32, 33). Plasmid pXO2 (96.2 kb) contains the *capA*, *capB*, and *capC* genes required for capsule biosynthesis and the *dep* gene involved in the depolymerization of the capsule (14, 24, 28, 32, 34, 41). In addition, both plasmids carry regulatory genes that control expression of the toxin and capsule genes: *atxA* and *pagR* on pXO1 (3, 10, 17, 20, 25, 30, 41, 42) and *acpA* and *acpB* on pXO2 (11, 43).

Although pXO1 and pXO2 play central roles in the pathogenesis of anthrax (24, 31, 44), little is known about the mechanism(s) of replication and copy number control of these plasmids. In culture, the pXO1 plasmid is extremely stable and is rarely cured spontaneously, while the pXO2 plasmid is not as stable and much more likely to be cured (14, 24, 31). A recent report suggested that differences in pXO2 copy number in naturally occurring strains may, at least in part, be related to differences in virulence (9). pXO1 and pXO2 replication and maintenance are not limited to *B. anthracis*. Although self-transmission of the plasmids has not been demonstrated, pXO1 and pXO2 can be mobilized into the closely related species *B. cereus* and *B. thuringiensis* by conjugative plasmids found in the *B. cereus* group (2, 15, 23, 24). Interspecies transduction of pXO2 into *B. cereus* has also been reported (14).

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The pXO2 plasmid contains sequences that share homology with the replication regions of plasmids of the pAMβ1 family, such as pAW63, pAMβ1, pIP501, and pSM19035, which are found in gram-positive organisms, suggesting that pXO2 also belongs to this plasmid family (4, 7, 26, 34, 45). These conjugative plasmids are promiscuous and have a broad host range (7). They replicate by a theta-type mechanism, and their replication proceeds unidirectionally from the origin (6, 7). Sequence alignments have shown that the predicted replication initiator protein of pXO2 termed RepS (ORF-38; 512 amino acids; nucleotides [nt] 34115 to 32580 of pXO2, GenBank accession no. NC_002146) has 96% identity with the Rep63A protein of the *B. thuringiensis* plasmid pAW63 (34, 45). The RepS protein of pXO2 also has approximately 40% identity with the Rep proteins of plasmids pAMβ1 and pRE25 of *Enterococcus faecalis*, pIP501 and pSM19035 of *Streptococcus agalactiae*, and pPLI100 of *Listeria innocua* on the basis of BLAST alignments (1). Similarly, the putative origin of replication (*ori*) of pXO2 (nt 32583 to 32524) is highly homologous to the postulated *ori* of pAW63 (34, 45), and the *ori* of pAMβ1 (4–7, 26, 27).

The replication regions of the pIP501, pSM19035, and pAMβ1 have been identified by the isolation of minimal replicons. The best-studied plasmid of this group is pAMβ1. The RepE protein of pAMβ1 has been isolated and shown to bind specifically to the double-stranded (ds) DNA at the origin and nonspecifically to single-stranded (ss) DNA (27). Binding of the RepE protein to the ds origin results in the formation of an open complex. RepE stays bound to the two melted single strands of the origin region. The pAMβ1 *ori* and the putative *ori* of pAW63 are located immediately downstream of the sequence coding for RepE (6, 27, 34, 45). The mRNA of the RepE protein of pAMβ1 also plays a role in providing the RNA primer for the initiation of DNA replication. Transcription of the Rep mRNA terminates approximately 20 nt downstream of the replication start site (5). At the origin, the 3′ end

of the RepE mRNA pairs with one strand of the DNA generating an R-loop structure. An RNase H-like activity in the cell or the RepE protein itself (it has been postulated to have an RNase H activity) may then cleave the RNA at the initiation site, and the RNA primer paired to the DNA serves as a primer for leading strand replication by DNA polymerase I (22, 27). After limited synthesis by DNA polymerase I, it is postulated to be replaced by the replisome that carries out coordinated leading and lagging strand synthesis (22, 27). Minimal information is available on the replication properties of pXO2 and the closely related pAW63 plasmid.

We have initiated studies to characterize the replication properties of the pXO2 plasmid. In this report, we describe the isolation and identification of a minireplicon of pXO2. Our results demonstrate that a 2,429-bp region (GenBank accession no. AF188935, pXO2 positions 32423 to 34851) containing the *repS* gene and the putative origin is sufficient for replication of the miniplasmid pXO2. We also report the overexpression and purification of the RepS initiator protein and demonstrate that RepS interacts specifically with the putative pXO2 origin.

