A *Bacillus anthracis*-Based In Vitro System Supports Replication of Plasmid pXO2 as Well as Rolling-Circle-Replicating Plasmids[▽]

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Capsule-encoding virulence plasmid pXO2 of *Bacillus anthracis* is predicted to replicate by a unidirectional theta-type mechanism. To gain a better understanding of the mechanism of replication of pXO2 and other plasmids in *B. anthracis* and related organisms, we have developed a cell-free system based on *B. anthracis* that can faithfully replicate plasmid DNA in vitro. The newly developed system was shown to support the in vitro replication of plasmid pT181, which replicates by the rolling-circle mechanism. We also demonstrate that this system supports the replication of plasmid pXO2 of *B. anthracis*. Replication of pXO2 required directional transcription through the plasmid origin of replication, and increased transcription through the origin resulted in an increase in plasmid replication.

In vitro replication systems provide important tools for the study of DNA replication. Rolling-circle (RC)-replicating plasmids are ubiquitous in gram-positive bacteria, including the members of the *Bacillus cereus* group. *B. cereus*, *Bacillus thuringiensis*, and *Bacillus mycoides* contain indigenous RC-replicating plasmids (2, 12, 18), while RC-replicating plasmids of *Staphylococcus aureus* such as pT181, pC194, and pE194 can also replicate and be established in *Bacillus anthracis* (1, 24). Members of this group of organisms also contain large plasmids that presumably replicate by the theta-type mechanism (3, 15, 17, 31, 33–35, 38, 41, 43, 44). *B. anthracis* contains two large virulence plasmids, pXO1 and pXO2, and related plasmids have also been identified in other members of the *B. cereus* group (3, 15, 17, 31, 33–35, 38, 41, 43, 44). Plasmid pXO1 (181.6 kb) encodes the anthrax toxin proteins termed the protective antigen, lethal factor, and the edema factor (14, 16, 24, 25, 32). Plasmid pXO2 (96.2 kb) contains genes involved in capsule production (24, 32).

Plasmid pXO2 contains sequences that resemble those present in the replication regions of gram-positive plasmids such as pAM β 1, pAW63, pIP501, and pSM19035, suggesting that pXO2 also belongs to the pAM β 1 family of plasmids (35). These conjugative plasmids replicate by a theta-type mechanism, and their replication proceeds unidirectionally from the origin (9). We have isolated a pXO2 minireplicon containing the *repS* gene and the origin of replication (*ori*) (42). The RepS protein of pXO2 is 96% identical to the Rep63A protein of plasmid pAW63 and approximately 40% identical to the Rep proteins of plasmids pAM β 1 and pRE25 of *Enterococcus faecalis*, pIP501 and pSM19035 of *Streptococcus agalactiae*, and pPLI100 of *Listeria innocua*. Similarly, the putative *ori* of pXO2 (nucleotide [nt] positions 32524 to 32583) is 95% homologous to the postulated *ori* of pAW63 (34, 44) and has a

more limited homology with the *ori* of pAM β 1 (42). The RepE protein of pAM β 1 has been isolated and shown to bind specifically to double-stranded DNA at the origin and nonspecifically to single-stranded DNA (30). The pAM β 1 *ori* and the putative *ori* of pAW63 are located immediately downstream of the RepE coding sequence (8, 30, 34, 44). The mRNA of the RepE protein of pAM β 1 also plays a role in providing the RNA primer for the initiation of DNA replication in vivo. Transcription of the Rep mRNA terminates approximately 20 nt downstream of the replication start site (7). At the origin, the 3' end of the RepE mRNA is expected to pair 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 (PolI) (20, 30). After limited synthesis by DNA PolI, it is postulated to be replaced by the replisome that carries out coordinated leading- and lagging-strand synthesis (20, 30). On the basis of sequence homology, pXO2 is expected to replicate by a mechanism similar to that of the pAM β 1 family of plasmids.

