Identification and Characterization of the Replicons of the *Bacillus anthracis* Virulence plasmids pXO1 and pXO2

by

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The pXO1 and pXO2 plasmids of *B. anthracis* are both necessary for full virulence, and understanding the mechanisms by which these plasmids replicate would be helpful in combating anthrax and the spread or use of these plasmids in other systems. A 5-kilobase region of the pXO1 plasmid was cloned into an *E. coli* vector and replicates when introduced into *B. anthracis*. Deletion analysis indicated that a 158bp region containing a stem-loop structure contains the origin of replication. Mutational analysis showed that open reading frame 45 (*repX*) of pXO1 is required for the replication of the miniplasmid in *B. anthracis*. Interestingly, *repX* showed limited homology to bacterial FtsZ proteins that are involved in cell division. A mutation in the predicted GTP binding domain of RepX abolished its replication activity. RepX was purified as an MBP- as well as His- N-terminal fusion by overexpression in *E. coli* and had strong GTPase activity. RepX also bound to DNA weakly and non-specifically.

A potential origin of replication (ori) and replication initiator gene, *repS* of plasmid pXO2 was cloned into an *E. coli* vector (pBSCm) which was shown to replicate in *B. anthracis*, *B. cereus*, and *B. subtilis*. The mini pXO2 replicon could not be established in *B. subtilis polA* mutant, suggesting that DNA Pol I is required for plasmid replication. A countertranscript encoded by the *repS* promoter region was identified which may control pXO2 copy number by inhibiting *repS* expression. RepS of pXO2 was overexpressed

and purified from *E. coli* as an MBP fusion at the amino terminal end. DNA binding experiments using double-stranded (ds) and single-stranded (ss) substrates showed that MBP-RepS specifically binds to a 60-bp ds sequence containing the putative pXO2 origin of replication and that a central 20-bp region containing the putative start site for replication and the 5' side of the origin is important for this binding. MBP-RepS also bound to ss DNA non-specifically.

A cell-free system from plasmid-negative *B. anthracis* cells that promotes robust replication of rolling-circle replicating (RCR) plasmids was developed and adapted to study the replication of plasmid pXO2 in vitro. This system showed that pXO2 replication requires RepS supplied in trans and directional transcription into the origin.

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1.0 GENERAL INTRODUCTION

1.1 BACILLUS ANTHRACIS AND ITS VIRULENCE PLASMIDS pXO1 AND pXO2

1.1.1 *B. anthracis* and disease.

Bacillus anthracis is a Gram positive spore-forming rod highly related to *B. cereus* and *B. thuringiensis* and is the causative agent of anthrax (Rasko et al., 2005). *B. anthracis* occurs naturally in soil and is endemic in many places of the world such as Asia, Africa, and South America and therefore is an economic problem causing many casualties in cattle; the spores have successfully been used as a bioweapon/bioterrorism agent causing disease and death in humans (Atlas, 2002). Anthrax occurs in all mammals, and three forms of the disease occur dependent upon route of infection: cutaneous, gastrointestinal, and inhalation. Of the three, inhalation anthrax is the most life threatening. In each case, *B. anthracis* spores are taken up by macrophages where the spores germinate and escape (Mock and Fouet, 2001). The vegetative cells produce a tripartite toxin, which is encoded by the virulence plasmid pXO1, and a poly-D-glutamic acid capsule, which is encoded by the virulence plasmid pXO2. Both pXO1 and pXO2 are required for disease in humans. The toxin contains a binding subunit, protective antigen (PA), which interacts with one of the two other subunits of the toxin: edema factor (EF) or lethal factor (LF). The complete toxin can then bind and enter cells where lethal factor acts a zinc metalloprotease cleaving MAPKKs interfering with the MAPK pathway and thus dampening the immune response against *B. anthracis*. Edema factor acts as an adenylate cyclase to increase cAMP levels also leading to dampening of the immune response. The poly-D-glutamic acid capsule serves to evade the immune

system by inhibiting phagocytosis. Death is thought to be caused by septic shock (Mock and Fouet, 2001).

Although vaccines exist, they are difficult to produce and often cause reactogenicity in humans (Spencer, 2003). Additionally, strains resistant to penicillin occur naturally, and other drugs are required to combat the disease with the possibility of emergence or intentional introduction of resistance in the future (Chen et al., 2004; Spencer, 2003).

1.1.2 *B. anthracis* virulence plasmids.

Bacillus anthracis requires both of its circular virulence plasmids pXO1 and pXO2 to be fully virulent, and the presence of these plasmids has often been used to identify *B. anthracis* (Mock and Fouet, 2001). The 181-kb plasmid pXO1 contains a 45-kb pathogenicity island encoding the toxin genes, PA, EF, and LF, and their regulators; pXO1 encodes proteins capable of regulating genes on pXO2 and the chromosome as well (Fouet and Mock, 2006). Recently, highly similar plasmids have been found in close relatives such as *B. cereus* which can also cause anthrax-like disease in humans (Hoffmaster et al., 2004; Pannucci et al., 2002a; Pannucci et al., 2002b; Rasko et al., 2004). Additionally, the presence of a nearly identical plasmid to pXO1 in a clinical *B. cereus* isolate (Hoffmaster et al., 2004) indicates that transfer of the plasmid is possible in nature. Sequence analysis of the pXO1 plasmid has revealed a few ORFs encoding proteins which could be involved in plasmid transfer or conjugation such as proteins similar to topoisomerase 1 and traG/D (Okinaka et al., 1999b).

The 96-kb plasmid pXO2 contains the capsule biosynthetic operon, and murine studies have shown that when isogenic strains of *B. anthracis* are transduced with pXO2 plasmids from different host strains, LD_{50}'s vary (Welkos et al., 1993) indicating the important role that pXO2 plays in modulating virulence in the host. Recently, sequences sharing homology to the *cap* operon and newly discovered replication initiator protein (see Chapter 2) have been found in other *Bacillus* species indicating that pXO2 may transfer naturally (Luna et al., 2006; Pannucci et al., 2002b; Van der Auwera et al., 2005). A 42-kb region involved in plasmid transfer is shared by both pXO2 and the highly related conjugative plasmid, pAW63 (Luna et al., 2006). However, a single gene within this locus in pXO2 contains a frameshift mutation (Luna et al., 2006) which may render this region inactive for conjugation.

Neither pXO1 nor pXO2 have been observed to be self-transmissible. However, both plasmids can be transferred with the help of another conjugative plasmid, pXO12 (Koehler, 2002), leaving open the possibility that other conjugative plasmids may exist which are capable of transferring pXO1 and/or pXO2 to new species in the environment. In summary, these plasmids have a high potential to be transferred to other host organisms. Not only can these plasmids lead to anthrax-like disease in a new host organism (Hoffmaster et al., 2004), but also these plasmids could modulate the pathogenesis of the new organism by regulating specific genes on its chromosome.

1.2 PLASMID REPLICATION

At a basic level, plasmid replication is the most important process in allowing an organism to maintain virulence or to survive in a particular environmental niche and to spread its genetic information to other organisms. The main determinant of plasmid host range is found in the plasmid's ability to replicate and be maintained within its host. The virulence plasmids pXO1 and pXO2 are crucial in anthrax virulence and pathogenesis by expressing specific virulence factors, controlling chromosomally encoded factors, and apparently transferring to new host species. Therefore, understanding how these plasmids replicate and are maintained would contribute to understanding the pathogenesis of *B. anthracis* and could lead to alternative therapies. For example, targeting plasmid-specific replication machinery could result in a broader-range therapy against any organism harboring the virulence plasmids. Aside from therapeutic benefits, understanding replication could provide insight into the evolution of *B. anthracis* and related organisms and enhance prediction of how these plasmids and therefore virulence might spread to other organisms.

1.2.1 GENERAL REPLICON FUNCTION AND FEATURES

The main event during the initiation of plasmid replication is the recruitment of host replication proteins to a specific sequence of DNA. This recruitment, however, differs depending on the plasmid origin architecture and the encoded initiator proteins. For example, some replication initiator proteins can directly interact with host DnaA, the initiator protein for chromosomal replication. Other plasmid-encoded Rep proteins

simply induce strand opening and recruit single-stranded binding proteins, and, if the ori contains DnaA sites, DnaA is recruited. The presence of DnaA on single-stranded DNA recruits other host replication machinery including the replicative helicase, primase, clamp loaders, clamps, and DNA polymerase III holoenzyme (del Solar et al., 1998; Schaeffer et al., 2005). In addition to interacting with DnaA, some Rep proteins can interact directly with the replicative helicases and primases leading to recruitment of a complete replisome. Alternatively, replicons exist that first utilize DNA polymerase I to initiate DNA synthesis by synthesizing a stable RNA-DNA primer recognized by DNA polymerase I; downstream sequences act as primosome assembly sites recruiting the more processive DNA polymerase III to complete replication (Bruand et al., 1995; Minden and Marians, 1985). As briefly discussed below, plasmids exist which encode multiple Rep proteins with helicase and primase functions allowing for independence from some of the host replication machinery.

Most replicons contain 3 main features: the origin of replication (ori), which is the sequence of DNA at which replication is initiated, a replication initiator protein (Rep), which specifically binds to the origin of replication, and elements involved in copy control (cop) (del Solar et al., 1998). Origin sequences are AT rich allowing for ease of strand opening; additionally some origins contain iteron sequences allowing initiator recognition and binding. Often in chromosomes an asymmetry of gene placement is observed such that the majority of genes are on the leading strand; this trend is thought to prevent head-on collisions of DNA polymerase with RNA polymerase and therefore to prevent abortive replication (Rocha, 2004). Replicons typically can be grouped into

families based on homology of the replication initiator protein and ori; these families share similar modes of replication and copy control mechanisms. Three modes of replication exist for circular plasmids: theta, rolling circle, and strand displacement (del Solar et al., 1998).

1.2.2 Theta

Theta replication is a mechanism utilized by chromosomes and most large plasmids. Most plasmids replicating via the theta mode encode a Rep protein, the main exception being the ColE1-type replicons. The Rep protein binds to the ori inducing strand opening and recruiting host machinery. An RNA primer is used to prime DNA synthesis, and leading and lagging strand synthesis is coupled. Replication can occur bidirectionally or unidirectionally depending on the presence or absence of an arrest site for lagging strand synthesis (del Solar et al., 1998). Within the theta replicons, up to six groups of replicons have been identified. These differ in the presence of a plasmid-encoded Rep protein and iterons within the ori and on their requirements for DNA polymerase I and RNAP.

1.2.3 Rolling Circle

Rolling circle replication seems to be limited to plasmids less than 10-kb. The ori contains iterons for Rep binding and inverted repeats that form a distinct cruciform structure at which the Rep nicks the DNA resulting in a covalent bond with the ori (Khan, 1997). The nick frees a 3' hydroxyl group used to prime leading-strand (first)

strand synthesis. Synthesis is unidirectional and uncoupled and, therefore, a full length single-stranded intermediate is formed (Khan, 1997). Not only is the Rep protein important for initiation of replication, but it is also utilized in termination of leading strand synthesis. Second strand synthesis requires RNAP for priming and is also continuous (del Solar et al., 1998; Khan, 1997).

1.2.4 Strand Displacement

Replication by strand displacement requires 3 plasmid-encoded proteins for initiation of leading strand synthesis: an initiator, primase, and helicase. Leading strand synthesis can occur at 2 sites on opposite strands, and therefore, single-stranded intermediates can be formed from either DNA strand as well as double-stranded and partially-double stranded circles. Conversion of any single-stranded intermediates occurs via the same mechanism as first-strand synthesis unlike rolling-circle replication (del Solar et al., 1998).

1.3 PLASMID COPY CONTROL AND PARTITIONING

Every plasmid has a characteristic average number of copies per cell. Copy control prevents a plasmid from over-replicating leading to host toxicity and under-replicating leading to loss from a population. This number is determined by regulation of the initiation of replication; in all known cases regulation factors are encoded by the plasmid (del Solar and Espinosa, 2000). Three main regulatory circuits exist to control copy number utilizing antisense RNA, both protein and antisense RNA, or iterons.

Antisense RNAs can control replication by transcriptional attenuation of the *rep* gene, translational inhibition of *rep*-mRNA, or by inhibiting primer maturation (Brantl, 2002). In some cases additional control is also mediated by proteins which bind to the Rep promoter and inhibit transcription (del Solar and Espinosa, 2000).

Iterons can be involved in 2 different processes of control dependent upon their location: autoregulation of the expression of Rep or control of active Rep protein. During autoregulation, iterons in the promoter of the *rep* gene are bound by Rep protein resulting in transcriptional inhibition or *rep* gene expression. Binding of iterons by the Rep protein can also cause nonproductive Rep protein interactions when the copy number of the plasmid is high. These interactions can lead to “handcuffing” of Rep molecules between different plasmid copies and cause a steric hindrance of initiation of replication (Paulsson and Chattoraj, 2006).

Plasmid partitioning determinants are usually located in close proximity to the origins of replication. These typically consist of two plasmid-encoded proteins involved in active partitioning of plasmids to new daughter cells: a protein that binds to a cis-acting centromere-like region (*parS*) in the plasmid recruiting the second protein, an ATPase that actively segregates the plasmid to daughter cells (Moller-Jensen et al., 2000).

1.4 REPLICONS SIMILAR TO PXO2

Initial analysis of the pXO2 sequence revealed a region sharing similarity to the pAMβ1 family of replicons (Bruand et al., 1993). Significantly, ORF38 shared homology with the replication initiator proteins of this family, and a downstream sequence shared homology with the origin of replication. Not only did homology exist with this family, but also, the rep gene/ori organization was highly similar to this family of replicons. Within the pAMβ1 family of replicons are plasmids that replicate in the *Enterococcus*, *Streptococcus*, and *Bacillus* genera resulting in the description of these plasmids as being promiscuous (Bruand et al., 1993; Le Chatelier et al., 1993; Luna et al., 2006; Van der Auwera et al., 2005; Wilcks et al., 1999). The pAMβ1 replicons encode one initiator protein which binds to the double-stranded (ds) origin, induces strand opening, coats the resulting single-stranded DNA, and is thought to be involved in primer maturation (Le Chatelier et al., 2001). These replicons replicate unidirectionally via the theta mode and require DNA polymerase I for initial steps in replication (Bruand et al., 1991; Le Chatelier et al., 1993). The *rep* gene is always located upstream of the origin of replication. Directional transcription into the ori is required for priming of leading strand synthesis, and this primer is provided by the 3' end of the *rep* transcript (Bruand and Ehrlich, 1998). It is thought that primer maturation occurs either via RNA pol collision with the ori-bound Rep protein and subsequent cleavage of the transcript by RNA pol or RNAseH or that the Rep protein itself may cleave its own transcript to from a mature primer (Le Chatelier et al., 2001). Once the mature primer is formed, DNA polymerase I is recruited and limited replication occurs until a primosome assembly site (*pas*) is reached at which DNA pol I is replaced by the more processive DNA pol III for leading

strand synthesis and also at which lagging strand synthesis is initiated (Bruand et al., 1995; Janniere et al., 1997). Copy number of this family of plasmids is low, 3-4/chromosome, and is controlled by both a protein, Cop, which binds to the Rep promoter and decreases transcription 8-10 fold and a countertranscript within the *rep* promoter which attenuates the *rep* transcript (Brantl and Behnke, 1992a; Brantl and Wagner, 1996; Le Chatelier et al., 1994; Le Chatelier et al., 1996). No homolog to the *cop* gene was found on pXO2; however, sequences similar to the countertranscript were found upstream of the homologous *rep* gene.

Genes homologous to the potential partitioning proteins of pAW63 and the conjugative pAD1 family of replicons (Francia et al., 2004; Wilcks et al., 1999) were found upstream of the putative *rep* gene of pXO2 along with multiple iterons which could act as the centromer-like region for plasmid segregation.

1.5 PXO1 REPLICON

Even though the sequence of pXO1 was known for many years, its replicon could not be identified due to a lack of similarity to the known replicons of other plasmids (Berry et al., 2002; Hoffmaster et al., 2004; Okinaka et al., 1999b; Rasko et al., 2004). Work described in this dissertation (see Chapter 5) has identified an autonomous replicating region of pXO1 which requires a protein, RepX, simlar to the FtsZ family of segregation proteins. At the time of publication of our paper, no replicon had been characterized with a Rep protein similar to RepX although many large plasmids ranging from 180-400-kb contained homologs of RepX (Berry et al., 2002; Han et al., 2006; Hoffmaster et al., 2004; Rasko et al., 2004).

FtsZ is a GTPase that shares homology with tubulin and forms a ring around the mid-cell during cell-division and sporulation providing the framework (Z-ring) for the docking of proteins involved in cell septation (Goehring and Beckwith, 2005). Hydrolysis of GTP by FtsZ is required for cell viability (Mukherjee et al., 1993) and provides the dynamic nature of the protein possibly needed for invagination of the septum (Goehring and Beckwith, 2005; Rothfield et al., 1999). Hydrolysis of GTP requires 2 molecules of FtsZ. The N-terminus of FtsZ contains a highly conserved motif involved in GTP binding, and and towards the middle of FtsZ are residues involved in FtsZ interaction causing hydrolysis (Romberg and Levin, 2003). The C-terminal portion of the FtsZ protein is less conserved, but importantly, has a 15aa motif involved in interactions with other host proteins such as FtsA and ZipA (Goehring and Beckwith, 2005; Romberg and Levin, 2003). Although FtsZ seems to be occluded by the presence of DNA (Goehring and

Beckwith, 2005), proper Z-ring formation requires the initiation of DNA replication (Romberg and Levin, 2003) closely linking FtsZ function with DNA replication. Also, FtsK, a protein involved in chromosomal segregation and formation of the division septum, has been shown to interact with FtsZ in bacterial two hybrid assays (Goehring and Beckwith, 2005) again tying FtsZ closely to DNA replication. Interestingly, a protein upstream of RepX encoded by ORF42 of pXO1 contains a sequence motif with limited homology to the linker and a portion of the C-terminal region of FtsK (Bigot et al., 2004).

1.6 STATEMENT OF THE PROBLEM

Fully virulent *B. anthracis* contains two megaplasmids, pXO1 and pXO2, encoding the toxin and capsule required for causing the disease anthrax (Koehler, 2002). *B. anthracis* occurs naturally causing disease in humans and animals and has been used as a powerful and lethal bioterrorism and biowarfare agent (Atlas, 2002). Transfer of these plasmids has been observed in the presence of helper plasmids (Battisti et al., 1985; Green et al., 1985; Green et al., 1989; Reddy et al., 1987). Recently, nearly identical plasmids have been isolated from other *Bacillus* species indicating that these plasmids can be transferred naturally and can replicate in other host organisms (Hoffmaster et al., 2004; Luna et al., 2006). The ability of these plasmids to replicate in these host organisms is of primary concern when considering the potential for the spread of these plasmids in nature and the maintenance of virulence within *B. anthracis*. Although the importance of pXO1 and pXO2 in *B. anthracis* pathogenesis has been well established and the sequences of both plasmids are known, the replication properties of these plasmids had not been characterized previous to this study.

The goals of this study were to identify and characterize the plasmid-encoded factors involved in replication of pXO1 and pXO2. Specific aim one involved identifiying the pXO2 replicon and testing for its ability to replicate in various *Bacillus* species. Purification and characterization of the DNA binding properties of the RepS initiator protein of pXO2 was also done. Specific Aim two involved developing an in vitro replication system from *B. anthracis* consisting of cell free protein extracts which support rolling circle replication of pT181 and replication of pXO2. The in vitro system

was then used to further characterize the requirements of pXO2 replication such as the necessity of the origin of replication, the initiator protein, RepS, and directional transcription into the origin. Specific aim 3 involved identification of the pXO1 replicon and characterization of the biochemical and DNA binding properties of the replication initiator protein, RepX. Analysis of the origin sequence involved in replication was also attempted.

1.7 THESIS OUTLINE

Chapter 1 consists of a general introduction discussing *B. anthracis* pathology and plasmid biology and general concepts about plasmid replication, copy control, and partitioning. Both the *B. anthracis* pXO1 and pXO2 plasmids are required for full virulence. Plasmids 99% identical to pXO1 have been found in related organisms causing anthrax-like disease. Discovering the replication requirements of these plasmids could not only help to combat disease but also the spread of these plasmids.

Chapter 2 describes initial isolation of the pXO2 replicon, identification of the origin of replication, and purification and characterization of the DNA binding properties of the RepS replication initiator protein.

Chapter 3 describes the identification of the nucleotide sequence to which RepS binds, of a potential copy control mechanism for the pXO2 replicon and of a potential partitioning system.