MATERIALS AND METHODS

Cloning of the pXO2 minireplicon in *Escherichia coli*. DNA enriched for pXO2 was isolated from *B. anthracis* strain 9131 containing pXO2 (13, 14). After digestion of the plasmid pXO2 DNA with NsiI, a 4,970-bp DNA fragment (GenBank accession no. AF188935, nt 31241 to 36210) was purified from a 0.7% agarose gel using Zymoclean (Zymo Research, Orange, Calif.). This fragment contains the *repS* and *repB* open reading frames (ORFs), the putative origin of replication of pXO2, and additional upstream and downstream sequences. The NsiI fragment was ligated into PstI-cleaved pBSIIKS (Stratagene, La Jolla, Calif.) and transformed into *E. coli* (38). Finally, the spectinomycin resistance cassette *aad9* from pJRS312 (37) was inserted into the BamHI site of the vector to yield pUTE439 (9,811 bp). The sequence of the cloned pXO2 DNA was confirmed using automated DNA sequencing.

We made a subclone of pUTE439 to further reduce the size of miniplasmid pXO2. For this, a 1,463-bp MspI-HindIII fragment containing the chloramphenicol resistance gene from plasmid pC194 of *Staphylococcus aureus* (nt 973 to 2435; GenBank accession no. NC_002013) was ligated into pBSIIKS digested with AccI and HindIII. The recombinant plasmid pBSCm (4,424 bp) was recovered by electroporating *E. coli* DH5α with selection for the ampicillin resistance marker. We then amplified a 2,429-bp region of pXO2 (nt 32423 to 34851) containing the *repS* gene and the putative pXO2 *ori*. The sequences of the primers used were 5′-CCG GAT CCG TGT TGA AAT GAT TCA GAC CAG TG-3′ for the forward primer (nt 34851 to 34828) and 5′-CCG GATCCC ACA TAC CAT AAT GAG AAT ATA ACC-3′ for the reverse primer (nt 32423 to 32447). The PCR primers contained BamHI linkers at their ends to facilitate cloning. The reaction mixtures contained a 200 μM concentration of each deoxynucleoside triphosphate (dNTP), 50 ng of pUTE439 DNA, a 1 μM concentration of each primer, and 5 U of the *Pfu* polymerase (Stratagene). The amplification conditions were as follows: (i) 3 min at 94°C; (ii) 25 cycles, with 1 cycle consisting of 1 min at 94°C, 1 min at 60°C, and 6 min at 72°C; and (iii) 10 min at 72°C. The amplified product was gel purified and digested with BamHI. The amplified DNA was then ligated into the BamHI site of the pBSCm plasmid, and the recombinant pBSCmrepS plasmid (6,853 bp) was recovered by transforming *E. coli* DH5α.

Mutagenesis of the *repS* gene. A frameshift mutation in the *repS* gene was introduced by using the Stratagene QuikChange site-directed mutagenesis kit according to the manufacturer's instructions. Two complementary primers were designed containing pXO2 nt 34011 to 34045 but lacking the A nucleotide at position 34029. This deletion was expected to destroy a BsaBI site and introduce a frameshift at codon 29 of the RepS ORF. The sequences of the primers used were 5′-CAA AAG CTG GAT TAG TTC TAT TGC TAA TCA AGA G-3′ and 5′-CTC TTG ATT AGC AAT AGA ACT AAT CCA GCT TTT G-3′. The reaction mixture (50 μl) contained 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, 100 μg of nuclease-free bovine serum albumin per ml, a 200 μM concentration of each dNTP, 75 ng of pBSCmrepS plasmid DNA, 125 ng of each primer, and 2.5 U of *Pfu*Turbo (Stratagene). The amplification conditions were as follows: (i) 30 s at 95°C; (ii) 13 cycles, with 1 cycle consisting of 30 s at 95°C, 1 min at 55°C, and 7 min at 68°C; and (iii) 10 min at 68°C. The reaction mixture was treated with 20 U of DpnI for 1 h at 37°C to remove the parental, methylated template DNA, followed by phenol-chloroform–isoamyl alcohol extraction and ethanol precipitation. The mutagenized plasmid was recovered by transforming *E. coli* DH5α, and miniplasmid preparations were screened by digestion with BsaBI. The deletion of a single nucleotide resulting in a frameshift mutation in the *repS* gene was confirmed by automated DNA sequencing.

Cloning of the pXO2 *repS* gene for overexpression. The RepS ORF of pXO2 (pXO2 positions 34115 to 32580) consists of 1,536 bp and is predicted to encode a protein of 512 amino acids with a molecular weight of 57,000. The RepS ORF was amplified from pUTE439 using PCR to encode amino acids 2 to 512 of RepS. The following primers containing BamHI linkers at their ends (shown in lowercase) were used: 5′-ccggatccaatacagtacaaaaagctatcg-3′ for the forward primer (nt 34112 to 34091 of pXO2) and 5′-ccggatccCACATACCATAATGAG AATATAACC-3′ for the reverse primer (nt 32423 to 32447 of pXO2, 154 bp downstream of the termination codon of RepS). The reaction mixtures contained a 200 μM concentration of each dNTP, 10 ng of pUTE439 plasmid DNA, a 1 μM concentration of each primer, and 5 U of the *Pfu* polymerase (Stratagene). The amplification conditions were as follows: (i) 3 min at 94°C; (ii) 25 cycles, with 1 cycle consisting of 1 min at 94°C, 1 min at 65°C, and 6 min at 72°C; and 10 min at 72°C. The amplified product was gel purified and digested with BamHI. The *repS* gene was then ligated in frame to the maltose binding protein (MBP) epitope at the BamHI site of the pMAL-p2X vector from New England Biolabs (Cambridge, Mass.). The ligation mixture was electroporated into *E. coli* DH5α, and the appropriate clones were isolated. The sequence of the cloned RepS ORF was confirmed by automated DNA sequencing.