In order to understand the replication properties of plasmids of *B. anthracis* and related organisms, the availability of a cell-free system is highly desirable. A major obstacle to the development of active cell-free systems from *B. anthracis* and related organisms is the poor lysis of these organisms due to the nature of their cell wall and S layers. Development of active in vitro replication systems requires gentle lysis conditions, as well as high protein concentrations. In this report, we describe the development of a system based on cell extract from *B. anthracis* that can support the replication of plasmid pT181, which replicates by a rolling-circle (RC) mechanism (11, 21). Plasmid pXO2 of *B. anthracis*, which is expected to replicate by the theta-type mechanism (42), was also found to replicate in the cell extracts. By using this system, we demonstrate that pXO2 replication requires RNA synthesis through its *ori* and that increased transcription through the *ori* increases the efficiency of replication. This in vitro system should be useful for

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FIG. 1. Plasmids containing the various pXO2 ori derivatives. (A) The pBSMCS vector used for ori clonings contains a spectinomycin resistance (*Spec*^r) gene (*aad9*) for selection in *B. anthracis*, an ampicillin resistance gene for selection in *E. coli*, the kanamycin promoter (*PaphA3*), and a multiple cloning site. (B) Plasmid pBSMCS derivatives containing various pXO2 ori derivatives. Arrows indicate the direction of transcription or leading-strand DNA; stem-loops indicate the location of the *E. coli* *rrnB* transcriptional terminator. *Pbla*, *bla* promoter; *repS*, *repS* open reading frame of pXO2. The sizes of the plasmids are shown on the right.

the study of the mechanism of replication of the virulence plasmids of *B. anthracis*, as well as other plasmids of the *B. cereus* group.

MATERIALS AND METHODS

Preparation of cell extracts from *B. anthracis* and in vitro plasmid replication.

Cell extracts were prepared as described earlier for *S. aureus* (27), with some modifications. A 1-liter culture of plasmid-negative *B. anthracis* strain UM23C1-1 (13) was grown in brain heart infusion broth to an A_{260} of 0.6, and cells were pelleted and washed with 100 ml of TEG (25 mM Tris-HCl [pH 8.0], 5 mM EGTA). Cells were resuspended in 6 ml of TEG and lysed with three or four passes through a French press at 20,000 lb/in². KCl was added to a final concentration of 100 mM, and the lysate was centrifuged at 33,000 rpm for 1 h in an ultracentrifuge with a Beckman SW41Ti rotor. To remove contaminating DNA and RNA, 1/10 of the volume of a 30% solution of streptomycin sulfate was slowly added to the lysate and stirred on ice for 30 min. The lysate was then centrifuged at 13,000 rpm for 10 min in a Sorvall SS34 rotor. Proteins were precipitated with ammonium sulfate to 70% saturation, and the pellet was resuspended in 1 ml of TDE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 100 mM KCl, 1 mM dithiothreitol, 10% ethylene glycol) and dialyzed in the same buffer. In vitro replication reactions were carried out as described earlier (23), in a buffer containing ribonucleotide triphosphates (rNTPs), deoxynucleoside triphosphates (one labeled with ³²P), cell-free protein extracts (72 μ g), 500 ng of pT181*cop608* (a copy mutant of pT181) DNA or 400 to 600 ng of various pXO2 ori plasmids (isolated with the QIAGEN maxiprep kit) as the template, and 200 ng of the pT181 initiator protein RepC or $\sim$ 1 μ g of the pXO2 initiator protein RepS fused to the maltose binding protein (MBP) epitope (42). After incubation at 32°C for 60 min, the reaction products were analyzed by agarose gel electrophoresis, followed by autoradiography (23). The MBP-RepC and MBP-RepS proteins were purified by overexpression and affinity chromatography as described earlier (10, 42).

Generation of various pXO2 ori-containing plasmids. Some of the plasmids generated in this study are shown in Fig. 1A and B. The putative ori of pXO2 is located immediately downstream of the *repS* gene, and the 3' end of the *repS* transcript is likely to provide the RNA primer for the initiation of pXO2 replication (42). To separate the pXO2 ori from the *repS* gene, plasmid pBSoriF, which contained the pXO2 ori downstream of the *aphA3* (kanamycin resistance gene) promoter, was generated. For this, the *aphA3* promoter from plasmid pUC4 Ω kan (4, 36) was amplified with the following primers containing BamHI and EcoRI linkers: forward primer, 5'-CCGGATCCCGAACCATTGTGAGGTG ATAGGTAAG-3'; reverse primer, 5'-CCGGAATTCCTCAAGAAGCTAATT ATAACAAGAC-3'. The spectinomycin resistance gene *aad9* was released from pJRS312 (39) with HindIII and ligated into the HindIII site of plasmid