Because replicons often require a range of host and plasmid-encoded factors, characterizing replication requirements in vivo can be difficult without isogenic mutant strains. In vitro replication systems can be useful in identifying host and plasmid encoded factors involved in replication since separate proteins can be targeted.

Chapter 4 describes the development of a cell-free extract system from *B. anthracis* useful for studying both rolling circle and pXO2 replication.

Chapter 5 describes the isolation of the minireplicon of pXO1 and identification of a key open reading frame encoding RepX, a protein similar to the FtsZ GTPases, necessary for replication and a DNA sequence containing a large inverted repeat involved in replication.

Chapter 6 discusses the purification of pXO1-encoded RepX, initial characterization of its biochemical activities, and its DNA binding properties.

Chapter 7 discusses the results of the research described, questions raised, and future goals.

2.0 ISOLATION OF A MINIREPLICON OF THE VIRULENCE PLASMID PXO2 OF BACILLUS ANTHRACIS AND CHARACTERIZATION OF THE PLASMID-ENCODED REPS REPLICATION PROTEIN

Work described in this chapter was published in the Journal of Bacteriology (2004, volume 186, pages 2717-2723) with authors Eowyn Tinsley, Asma Naqvi, Agathe Bourgogne, Theresa M. Koehler, and Saleem Khan.

2.1 INTRODUCTION

Bacillus anthracis is a gram-positive bacterium that is the etiological agent of anthrax (reviewed in references Hanna, 1998; Koehler, 2002; Mock and Fouet, 2001). 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 (Hanna, 1998; Helgason et al., 2000; Koehler, 2002; Mock and Fouet, 2001; Okinaka et al., 1999b; Read et al., 2002; Uchida et al., 1986). Plasmid pXO1 (181.6 kb) encodes the anthrax toxin proteins termed protective antigen, lethal factor, and edema factor (Guidi-Rontani et al., 1999; Hoffmaster and Koehler, 1999; Koehler, 2002; Okinaka et al., 1999a; Okinaka et al., 1999b). 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 (Green et al., 1989; Koehler, 2002; Makino et al., 1989; Okinaka et al., 1999a; Pannucci et al., 2002b; Uchida et al., 1993). In addition, both plasmids carry regulatory genes that control expression of the toxin and capsule genes: *atxA* and *pagR* on pXO1 (Bourgogne et al., 2003; Dai et al., 1995; Guignot et al., 1997; Hoffmaster and Koehler, 1999; Koehler et al., 1994; Mignot et al., 2003; Uchida et al., 1993; Uchida et al., 1997) and *acpA* and *acpB* on pXO2 (Drysdale et al., 2004; Vietri et al., 1995).

Although pXO1 and pXO2 play central roles in the pathogenesis of anthrax (Koehler, 2002; Mock and Fouet, 2001; Welkos et al., 1993), 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 (Green et al., 1985; Koehler, 2002; Mock and Fouet, 2001). 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 (Coker et al., 2003). 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(Battisti et al., 1985; Green et al., 1989; Koehler, 2002). Interspecies transduction of pXO2 into *B. cereus* has also been reported (Green et al., 1985).

The pXO2 plasmid contains sequences that share homology with the replication regions of plasmids of the pAMß1 family, such as pAW63, pAMß1, pIP501, and pSM19035, which are found in gram-positive organisms, suggesting that pXO2 also belongs to this plasmid family (Brantl et al., 1990; Bruand et al., 1993; Le Chatelier et al., 1993; Pannucci et al., 2002b; Wilcks et al., 1999). These conjugative plasmids are promiscuous and have a broad host range (Bruand et al., 1993). They replicate by a theta-type mechanism, and their replication proceeds unidirectionally from the origin(Bruand et al., 1991; Bruand et al., 1993). 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 (Pannucci et al., 2002b; Wilcks et al., 1999). The RepS protein of pXO2 also has approximately 40% identity with the Rep proteins of plasmids pAMß1 and pRE25 of

Enterococcus faecalis, pIP501 and pSM19035 of *Streptococcus agalactiae*, and pPLI100 of *Listeria innocua* on the basis of BLAST alignments(Altschul, 1990). Similarly, the putative origin of replication (*ori*) of pXO2 (nt 32583 to 32524) is highly homologous to the postulated *ori* of pAW63, and the *ori* of pAMß1 (Brantl et al., 1990; Bruand and Ehrlich, 1998; Bruand et al., 1991; Bruand et al., 1993; Le Chatelier et al., 1993; Le Chatelier et al., 2001).

The replication regions of the pIP501, pSM19035, and pAMß1 have been identified by the isolation of minimal replicons. The best-studied plasmid of this group is pAMß1. The RepE protein of pAMß1 has been isolated and shown to bind specifically to the double-stranded (ds) DNA at the origin and nonspecifically to single-stranded (ss) DNA (Le Chatelier et al., 2001). 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 pAMß1 *ori* and the putative *ori* of pAW63 are located immediately downstream of the sequence coding for RepE (Bruand et al., 1991; Le Chatelier et al., 2001; Pannucci et al., 2002b; Wilcks et al., 1999). The mRNA of the RepE protein of pAMß1 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 (Bruand and Ehrlich, 1998). At the origin, the 3' end of the RepE mRNA pairs with one strand of the DNA generating an R-loop structure. An RNaseH-like activity in the cell or the RepE protein itself (it has been postulated to have an RNaseH 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 (Janniere et al., 1997). 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 (Janniere et al., 1997; Le Chatelier et al., 2001). 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.

2.2 MATERIALS AND METHODS

Cloning of the pXO2 minireplicon in *Escherichia coli*. DNA enriched for pXO2 was isolated from *B. anthracis* strain 9131 containing pXO2 (Etienne-Toumelin et al., 1995; Green et al., 1985). 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* (Sambrook, 1989). Finally, the spectinomycin resistance cassette *aad9* from pJRS312 (Saile and Koehler, 2002) 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 deoxynucleotide 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'-ccggatccCACATACCATAATGAGAATATAACC-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-ß-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 (Chang et al., 2000). 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 (Chang et al., 2000). 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 (Sambrook, 1989). 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 (Le

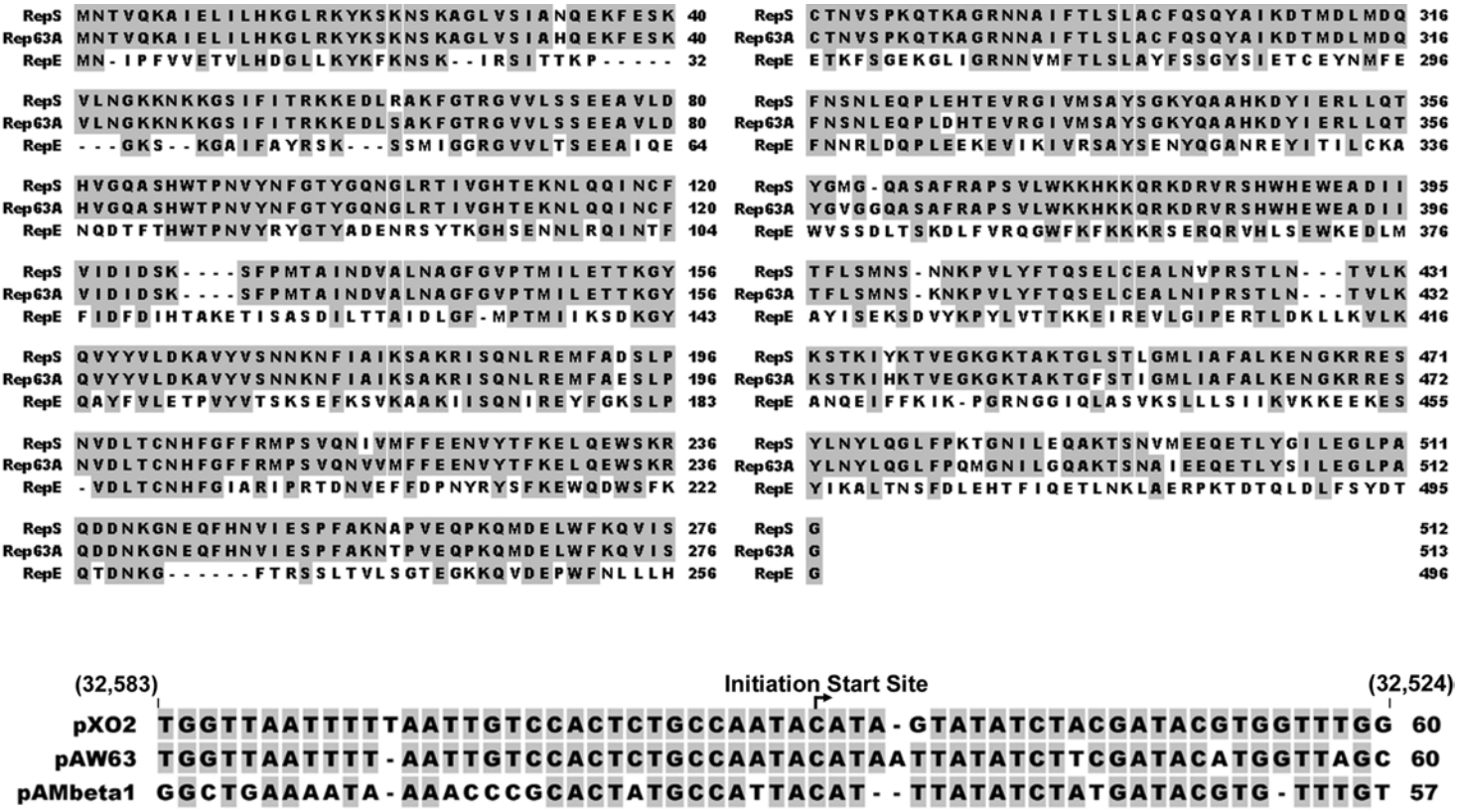
Chatelier et al., 2001). The reaction mixtures were incubated at room temperature for 15 min, and the DNA-protein complexes were resolved by electrophoresis on 6% native polyacrylamide gels. The gels were dried and subjected to autoradiography. In competition DNA binding experiments, various amounts of cold competitor oligonucleotides were also included in the above reaction mixtures.

2.3 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 pAMß1 of *E. faecalis* (Fig. 1). Similarly, the putative origin of pXO2 is highly homologous to the postulated *ori* of pAW63 and the *ori* of pAMß1 (Fig. 1). We generated plasmid pBSCmrepS (6,853 bp) containing a 2,429-bp region of pXO2 (nt 32423 to 34851, including the 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 (Dunny et al., 1991; Marrero and Welkos, 1995; Quinn and Dancer, 1990). 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).

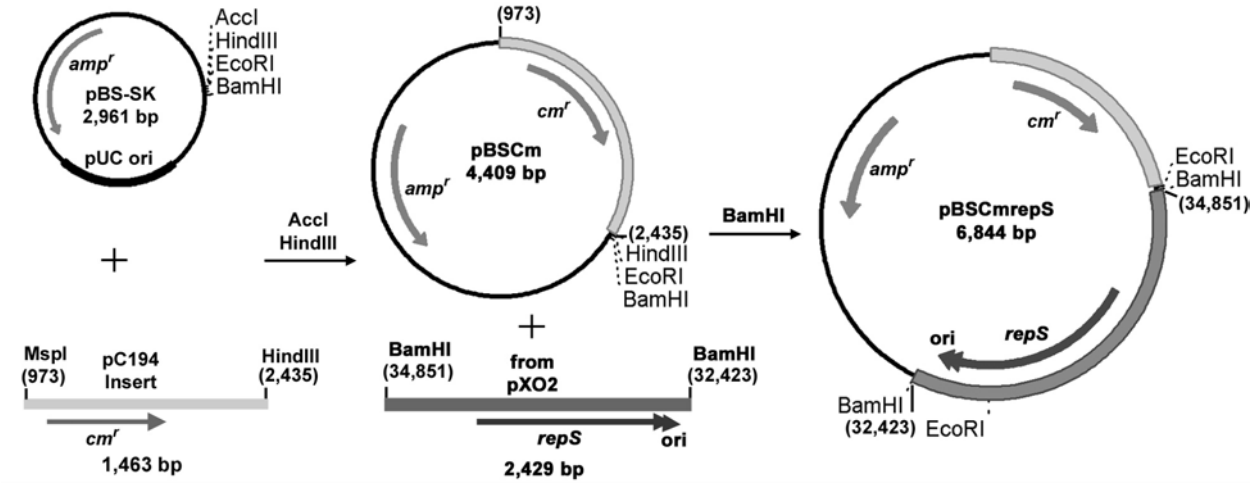
The pBSCmrepS plasmid was also introduced into *B. cereus* and *B. subtilis* by electroporation (Dunny et al., 1991; Ito and Nagane, 2001). The restriction patterns of plasmid DNA isolated from these gram-positive hosts were identical to those from *B.*

Figure 1. Alignment of the RepS protein of pXO2 with similar replicons



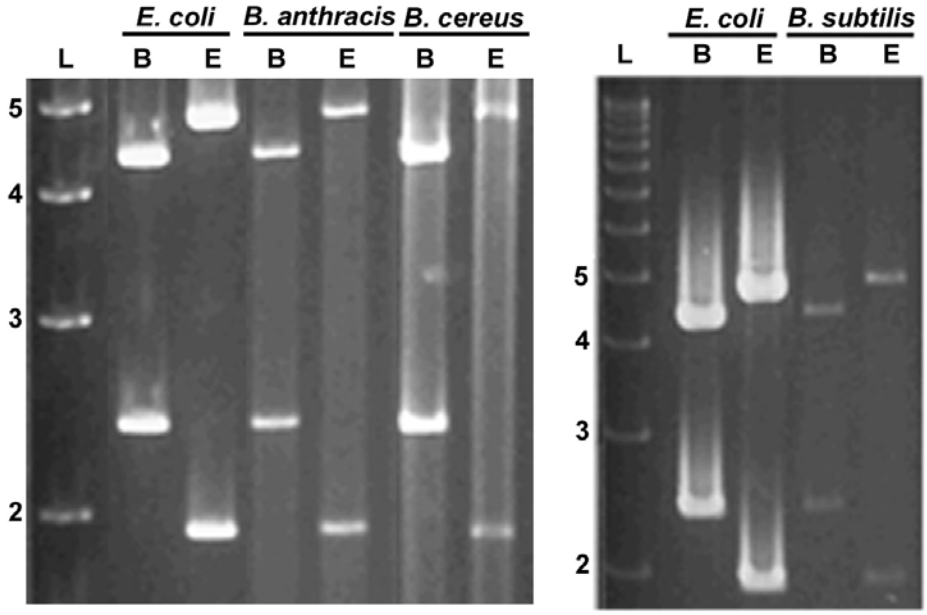
Alignment of the RepS protein of pXO2, Rep63A protein of plasmid pAW63, and RepE protein of pAMß1 as well as the origins of replication of these three plasmids (Altschul, 1990; Bruand et al., 1993; Le Chatelier et al., 2001; Pannucci et al., 2002b; Wilcks et al., 1999). The alignment was done using the 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.

Figure 2. Construction of plasmid pBSCmrepS containing the pXO2 minireplicon



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.

Figure 3. Restriction analysis of the pBSCmrepS plasmid

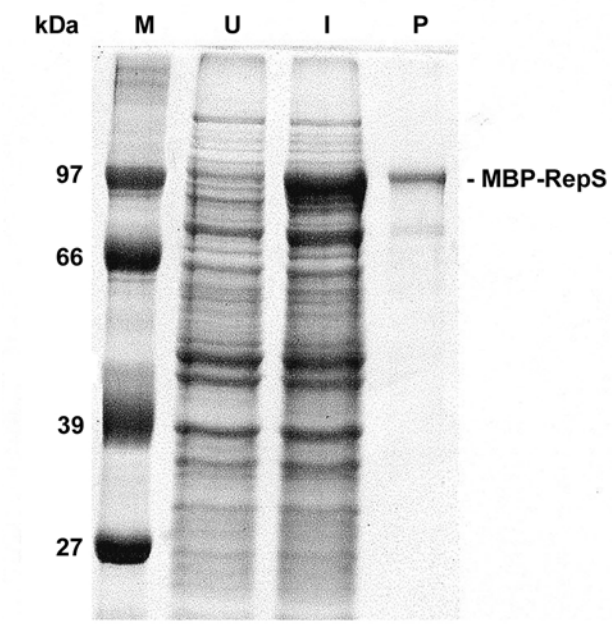


Restriction analysis of the pBSCmrepS plasmid isolated from *E. coli*, *B. anthracis*, *B. cereus*, and *B. subtilis*. Plasmid was digested with BamHI (B) or EcoRI (E). L lanes contain size markers (in kilobases).

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 (Wilcks et al., 1999). 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 amylose 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.

Figure 4. Purification of the RepS protein of pXO2



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).

ds and ss DNA binding activity of the RepS protein. On the basis of sequence homology to the pAW63 and pAMß1 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 pAMß1 plasmid, the 5' and central regions of the *ori* have been shown to be critical for RepE binding (Le Chatelier et al., 2001). 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).

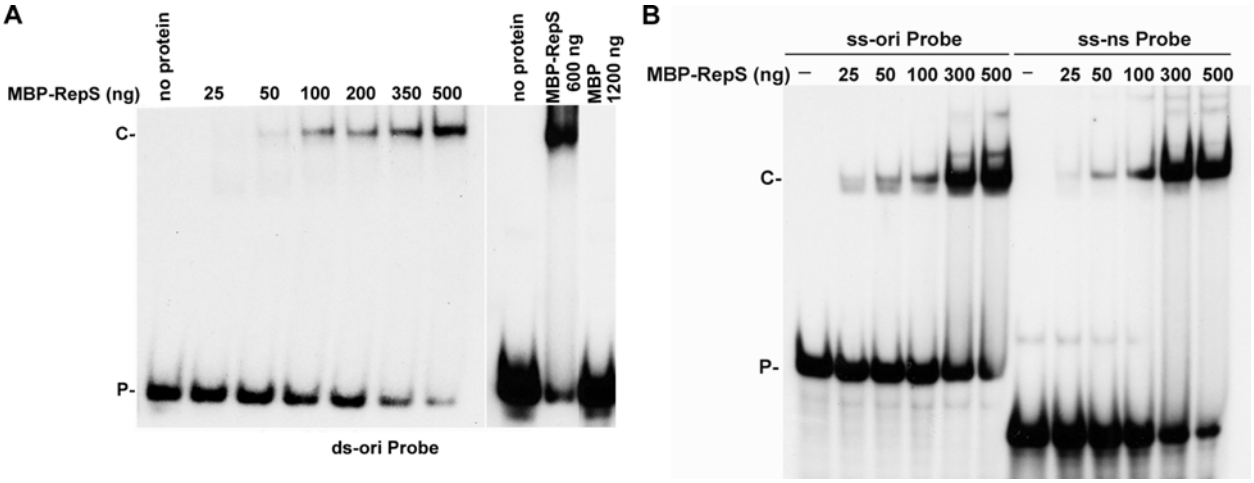
We also used additional 60-bp oligonucleotides in competition EMSA that include 20 nt of the *ori* and the adjacent 40 nt on either side of the *ori* (Table 1). The 5' region of *ori* is located immediately adjacent to the *repS* gene, while its 3' region is distal to *repS*. The RepS-*ori* complex was 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 contributes 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

Table 1. Oligonucleotides used in the pXO2 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′

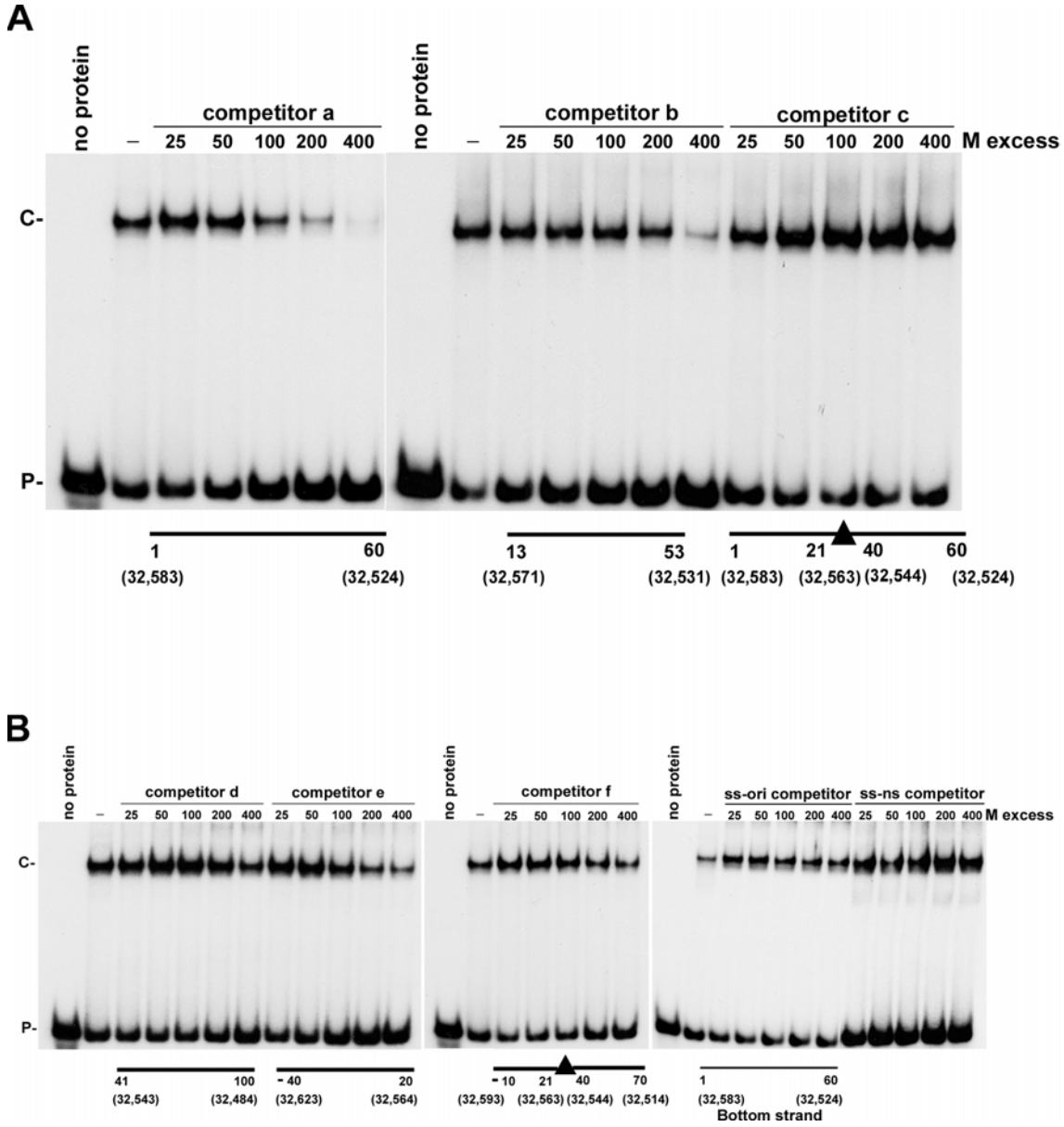
[a] Only the top strands are shown.