Overexpression and purification of the MBP-RepS protein. To improve the integrity and yield of the MBP-RepS protein, the MBP-RepS expression plasmid was introduced into *E. coli* BL21. Cells were grown in Luria-Bertani broth supplemented with 10 mM glucose at 37°C to the mid-exponential phase, and the MBP-RepS protein was overexpressed by induction with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 30°C for 2 h. The cells were lysed by several freeze-thaw cycles in a buffer containing 20 mM Tris-HCl (pH 8), 0.1 mM EDTA, 1 M NaCl, 10% glycerol, and Complete protease inhibitor cocktail tablets (Roche Molecular Biochemicals, Indianapolis, Ind.) as described earlier (8). The MBP-RepS protein was purified by chromatography on an amylase affinity column, and the protein was eluted using the above buffer in the presence of 10 mM maltose (8). The purity of the protein was tested by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and staining with Coomassie brilliant blue. We also purified the native MBP by similar procedures using the pMAL-p2X overexpression plasmid (not shown).

DNA binding assays. The binding of the RepS protein to various DNA substrates was studied using electrophoretic mobility shift assays (EMSA). ds or ss oligonucleotides were labeled at the 5′ ends with ^{32}P using T4 polynucleotide kinase (38). Approximately 1 ng of various probes was incubated with the indicated amounts of MBP-RepS in a reaction buffer consisting of 10 mM Tris-HCl (pH 7.5), 70 mM NaCl, 2.5 mM $MgCl_2$, 50 ng of poly(dI-dC), 1 mM dithiothreitol, and 10% ethylene glycol (27). The reaction mixtures 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. In competition DNA binding experiments, various amounts of cold competitor oligonucleotides were also included in the above reaction mixtures.

RESULTS AND DISCUSSION

Replication of miniplasmid pXO2 in *B. anthracis* and other gram-positive bacteria. Sequence alignment showed that the RepS protein of pXO2 has 96% identity with the Rep63A protein of plasmid pAW63 of *B. thuringiensis* (Fig. 1). BLAST alignment also showed that RepS has 39% identity and 56% similarity to the better-studied RepE protein of plasmid pAMβ1 of *E. faecalis* (Fig. 1). Similarly, the putative origin of pXO2 is highly homologous to the postulated *ori* of pAW63 and the *ori* of pAMβ1 (Fig. 1).

We generated plasmid pBSCmrepS (6,853 bp) containing a 2,429-bp region of pXO2 (nt 32423 to 34851, including the