pBlueScript II SK (Stratagene) to generate pBSspc. The PCR product containing the *aphA3* promoter was digested with BamHI and EcoRI and ligated into similarly digested plasmid pBSspc to generate pBSprm. A 127-bp region of pXO2 (bp 32492 to 32618) containing the predicted ori was amplified with the following primers containing EcoRI linkers: forward, 5'-CCGGAATTCGAAA CACTATACGGCATATTGGAAGG-3'; reverse, 5'-CCGGAATTCCTAGTG AATCCTGTAATTCGAAGACTG-3'. The resulting fragment was digested with EcoRI and ligated into pBSprm to generate pBSprmori (data not shown). This plasmid contained the *aphA3* promoter upstream of the pXO2 ori such that the direction of transcription is colinear with the predicted direction of leading-strand synthesis. Plasmid pBSprmori was cut with BsmI, and an oligonucleotide containing multiple cloning sites having the sequence 5'-CTGCAGCCTAGGA GATCTGGCGCGCCACCGGTCCCGGGGAATGCA-3' was ligated into this site to generate pBSoriF (Fig. 1B). This plasmid is similar to pBSprmori but also contains the multiple cloning sites. A pBSoriF derivative lacking the pXO2 ori was also generated by digesting this plasmid with EcoRI to release a small fragment containing the ori and religating the remainder of the plasmid to generate pBSMCS (Fig. 1A). Plasmid pSK236 is an *Escherichia coli*-*S. aureus* shuttle vector (Amp^r Cm^r) consisting of plasmids pUC19 and pC194 joined at their HindIII sites (unpublished data). The *repS* gene with its promoter (pXO2 bp 32562 to 34828) was amplified by PCR with the following primers containing BamHI linkers: forward, 5'-CCGGATCCGTGGGACAAATTAATAACCA GCTG-3'; reverse, 5'-CCGGATCCGTGTTGAAATGATTCAGACCAAGTG-3'. The PCR product was digested with BamHI and ligated into the BamHI site of pSK236 to yield pSK236*repS* (data not shown), which is expected to express the RepS protein.

The in vivo and in vitro replication requirements for directional transcription into the pXO2 ori were investigated by generating plasmids containing promoters and transcription terminators at various locations with respect to the ori. The *E. coli* *rrnB* terminator (37) was amplified by PCR with pBR322 DNA as the template and the following primers: forward, 5'-TTGGCGCGCCGCTGTTTT GGCGGATGAG-3'; reverse, 5'-TTGGCGCGCCCAAAAAGAGTTGTAGA AACGCAAAA-3'. The PCR product was ligated into the *AscI* site of pBSoriF. A 658-bp EcoRV-BglII fragment containing the pXO2 ori and the *rrnB* terminator was isolated from this plasmid, filled in with the Klenow fragment of DNA PolI, and then ligated into the *SmaI* site of pBSMCS (Fig. 1A), yielding plasmids pBSoriFt and pBSoriRt, which contain the ori in two different orientations with respect to the *aphA3* promoter (Fig. 1B). pBSoriFt contains the *rrnB* terminator between the *aphA3* promoter and the ori and is expected to reduce or block transcription through the ori (Fig. 1B). Plasmid pBSoriRt contains the *aphA3* promoter adjacent to the pXO2 ori such that transcription is colinear with lagging-strand synthesis, and the *rrnB* terminator is positioned to block any basal-level transcription from the vector sequences into the ori that may be colinear with leading-strand synthesis (Fig. 1B). Lastly, pBSoriFtbla was gener-

ated by cloning the *rrnB* terminator along with the *bla* promoter from pBR322 (19, 28) into the *AscI* site of pBSoriF. The following primers were used to isolate the *rrnB* terminator and the *bla* promoter: forward, 5'-TTGGCGCGCCGCTG TTTTGGCGGATGAG-3'; reverse, 5'-TTGGCGCGCCGGTATTGTCTCAT GAGCGG-3'. In plasmid pBSoriF_{tbla}, transcription is expected to initiate from the *bla* promoter and proceed through the *ori* in the direction of leading-strand synthesis (Fig. 1B). Various plasmids were introduced into *B. anthracis* strains by electroporation as previously described (26).