Figure 5. Binding of the RepS protein to ds pXO2 origin DNA (A) or to ss DNA (B)



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.

Figure 6. Competition EMSA experiments pXO2 ori



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.

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 demonstrate 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 pAMß1 *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 pAMß1 *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 pAMß1 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 (Le Chatelier et al., 2001).

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 pAMß1 has been isolated and shown to bind specifically to the ds DNA at the origin and nonspecifically to ss DNA (Le Chatelier et al., 2001). Interestingly, RepE of pAMß1 binds to ss DNA (both *ori* specific and nonspecific) with a higher affinity than to specific, ds *ori* DNA (Le Chatelier et al., 2001). 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.

3.0 MAPPING OF THE REPS BINDING SITE, IDENTIFICATION OF POTENTIAL COPY CONTROL ELEMENTS AND PARTITIONING SYSTEM OF PXO2

3.1 INTRODUCTION

Previous work (see Chapter 2) identified a minimal sequence involved in pXO2 replication sharing sequence similarity to the pAMβ1 family of replicons. The requirement for the replication initiator protein, RepS, was established, and a core DNA sequence involved in RepS binding to the pXO2 origin of replication was identified using EMSAs. However, additional analysis of the replicating region of pXO2 was necessary to help identify potential copy control mechanisms of the replicon as well as a partitioning system contributing to plasmid stability. Further, identification of the exact sequence to which RepS binds could confirm EMSA results and would help in future characterization of the effects of RepS on origin architecture.

3.2 MATERIAL AND METHODS

DNase I Footprinting. To generate a probe for DNase I footprinting, restriction digestion of pBSCmrepS was performed with EcoRI and SpeI. The resulting 420bp fragment containing the 3' end of the *repS* gene and the origin of replication was cloned into the EcoRI/SpeI sites of pBS-SK to yield pBS420. This plasmid (50 ug) was digested with EcoRI and SpeI, the ends were alkaline phosphatase treated (6-9U) followed by end-labeling with T4 PNK (15U). The resulting fragment was FatI digested to yield a 136bp fragment labeled on the bottom strand. To isolate 136bp fragment labeled on the top strand, pBS420 was digested with FatI, CIP treated, labeled, and digested with SpeI. Probes were cleaned with a G50 sepharose column and run on 2% agarose gel followed by gel extraction using Qiaquick Gel Extraction kit from Qiagen. EMSA reactions were performed as previously described with 0.2 μl of probe and 0, 100 ng, 500 ng, or 1 μg of MBP-RepS protein per reaction. DNaseI buffer was added directly to the EMSA reactions to yield a 1X concentration and reactions (30 μl total volume) were equilibrated at room temperature for 10 minutes. The resulting complexes were digested with 1U of DNaseI for 1 minute at room temperature. An equal volume of stop solution (200 mM NaCl, 20 mM EDTA, 1% SDS, 100 μg/ml tRNA, 50 μg/ml ProteinaseK) was added per reaction and incubated at 37˚C for 10min. The volume was increased to 100 μl and reactions were phenol/chloroform extracted, ethanol precipitated, and resuspended in 5 μl of sequencing buffer (96% formamide, 10 mM EDTA, 10 mM NaOH, and 0.005% BFB/xylene cyanol). Samples were boiled for 2 min and loaded onto 8% polyacrylamide 8 M urea sequencing gels which had been prerun for 30 min at 60W (1700V). Sequencing gels were run at 60W for 1.5 h, fixed

with methanol/acetic acid, transferred to Whatman paper, dried, and exposed to film. Sequencing reactions were performed using Sequenase Version 2.0 DNA Sequencing Kit from USB according to the manufacturer's instructions.

RNA extraction from *B. anthracis*. A small culture of *B. anthracis* was grown in BHI overnight. A flask of 100 ml of BHI was inoculated with 2 ml of the overnight culture and allowed to grow to an OD_{600}=0.6. A volume of 12.5 ml of 5% water saturated phenol in ethanol was added to the culture, and the media was filtered off. Cells were resuspended in 8 ml TE containing 4 mg/ml lysozyme and incubated on ice with mixing for 45 min. One-tenth the volume of 10% SDS (800 μl) was added to the cells and incubated at 64˚C for 2 minutes. A total of 880 μl of 1 M sodium acetate pH 5.2 was added to the cells, mixed, and aliquoted to Eppendorf tubes containing an equal volume of phenol. This solution was mixed and incubated at 64˚C for 6 min with occasional mixing. Samples were chilled on ice followed by spinning at 14 K rpm at 4˚C for 10min. Phenol extraction was repeated for samples until the top layer was clear. The top layer was chloroform extracted, followed by ethanol precipitation, and the resulting pellets were resuspended in a total volume of 100 μl of DEPC-treated water by transferring from one tube to the next. DNase treatment was done with 14 U DNaseI/250 μg total RNA and RNasin at room temperature for 15 min. RNA was also cleaned using the RNeasy kit from Qiagen, whene necessary. Yield were between 700 ng/μl and 1 ug/μl of RNA after cleaning. Samples were stored at -80˚C.

Northern blot analysis. 20 μg of RNA was precipitated and run on 1% formaldehyde agarose gels at 200V for 3 h. Gels were soaked in 50 mM NaOH and 10 mM NaCl for

15 minutes and neutralized by soaking in 0.1 M Tris-HCl pH 7.5 for 45 min followed by soaking in 10X SSC for 1 h. Transfer to genescreen was done overnight via capillary action. Probes were prepared either by using in vitro transcription reactions or by end-labeling oligonucleotides. In vitro transcription reactions were done using pBS vectors containing various portions of the *repS* gene and the promoter. For in vitro transcription reactions, 200 ng linearized template DNA was added to 5X transcription buffer (0.2 M Tris-HCl (pH 8.0), 40 mM MgCl2, 10 mM spermidine-$(HCl)_3$, and 125 mM NaCl), 0.4 mM of CTP, GTP, and UTP, 10 μM ATP, 5 mM DTT, 20 U T7 or T3 polymerase, and 5 μl of αP^{32} ATP (3,000Ci/mmol), and 20 U RNasin in a total reaction volume of 25 μl. Reactions were carried out at 30˚C for 1h, and treated with 1U RQ1 DNase (Promega) at 37˚C for 20min. Volume was increased to 50ul, and samples were cleaned with a G50 column. The specific activities of the probes were normally in the high 10^6-low 10^7cpm/μg of the DNA. Hybridization using the RNA probes was done at 55˚C in formamide/dextran sulfate buffer (Sambrook, 1989). Two room temperature washes were done in 2X SSC and 0.1% SDS followed by multiple washes at 60˚C in 0.1X SSC and 0.1% SDS.

3.3 RESULTS

DNase Footprinting of RepS binding to the pXO2 origin of replication. Previous work using mobility shift assays had identified a core region within the 60bp ds origin of replication of pXO2 to which the replication initiator protein, RepS, bound specifically (see Chapter 2). This region consisted of the first 40 nt of the above sequence. To further identify the residues important for RepS binding, DNase footprinting assays were done using probes containing 136bp of the 3' end of the *repS* gene and the entire 60bp origin of replication (Fig. 7). Protection of the top strand was seen for nt 61-74 and for the bottom strand nt 67-89. It was expected that binding on the top and bottom strands would be similar. The difference in protection was probably due to the failure of the DNase to completely digest the top strand (Fig.7 no RepS lane) near the area of protection thus making what constituted full protection for the top strand difficult. Not only did these results confirm the EMSA data for pXO2, but also, they correlated well with the binding pattern seen for the initiators of the pAMβ1 family of replicons which bind just upstream of the initiation site of leading strand synthesis (Le Chatelier et al., 2001).

Figure 7. DNase Footprinting of RepS on the pXO2 ori.

A

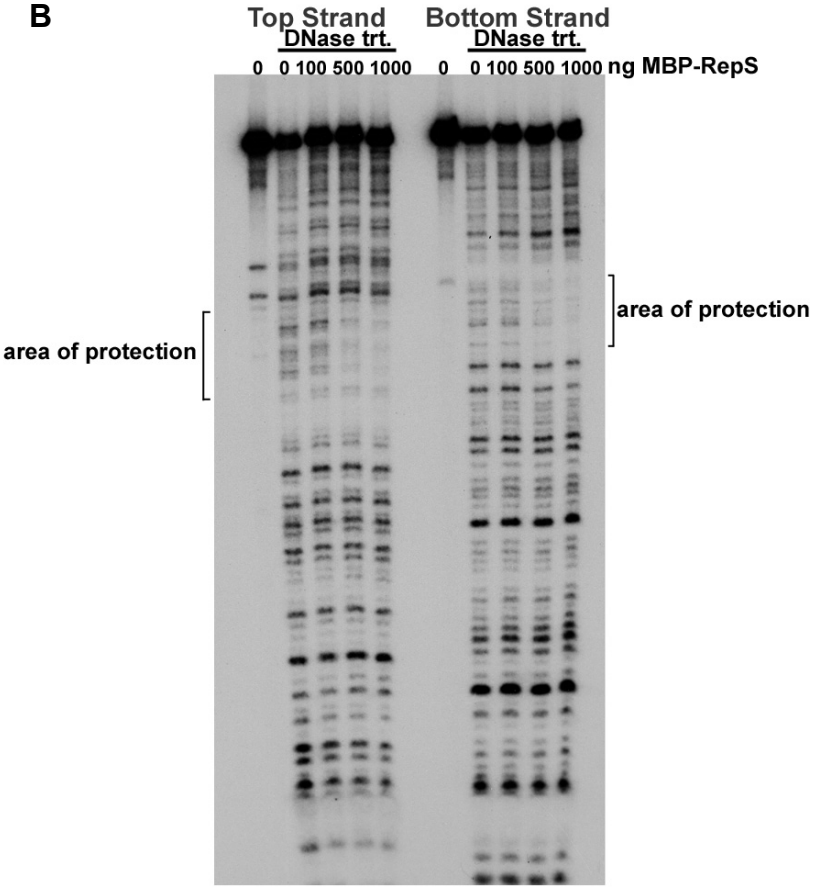
Top Strand Used:

GCAATGTCAT GGAGGAACAA GAAACACTAT ACGGCATATT GGAAGGGCTA
CCAGCTGGTT AATTTTTAAT TGTCCACTCT GCCAATACAT AGTATATCTA
CGATACGTGG TTTGGTTAGC CAGTCTTGGA ATTACAGGAT TCA

Bottom Strand Used:

TGAATCCTGT AATTCCAAGA CTGGCTAACC AAACCACGTA TCGTAGATAT
ACTATGTATT GGCAGAGTGG ACAATTAAAA ATTAACCAGC TGGTAGCCCT
TCCAATATGC CGTATAGTGT TTCTTGTTCC TCCATGACAT TGC

B



A. A. DNA probes used in DNaseI footprinting assays.

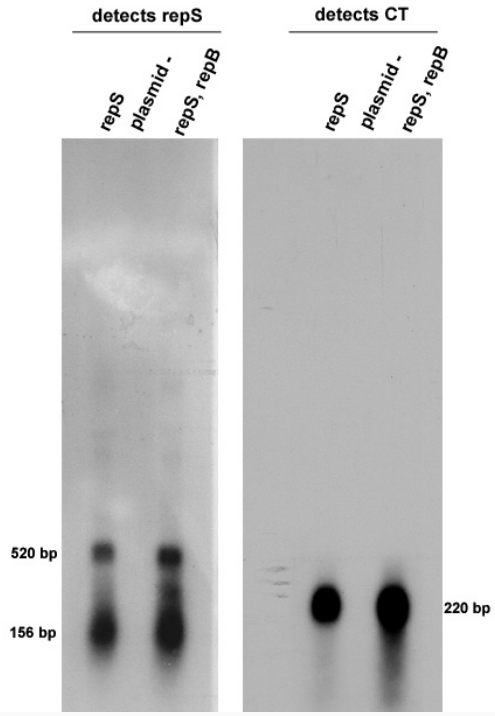
B. Areas of protection observed upon RepS binding to the pXO2 ori in DNAse footprinting assays.

C. Summary of EMSA and DNase footprinting assays of RepS binding to the ds ori of pXO2. Solid lines over nt indicate areas of binding/protection.

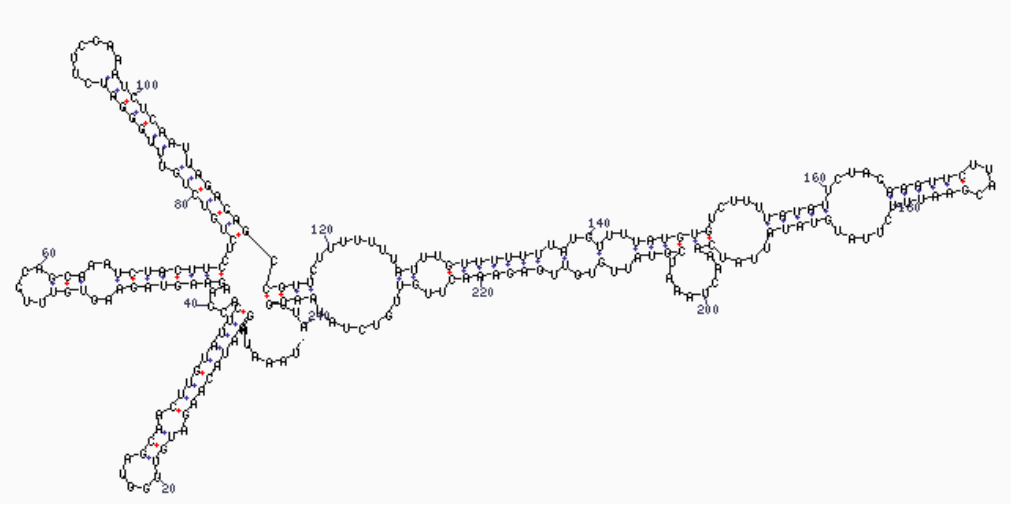
Northern blot analysis of the *repS* gene. Because the pAMβ1 family of replicons requires transcription into the ori from the *rep* gene for replication to occur (Bruand and Ehrlich, 1998), Northern blot analysis of the *repS* gene of pXO2 was done using probes that would detect full length *repS* mRNA and potential leader sequence of mRNA (Fig. 8A). However, full length transcripts were not detected, and, instead, two shorter products were seen when Northerns were probed for full *repS* (Fig. 8A). Attenuation of the *repS* transcript was likely since copy number of this family of replicons is controlled in part by transcriptional attenuation of the *rep* gene via a countertranscript present in the promoter region of the *rep* gene (Brantl and Wagner, 1996; Le Chatelier et al., 1996). Northern blot analysis was done using either probes that would detect transcripts present in the 5' end and promoter region of *repS*, and a countertranscript (CT) of 220 nt was detected. Further analysis of this transcript using oligonucleotide probes revealed that the countertranscript was present 650-bp upstream of the *repS* initiation ATG codon. The CT sequence within this region shared some homology with the *rep* CT sequences and was capable of forming a stem loop structure (Fig. 8B) similar to the CT structures involved in transcriptional attenuation of the pAMβ1 family of replicons (Le Chatelier et al., 1996). Additional probes revealed that both the attenuated products and CT sequences overlapped indicating that the CT could play a role in attenuation of the *repS* transcript.

Figure 8. Northern Blot analysis of the *repS* region of pXO2.

A)



B)



A. Northern blots detecting repS mRNA and countertranscript (CT); 3 strains were tested: *repS*, strain containing pXO2 minireplicon encoding the wt *repS* ORF only; plasmid -, strain containing no plasmids; *repS*, *repB* strain containing a minireplicon encoding both *repS* and *repB* of pXO2. **B**. Predicted folding of *repS* CT using mfold (M. Zuker, 1999).

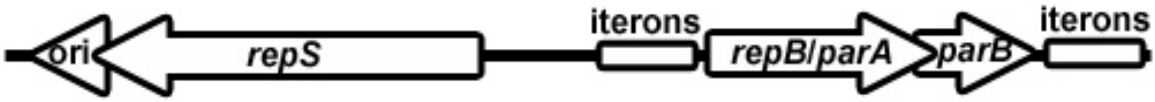
Prediction of a partitioning system for pXO2. Our studies demonstrated that the RepB protein is dispensable for replication of the pXO2 miniplasmid (Chapter 2). Other studies on similar proteins listed genetic evidence that this protein was required for replication (Wilcks et al., 1999) or involved in copy control and partitioning (Weaver et al., 1993). Studies on the aa sequence of RepB of pXO2 revealed that it shares homology with the ParA proteins containing Walker A ATPase motifs and that ORF40 shared homology to the ParB proteins involved in iteron binding (Gerdes et al., 2000). The ParA proteins are involved in moving the DNA into daughter cells via interaction with a ParB protein which binds to iterons (parS) present on the plasmid (Gerdes et al., 2000). The ParA/ParB/parS region is often located close to the origin of replication, and is organized such that ParA is immediately upstream of ParB, and the iterons fall within the promoter regions of ParA (Gerdes et al., 2000). RepB of pXO2 lies just downstream of RepS. Analysis of the pXO2 sequence near RepB (the ParA homolog) revealed that multiple iterons existed upstream of the RepB gene and downstream of ORF40. Additionally, RepB was upstream of ORF40. Thus the proximity of RepB to the ori of pXO2 and the gene organization of the iterons, RepB, and ORF 40 reveal a conserved gene organization similar to the ParA/ParB loci (Fig. 9). Complete cloning of this region of pXO2 has been performed; however, stability studies will be done in the future to determine if this region is involved in plasmid stability.

Figure 9. Identification of a putative partitioning system of pXO2.

A)

ATAATGTGTA AATGTGTACA TTTACACATT TACACATTTA CACATTTACA CATTTTTTAT
TATTACACAT TTACACATGT AAATGTGTAA ATGTGTAAAT GTGTAAATGT GTAAAAAATA

B)



A. Example of iterons present in the putative *par* locus of pXO2. **B.** pXO2 contains a locus organized similarly to the *parA/B* partitioning loci.