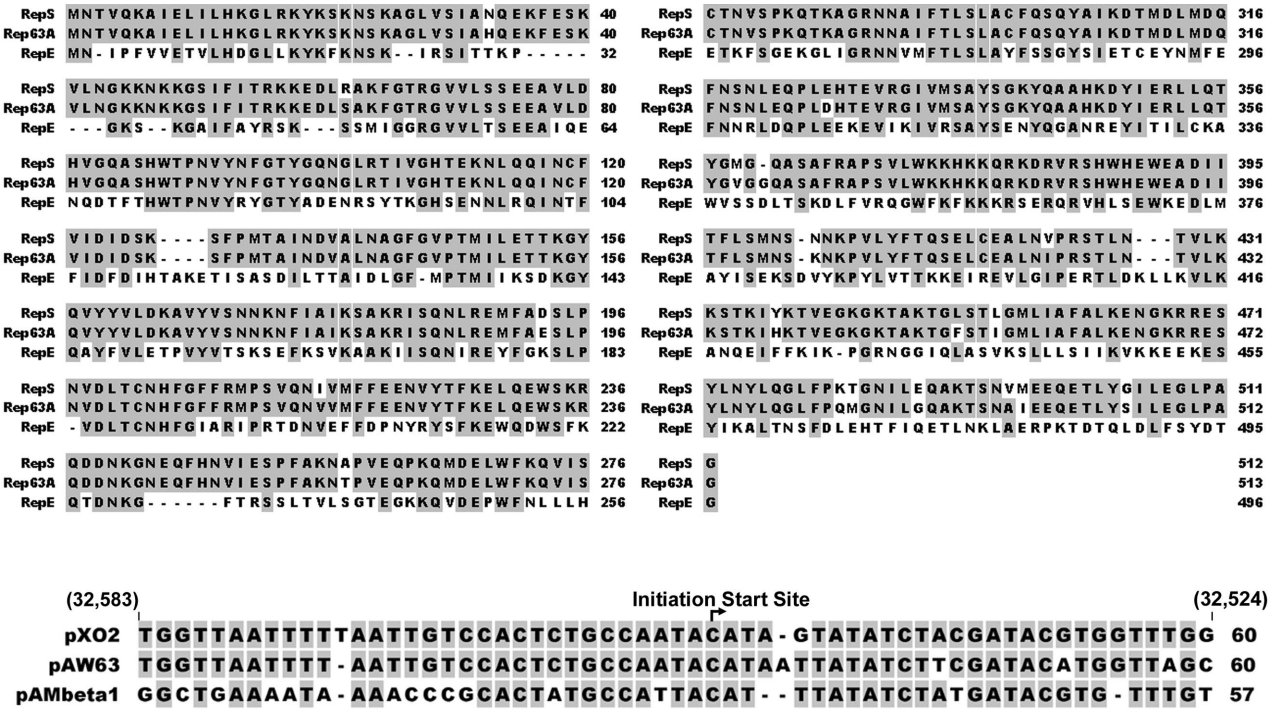

FIG. 1. Alignment of the RepS protein of pXO2, Rep63A protein of plasmid pAW63, and RepE protein of pAMβ1 as well as the origins of replication of these three plasmids (1, 7, 27, 34, 45). The alignment was done using the ClustalW program, and the shaded letters indicate identical amino acids or nucleotides. Gaps introduced to maximize alignment are indicated by hyphens. Nucleotide coordinates of the pXO2 origin of replication are indicated in parentheses.

RepS ORF and the putative *ori*) and a 1,463-bp fragment from plasmid pC194 containing the Cm^r gene (Fig. 2). The pBSCmrepS plasmid was isolated from *E. coli* and introduced into the plasmid-free *B. anthracis* strain UM23C1-1 using electroporation and selection for chloramphenicol resistance (12, 29, 35). Plasmid DNA was isolated and digested with BamHI and EcoRI. The digestion pattern of the plasmid DNA from the *B. anthracis* isolates was identical to that of pBSCmrepS from *E. coli* (Fig. 3). These results indicate that the functional replicon of pXO2 is contained within a 2,429-bp region. This region includes the RepS ORF (nt 34115 to 32580) and the putative *ori* of pXO2 present immediately downstream of *repS* (nt 32583 to 32524).

The pBSCmrepS plasmid was also introduced into *B. cereus* and *B. subtilis* by electroporation (12, 21). The restriction patterns of plasmid DNA isolated from these gram-positive hosts

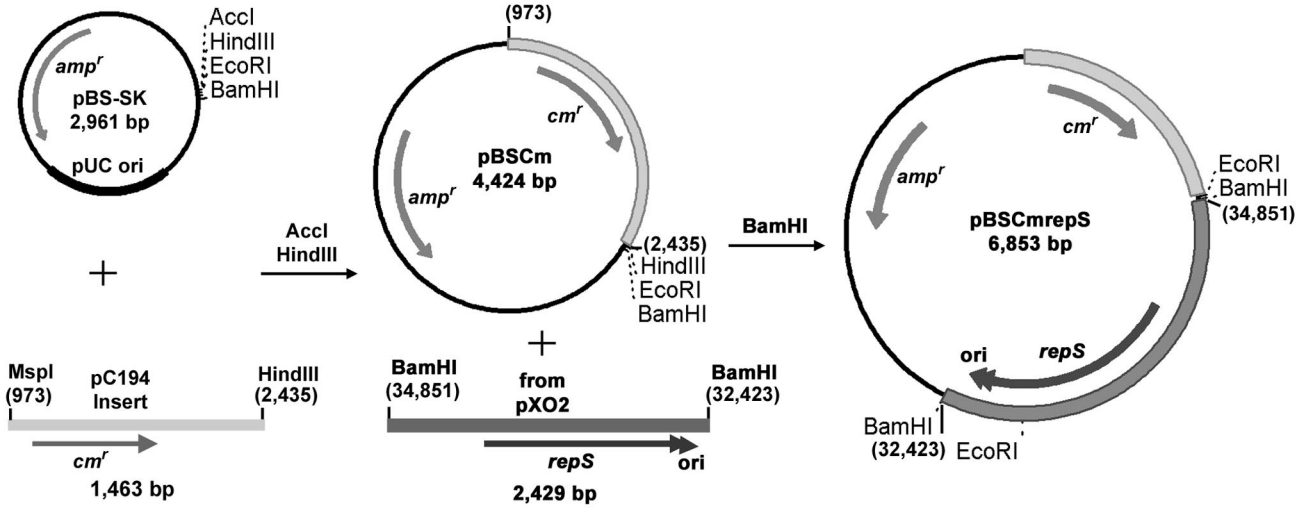

FIG. 2. Schematic representation of the construction of plasmid pBSCmrepS containing the pXO2 minireplicon. The numbers in parentheses correspond to the nucleotide coordinates of pC194 or pXO2. The direction of transcription of the various genes is indicated by the arrows.