Northern blot analysis. To identify the *repS* transcript, RNA was prepared from 100-ml brain heart infusion broth cultures of various *B. anthracis* strains grown to an optical density at 600 nm of 0.6. A volume of 12.5 ml of 5% water-saturated phenol in ethanol was added to the culture, and the medium was filtered off. Cells were then resuspended in 8 ml of Tris-EDTA containing 4 mg/ml lysozyme and incubated on ice with mixing for 45 min. RNA was then isolated by the hot-phenol extraction method as previously described (<http://cat.ucsf.edu/pdfs/TotalRNAIsolation.pdf>). The RNA pellets were resuspended in a total volume of 100 μ l of diethyl pyrocarbonate-treated water. RNA preparations were then treated with 14 U of DNase I per 250 μ g of total RNA in the presence of RNasin at room temperature for 15 min. RNA was also cleaned up with the RNeasy kit from QIAGEN when necessary. Samples were stored at -80°C . For Northern blot analysis, 20 μ g RNA was precipitated with ethanol and electrophoresed on 1% formaldehyde agarose gels at 200 V for 3 h. RNA was then transferred to GeneScreen as previously described (40). To identify the *repS* transcript or a possible countertranscript (CT), RNA probes were generated. An 820-bp BamHI/BsaBI fragment (pXO2 positions 34032 to 34851) containing 736 bp upstream of the RepS initiation codon and 84 bp of its coding sequence was isolated from plasmid pBSCmrepS (42) and ligated into the corresponding sites of plasmid pBlueScript II SK (Stratagene, La Jolla, CA). This vector contains the T7 and T3 promoter sequences on either side of the multiple cloning sites. RNA probes were then generated in vitro with the T7 and T3 RNA polymerases from Invitrogen according to the supplier's instructions. The specific activities of the RNA probes generally ranged from 10^6 to 10^7 cpm/ μ g of RNA. The probes were then hybridized to Northern blots at 55°C in formamide-dextran sulfate buffer (40). The blots were washed twice at room temperature with $2\times$ SSC ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate, followed by multiple washes at 60°C in $0.1\times$ SSC–0.1% sodium dodecyl sulfate. The Northern blots were then subjected to autoradiography. The sizes of the RNA bands on the Northern blots were estimated by using an RNA ladder ranging from 0.16 to 1.77 kb from Invitrogen.

RESULTS AND DISCUSSION

***B. anthracis* cell extracts support the replication of RC-replicating plasmid pT181.** We have previously shown that plasmid pT181 can replicate in *B. anthracis* (1). This plasmid replicates by an RC mechanism and has been extensively characterized for its replication properties with a cell extract from *S. aureus* (11, 22, 23). We therefore tested whether *B. anthracis* cell extracts can support the replication of this plasmid. Plasmid pT181 replicated efficiently, as shown by the presence of labeled supercoiled (SC) and open-circular (OC) DNAs along with replication intermediates (RI) (Fig. 2). The replication products were similar to those obtained with the *S. aureus* cell extracts (Fig. 2). Replication of the pT181_{cop608} DNA required its initiator protein (Fig. 2), and no replication was obtained with plasmid pSK265, which lacks the pT181 *ori* (data not shown). Replication was not significantly affected in the presence of rifampin and/or in the absence of rNTPs. This is consistent with previous studies showing that RC replication of plasmid pT181 does not require transcription (23, 27). These results demonstrate that the *B. anthracis* extracts specifically support pT181 replication in the presence of its initiator protein. Thus, these extracts are active in supporting plasmid RC replication.

In vitro replication of the pXO2 plasmid in *B. anthracis* extracts. As mentioned above, the 3' end of the *repS* transcript is predicted to provide the RNA primer for the initiation of

FIG. 2. In vitro replication of plasmid pT181_{cop608}. The top panel shows an ethidium bromide-stained agarose gel, and the bottom panel is an autoradiograph of the gel. The complete reaction mixture included all of the components described in the text. A minus sign indicates an omitted component, and a plus sign indicates an additional component.

pXO2 replication. Plasmid pSK236_{repS}, which is expected to express the RepS protein, was introduced into plasmidless *B. anthracis* strain UM23C1-1 (13) to obtain a RepS-expressing strain of *B. anthracis*. Plasmids pBSoriF, containing a 127-bp region of the putative pXO2 *ori* with the *aphA3* promoter in the same orientation as for leading-strand synthesis, and pBSMCS, which lacks the pXO2 *ori* (Fig. 1A), were introduced into the above *B. anthracis* strain by electroporation (26). Transformants (approximately 20 each in three independent experiments) were obtained only with pBSoriF, and agarose gel electrophoresis showed the presence of an appropriate-size plasmid (not shown). These results showed that the functional pXO2 *ori* is contained within a 127-bp region and can be uncoupled from the *repS* gene, provided that a promoter drives transcription through the *ori* (see below).