4.0 DEVELOPMENT OF AN IN VITRO SYSTEM FOR PLASMID REPLICATION FROM *BACILLUS ANTHRACIS*

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Key words: Plasmid replication, in vitro system, rolling-circle replication, *Bacillus anthracis*, pXO2 plasmid

4.1 ABSTRACT

In vitro systems facilitate studies on the mechanisms involved in plasmid DNA replication. We have developed a cell-free system from Bacillus anthracis that can faithfully replicate plasmid DNA in vitro. The newly developed system was shown to support the in vitro replication of the pT181 plasmid that replicates by the rolling-circle mechanism. We also demonstrate that this system supports the replication of the minireplicon of the pXO2 virulence plasmid of B. anthracis. Replication of pXO2 required directional transcription through the plasmid origin of replication.

4.2 INTRODUCTION

In vitro replication systems provide important tools for the study of DNA replication. Rolling-circle-replicating (RCR) plasmids are ubiquitous in Gram-positive bacteria, including the members of the *B. cereus* group. *Bacillus cereus, Bacillus thuringiensis*, and *Bacillus mycoides* contain indigenous RCR plasmids (Andrup et al., 2003; Khan, 1997) while RCR plasmids of *Staphylococcus aureus* such as pT181, pC194 and pE194 can also replicate and be established in *Bacillus anthracis* (Anand et al., 2004). Members of this group of organisms also contain large plasmids that presumably replicate by the theta-type mechanism (Berry et al., 2002; Han et al., 2006; Hoffmaster et al., 2004; Luna et al., 2006; Okinaka et al., 1999b; Pannucci et al., 2002a; Pannucci et al., 2002b; Rasko et al., 2004; Schwarz et al., 2001; Van der Auwera et al., 2005; Wilcks et al., 1999). *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 (Berry et al., 2002; Han et al., 2006; Hoffmaster et al., 2004; Luna et al., 2006; Okinaka et al., 1999b; Pannucci et al., 2002a; Pannucci et al., 2002b; Rasko et al., 2004; Schwarz et al., 2001; Van der Auwera et al., 2005; Wilcks et al., 1999). In order to understand the replication properties of various plasmids, the availability of a cell-free system is highly desirable. A major obstacle to the development of active cell-free systems from *Bacillus anthracis* and related organisms is the poor lysis of these organisms due to their cell wall and S-layers. Development of active in vitro replication systems requires gentle lysis conditions that avoid fragmentation of chromosomal DNA that results in high background signals. At the same time, high protein concentrations are usually desirable for the establishment of active in vitro replication systems. In this

study, we describe the development of a cell-free extract system from *B. anthracis* that can support replication of the pT181 plasmid that replicates by a rolling-circle (RC) mechanism as well as the pXO2 plasmid of *B. anthracis* which is expected to replicate by the theta-type mechanism. Using this system, we demonstrate that pXO2 replication requires RNA synthesis for initiation and increased transcription through its origin of replication increases the efficiency of replication. This in vitro system should be useful in the study of replication of plasmids belonging to the *B. cereus* group and possibly other related organisms.

4.3 RESULTS AND DISCUSSION

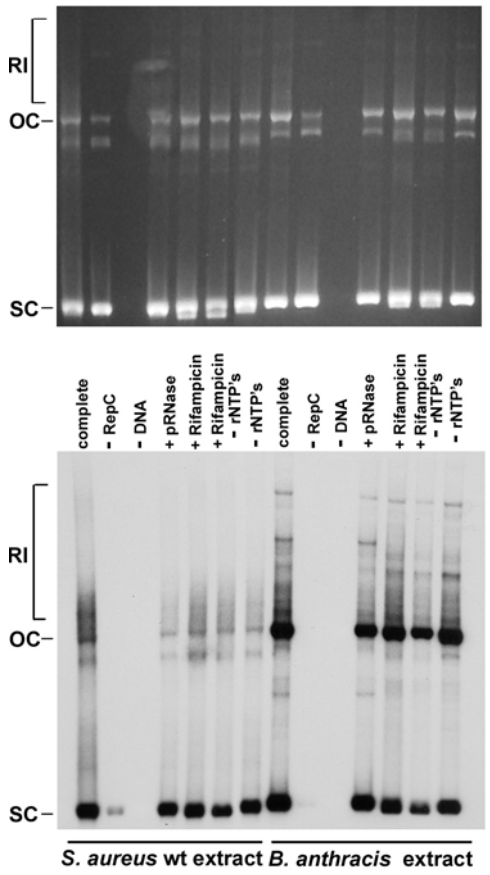
Development of a cell-free extract from *B. anthracis* UM23C1-1. A one liter culture of the plasmid-negative *B. anthracis* strain UM23C1-1 (Green et al., 1985) was grown to an A_{260} of 0.6, cells were pelleted, and washed with 100 ml of TEG (25 mM Tris-HCl, pH8.0 and 5 mM EGTA). Cells were resuspended in 6 ml of TEG and lysed using a French Press at 20,000 lb/in^2 with 3-4 passes. 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 using a Beckman SW41Ti rotor. To remove contaminating DNA and RNA, one-tenth 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 Sorval SS34 rotor. Proteins were precipitated with ammonium sulfate to a saturation of 70%, 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 DTT and 10% ethylene glycol) and dialyzed in the same buffer.

***B. anthracis* cell-free extracts support replication of the RCR plasmid pT181.** We have previously shown that the pT181 plasmid can replicate in *B. anthracis* (Anand et al., 2004). This plasmid replicates by a RC mechanism and has been extensively characterized for its replication properties utilizing a cell-free extract from *S. aureus* (Khan et al., 1981; Koepsel et al., 1985). We, therefore, tested whether the *B. anthracis* cell-free extracts can support replication of this plasmid. One-half microgram of pT181*cop608* (a copy mutant of pT181) DNA was incubated as described earlier (Khan et al., 1981) in a buffer containing rNTPs, dNTPs (one labeled with ^{32}P), an ATP-

regenerating system, cell-free protein extracts (72 µg), 200 ng of the pT181 initiator protein RepC, and the products were analyzed by agarose gel electrophoresis followed by autoradiography (Khan et al., 1981). *B. anthracis* cell-free extracts supported replication of the pT181 DNA as seen by the presence of labeled supercoiled (SC) and open-circular (OC) DNA along with replication intermediates (RI) (Fig. 10). The replication products were similar to those obtained with the *S. aureus* cell-free extracts (Fig. 10). Replication of the pT181*cop608* DNA required its initiator protein (Fig. 10), and no replication was obtained with a plasmid that lacked the pT181 origin (data not shown). Replication was not significantly affected in the presence of rifampicin and/or in the absence of rNTPs, similar to previous studies with pT181 (Khan et al., 1981; Koepsel et al., 1985). 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. We have recently identified the replication region of the pXO2 plasmid of *B. anthracis* (Tinsley et al., 2004). These studies showed that pXO2 replication in vivo requires the plasmid-encoded initiator protein RepS, and the putative origin of replication (ori) of pXO2 is located immediately downstream of the *repS* gene (Tinsley et al., 2004). Plasmid pXO2 belongs to the pAMβ1 family of plasmids that replicate by a unidirectional theta-mode (Bruand et al., 1993). Thus, the 3' end of the *repS* transcript is likely to provide the RNA primer for the initiation of pXO2 replication (Bruand and Ehrlich, 1998). Plasmid

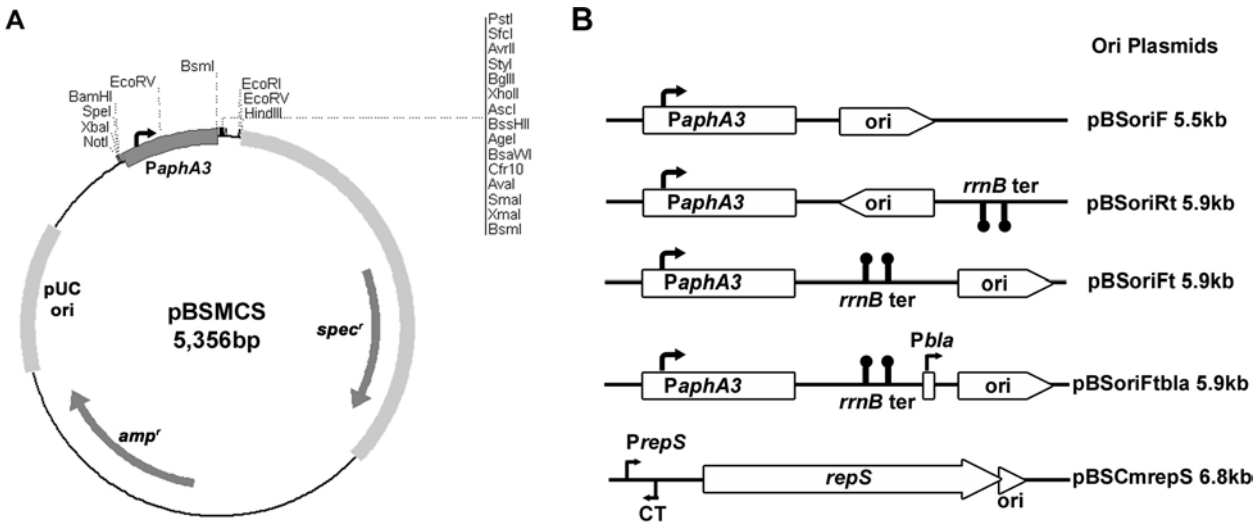
Figure 10. In-vitro replication of the RC pT181 derivative cop608.



Top panel: ethidium bromide agarose gel of the replication products from in-vitro reactions. Bottom panel: autoradiograph of the top panel; S. aureus wt extract and B. anthracis extract, in-vitro reactions replicated with extracts obtained from *S. aureus* and *B. anthracis* respectively. OC, open circular DNA; RI, replication intermediate; SC, supercoil DNA. Complete, reaction contains DNA, rNTP's, extract, and RepC; - RepC, reactions lack RepC only; - DNA, reactions lack DNA only; + pRNAse, complete reaction with 100ug/ml pancreatic RNAse; + Rifampicin, complete reaction with 100ug/ml rifampicin; +Rifampicin/-rNTP's, complete reaction with 100ug/ml rifampicin but lacking rNTP's; -rNTP's, complete reaction lacking rNTP's.

pBSCmrepS containing the *repS* gene as well as the downstream pXO2 ori replicates in *B. anthracis* in vivo (Tinsley et al., 2004). To separate the pXO2 *ori* from the *repS* gene, plasmid pBSoriF was generated that contained the pXO2 ori downstream of the *aphA3* (kanamycin resistance gene) promoter. For this, the *aphA3* promoter from plasmid pUC4Ωkan (obtained from T. Koehler) (Perez-Casal et al., 1991) was amplified with the following primers containing *Bam*HI and *Eco*RI linkers: forward primer 5'-CCGGATCCCGAACCATTTGAGGTGATAGGTAAG -3' and reverse primer 5'-CCGGAATTCCCCAAGAAGCTAATTATAACAAGA C -3'. The spectinomycin resistance gene, *aad9,* was released from pJRS312 (obtained from T. Koehler) using *Hin*dIII and ligated into the *Hin*dIII site of pBS-SK (Stratagene) to generate pBSSpc. The PCR product containing the *aphA3* promoter was digested with *Bam*HI and *Eco*RI and ligated into similarly digested pBSSpc plasmid to generate pBSprm. A 127-bp region of pXO2 (bp 32,492-32,618) containing the ori was amplified using the following primers containing *Eco*RI linkers: forward, 5'-CCGGAATTCGAAACACTATACGGCATATTGGAAGG -3' and reverse, 5'- CCGGAA TTCCTAGTGAATCCTGTAATTCCAAGACTG -3'. The resulting fragment was digested with *Eco*RI and ligated into pBSprm to generate pBSprmori. This plasmid contained the *aphA3* promoter upstream of the pXO2 ori such that the direction of transcription is co-linear with the leading strand synthesis. The plasmid pBSprmori was cut with *Bsm*I, and an oligonucleotides containing multiple cloning sites (MCS) having the sequence 5'-CTGCAGCCTAGGAGATCTGGCGCGCCACCGGTCCCGGGGAATGCA-3' was ligated into this site to generate pBSoriF (see Fig. 11B). This plasmid is similar to pBSprmori but also contains the MCS. A pBSoriF derivative lacking the pXO2 origin was also

Figure 11. Vector (A) and pXO2 origin constructs (B).



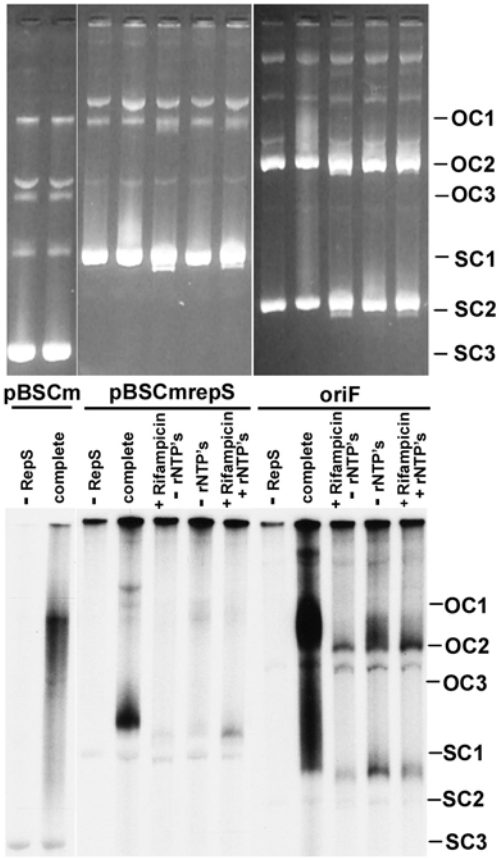
A. pBSMCS, vector used for ori clonings, pBluescript containing a spectinomycin resistance gene (spec^r/*aad9*) for selection in *B. anthracis*, the kanamycin promoter (P*aphA3*) and a multiple cloning site (MCS). **B.** Arrows indicate direction of transcription or leading strand synthesis; stem loops indicate placement of the *rrnB* transcriptional terminator; P*aphA3*, kanamycin promoter; ori, pXO2 origin of replication; *rrnB* ter, *E. coli rrnB* transcriptional terminator; P*bla*, *bla* promoter; CT, countertranscript; *repS*, *repS* ORF of pXO2.

generated by digesting this plasmid with *Eco*RI to release a small fragment containing the ori and religating the remainder of the plasmid to generate pBSMCS (Fig. 11A). Plasmid pSK236 is an *E. coli-S. aureus* shuttle vector consisting of the pUC19 and pC194 plasmids joined at their *Hin*dIII sites (unpublished data). The *repS* gene with its promoter (pXO2 bp 32,562-34,828) was amplified with the following primers containing *Bam*HI linkers: forward, 5'- CCGGATCCGTGGACAATTAAAAATTAACCAGCTG -3' and reverse 5'- CCGGATCCGTGTTGAAATGATTCAGACCAGTG -3'. The PCR product was digested with *Bam*HI and ligated into the *Bam*HI site of pSK236 to yield pSK236*repS* which is expected to express the RepS protein. This plasmid was introduced into the plasmidless *B. anthracis* strain UM23C1-1 (Green et al., 1985) to obtain a RepS-expressing strain of *B. anthracis*. The plasmids pBSoriF, containing the pXO2 ori with the *aphA3* promoter in the same orientation as leading strand synthesis and pBSMCS which lacks the pXO2 ori were introduced into the above *B. anthracis* strain. Transformants were obtained only with pBSoriF and agarose gel electrophoresis showed the presence of an appropriate size plasmid (not shown). These results showed that pBSoriF contains a functional origin of replication of pXO2 which is contained within a 127-bp region.

We tested the in vitro replication of the pXO2 miniplasmid (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. The MBP-RepS protein was purified by overexpression in *E. coli* and affinity chromatography as described earlier (Tinsley et al., 2004). In the absence of RepS, a low level of background signal was obtained in the in

vitro replication reaction with the pBSCmrepS plasmid (Fig. 12). Addition of MBP-RepS resulted in a significant increase in the signal and a DNA band migrating slightly slower than the SC form of the plasmid and presumably corresponding to a replication intermediate was observed (Fig. 12). Replication of pBSCmrepS was severely inhibited in the presence of rifampicin or in the absence of added rNTPs (CTP, GTP and UTP) or both (Fig. 12). These results demonstrated that RNA transcription is necessary for the replication of the pBSCmrepS plasmid. Since the pXO2 plasmid is predicted to replicate by a unidirectional theta-mode that utilizes an RNA primer for initiation at the ori (Bruand and Ehrlich, 1998), the above results are consistent with this possibility. Furthermore, based on the similarity of the pXO2 ori with those of the plasmids of the pAMβ1 family (Bruand et al., 1993), it is likely that the RepS mRNA provides the primer for the initiation of plasmid replication (see below). The replication signal obtained with the pBSCmrepS plasmid was weak, consistent with the low copy number of this plasmid in vivo (Koehler, 2002). The pBSoriF plasmid replicated in vitro much more efficiently than pBSCmrepS in the presence of the RepS protein (Fig. 12), demonstrating that transcription from another promoter can stimulate initiation of replication from the pXO2 ori. Since in vitro replication of pBSCmrepS was weak, it was possible that transcription of the *repS* gene (which may generate a primer for replication) is attenuated by a countertranscript within the promoter region as is the case with the pAMβ1-type plasmids (Le Chatelier et al., 1996). Northern blot analysis showed very low levels of a full-length *repS* transcript and high levels of 156 nt and 520 nt products (data not shown). Also, an antisense transcript of 220 nt was found to be encoded from the *repS* promoter region (not shown). Thus, since the *aphA3* promoter is likely to direct much

Figure 12 The *B. anthracis* extract supports in-vitro replication of pXO2 ori-containing plasmids when RepS and transcription are present.



Reactions as described in Fig. 10. pBSCm, vector control; pBSCmrepS, pXO2 wt minireplicon; pBSoriF, pXO2 ori cloned downstream of the *aphA3* promoter such that leading strand synthesis is colinear with *aphA3* transcription. OC1 and SC1, open circular and supercoil forms of pBSCmrepS respectively, OC2 and SC2, pBSoriF, OC3 and SC3, pBSCm.

higher levels of transcripts through the ori as compared to the *repS* promoter, increased replication levels of pBSoriF in vitro suggest that the level of transcription proceeding through the ori may determine the replication efficiency of the pXO2 plasmid. Although likely, whether such transcripts directly provide the primer for the initiation of pXO2 replication remains to be demonstrated.

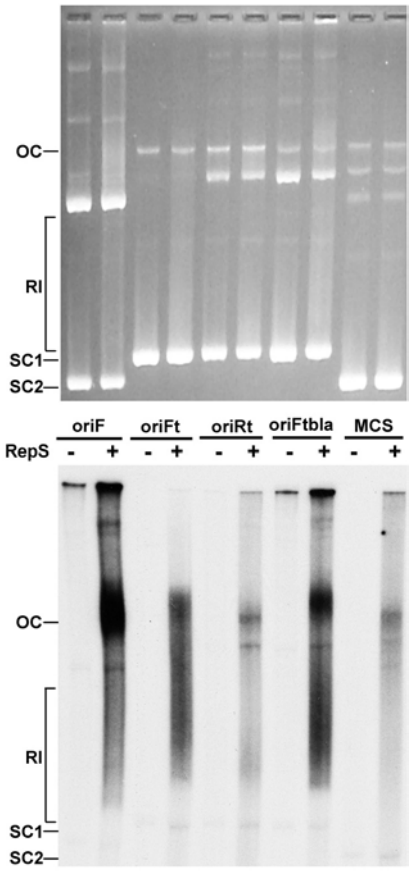
Requirement for directional transcription into ori for pXO2 replication. Plasmids of the pAMβ1 family require directional transcription into the ori for replication in vivo (Bruand and Ehrlich, 1998). Furthermore, the 3' end of the Rep mRNA is likely to provide the primer for replication (Bruand and Ehrlich, 1998). The in vivo requirement for directional transcription into the pXO2 ori was investigated by testing the ability of plasmids pBSoriF and pBSoriRt (Fig. 11B) to replicate in *B. anthracis* in the presence of the pSK236*repS* plasmid expressing the RepS protein. To generate pBSoriRt, the *E. coli rrnB* terminator was amplified by PCR using the pBR322 DNA as the template and the following primers: forward, 5'- TTGGCGCGC CGCTGT TTTGGCGGATGAG -3' and reverse, 5'- TTGGCGCGCCCAAAAAGAGTTTGTAGAAACGCAAAA -3'. The PCR product was ligated into the *Asc*I site of pBSoriF. A 658-bp *Eco*RV and *Bgl*II fragment containing the pXO2 ori and the *rrnB* terminator was isolated from this plasmid, filled-in using the Klenow fragment of DNA PolI and then ligated into the *Sma*I site of pBSMCS (Fig. 11A) yielding plasmids pBSoriFt and pBSoriRt which contain the ori in two different orientations with respect to the *aphA3* promoter (Fig. 11B). The pBSoriRt plasmid contains the *aphA3* promoter adjacent to the pXO2 ori such that transcription is co-linear 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 co-linear with leading strand synthesis (Fig. 11B). 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 the above strain. These results showed that directional transcription into the pXO2 ori is essential for replication.