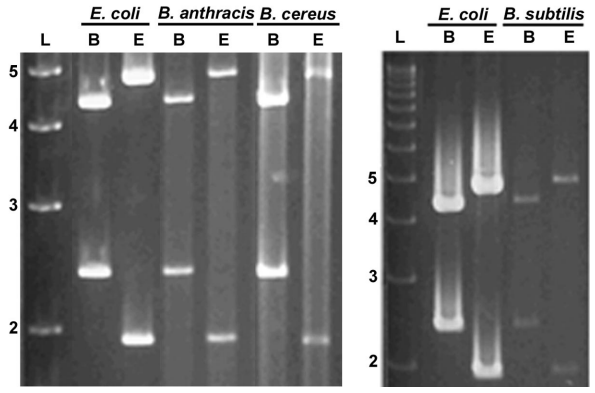

FIG. 3. Restriction analysis of the pBSCmrepS plasmid isolated from *E. coli*, *B. anthracis*, *B. cereus*, and *B. subtilis*. Plasmid was digested with BamHI (B) or EcoRI (E). L lanes contain size markers (in kilobases).

were identical to those from *B. anthracis* and *E. coli* (Fig. 3). These results show that pXO2 miniplasmid has a broad host range, since it can be established in at least three different species, *B. anthracis*, *B. cereus*, and *B. subtilis*. To test whether the RepS protein was essential for pXO2 replication, we generated a frameshift mutation at codon 29 of RepS in the context of the pBSCmrepS plasmid to generate pBSCmrepS*mut*. In three independent experiments, no Cm^r colonies were obtained when plasmid pBSCmrepS*mut* was electroporated into *B. anthracis*, *B. cereus*, and *B. subtilis*. These results showed that, as expected, RepS is essential for pXO2 replication. Previous studies with the pAW63 plasmid have suggested that in addition to the Rep63A protein (homolog of pXO2 RepS), the Rep63B protein (homolog of RepB of pXO2) may also be involved in plasmid replication (45). Our studies demonstrate that the RepB protein is dispensable for replication of the pXO2 miniplasmid.

Overexpression and purification of the MBP-RepS protein. The MBP-RepS protein was purified by chromatography on an amylase affinity column. The purity of the protein was tested by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and staining with Coomassie brilliant blue. The purified protein contained the full-length MBP-RepS protein of approximately 100 kDa as well as some breakdown products (Fig. 4). Protease inhibitors were used throughout the purification procedures, and various times and temperatures for IPTG induction were attempted. However, the breakdown products were always observed. Presumably, the MBP-RepS protein is subject to partial breakdown in vivo as well as during the purification procedures.

ds and ss DNA binding activity of the RepS protein. On the basis of sequence homology to the pAW63 and pAMβ1 origins, a region immediately downstream of the RepS ORF was postulated to contain the *ori* of pXO2. We utilized a 60-bp ds oligonucleotide containing the putative pXO2 *ori* (oligonucleotide a, nt 32583 to 32524) to study the DNA binding activity of the RepS protein. In the case of the pAMβ1 plasmid, the 5′ and central regions of the *ori* have been shown to be critical for RepE binding (27). We, therefore, also utilized several pXO2 *ori* derivatives lacking the 5′, 3′, or central region of the 60-bp *ori* (Table 1) in competition EMSA studies. We also studied the binding of the MBP-RepS protein to ss origin DNA and to nonspecific ss DNA (Table 1).

When MBP-RepS was incubated with the 60-bp *ori* probe (oligonucleotide a, nt 1 to 60), a single DNA-protein complex was observed in a RepS dose-dependent manner (Fig. 5A). This band presumably corresponds to the RepS-*ori* complex. Purified native MBP did not bind to the *ori* (Fig. 5A), demonstrating that the DNA binding activity of the MBP-RepS fusion was due to the RepS protein. We also tested whether RepS can bind to ss DNA. For this purpose, two probes were used: one corresponding to the bottom strand of the origin (complementary to oligonucleotide a in Table 1; specific ss probe), while the second consisted of an unrelated 53-nt ss sequence (nonspecific ss probe). EMSA results showed that RepS bound to both specific and nonspecific ss DNA in a dose-dependent manner, generating a single DNA-protein complex (Fig. 5B). These results showed that RepS has both ds and ss DNA binding activities.