We have previously described the construction of plasmid pBSCmrepS containing the pXO2 replicon (42). We tested the in vitro replication of pBSCmrepS containing the *repS* gene and the wild-type pXO2 *ori*, as well as pBSoriF containing only the pXO2 *ori* downstream of the *aphA3* promoter. In the absence of the initiator protein RepS, very little or no signal

FIG. 3. Agarose gel electrophoresis of in vitro replication products of various pXO2 ori-containing plasmids. Top, ethidium bromide-stained gel; bottom, autoradiogram of the gel. (A) OC1 and SC1, OC and SC forms of pBSCmrepS, respectively; OC2 and SC2, OC and SC forms of pBSCm, respectively. (B) oriF, pBSoriF; OC1 and SC1, OC and SC forms of pBSCmrepS, respectively; OC2 and SC2, OC and SC forms of pBSoriF, respectively.

was obtained in the in vitro replication reaction with the pBSCmrepS plasmid (Fig. 3A, bottom). Addition of MBP-RepS generated a strong signal corresponding to a band that migrated more slowly than the SC pBSCmrepS template DNA isolated from *E. coli* (Fig. 3). This band may represent either RI or supercoiling differences between *E. coli* and the *B. anthracis* in vitro system. Vector plasmid pBSCm did not replicate to significant levels (Fig. 3A, bottom), demonstrating the dependence of in vitro replication on the pXO2 ori. Replication of pBSCmrepS was severely inhibited in the presence of rifampin, in the absence of added rNTPs (CTP, GTP, and UTP), or under both conditions (Fig. 3B). These results demonstrate that RNA transcription is necessary for the replication of plasmid pBSCmrepS. Since plasmid pXO2 is predicted to replicate by a unidirectional theta mode that uses an RNA primer for initiation at the ori (7), the above results are consistent with this possibility. Furthermore, on the basis of the similarity of the pXO2 ori with those of the plasmids of the pAM β 1 family (9), it is likely that the RepS mRNA provides the primer for the initiation of plasmid replication (see below). Plasmid pBSoriF replicated much more efficiently than pBSCmrepS in the presence of the RepS protein (Fig. 3B), demonstrating that transcription from another promoter can stimulate initiation of replication from the pXO2 ori. The lower replication of plasmid pBSCmrepS containing the wild-type pXO2 ori may be due to limited initiation of replication from the ori in vitro and is consistent with the low copy number

FIG. 4. Northern blot analysis of *repS* transcripts. Twenty micrograms of RNA from *B. anthracis* strain UM23C1-1 either lacking any plasmid (plasmid⁻) or containing plasmid pBSCmrepS (*repS*) was analyzed. The Northern blots were hybridized to RNA probes that detect either the *repS* transcript or the CT.

of this plasmid in vivo (24). It was possible, as is the case for the pAM β 1-type plasmids (29), that transcription of the *repS* gene (which may generate a primer for replication) in pBSCmrepS is attenuated by a CT within the promoter region (see below). Since the *aphA3* promoter is likely to generate higher levels of transcripts through the ori compared to the *repS* promoter, increased replication of pBSoriF in vitro is consistent with the possibility that the level of transcription proceeding through the ori may determine the replication efficiency of plasmid pXO2. Although it is likely that such a transcript directly provides the primer for the initiation of pXO2 replication, it remains to be demonstrated.

Transcriptional attenuation of the *repS* transcript. The pAM β 1 family of replicons requires transcription into the ori from their *rep* gene for replication to occur (9). Furthermore, *rep* gene transcription is regulated by CT-driven attenuation (7, 31). We performed Northern blot analysis to identify the *repS* transcript and/or its attenuated products. Northern blots containing total RNA from plasmidless *B. anthracis* strain UM23C1-1 or its derivative containing pXO2 miniplasmid pBSCmrepS (44) were hybridized to an RNA probe that would detect both full-length and attenuated *repS* transcripts. A very weak signal was observed for slowly migrating bands (Fig. 4) that may correspond to the full-length *repS* transcript, which is expected to have a size of more than 1.5 kb. However, two major bands corresponding to approximately 520 and 156 nt were observed (Fig. 4). These bands likely correspond to attenuated *repS* transcripts. This is consistent with previous studies with the plasmids of the pAM β 1 family, whose replication is controlled in part by transcriptional attenuation of their *rep* genes via a CT encoded by the complementary strand of their promoter regions (8, 31). To test whether the *repS* gene also encodes a CT, the Northern blots were also hybridized to an RNA that would detect any CTs. High levels of an ~220-nt