We then tested the requirement for directional transcription into the ori for pXO2 replication in vitro. The pBSoriFt contains the *rrnB* terminator between the *aphA3* promoter and the ori and is expected to reduce/block transcription through the ori (Fig. 11B). Lastly, pBSoriFtbla was generated by cloning the *rrnB* terminator along with the *bla* promoter from pBR322 into the *Asc*I site of pBSoriF. The following primers were used to isolate the *rrnB* terminator and the *bla* promoter: forward, 5'-TTGGCGCGCCGCTGT TTTGGCGGATGAG -3' and reverse, 5'-TTGGCGCGCCGGTTATTGTCTCATGAGCGG -3'. In the pBSoriFtbla plasmid, transcription is expected to initiate from the *bla* promoter and proceed through the ori in the direction of leading strand synthesis (Fig. 11B).

As expected, plasmid pBSoriF replicated efficiently in *B. anthracis* extracts in the presence of MBP-RepS generating predominantly the OC form of DNA (Fig. 13). The presence of the *rrnB* terminator in plasmid pBSoriFt significantly reduced its replication (Fig. 13), suggesting that transcription through the ori is important for pXO2 replication. Limited levels of RepS-dependent DNA synthesis observed with pBSoriFt probably result either from limited transcription through the *rrnB* terminator or the signal may

Figure 13. Directional transcription into the pXO2 ori is important for replication.



Constructs as described in Fig. 11. OC, open circular DNA; RI, replication intermediates; SC1, supercoil form of oriFt, oriRt, and oriFtbla; SC2, supercoil form of MCS and oriF.

represent non-specific incorporation as seen with the pBSCm plasmid that lacks the pXO2 ori (Figs. 12 and 13). The presence of the *bla* promoter downstream of the *rrnB* terminator in the pBSoriFtbla plasmid resulted in increased replication. Only low-level background DNA synthesis was observed with the vector pBSMCS plasmid that lacks the pXO2 ori and the pBSoriRt plasmid which contains the ori but lacks directional transcription into the ori (Fig.13). 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 from *B. anthracis* or related organisms. This system supports the replication of both RCR plasmids as well as those that replicate by the theta-type mechanism. We have shown the utility of this system by demonstrating that this system can also support the replication of plasmids containing the pXO2 ori. Based on conservation of the ori sequence and genes encoding the initiator proteins, the pXO2 replicon belongs to the pAMβ1-type plasmids of Gram-positive bacteria that replicate by a unidirectional theta-type mechanism (Bruand et al., 1991; Bruand et al., 1993). In addition to the pAMβ1 plasmid of *Enterococcus faecalis*, this family also includes pRE25, pIP501, pSM19035 and pPLI100 (Brantl and Behnke, 1992b; Brantl et al., 1990; Schwarz et al., 2001). It is likely that our in vitro system will support replication of these plasmids as well. We have demonstrated that pXO2 replication requires directional transcription through the ori and our results are consistent with unidirectional theta-type replication of this plasmid. The in vitro replication system from *B. anthracis* should be of great value for the study of

replication of plasmids from *B. anthracis* and related organisms, including the pXO1 and related plasmids that encode the anthrax toxin.

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5.0 A NOVEL FTSZ-LIKE PROTEIN IS INVOLVED IN REPLICATION OF THE ANTHRAX TOXIN-ENCODING PXO1 PLASMID IN BACILLUS ANTHRACIS

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5.1 INTRODUCTION

Bacillus anthracis is a gram-positive, spore-forming bacterium that is the etiological agent of anthrax in humans (reviewed in references (Hanna, 1998; Koehler, 2002; Mock and Fouet, 2001). *B. anthracis* is genetically very closely related to *Bacillus cereus* and *Bacillus thuringiensis* (Helgason et al., 2000; Jensen et al., 2005; Schuch et al., 2002), and most of the chromosomal virulence-promoting genes of *B. anthracis* are also present in the latter organisms. A major difference between *B. anthracis* and related organisms is the presence of two large plasmids, pXO1 and pXO2, which are required for the virulence of this organism (Koehler, 2002; Mock and Fouet, 2001; Read et al., 2002; Van der Auwera et al., 2005). Recently, a strain of *B. cereus* has been described that contains a plasmid almost identical to pXO1 and causes an anthrax-like disease (Hoffmaster et al., 2004). The pXO1 plasmid (181.6 kb) encodes the anthrax toxin proteins termed the protective antigen, lethal factor, and the edema factor (Guidi-Rontani et al., 1999; Koehler, 2002; Mock and Fouet, 2001). This plasmid also contains genes that are involved in germination of the spores and genes such as *atxA* and *pagR* that regulate the expression of the anthrax toxin and other virulence factors (Bourgogne et al., 2003; Guidi-Rontani et al., 1999; Koehler, 2002; Pannucci et al., 2002a). Plasmid pXO1 also contains a number of genes resembling those involved in the horizontal transfer of plasmids (Green et al., 1989; Koehler, 2002; Pannucci et al., 2002a), suggesting that this plasmid may mediate its own transfer to other related organisms.

Very little is known about the replication properties of the pXO1 plasmid. Studies of the identification of the pXO1 replicon have been hampered since this plasmid does not

encode proteins that share significant similarity with known replication initiator proteins encoded by other plasmids (Chattoraj, 2000; del Solar et al., 1998; Khan, 1997; Osborn, 1999). Understanding the replication properties of pXO1 is critical for analyzing the potential of this plasmid to replicate and transfer the anthrax toxin-encoding genes in nature.

In this study, we describe the isolation of the replication region of the pXO1 plasmid. Interestingly, the putative replication initiator protein (RepX) of pXO1 shares limited homology with the FtsZ proteins of eubacteria that are involved in cell division. Genes nearly identical to *repX* were also present on plasmids in related organisms such as *B. cereus* and *B. thuringiensis*. Our results suggest that RepX may define a novel family of plasmid-encoded initiator proteins involved in the replication of virulence plasmids in *B. anthracis* and other members of the *B. cereus* group.

5.2 MATERIALS AND METHODS

Strains and plasmid construction. The *B. anthracis* Sterne 34F2 strain containing the pXO1 plasmid but lacking pXO2, as well as recombinant plasmids containing various regions of pXO1, was obtained from Theresa Koehler. A 10,138-bp PstI fragment of pXO1 (nucleotides [nt] 52397 to 62534 containing open reading frames [ORFs] 43 to 48 and a portion of ORF 42) was cloned into the PstI site of the *Escherichia coli* vector pBSCm containing the ColE1 replicon of *E. coli* and ampicillin and chloramphenicol resistance genes (Tinsley et al., 2004) to generate plasmid pC43-48P. The recombinant plasmid was recovered by transforming *E. coli* DH5α (Sambrook, 1989). The pC43-48P plasmid was then used as the source for subsequent subclonings. Plasmid pC43-48P was digested with XbaI and BamHI, and the 3,482-bp fragment (pXO1 nt 56685 to 60166 containing ORFs 45 and 46 and a portion of ORFs 44 and 47) was cloned into BamHI- and XbaI-digested pBSCm to generate plasmid pC45-46XB. Plasmid pC43-48P was digested with EcoRV and PstI, and the 3,441-bp fragment (nt 59094 to 62534 containing ORFs 47 and 48 and a portion of ORF 46) was ligated into pBSCm digested with EcoRV and PstI to generate pC47-48EP. Plasmid pC43-48P was digested with SacI to remove a 3,999-bp fragment containing ORFs 45 to 48, and the DNA with the remaining 6,187-bp region of pXO1 was religated to generate pC43-44S (pXO1 nt 52397 to 58583 containing ORFs 43 and 44 and a portion of ORFs 42 and 45). Similarly, BamHI was used to remove a 2,376-bp fragment containing ORFs 47 and 48 from pC43-48P, and the plasmid with the remaining 7,770-bp region of pXO1 was religated to generate pC43-46B (pXO1 nt 52397 to 60167 containing ORFs 43 to 46 and portions of ORFs 42 and 47). The plasmid pC43-45SMB was generated by deleting a

large segment of ORF 42 by digesting pC43-46B with SwaI and MscI and religating the plasmid containing the 5,304-bp region of pXO1 (nt 54863 to 60167). The plasmid pCΔ44 was constructed by introducing a frameshift into ORF 44 (see below) and by digesting the resulting plasmid with XbaI, thus releasing a 297-bp internal fragment from ORF 44 (nt 56388 to 56684); the resulting 5,803 and 3,494-bp fragments were religated, maintaining the correct orientation of the gene in the plasmid (pXO1 nt 54863 to 56387 and 56685 to 60166). For complementation experiments, a new shuttle vector (pBSKm) was produced by inserting a HindIII fragment from pUC4Ωkan (Perez-Casal et al., 1991) containing the *aphA3* (Km^r) gene into the HindIII site of pBSIISK. The plasmid pK43-46SM was generated by digesting pC43-46B with SpeI and SwaI to delete the majority of ORF 42, yielding a 5,304-bp region of pXO1 (nt 54863 to 60166), and ligating this fragment to SpeI/SmaI-digested pBSKm. This plasmid was used as the source of RepX for the complementation of plasmids lacking a functional *repX* gene. The plasmid pXori1 was obtained by digesting pCΔ44mut43 with EcoRV and cloning the 738-bp fragment (nt 55617 to 56355) into the EcoRV site of pBSCm. The plasmid pXori was generated by amplifying a 158-bp region of pXO1 (nt 55726 to 55883) using the following primers containing BamHI linkers: 5'-CCGGATCCGATGCAAATTGTAAATTCATTATC-3' and 5'-CCGGATCCGGTGTTAGAATAGCGATTGAAC-3'. The reaction mixtures (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/ml nuclease-free bovine serum albumin, 200 µM of each deoxynucleotide triphosphate, 10 ng of plasmid DNA, 1 µM of each primer, and 2.5 units of *Pfu* polymerase (Stratagene, La Jolla, CA). The conditions of PCR amplification were as follows: 95°C for 3 min; 95°C for 1 min, 65°C for 1 min, and 72°C for 1 min for 25

cycles; and 72°C for 10 min. The PCR product and pBSCm were digested with BamHI, and the DNA was ligated to generate pXori. All the above plasmids were passaged through *E. coli* GM2163 (*dam dcm* mutant), which facilitated the subsequent introduction of plasmid DNA into *B. anthracis* (Marrero and Welkos, 1995). Plasmid DNAs (10 to 15 µg) were then electroporated into the plasmidless *B. anthracis* strain UM23C1-1 (Dunny et al., 1991; Green et al., 1985; Koehler et al., 1994) to test for the presence of a functional replicon. Plasmids which did not generate any *B. anthracis* UM23C1-1 transformants in at least three independent experiments were subsequently electroporated into *B. anthracis* containing the RepX-expressing pK43-46SM plasmid to test for complementation.

Mutagenesis of various pXO1 ORFs. Translational frameshifts were introduced into ORFs 43, 44, 45, and 46 using the Stratagene QuikChange kit according to the manufacturer's instructions. ORF 43 was mutagenized using two complementary mutagenic primers containing pXO1 nt 55715 to 55749 but lacking the A residue at nt 55728, which should result in the loss of an SfaNI site. ORF 44 was mutagenized using two complementary mutagenic primers containing pXO1 nt 57160 to 57194 but lacking the A residue at position 5716,7 which is expected to result in the loss of a DraI site. Similarly, ORF 45 was mutagenized using primers containing pXO1 nt 58907 to 58942 but lacking a T at position 58934, resulting in the loss of a StyI site. ORF 46 was mutagenized using primers containing pXO1 nt 59327 to 59359 and introducing GC between nt 59347 and 59348, thus generating a new HindIII site. Use of the above primers is expected to result in frameshifts at amino acid positions 7, 19, 11, and 3 of ORFs 43, 44, 45, and 46, respectively. A GTP binding mutant of ORF 45 was made

using primers containing pXO1 nt 58579 to 58611 with bp 58591 to 58593 changed from CAG to GCA, which is expected to result in a Thr→Ala mutation at amino acid 125 and the loss of an AlwNI site. The sequences of the primers for mutagenesis of the various ORFs were as follows:

ORF 43, 5'-GATAATGAATTTACAATTTGCTCAACAACGAATG-3' and

5'-CATTCGTTGTTGAGCAAATTGTAAATTCATTATC-3'

ORF 44, 5'-CTCTTTTAACGAAAAGATTTCTGTGTGCCTTTAC-3' and

5'-GTAAAGGCACACAGAAATCTTTTCGTTAAAAGAG-3'

ORF 45, 5'-GAATCCAAATTTCAAACTAATATTTCCTGGCTTTC-3' and

5'-GAAAGCCAGGAAATATTAGTTTGAAATTTGGATTC-3'

ORF 46, 5'-GGTGGTGTGAAAGCTTAGTGTCTAATATATCAATG-3' and

5'-CATTGATATATTAGACACTAAGCTTTCACACCACC-3';

GTP binding mutant of ORF 45, 5'GTGGTCTTGGTGGAGGAAGCAGAACTGGAGCTC-

3' and 5'-GAGCTCCAGTTCTGCTTCCTCCACCAAGACCAC-3'.

The reaction mixtures (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/ml nuclease-free bovine serum albumin, 200 µM of each deoxynucleotide triphosphate, 75 ng of plasmid DNA, 125 ng of each primer, and 2.5 units of *Pfu* Turbo polymerase (Stratagene, La Jolla, CA). The

conditions of PCR amplification were as follows: 95°C for 30 s; 95°C for 30 s, 55°C for 1 min, and 68°C for 10 min for 12 cycles for ORFs 44 and 45, 14 cycles for ORFs 43 and 46, and 16 cycles for the GTP binding mutant; and 68°C for 10 min. The reaction products were treated with 20 units 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 plasmids were recovered by transforming *E. coli* DH5α, and miniplasmid preparations were screened by digestion with SfaNI for ORF 43, DraI for ORF 44, StyI for ORF 45, HindIII for ORF 46, and AlwNI for the GTP binding mutant of ORF 45. The sequences of all the above mutants were confirmed by automated DNA sequencing.

5.3 RESULTS

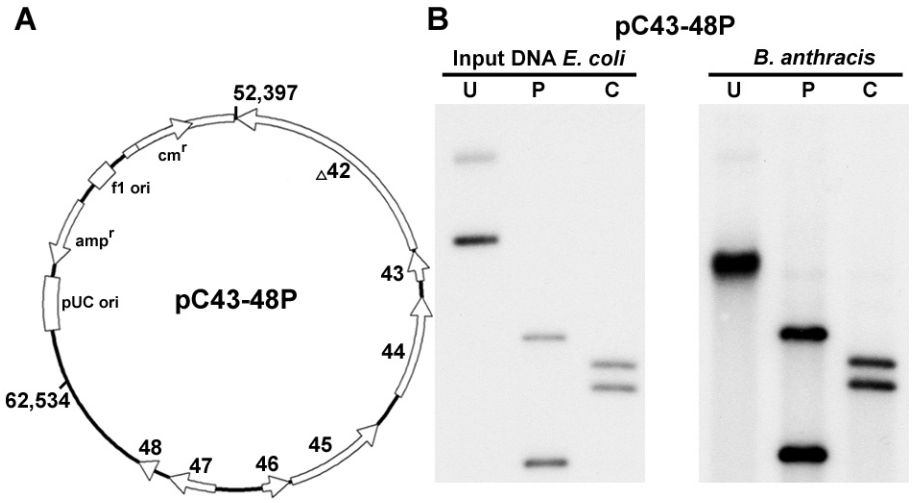
Analysis of the pXO1 sequence for the identification of the putative replicon. Most plasmids with sequenced genomes contain genes encoding a replication initiator protein. An alignment of such genes has shown that various plasmids can be grouped into plasmid families which share significant homologies in their replication initiator genes and the origin of replication (Chattoraj, 2000; del Solar et al., 1998; Khan, 1997). However, in our homology searches using a variety of software we failed to identify homologs of known replication initiator proteins in the pXO1 sequence. The genes encoding replication initiator proteins of both chromosome and plasmids are generally located in the vicinity of their replication origins (Chattoraj, 2000; del Solar et al., 1998; Khan, 1997). In order to identify the putative replicon of the pXO1 plasmid, we relied upon GC skew, strand-specific biases such as gene orientation, plasmid-specific oligomer skew analysis, and origin comparisons (Hallin and Ussery, 2004; Rocha, 2004; Worning et al., 2006) provided by the Genome Atlas Database at http://www.cbs.dtu.dk/services/GenomeAtlas/ to predict the location of an origin of replication (ori). This suggested two possible locations of the putative ori, the region of pXO1 from nt 40000 to nt 60000 and near nt 150000. The pXO1 region near position 150000 falls within the pathogenicity island encoding the anthrax toxin, which is potentially a mobile element and unlikely to contain the ori (Okinaka et al., 1999b). Therefore, we postulated that the region near position 60000 is more likely to contain the pXO1 ori. A close examination of the pXO1 sequence also showed that the region between nt 50000 and 60000 contained many ORFs that were conserved in other large plasmids of the *B. cereus* family and therefore may be involved in essential plasmid

functions such as replication and maintenance. Based on the above analyses, we focused on this region in our attempts to identify the pXO1 replicon.

Cloning of a 10,138-bp region containing the pXO1 replicon. We generated several plasmid constructs that contained sections of the nt 52000 to 62000 region of the pXO1 plasmid. A 10,138-bp PstI fragment of pXO1 (NC_001496; positions 52397 to 62534 containing ORFs 43 to 48 and a portion of ORF 42) was cloned into the *E. coli* vector pBSCm to generate the plasmid pC43-48P (Fig. 14A). The resulting plasmid was isolated from the *dam dcm* mutant strain of *E. coli* GM2163 and electroporated into the plasmid-free *B. anthracis* strain UM23C1-1. Plasmid DNA from Cm^r *B. anthracis* transformants was isolated and digested with PstI or ClaI. Agarose gel electrophoresis followed by Southern blot analysis showed that both the supercoiled form of the pC43-48P DNA and its restriction digests were identical to the plasmid DNA isolated from *E. coli* (Fig. 14B). The slight difference in the migration of supercoiled plasmid DNA isolated from *E. coli* and *B. anthracis* was seen only when the DNA was prepared from *B. anthracis* using a maxiprep procedure and not when a miniprep procedure was used (data not shown). No *B. anthracis* transformants were obtained when the pBSCm plasmid was introduced into *B. anthracis*. These results suggested that the above 10,138-bp region contains the functional replicon of the pXO1 plasmid.