Binding of RepS to ds DNA is origin specific. We also tested the specificity of RepS binding to DNA using competition EMSA experiments. The DNA-protein complex formed in the presence of the ds *ori* probe was disrupted in the presence of excess cold ds *ori* oligonucleotide (oligonucleotide a) (Fig. 6A). A 100-fold molar excess of cold ds *ori* disrupted more than 50% of the binding. The central 41-bp sequence of the *ori* (oligonucleotide b, nt 13 to 53) also disrupted the RepS-*ori* complex, although at a higher molar excess (Fig. 6A). On the other hand, a 40-bp region of *ori* that lacks the central 20 nt (oligonucleotide c) did not significantly affect the RepS-*ori* complex (Fig. 6A).

We also used additional 60-bp oligonucleotides in competition EMSA that include 20 nt of the *ori* and the adjacent 40 nt on either side of the *ori* (Table 1). The 5′ region of *ori* is

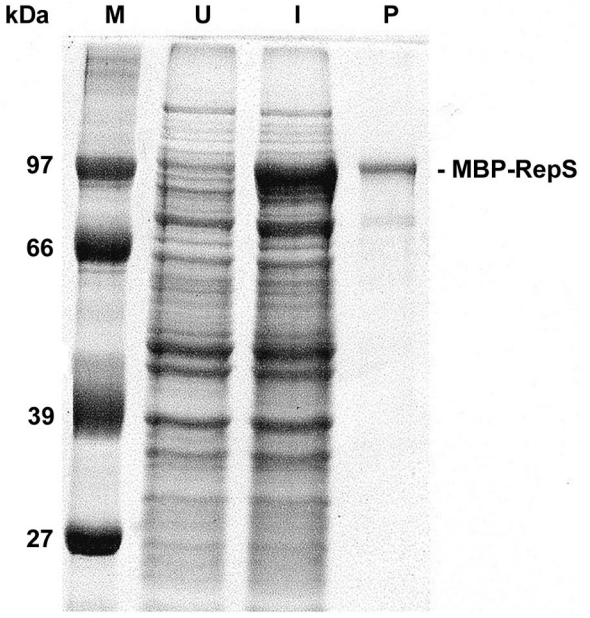

FIG. 4. Purification of the RepS protein of pXO2. Lanes: U, lysates from uninduced *E. coli* cells; I, lysates from IPTG-induced cells overexpressing the MBP-RepS protein; P, purified MBP-RepS; M, protein molecular mass standards (in kilodaltons).

TABLE 1. Oligonucleotides used in the EMSA studies[a]

Oligonucleotide	Sequence
a	5′-TGGTTAATTTTTAATTGTCCACTCTGCCAATACATAGTATATCTACGATACGTGGTTTGG-3′
b	5′-AATTGTCCACTCTGCCAATACATAGTATATCTACGATACGT-3′
c	5′-TGGTTAATTTTTAATTGTCCATCTACGATACGTGGTTTGG-3′
d	5′-ATCTACGATACGTGGTTTGGTTAGCCAGTCTTGGAATTACAGGATTCACTAGTTTAGGAT-3′
e	5′-AACAAGAAACACTATACGGCATATTGGAAGGGCTACCAGCTGGTTAATTTTTAATTGTCC-3′
f	5′-GGCTACCAGCTGGTTAATTTTTAATTGTCCATCTACGATACGTGGTTTGGTTAGCCAGTC-3′
ss nonspecific oligonucleotide	5′-GATCCAACCGGCTACTCTAATAGCCGGTTGGACGCACATACTGTGTGCATATG-3′

[a] Only the top strands are shown.

located immediately adjacent to the *repS* gene, while its 3′ region is distal to *repS*. The RepS-*ori* complex was not appreciably affected in the presence of an excess of oligonucleotide d that lacks nt 1 to 40 of the 60-bp *ori*, whereas oligonucleotides e and f that lack the central 20 nt of the *ori* but contain the 5′ 20 bp of the *ori* (nt 1 to 20 in Fig. 1) were more effective as competitors (Fig. 6B). Also, the binding of RepS to *ori* was not detectably affected in the presence of unrelated 44- and 65-bp ds oligonucleotides (not shown). We conclude from the above experiments that the central 20 nt of the *ori* (nt 21 to 40 [Fig. 1]) are critical for the recognition of the *ori* by the RepS protein. Furthermore, nt 1 to 20 at the 5′ end of the *ori* also contribute to RepS binding. On the other hand, the 3′ region of *ori* (nt 41 to 60 in Fig. 1) does not appear to be critical for RepS binding. Finally, neither the ss bottom strand of the origin DNA nor a nonspecific ss DNA disrupted RepS-*ori* binding (Fig. 6B). The top strand of the *ori* also did not affect RepS-*ori* binding (not shown). Taken together, the above results suggest that RepS interacts with the ds *ori* in a sequence-specific manner and that its affinity for ds *ori* DNA appears to be much stronger than its affinity for ss DNA.