transcript were detected (Fig. 4). Further analysis of attenuated *repS* transcripts and the CT with oligonucleotide probes revealed that these transcripts overlapped and the CT was encoded by a region a few hundred nucleotides upstream of the RepS initiation codon. The RepS protein is encoded by pXO2 nt 34115 to 32580 of the bottom strand of the DNA (37). pXO2 nt 34379 to 34618, which include the CT encoding region identified by Northern blot analysis, can form a stem-loop structure (not shown) similar to the CT structures involved in transcriptional attenuation of the pAM β 1 family of replicons (31). These results suggest that the CT encoded by the upstream region of the *repS* gene of pXO2 may play a role in attenuation of the *repS* transcript. Future studies should identify the precise mechanism involved in this process.

Requirement for directional transcription into ori for pXO2 replication. The in vivo and in vitro requirement for directional transcription into the pXO2 ori for its replication was investigated by using plasmids containing promoters and transcription terminators at various locations with respect to the ori. Plasmid pBSoriF, which contains the *aphA3* promoter driving transcription into the ori in the direction of leading-strand synthesis, but not pBSoriRt, could be established in *B. anthracis* strain UM23C1-1 in the presence of plasmid pSK236*repS* expressing the RepS protein (data not shown). These results showed that directional transcription into the pXO2 ori is essential for in vivo replication. We then tested various pXO2 ori plasmids for the ability to replicate in vitro. As expected, plasmid pBSoriF replicated efficiently in *B. anthracis* extracts in the presence of MBP-RepS, generating predominantly the OC form of DNA along with low levels of RI (Fig. 5). The presence of the *rrnB* terminator in plasmid pBSoriFt significantly reduced replication (Fig. 5), suggesting that transcription through the ori is important for pXO2 replication. Limited RepS-dependent DNA synthesis observed with pBSoriFt probably results from limited transcription through the *rrnB* terminator. This is consistent with our observation that this plasmid can be established in *B. anthracis* UM23C1-1 in the presence of a RepS-expressing plasmid (approximately 30 transformants each were obtained in the three independent experiments; data not shown). The presence of the *bla* promoter downstream of the *rrnB* terminator in plasmid pBSoriFtbla resulted in increased replication. Only low-level background DNA synthesis was observed with vector plasmid pBSMCS, which lacks the pXO2 ori, and plasmid pBSoriRt, which contains the ori but lacks directional transcription into the ori (Fig. 5). The above results further suggest that directional transcription through the pXO2 ori is important for plasmid pXO2 replication.

In summary, we have developed an active in vitro system for plasmid replication from a plasmid-negative *B. anthracis* strain. To our knowledge, this is the first such system based on *B. anthracis* or related organisms. This system supports the replication of plasmid pT181, which replicates by an RC mechanism, as well as pXO2 miniplasmids, which are postulated to replicate by the theta-type mechanism. In addition to pXO2, the pAM β 1 family of plasmids also includes pRE25, pIP501, pSM19035, and pLI100 (5, 6, 41). It is likely that this in vitro system will also support the replication of the above plasmids. We have also demonstrated that pXO2 replication requires directional transcription through the ori, and our results are consistent with unidirectional theta-type replication of the

FIG. 5. Directional transcription into the pXO2 ori is important for replication. Various plasmids were replicated in vitro, and the products were analyzed by agarose gel electrophoresis. Top panel, ethidium bromide-stained gel; bottom panel, autoradiogram of the gel. SC1, SC form of oriFt, oriRt, and oriFtbla; SC2, SC form of pBSMCS and oriF. MCS, multiple cloning site.

pXO2 miniplasmids. This in vitro replication system based on *B. anthracis* should be of great value for the study of the replication of plasmids from *B. anthracis* and related organisms, including pXO1 and related plasmids that encode the anthrax toxin.

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