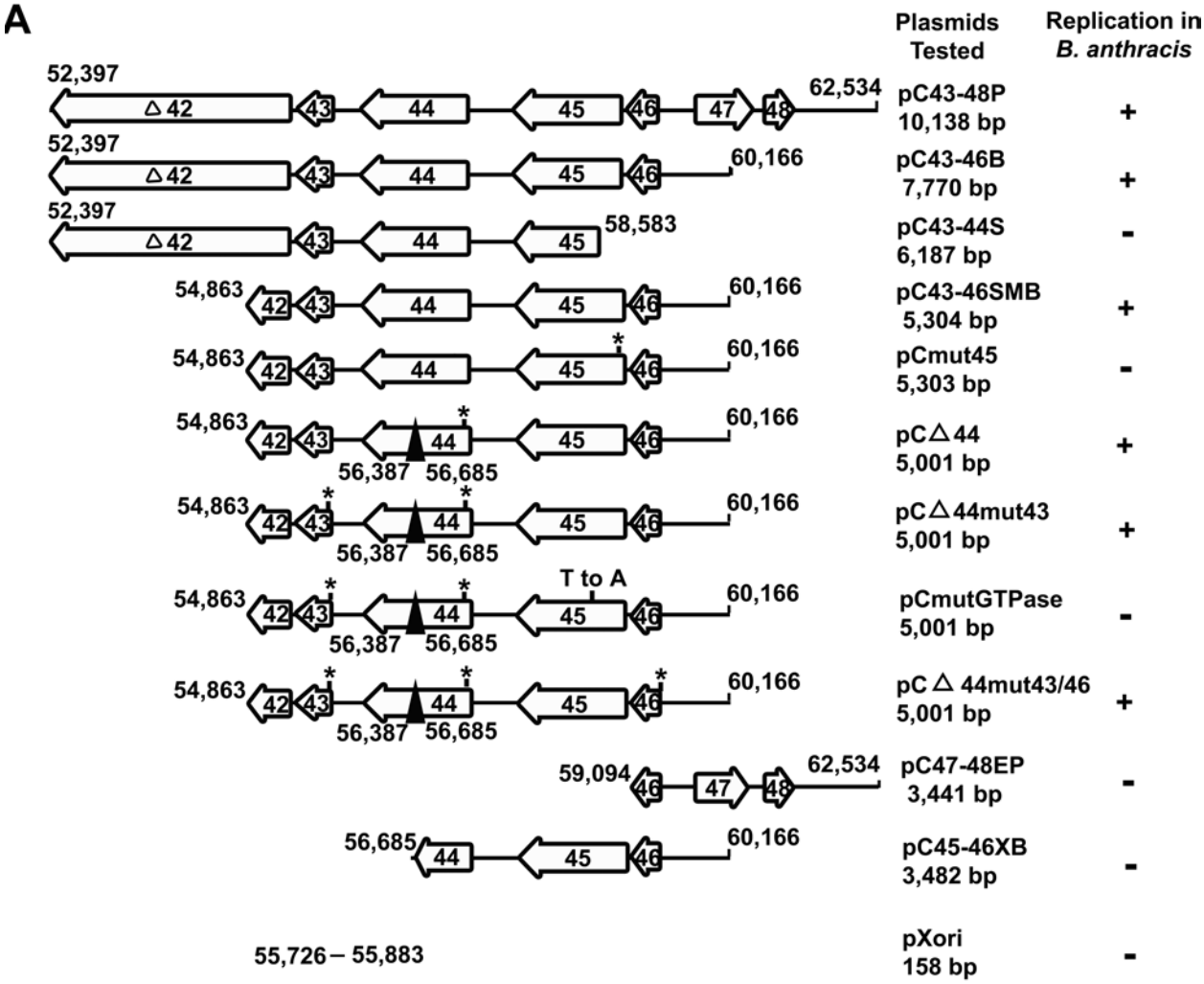
Deletion analysis of the pXO1 replicon. We subcloned various subregions of the 10,138-bp pXO1 replicon into the pBSCm plasmid of *E. coli* to further identify the elements involved in plasmid replication (Fig. 15A). All constructs were isolated from *E. coli* GM2163 and electroporated into *B. anthracis* UM23C1-1. Both pC43-46B

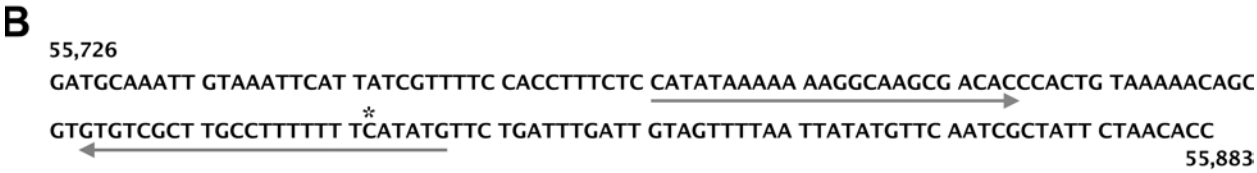
Figure 14. Replication of a mini-pXO1 plasmid in *B. anthracis*



A. The pC43-48P plasmid containing a 10-kb region of pXO1 (positions 52397 to 62534). Plasmid pXO1 ORFs and their direction of transcription are shown. **B.** Southern blot analysis of plasmid DNA isolated from a *B. anthracis* strain transformed with the pC43-48P plasmid. Input DNA from *E. coli* is shown in the left panel, and the DNA isolated from *B. anthracis* is in the right panel. U, uncut plasmid DNA; P, PstI-cleaved DNA; C, ClaI-cleaved DNA.

Figure 15. The pXO1 replicon and its putative origin of replication





A. Regions of the pXO1 replicon cloned into an *E. coli* plasmid. Boxes with arrows indicate the various ORFs and their directions of transcription. The numbers correspond to the nucleotide coordinates of pXO1. Asterisks correspond to the deletion of 1 bp in various ORFs except for ORF46 in pCΔ44mut43/46, in which 2 bp have been inserted. Plasmid pK43-46SM (not shown) is similar to pC43-46SMB except that it contains a Km^r marker in place of the Cm^r gene. The sizes of the pXO1 replication regions and their ability to replicate in a plasmid-negative derivative of *B. anthracis* are indicated on the right. **B.** Nucleotide sequence of the putative origin of replication of pXO1. The arrows show an IR sequence. The asterisk corresponds to an extra C residue in one arm of the IR. The numbers correspond to the nucleotide coordinates of pXO1.

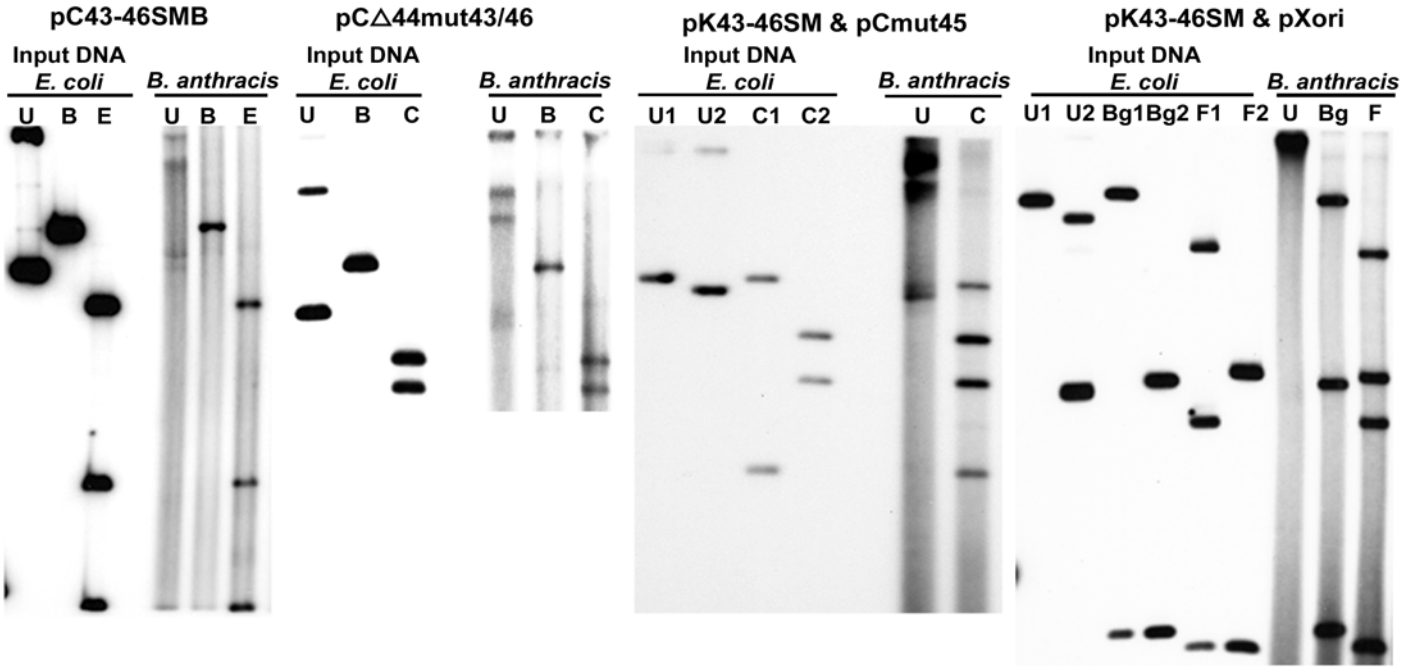
(containing a 7,770-bp region of pXO1 from nt 52397 to nt 60166 that includes ORFs 43 to 46 and a majority of ORF 42, 983 amino acids out of 1,109) and pC43-46SMB (containing a 5,304-bp region of pXO1 from position 54863 to 60166 including ORFs 43 to 46 and a small portion of ORF 42, 160 amino acids out of 1,109) yielded Cm^r transformants, and analysis of plasmid DNA by agarose gel electrophoresis followed by Southern blot hybridization showed that these plasmids replicated in *B. anthracis* (Fig. 16 and data not shown). The restriction digests of plasmid DNAs isolated from *B. anthracis* were also identical to the input plasmid DNA isolated from *E. coli* (Fig. 16). On the other hand, plasmids pC45-46XB, pC47-48EP, and pC43-44S (Fig. 15A) failed to generate any *B. anthracis* transformants in at least three independent experiments. The above results showed that the functional pXO1 replicon is present within a 5,304-bp region (positions 54863 to 60166) contained on pC43-46SMB. Generally, for the results shown in Fig. 15A, 10 to 30 *B. anthracis* transformants were obtained with plasmid constructs that replicated in this organism, while no transformants were obtained with nonreplicating plasmids (in at least three independent experiments).

The product of ORF 45 is required for pXO1 replication. Experiments were carried out to identify the gene encoding the replication initiator protein of plasmid pXO1. The pC43-46SMB plasmid which replicates in *B. anthracis* contains two major pXO1 ORFs, 44 and 45 (Fig. 15A). In addition, it contains two smaller ORFs, 43 and 46 (encoding putative proteins of 128 and 119 amino acids, respectively), and two truncated ORFs, 42 and 47 (Fig. 15A). We, therefore, considered it highly likely that ORF 44 or 45 may encode the essential replication initiator protein of pXO1. To identify this protein, we generated the deletion mutant pCΔ44, which lacks 99 amino acids (positions 180 to

278) and also contains a translational frameshift in ORF44. We also introduced a frameshift in ORF 45 in a separate plasmid, pCmut45. The above mutant plasmids were isolated from *E. coli* and introduced into *B. anthracis*. Cm^r *B. anthracis* transformants were obtained with plasmid pCΔ44 but not with pCmut45 in five independent experiments. The presence of the appropriate plasmid DNA in *B. anthracis* transformed with pCΔ44 was confirmed by agarose gel analysis and Southern blot hybridization (data not shown). To rule out the involvement of smaller ORFs 43 and 46 in pXO1 replication, we also generated frameshift mutations in these ORFs in the context of the above pCΔ44 plasmid and found that the pCΔ44mut43/46 plasmid was able to replicate in *B. anthracis* (Fig. 16).

The above studies suggested that ORF 45 (encoding RepX) is required for pXO1 replication. Homology searches revealed that RepX has similarity to the GTPases of the FtsZ family (see below). We generated a Thr→Ala mutation at amino acid position 4 of the putative GTP binding domain (GGGTGT/SG) of *repX* using pCΔ44mut43 as a template. In the case of *E. coli* FtsZ, such a mutation is lethal and leads to a marked decrease in its GTPase activity although FtsZ retains its ability to form multimers (Mukherjee et al., 1993). The resulting plasmid, pCmutGTPase, did not generate any transformants in *B. anthracis* in five independent experiments. However, both pCmut45 and pCmutGTPase replicated when introduced into a *B. anthracis* strain containing the Km^r plasmid pK43-46SM, which differs from pC43-46SMB only with respect to the resistance marker used (Fig. 16 and data not shown). Taken together, the above results suggest that ORF 45 encodes the essential RepX protein that may

Figure 16. Southern blot analysis of plasmid DNA isolated from *B. anthracis* strains transformed with various pXO1 miniplasmids



Input DNAs from *E. coli* are shown on the left, and the DNA isolated from *B. anthracis* is on the right in each panel. U, uncut plasmid DNA; B, BamHI; C, ClaI; E, EcoRI; Bg, BglI; F, FspI. The two extreme right panels show a *B. anthracis* strain containing a functional pXO1 replicon expressing RepX (pK43-46SM) that can complement the replication of the pCmut45 (third panel from left) or pXori (fourth panel from left) plasmids in *trans*. U1 and C1, uncut or ClaI-digested pK43-46SM DNA, respectively; U2, uncut pCmut45 (third panel) or pXori (fourth panel) DNAs; C2, ClaI-digested pCmut45 DNA (third panel). In the fourth panel, Bg1 and F1 represent pK43-46SM DNA digested with BglI and FspI, respectively, while Bg2 and F2 represent pXori DNA digested with the same enzymes.

correspond to the replication initiator protein of plasmid pXO1, and GTP binding/hydrolysis may be important for its function.

Identification of the putative origin of replication of plasmid pXO1. The plasmid pC45-46XB containing the *repX* gene (Fig. 15A) does not replicate in *B. anthracis*, suggesting that it may lack the origin of replication of pXO1. To test this possibility, the Cm^r plasmid pXori (containing nt 55726 to 55883; Fig. 15B), which does not replicate in *B. anthracis* UM23C1-1, was introduced into a *B. anthracis* strain containing pK43-46SM that expresses RepX. Agarose gel electrophoresis and Southern blot hybridization of plasmid DNAs isolated from Km^r and Cm^r doubly resistant colonies and digested with two different restriction enzymes showed the presence of both pXori and pK43-46SM plasmids (Fig. 16). Note that only nicked open-circular DNA was observed in the lane containing undigested plasmid DNA from *B. anthracis*. In similar experiments, pK43-46SM failed to complement the replication of the pC45-46XB plasmid (not shown). Plasmid pXori1 (containing a 738-bp fragment, Fig. 15A; nt 55617 to 56355), which is unable to replicate in *B. anthracis*, was also found to replicate in the presence of the pK43-46SM plasmid (data not shown). The above results suggested that the functional pXO1 origin is contained within a 158-bp region (nt 55726 to 55883) present in pXori. Furthermore, these results also showed that the RepX protein can act in *trans* and support the replication of an origin-containing plasmid.

5.4 DISCUSSION

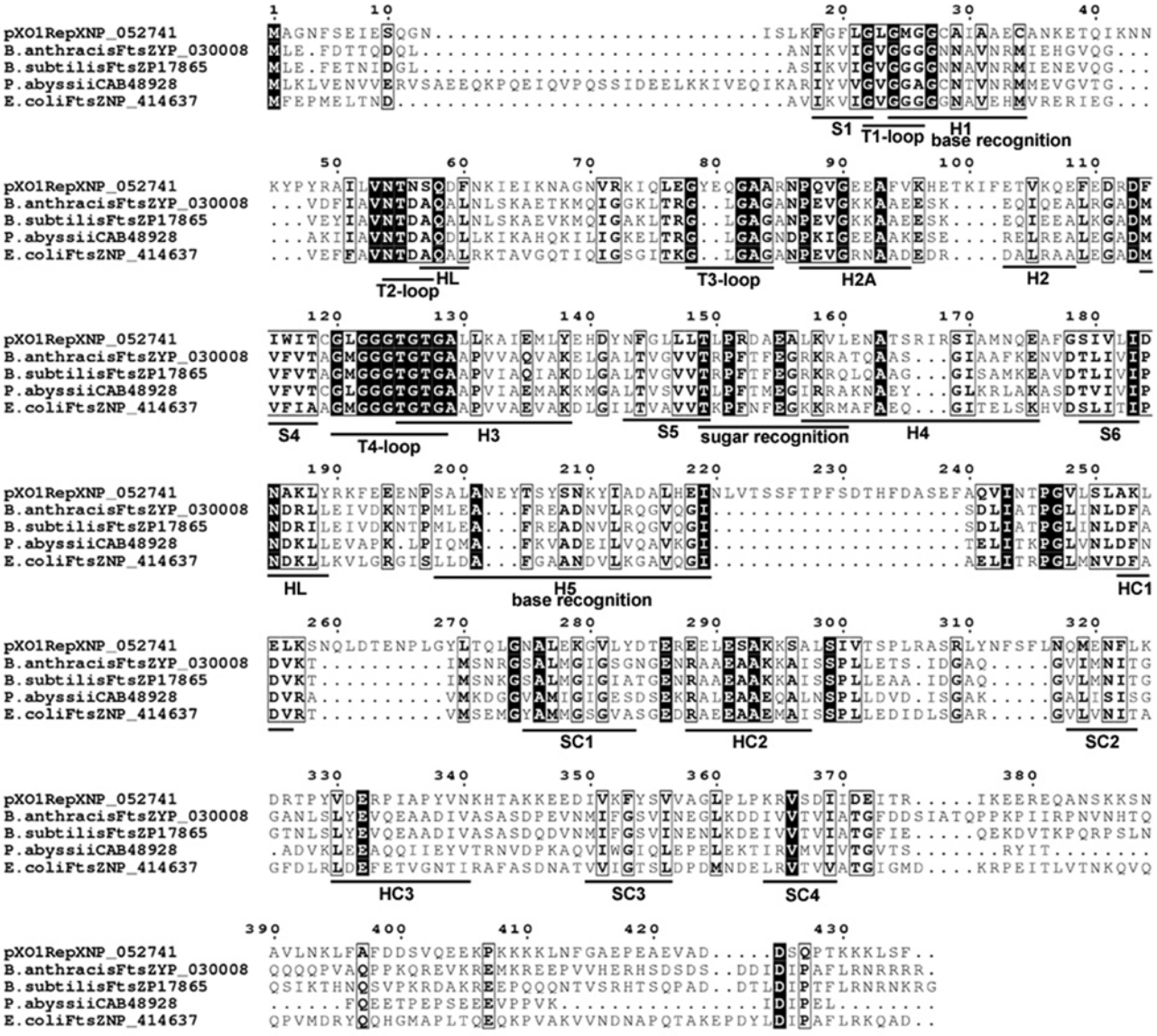
We have identified the replicon of the pXO1 plasmid to within a 5.3-kb region since plasmid pC43-46SMB (pXO1 nt 54863 to 60166) replicated autonomously in *B. anthracis* (Fig. 15A and 16). Within this region, the product of ORF 45 (RepX) is essential for pXO1 replication and may correspond to its replication initiator protein (see below). Since plasmid pC45-46XB containing an intact ORF 45 did not replicate in *B. anthracis* (Fig. 15A), it suggested that the pXO1 origin of replication may require sequences upstream of position 56685. Therefore, we tested the ability of plasmid pXori1 containing an upstream 738-bp region of pXO1 (nt 55617 to 56355) to replicate in the presence of the RepX-expressing plasmid pK43-46SM. Plasmid pXori1 replicated in *B. anthracis* (data not shown), demonstrating that the functional origin of replication of pXO1 is contained within the above 738-bp region. This region contains an inverted repeat (IR) of 24 nt (Fig. 15A), which is a common feature of origins of replication. Plasmid pXori containing a 158-bp region (nt 55726 to 55883) that includes the above IR was also found to replicate in the presence of the RepX-expressing plasmid (Fig. 16). These data showed that the functional pXO1 ori is contained within a 158-bp region (nt 55726 to 55883). The requirement of the above IR sequence in pXO1 origin function is currently unknown and will be the subject of future study. A few plasmids such as R6K and F contain multiple replicons/origins of replication. Whether the replicon identified in this study represents the only replicon/origin of plasmid pXO1 is currently unknown. Extensive homology searches by our laboratory as well as by others have failed to identify significant similarity between pXO1-encoded proteins and initiator proteins that have been shown to be involved in the initiation of plasmid or chromosome DNA

replication (Chattoraj, 2000; del Solar et al., 1998; Khan, 1997). Interestingly, plasmids in several members of the *B. cereus* group which are closely related to *B. anthracis* contain sequences that are nearly identical to the *repX* gene. Two fully sequenced plasmids of *B. cereus*, pBCXO1 and pBC10987, which are very similar to pXO1, contain orthologs of *repX* with 99.8% and 98.4% identity, respectively (Hoffmaster et al., 2004; Rasko et al., 2004). Furthermore, DNA hybridization and PCR studies have shown that the *repX* gene is also contained on two megaplasmids of approximately 330 kb present in *B. cereus* 43881 and *B. thuringiensis* 33679 (Berry et al., 2002; Pannucci et al., 2002a). We propose that RepX defines a novel family of replication initiator proteins that are involved in the replication of megaplasmids of *B. anthracis* and other members of the *B. cereus* group. Orthologs of various plasmid initiator proteins are spread throughout the bacterial kingdom (Chattoraj, 2000; del Solar et al., 1998; Khan, 1997; Osborn, 1999) (http://www.essex.ac.uk/bs/staff/osborn/DPR_home.htm). Therefore, it is intriguing that RepX is restricted to *B. anthracis* and closely related organisms. In addition to a plasmid-encoded initiator protein, plasmid replication requires a large number of chromosome-encoded proteins (for a review, see reference (del Solar et al., 1998). It is possible that one or more proteins required for the replication of pXO1-like plasmids are present only in *B. anthracis* and other members of the *B. cereus* group. This may explain why such plasmids encoding the anthrax toxin and other virulence factors are not widely distributed in nature. Future studies should reveal the molecular basis for the predicted narrow host range of such plasmids.