The results of our studies demonstrating that miniplasmid pXO2 containing the *repS* gene can replicate in *B. anthracis*, *B. cereus*, and *B. subtilis* suggest that RepS corresponds to the replication initiator protein of pXO2. This conclusion is supported by results of our DNA binding studies demonstrating that RepS binds efficiently to the putative origin of replication of pXO2. The pAMβ1 *ori* is located immediately downstream of the RepE-coding sequence and shares homology with the corresponding regions of pXO2 and pAW63 (Fig. 1). Our results suggest that the 60-bp region of pXO2 (nt 32583 to 32524) located immediately downstream of the RepS ORF corresponds to the pXO2 origin. Within this region, the central 20-bp region (pXO2 positions 32563 to 32544) is critical for RepS binding, since oligonucleotides lacking this region competed poorly with the 60-bp *ori* in EMSA (Fig. 6). On the basis of the homology of the pXO2 and pAMβ1 *ori*, the RNA-DNA transition point during the initiation of pXO2 replication is expected to correspond to the conserved C residue at position 33 of *ori* (nt 32551 of pXO2). Our observation that nt 21 to 40 of *ori* are critical for RepS binding are consistent with the possibility that this region may play an important role in the generation of a RepS-dependent RNA primer for pXO2 replication. Our data are also consistent with the results obtained with the pAMβ1 plasmid in which the 5′ and central regions of the *ori* were found to be important for RepE binding, whereas the 3′ region of the *ori* did not play a significant role in RepE binding (27).

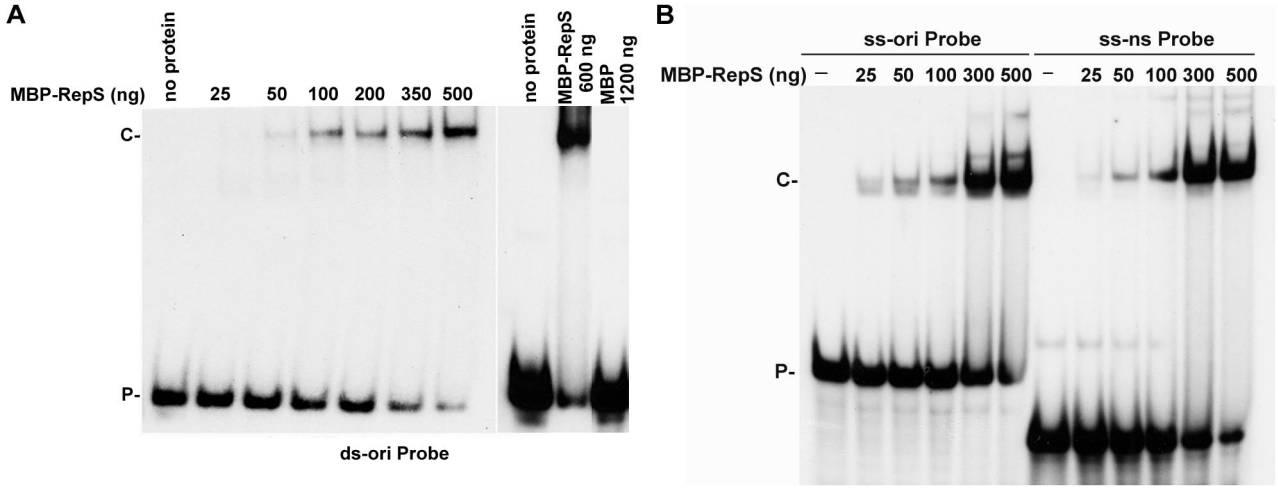

FIG. 5. Binding of the RepS protein to ds origin DNA (A) or to ss DNA (B). The indicated amounts of the MBP-RepS or MBP protein were incubated with 5′-end-labeled probes, and the DNA-protein complexes were resolved by electrophoresis on native 6% polyacrylamide gels. The probes used were as follows: a 60-bp region containing the putative *ori* of pXO2 (ds-ori; oligonucleotide a in Table 1); ss-ori, 60-nt bottom strand of the *ori*; ss-ns, a 53-nt nonspecific ss oligonucleotide. The positions of free probe (P) and RepS-DNA complex (C) are shown to the left of the gels.