An alignment of the RepX protein using the BLAST (Altschul, 1990), ClustalW, and EMBOSS programs (global, conserved domains and pairwise/local alignments) revealed

that it shares limited similarity with the FtsZ proteins of several bacteria. For example, RepX is approximately 20 to 22% identical and 32 to 38% similar to the chromosome-encoded FtsZ proteins of *E. coli*, *Bacillus subtilis*, *B. anthracis*, and *Pyrococcus abyssi*. The RepX protein consists of 435 amino acids while the various FtsZ proteins range in size from 350 to 400 amino acids (Lee et al., 2003). Several regions of identity/similarity between RepX and FtsZ proteins are located between amino acids 1 and 320 of RepX (Fig. 17). The predicted folded structure of RepX using the ESyPred3D program (Lambert et al., 2002); data not shown) is very similar to the structure of *Methanococcus jannaschii* FtsZ determined by X-ray crystallography (Lowe and Amos, 1998). Bacterial FtsZ proteins are functional homologs of eukaryotic tubulin and are critical for cell division (Carballido-Lopez and Errington, 2003; Weiss, 2004). FtsZ forms a dynamic ring structure (Z-ring) in a GTP-dependent manner that mediates cell division (Carballido-Lopez and Errington, 2003). The Z-ring then recruits cellular proteins such as FtsA, ZipA, MinC, and others that promote cytokinesis (Carballido-Lopez and Errington, 2003; Weiss, 2004). The FtsZ and tubulin proteins contain a conserved guanine nucleotide binding motif, GGGTGT/SG (Carballido-Lopez and Errington, 2003; Weiss, 2004). This motif is totally conserved in the RepX protein (amino acids 122 to 128; Fig. 17), and we have purified the RepX protein as a fusion with the maltose binding protein epitope at its amino-terminal end and found that it has a robust GTPase activity (data not shown). Our mutational and complementation analyses showed that GTP binding and/or GTPase activity of RepX is critical for its function as a replication protein (Fig. 15A and data not shown). Since pXO1 is not essential for *B. anthracis* growth (Koehler, 2002), and the

Figure 17. Alignment of RepX (ORF 45) with various FtsZ proteins



Amino acid numbering corresponds to that of RepX. The shaded regions indicate amino acid identity, while those in boxes indicate similarity. The various motifs of FtsZ are shown based on the crystal structure of the *Methanococcus jannaschii* FtsZ protein (Lowe and Amos, 1998).

chromosome of this organism encodes the FtsZ protein, RepX is unlikely to share the cell division function of the FtsZ proteins. The carboxyl-terminal region of FtsZ is involved in protein-protein interactions that play important roles in the recruitment of proteins to the septal ring (Weiss, 2004). This region of RepX does not share significant homology with FtsZ proteins and may be involved in its replication-specific function. Since pXO1 is a low-copy-number plasmid (one or two per chromosome), it is possible that RepX domains that are similar to FtsZ may position the replicated pXO1 plasmids at the septal ring and promote its segregation to the daughter cells. Future studies should reveal the precise role of RepX in plasmid pXO1 replication and segregation. RepX may provide a novel drug target for the elimination of anthrax toxin-producing plasmids from *B. anthracis* and other organisms into which pXO1 may either transfer naturally or be introduced intentionally by bioterrorists.

6.0 PURIFICATION OF THE PXO1 REPLICATION PROTEIN REPX, CHARACTERIZATION OF ITS BIOCHEMICAL PROPERTIES, AND FURTHER ANALYSIS OF THE PXO1 ORIGN OF REPLICATION

6.1 INTRODUCTION

The pXO1 replication protein, RepX, is required for replication of the minireplicon of pXO1 and shares limited homology with the FtsZ family of GTPases. The main amino acid identity is shared within the GTPase domain, and as mentioned previously (Chapter 5), mutating the motif involved in GTP binding and hydrolysis abrogates replication of the pXO1 replicon in-vivo. As the only plasmid-encoded protein required for replication, RepX is likely to serve as an initiator protein, which would require specific binding to the pXO1 ori for replication to occur. However, the FtsZ proteins are occluded by DNA though their organization and location for septation is tightly linked to DNA replication (Romberg and Levin, 2003). Therefore, this study addressed RepX biochemical activities and ability to bind to DNA and further assessed what DNA sequence was serving as the ori for pXO1.

6.2 MATERIALS AND METHODS

RepX Overexpression and Purification. RepX and RepXmut purification was done by modifying a published procedure (Lu et al., 1998). RepX was overexpressed as an N-terminal MBP tagged protein using pMmalp2X from NEB. The BL21 strain was used to reduce protein degradation, and one liter culture was grown to an OD_{600}=0.4. Induction was done using a final concentration of 1 mM IPTG (Invitrogen) for 2.5 h at 37˚C. Cells were washed with 200 ml of TE (pH 8) and stored at -80˚C. Cells were thawed on ice and resuspended to a final volume of 18 ml using lysis buffer TEN (0.05 M Tris pH 8, 0.1 M NaCl, 1 mM EDTA) containing PMSF. Two freeze/thaw cycles were done, and lysozyme was added to a final concentration of 4 mg/ml. Cells were incubated on ice with mixing every 7-8 min for 30 min followed by addition of $MgCl_2$ to a final concentration of 5 mM. Cells were again freeze/thawed twice. The lysate was ultracentrifuged at 33 K rpm for 1h at 4˚C, and 1.2ml of amylose resin was equilibrated in TEN buffer. The lysate was allowed to bind to the amylose for 1h and loaded onto a column. The resulting resin was washed with 2 column volumes of TEN without PMSF and, if proceeding to factor Xa cleavage, once with Xa cleavage buffer (20 mM Tris pH 6.8, 50 mM NaCl, and 1 mM $CaCl_2$). Otherwise, the column was incubated with RepX storage buffer (0.05 M Tris pH 8 , 0.1 M NaCl, 1 mM EDTA, 10 mM $MgCl_2$, 10% ethylene glycol , and 5 mM DTT) containing 10 mM maltose. For Xa cleavage, the amylose resin was resuspended in 1 ml of Xa cleavage buffer, and 24 µg of Xa protease was added. The resin was then incubated at 4˚C overnight with rocking. The resin was spun a 5 K for 5 min, and the supernatant was collected and dialyzed in RepX binding buffer (50 mM Tris pH 7.5, 50 mM KCl, and 5% glycerol) for 3h. The Cibicron

Blue HiTrap 1 ml column (GE/Amersham) was equilibrated with 20 ml RepX binding buffer, and the dialyzed protein was bound to the column for 1 h. The column was washed with 20 ml of RepX binding buffer and eluted with RepX storage buffer containing 2 M NaCl. Protein was dialyzed for 5-6 h with one buffer change in RepX storage buffer.

RepX NTPase assays. NTPase assays were done similarly to the assays described in (RayChaudhuri and Park, 1992). Reactions were done in a final volume of 20 μl in a final concentration of 1X Buffer B (50 mM Tris-HCl, pH 7.5, 50 mM KCl, 5% glycerol and 0.5 mM $MgCl_2$) and 1 μCi of [αP^{32}]dNTP or NTP. Various dilutions of RepX were made (0-12 μM) and added to the reaction and incubated at 32˚C for 1 h. An equal volume of stop solution (1% SDS and 20 mM EDTA) was added to each reaction and incubated at 75˚C for 2 min. A total of 8 μl of the resulting reaction mixture was spotted on PEI cellulose and the solvent front was allowed to run to the top in 0.5 M KH_2PO_4 pH 3.5. TLC plates were subjected to autoradiography.

RepX EMSA assays. Restriction fragments from pXO1 were purified from agarose gels using SpinX columns and end-labeled with PNK. A concentration of 0-250 μM of protein was added to 1X EMSA buffer (50 mM Tris pH7.5 (unless otherwise stated), 50 mM KCl, 0-5 mM $MgCl_2$, 1mM DTT and 10% ethylene glycol) per reaction. Reactions (total volume 25 μl) were incubated at room temperature for 30 min. If formaldehyde crosslinking was used, 1 μl of 5% formaldehyde was added per reaction and incubated for 5 min. A total of 2.5 μl 2.5 M glycine was added per reaction for 5 min at room

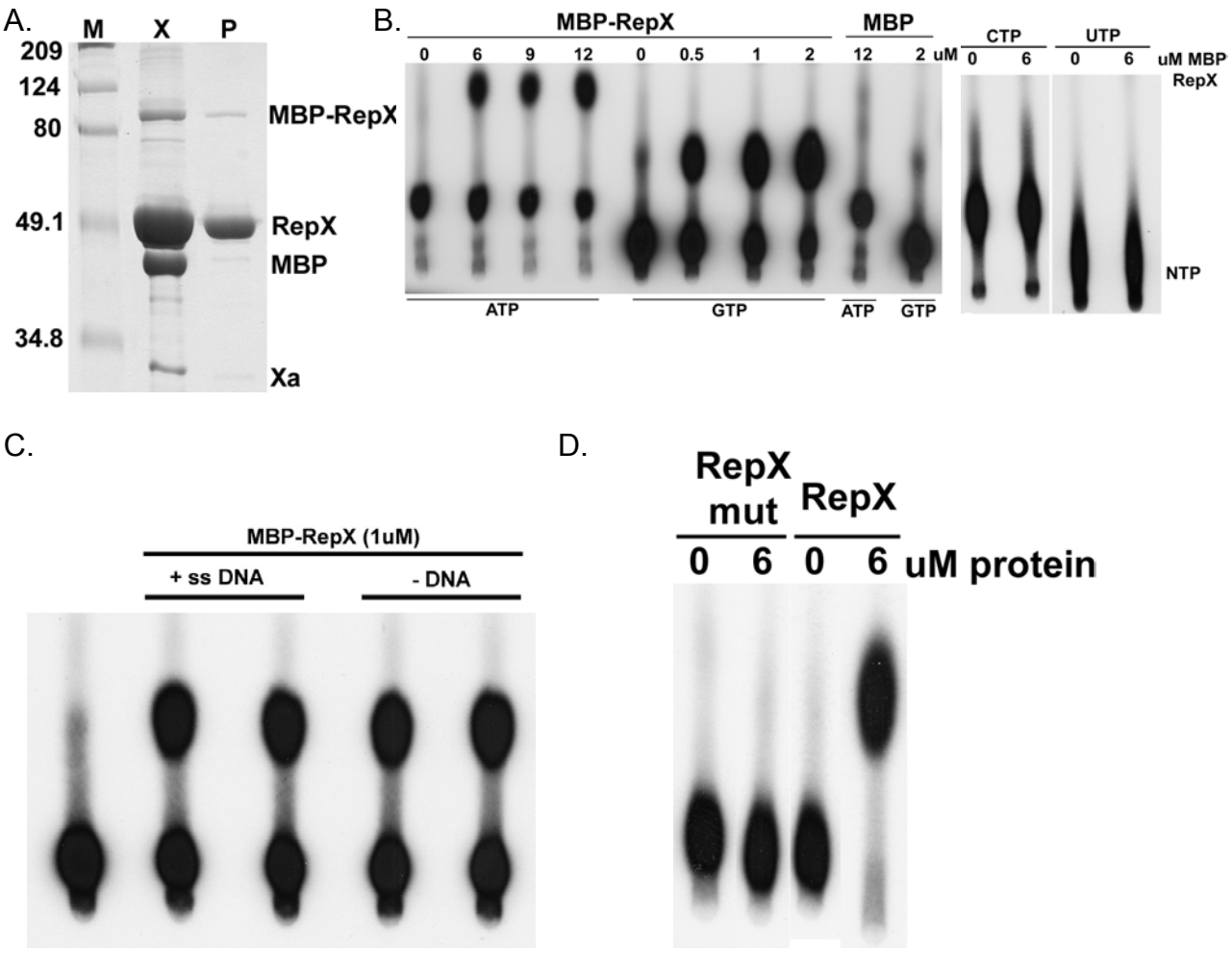
temperature to quench the crosslinking reaction. Samples were loaded onto 6% polyacrylamide gels and run at 140V at room temperature unless otherwise stated.

6.3 RESULTS

RepX purification. RepX was overexpressed as an N-terminal MBP fusion and affinity purified. When the protein was to be used in DNA binding assays, the MBP tag was cleaved using factor Xa protease in order to yield a native protein product (Fig. 18A). Inactive protein was removed using the HiTrap Cibicron Blue column which mimics dNTP's. Xa cleavage was not completely efficient, and therefore, a small amount of MBP-tagged RepX remained in the preparation (Fig. 18A). RepXmut, containing the T to A mutation in the GTPase motif mentioned in Chapter 5, was also purified using affinity chromatography, however, the yield was low and protein quality was poor.

NTPase Assays. Either MBP tagged RepX or Xa cleaved native RepX was used in NTPase assays. No difference in overall results was observed other than that the MBP tagged form of the protein showed a slightly higher NTPase activity than factor Xa-cleaved RepX. Various dilutions (0-12 μM) of RepX were used in NTPase assays which were incubated at 30˚C for 1 h, run on PEI cellulose, and exposed to autoradiography. RepX was capable of hydrolyzing GTP, ATP, dGTP, and dATP but not CTP or UTP (Fig. 18B). NTPase activity was not enhanced by the presence of ds or ss DNA (Fig. 18C). RepX seemed to prefer GTP over ATP as a substrate since a lower amount of protein was needed to hydrolyze GTP to completion (Fig.18B). RepXmut was incapable of NTP hydrolysis confirming that the GTP binding motif is important for hydrolysis (Fig. 18D).

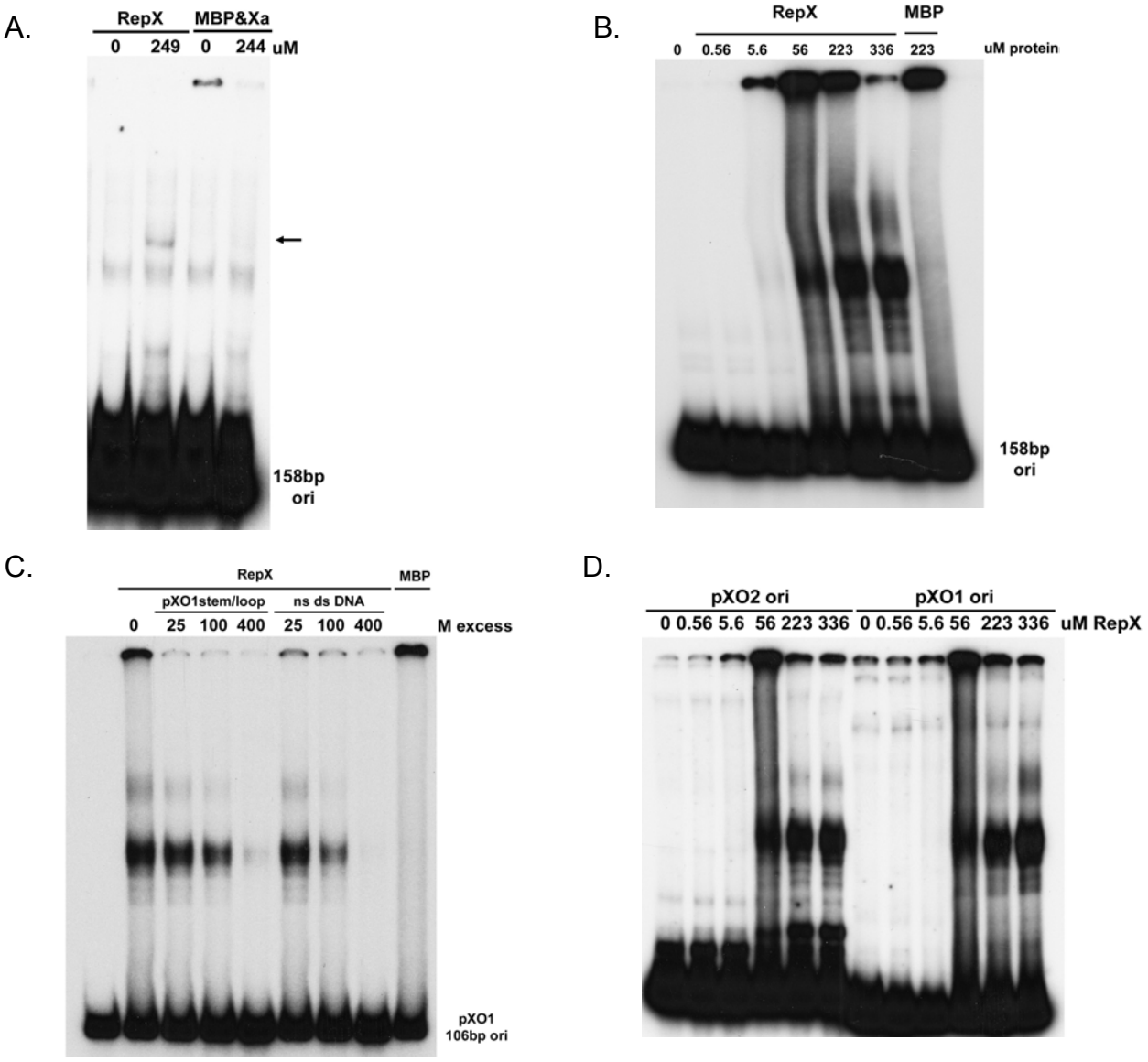
Figure 18. Purification of RepX and NTPase activity



A. Purification of RepX: M, molecular weight marker, X, Xa cleaved, P, purified protein from Cibicron Blue column. B. MBP-RepX hydrolyzes GTP and ATP but not CTP or UTP. C. MBP-RepX GTPase activity is not enhanced in the presence of DNA. D. RepXmut does not hydrolyze GTP.

Binding of RepX to DNA is non-specific. Because RepX is the only plasmid-encoded protein necessary for the replication of the pXO1 minireplicon, it was likely that RepX was acting as the replication initiator protein and therefore was binding specifically to the pXO1 origin of replication. To test this hypothesis, mobility shift assays were done using Xa cleaved RepX and various end labeled restriction fragments. RepX was capable of binding to the pXO1 ori although binding was extremely weak and required large amounts of protein for a detectable complex on polyacrylamide gels (Fig. 19A). Therefore, formaldehyde crosslinking was used to enhance a detectable complex on polyacrylamide gels. It has been documented that formaldehyde crosslinking maintains the specificity of a protein for its DNA substrate (Toth and Biggin, 2000). A detectable complex was observed with 5.6 μM of protein, and this complex was not observed when 223 μM of MBP was used, showing that the complex formation was indeed due to the presence of RepX (Fig. 19B). However, when cold ds oligonucleotides were used, both specific oligos containing the stem loop sequence of the putative pXO1 origin and non-specific oligos with or without stem-loop structures competed equally well for RepX binding (Fig. 19C). Additionally, RepX bound to the pXO2 ori with a similar affinity as it bound to the pXO1 ori (Fig. 19D).

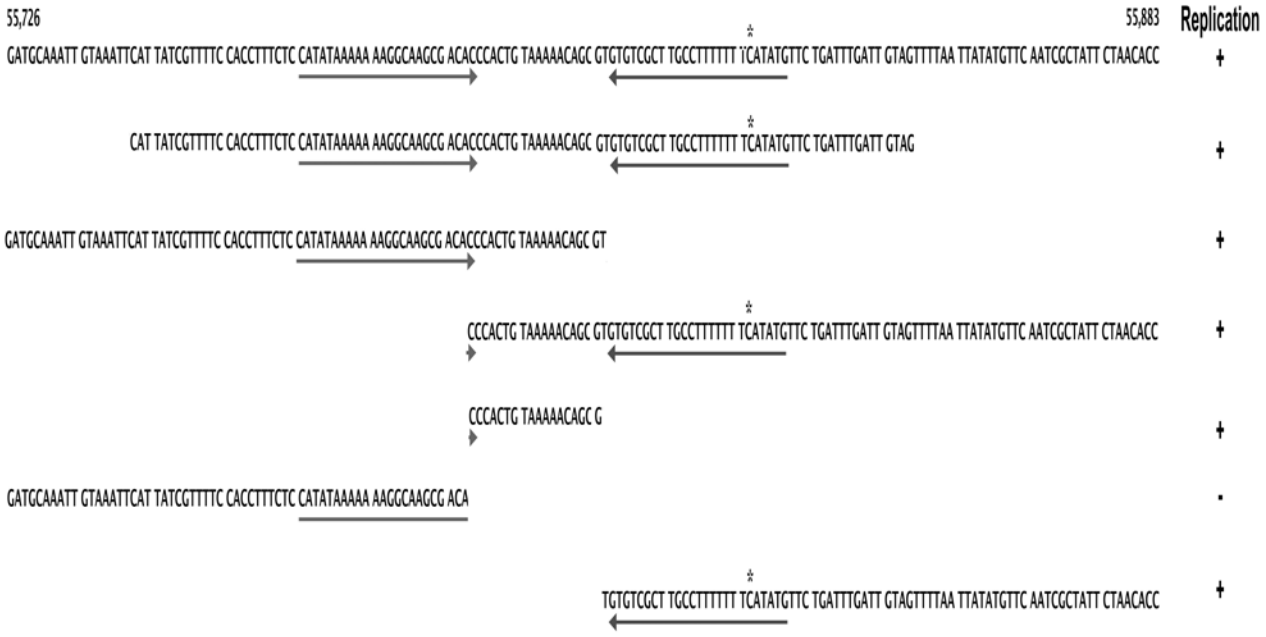
Figure 19. RepX binds to DNA non-specifically



A. RepX binds to the pXO1 origin of replication in the absence of formaldehyde crosslinking; the arrow indicates the RepX-DNA complex. B. Formaldehyde crosslinking enhances RepX binding to the pXO1 ori. C. RepX binds non-specifically to DNA: pXO1 stem/loop, the stem loop of the pXO1 origin of replication as a cold competitor; ns ds DNA, non-specific double-stranded DNA as a cold competitor. D. RepX binds non-specifically to DNA: pXO2 ori, the origin of replication of pXO2 as a probe; pXO1 ori, the origin of replication of pXO1 as a probe.