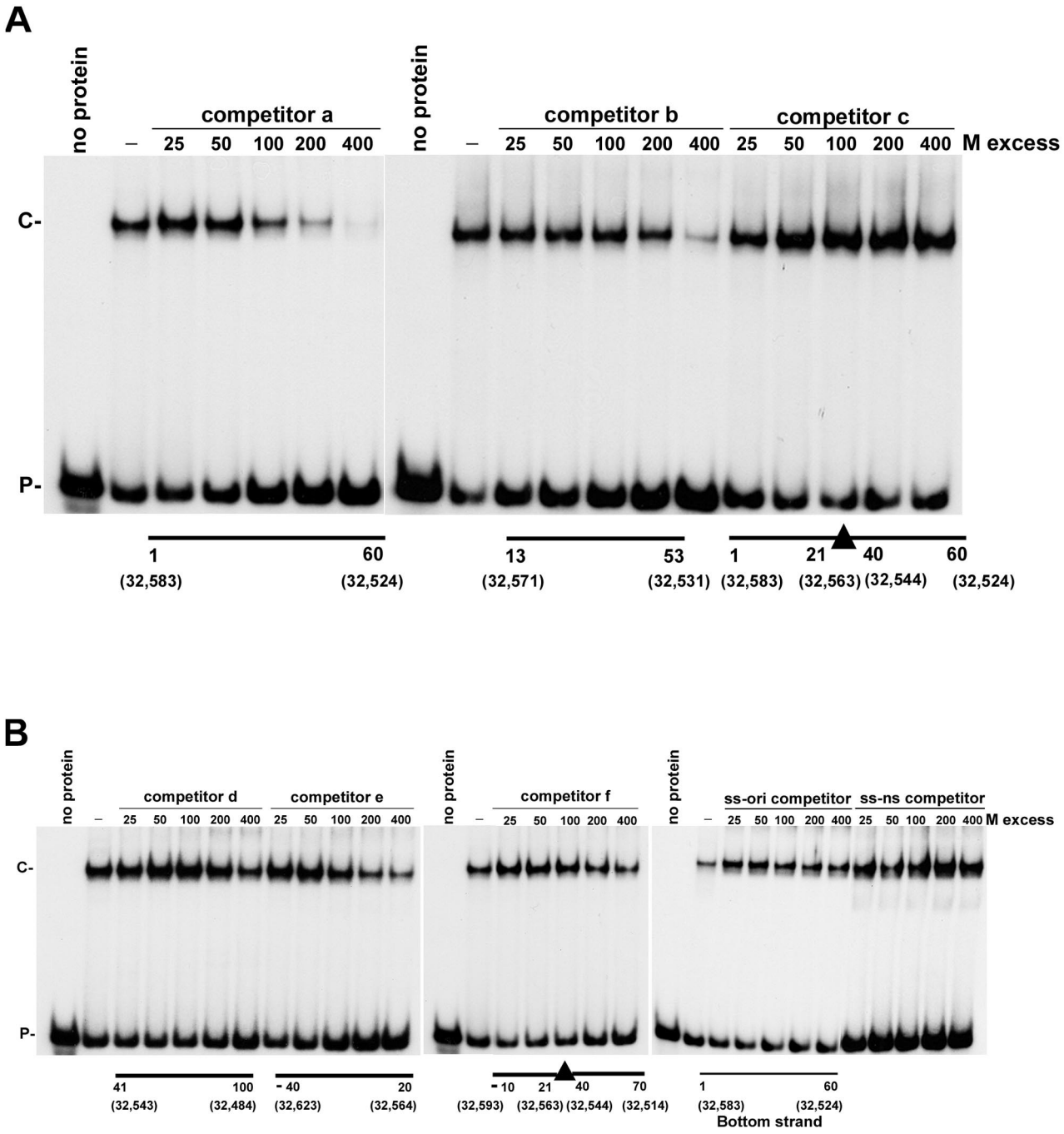

FIG. 6. Competition EMSA experiments using the 60-bp ds *ori* (oligonucleotide a) as the probe. ^{32}P-labeled ds *ori* DNA was incubated in the presence or absence of molar (M) excesses of ds oligonucleotide competitors a, b, and c (A) or ds oligonucleotides d, e, f, ss *ori*, and nonspecific ss oligonucleotide (B). The "wild-type" *ori* consists of 60 bp (nt 1 to 60). Positions with a minus sign refer to sequences upstream of position 1 of the *ori*. Numbers higher than 60 indicate sequences present downstream of the *ori*. The positions of free probe (P) and RepS-DNA complex (C) are shown to the left of the gels.

The RepS protein of pXO2 is likely to be involved in the generation of an RNA primer in a manner similar to RepE during the initiation of plasmid replication. The RepE protein of pAMβ1 has been isolated and shown to bind specifically to the ds DNA at the origin and nonspecifically to ss DNA (27). Interestingly, RepE of pAMβ1 binds to ss DNA (both *ori* specific and nonspecific) with a higher affinity than to specific, ds *ori* DNA (27). RepS protein of pXO2, on the other hand, shows a stronger interaction with the ds origin than to ss DNA (Fig. 6). Further biochemical studies should identify the mechanism of initiation of pXO2 replication and the significance of the differences in the relative ds and ss DNA binding affinities of the RepS and RepE initiator proteins.

Since the pXO2 plasmid is important for the virulence of

B. anthracis, further studies are necessary for a better understanding of its replication and transfer. Such studies will provide insight regarding the potential for generation of recombinant microorganisms in nature and may reveal new molecular targets for therapeutics that affect plasmid replication and/or maintenance during infection.

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