Further Analysis of the pXO1 origin of replication. Previous studies had revealed that a 158bp sequence containing a stem-loop structure was involved in pXO1 replication (Chapter 5). Since EMSA assays only yielded a RepX DNA complex that was non-specific, further characterization of the sequences involved in replication were done in vivo. Since the origin consisted primarily of a stem-loop structure, deletion of one or both arms of the stems was done and tested for replication. When the 3' arm and loop were deleted, no replication occurred. However, when the 5' arm and loop was deleted, replication occurred (Fig. 20). Interestingly, when the loop was present by itself, replication also took place indicating that the 3' arm is not necessary for replication. However, the 3' portion of the arm was capable of supporting replication without the loop indicating that the loop was dispensable for replication (Fig. 20). At this time it is unclear how the loop and 3' arm are involved in replication.

Figure 20. Origin constructs tested for replication in the presence of RepX in vivo.



7.0 GENERAL DISCUSSION

7.1 ISOLATION OF A MINIREPLICON OF THE VIRULENCE PLASMID PXO2 OF BACILLUS ANTHRACIS AND CHARACTERIZATION OF THE PLASMID-ENCODED REPS REPLICATION PROTEIN

Sequence analysis of the *B. anthracis* pXO2 megaplasmid revealed a region that shared homology with the broad host range family of replicons such as pAMβ1. This family encodes a single replication initiator protein which binds to an ori sequence downstream of the initiator gene and induces strand opening. These replicons require DNA polymerase I for replication and replicate unidirectionally requiring directional transcription into the ori from the *rep* gene for priming of leading strand synthesis (del Solar et al., 1998). The copy number of these plasmids is low because a countertranscript, produced in the promoter region of the *rep* gene, attenuates *rep* transcription. A second level of copy control involves a protein, Cop, encoded upstream of the *rep* gene which blocks transcription of the *rep* (Brantl, 1994). Sequence analysis of pXO2 revealed an ORF, *repS*, encoding a protein sharing 40% identity to the Rep proteins of the pAMβ1 family of replicons as well as a homologous ori sequence immediately downstream of repS. However, no homologous *cop* ORF was present indicating that pXO2 may differ slightly in copy control mechanisms.

The region of pXO2 sharing homology to the pAMβ1 family of replicons was cloned into an *E. coli* shuttle vector and shown to replicate in *B. anthracis*, *B. cereus*, and *B. subtilis*. Importantly, a *polA* mutant of *B. subtilis* did not support replication of the pXO2 replicon confirming that DNA polymerase I is important for replication. A frameshift mutation in the *repS* gene abrogated replication confirming its essentiality.

RepS was purified as an N-terminal tagged MBP fusion and tested for its ability to bind to DNA. RepS bound non-specifically to ss DNA and specifically to the 5' and central portions of the ds 60 bp ori sequence just upstream of the conserved replication initiation point similar to the RepE protein of pAMβ1. These binding properties allow for the protein to specifically recognize its double-stranded substrate for initiation of replication. Once properly bound to the ori, these proteins can induce topological changes promoting strand opening (Le Chatelier et al., 2001). After strand opening, the Rep proteins may facilitate primer formation and recruitment of host replication machinery.

Because pXO2 shares homology with the pAMβ1 family of replicons, RepS has DNA binding properties similar to these replicons, and replication requires DNA pol I, we conclude that pXO2 is a member of this family. This family of replicons replicates in a broad range of hosts including *Enterococcus*, *Streptomyces*, *Bacillus*, and *Escherichia* (Kurenbach et al., 2003). Plasmids encoding the *cap* genes of pXO2 and the *repS* gene have been found in closely related but distinct species of *Bacillus* (Luna et al., 2006) indicating the possibility that pXO2 can not only be transferred to new hosts but also can be maintained via this broad host range replicon. The fact that in our studies the minireplicon of pXO2 replicated in 3 different *Bacillus* species points to this possibility. A drug targeting a required element of replication could therefore be utilized in treating anthrax and anthrax-like diseases circumventing the need for organism identification.

7.2 MAPPING OF THE REPS BINDNIG SITE, IDENTIFICATION OF POTENTIAL COPY CONTROL ELEMENTS AND PARTITIOINING SYSTEM OF PXO2

Although mobility shift assays located a region of the ori needed for RepS binding, further mapping was necessary to identify the specific residues bound by RepS. DNaseI footprinting assays were done for both the top and bottom strands of the pXO2 ori. These assays confirmed the EMSA results and revealed the exact sequence of RepS binding which consists of a 22-bp region immediately upstream of the putative initiation site for replication, again the similarity of RepS to the Rep initiators of the pAMβ1 family which bind to a 25-bp sequence directly upstream of the initiation site.

Analysis of the pXO2 replicon revealed that unlike the pAMβ1-type replicons, it lacked a Cop repressor gene. Nothern blot analysis revealed that the *repS* transcript is attenuated and that a countertranscript is produced overlapping the *repS* transcript within the promoter region. The pAMβ1-type replicons encode a countertranscript which mediates transcriptional attenuation of the *rep* transcript (Brantl and Wagner, 1996; Le Chatelier et al., 1996), and it is likely that the same mechanism is used for the pXO2 replicon based on our Northern blot results. Assessment of the countertranscript sequence revealed that it was capable of forming 4 stem loops similar to the countertranscripts encoded by the pAMβ1 replicons. The fact that pXO2 appears to lack the *cop* repressor gene suggests that its copy control may be regulated in a slightly different manner. One function of the Cop protein is to bind to the *repS* promoter directly decreasing transcription by blocking RNA pol binding (Brantl, 1994). A second role of Cop is to prevent convergent transcription from the antisense promoter, and,

therefore, when copy number is high, CopR levels are high allowing for higher levels of antisense RNA to be produced. This in turn leads to CT binding to the *repS* transcript, transcriptional attenuation of *repS*, and a return to proper copy number in the cell. However, the CT is an extremely long-lived product (Brantl and Wagner, 1996); therefore, when the CT decreases the copy number, the resulting decrease in Cop protein levels also allows for a decrease in CT thus preventing plasmid loss (Brantl and Wagner, 1997). Since pXO2 does not encode Cop, this level of control may not be present; possibly, the CT of pXO2 is less stable than other CTs within this plasmid family thus negating the need for a second level of control.

A possible partitioning system for pXO2 was identified based on sequence homology to the ParA/ParB proteins of the pAD1 family of replicons. Not only was homology to the Par proteins similar, but also the genetic organization was similar. It is interesting that pXO2 contains a Rep and ori from one family and a possible partitioning system from a separate family of replicons. Other plasmids exist which share this same gene organization such as the conjugative plasmids pAW63 and pBT9727 from *B. thuringiensis* (Van der Auwera et al., 2005; Wilcks et al., 1999). This suggests that plasmids exist in *the B. cereus* family which share a common backbone with pXO2 but contain genes specific to their environmental niches probably obtained from the many mobile elements such as genes flanked by transposases including the *cap* genes of pXO2 (Van der Auwera et al., 2005).

7.3 IN-VITRO REPLICATION EXTRACT FROM BACILLUS ANTHRACIS SUPPORTS REPLICATION OF THE PT181 ROLLING CIRCLE REPLICON AND THE PXO2 REPLICON.

Although the requirements for pXO2 replication could be tested to some extent in vivo, it was desirable to develop an in vitro system that supported replication of the pXO2 minireplicon which would provide a way to easily test a range of variables without the complications and necessity of genetic knock-outs. A cell-free extract was prepared from *B. anthracis* using a French press for lysis, streptomycin sulfate to remove RNA and DNA, and ammonium sulfate to isolate a replication-active protein fraction. Initially, the rolling circle plasmid pT181 was used to confirm the activity of the extract since this replicon has been well-characterized in in vitro systems from *S. aureus* (Khan et al., 1981). The *B. anthracis* extract behaved similarly to the *S. aureus* extract in its ability to replicate pT181 in a specific manner when the RepC protein of pT181 was added. Additionally, little effect on replication was observed when Rifampicin was added or rNTPs were excluded showing that transcription is not required for at least leading strand synthesis of pT181.

The pXO2 minireplicon containing the wt *repS* gene and downstream ori sequence was tested in the presence of the *B. anthracis* extracts and purified MBP-RepS. An increase in replication above the background levels of vector alone was seen when the *repS* ORF, ori, and MBP-RepS were present. In order to dissect the requirements for the ori vs. the *repS* ORF, the pXO2 ori was cloned downstream of the constitutive *aphA3* promoter such that leading strand synthesis was primed from *aphA3*. Much higher

levels of replication above the background vector sequence were observed for this construct. This result confirmed that the ori is required for pXO2 replication and that MBP-RepS specifically recognizes the ori. When MBP-RepS was excluded from the reaction, a decrease in replication was observed for both the wt and oriF constructs confirming the necessity of RepS in pXO2 replication. To test for the requirement of transcription, a transcriptional terminator was placed between the promoter and the ori in the oriF construct. Lower levels of replication were observed in comparison to oriF, however, this construct was capable of replicating in-vivo indicating that the terminator was not eintirely efficient at preventing priming of the ori. Requirements for transcription were confirmed by adding rifampicin or excluding rNTP's from the reaction for both the oriF and wt constructs, and a large reduction in replication activity was observed. Since the pAMβ1 replicons require directional transcription into the ori for replication to occur, the ori was cloned such that lagging strand synthesis was being primed by the *aphA3* promoter instead of leading strand (oriRt). Radiolabel incorporation was reduced in this construct. When these constructs were tested in-vivo, the oriF and wt constructs replicated but not the oriR. Additionally, the oriF construct only replicated in a strain that encoded the RepS protein in trans again confirming that RepS is required but not necessary in *cis* as long as some priming occurs into the leading strand of the ori.

The *B. anthracis* cell extract was also active for the replication of the pXO2 replicon and therefore supported replication of both RCR and theta-replicating plasmids. Additionally, the use of the extracts allowed for the identification of specific requirements for pXO2 replication including the ori sequence, the RepS protein, and directional transcription. These requirements are consistent with the classification of the pXO2

replicon as a member of the pAMβ1 family. Although in vivo requirements for transcription using the wt construct were not easily testable, use of the ori constructs containing a strong constitutive promoter proved useful for both in vitro and in vivo analysis of requirements. Importantly, in vitro results correlated well with the in vivo data showing that the in vitro system accurately reflects the replication properties of both pXO2 and pT181.

7.4 A NOVEL FTSZ-LIKE PROTEIN IS INVOLVED IN REPLICATION OF THE ANTHRAX TOXIN-ENCODING PXO1 PLASMID IN BACILLUS ANTHRACIS

Analysis of the pXO1 megaplasmid revealed that it did not contain any orthologs of known initiator proteins. Because the pathogenicity island (PAI) of pXO1 is flanked by mobile genetic elements which could undergo rearrangement and loss, it was unlikely that the functional replicon was present in this region. Oligomer skew analysis, and origin comparisons (Hallin and Ussery, 2004; Rocha, 2004; Worning et al., 2006) provided by the Genome Atlas Database at http://www.cbs.dtu.dk/services/GenomeAtlas/ (Hallin and Ussery, 2004) and analysis of ORFs which were conserved in several related plasmids in the *B. cereus* group revealed that a region from nt positions 40,000-60,000 of pXO1 was likely to harbor a replicon. Conserved ORFs were considered to be important in the basic functions of the plasmids such as replication, copy control, partitioning, and transfer since other plasmid genes would may be unique to specific environmental niche.

A 10-kb region containing two highly conserved ORFs, 42 and 45, was cloned and found to replicate in a plasmid free strain of *B. anthracis* showing that this portion of pXO1 was capable of autonomous replication and therefore contained a functional origin of replication. Deletion and frame-shift analysis revealed that ORF45 (*repX*) and an intergenic sequence downstream of *repX* was required for replication. This gene was present on several large plasmids found in *B. cereus* and *B. thuringiensis* sharing 22-99% identity. In fact, since publication of our results, a mini-replicon containing a gene encoding a protein sharing 22% identity with RepX was shown to be required for

replication of pBtoxis, a 218-kb plasmid encoding the crystal toxins of *B. thuringiensis* (Tang et al., 2006). However, this study reported that both the FtsZ-like protein and the ORF immediately upstream were necessary for replication. The authors based this conclusion on deletion analysis in which deletion of the upstream ORF yielded an inactive replicon. Our Northern blot analysis of the RepX transcript suggests that RepX is part of an operon including the gene immediately upstream of *repX*. Therefore, deletion analysis alone as done by the Tang et al. paper would not accurately represent the requirements for replication; instead, in our study a frameshift mutation in the upstream gene which allows for transcription of *repX* showed that the upstream gene is dispensable for replication of pXO1 (Tinsley and Khan, 2006). Therefore, it is likely that these minireplicons define a new family of replicons requiring an FtsZ-like protein and a unique ori sequence to function.

Sequence analysis of RepX showed that it shared limited (20-30%) identity with the FtsZ GTPase proteins involved in cell septation. Interestingly, aa identity was most evident in the N-terminal region of the protein which functions as the GTP binding and hydrolysis domain in the FtsZ proteins. When a key Thr in the GTP binding motif was mutated to an Ala in RepX, replication was abrogated. Many replication proteins contain NTPase motifs, and NTP hydrolysis serves to provide energy to allow for strand opening at the origin of replication (del Solar et al., 1998). It is possible that the GTPase activity of RepX is used in this manner. The C-terminal portion of RepX shared little homology with the FtsZ proteins. However, the FtsZ proteins also differ from each other in this region, and the significance of this difference between RepX and the FtsZ

proteins is currently unknown. The C-terminus of FtsZ is involved in specific protein-protein interactions recruiting scaffolding proteins to midcell (Goehring and Beckwith, 2005; Rothfield et al., 1999; Weiss, 2004). Possibly, the C-terminus of RepX is involved in protein interactions acting to recruit host replication machinery to the ori of pXO1. Additionally, the differences in the C-terminal portion of RepX may allow for it to bind DNA unlike FtsZ proteins which are occluded by DNA (Romberg and Levin, 2003). FtsZ positioning at midcell is dependent on the location and replication status of the chromosome; positioning is tightly linked to initiation of DNA replication (Romberg and Levin, 2003). Therefore, the location and replication status of DNA plays a pivotal role in proper FtsZ function. The same may be true for RepX; the topological status of pXO1 DNA and the presence of the pXO1 ori may be required for proper RepX function. Our results demonstrate that both RepX and an adjacent ori sequence are required for replication. RepX may serve a dual function not only as a replication initiator but also as a partitioning protein. Similar to FtsZ polymerization at midcell, RepX may localize to midcell while interacting with pXO1 thus moving the plasmid DNA to midcell for proper partitioning to daughter cells. However, since partitioning is not a requirement for replication, it is unlikely that partitioning is the main function of RepX.

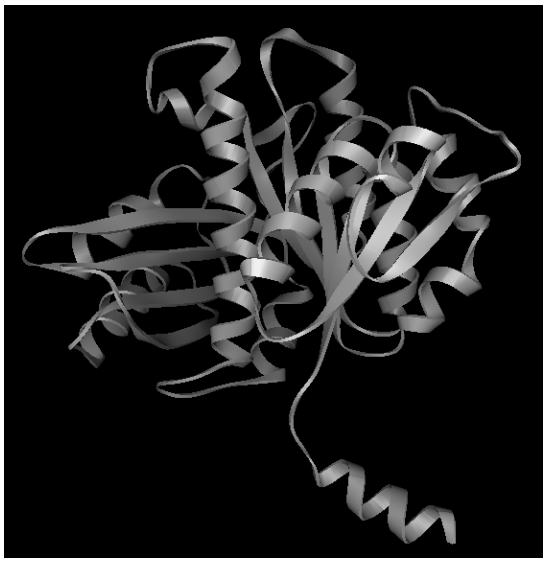
7.5 PURIFICATION OF THE PXO1 REPLICATION PROTEIN REPX, CHARACTERIZATION OF ITS BIOCHEMICAL PROPERTIES, AND FURTHER ANALYSIS OF THE PXO1 ORIGN OF REPLICATION

Further analysis of the pXO1 origin of replication in vivo revealed that when RepX was supplied in trans, the loop and 3' stem could individually promote plasmid replication. This is a surprising observation, and currently, how the ori functions and the minimal nt sequence required for replication is unknown.

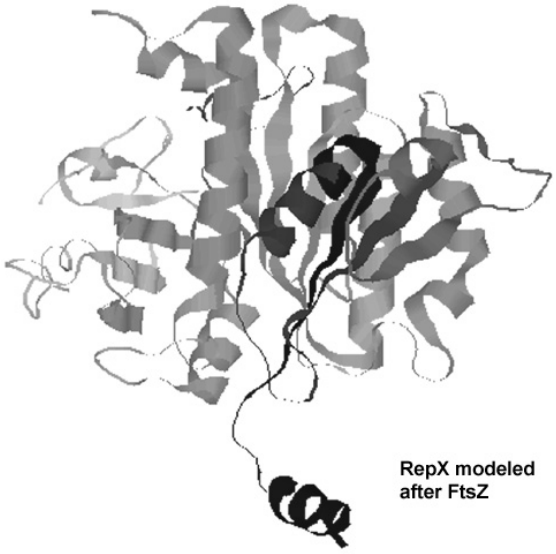
Structural analysis of the RepX protein revealed that RepX could form a similar 3D structure to that of the known crystal structure of FtsZ (Fig. 21) lending strength to the possibility that RepX shares similar biochemical activities to that of FtsZ. RepX of pXO1 was overexpressed as an N-terminally tagged MBP fusion and either purified via affinity chromatography or cleaved with factor Xa to yield the native protein. The frameshift mutation in the GTP binding motif (RepXmut) was also purified though yield and protein quality was poor. Purified protein was used in NTPase assays and EMSAs. Wild-type RepX, but not RepXmut, showed both GTPase and ATPase activities but not UTPase or CTPase activity. Unlike FtsZ, RepX was capable of ATP hydrolysis; the significance of this activity, if any, is currently unknown. Many replication initiator proteins are capable of hydrolyzing various NTPs although a binding preference for a specific nucleotide is usually observed (del Solar et al., 1998). More molecules of RepX were required for complete hydrolysis of ATP than of GTP. This indicates that RepX has a preference for GTP over ATP; however, it is unknown if this preference is during the binding or hydrolysis step. For FtsZ, GTPase activity is required for the dynamic nature

Figure 21. RepX modeled after FtsZ forms a similar 3D structure.

A.



B.



A. 3D Structure of FtsZ (Lowe and Amos, 1998)

(http://ca.expasy.org/uniprot/Q57816).

B. RepX modeled after the 3D structure of FtsZ using EsyPred3D (Lambert et al., 2002).

of the protein. FtsZ forms protofilaments which vary in shape and size depending upon the GTP vs. GDP content of the filaments (Romberg and Levin, 2003). GTP hydrolysis requires at least 2 subunits of FtsZ, one which binds GTP and the other which interacts with the GTP-bound FtsZ molecule to catalyze hydrolysis (Romberg and Levin, 2003). Currently, it is unknown if RepX displays similar properties such as the necessity of interaction for hydrolysis and the ability to form protofilaments.

RepX was capable of binding DNA in mobility-shift assays. This binding was weak and barely detectable with high amounts of protein. The presence of GTP or GDP in the binding reactions did not affect binding although this experiment was not performed upon formaldehyde crosslinking. Formaldehyde crosslinking allowed detection of a complex with lower levels of protein. DNA binding by RepX was nonspecific since RepX was capable of binding both the pXO1 and pXO2 ori sequences and since non-specific cold competitor DNA consisting of a multiple cloning site competed equally well as specific cold competitor for RepX binding. As a replication initiator, specific interaction of RepX with the pXO1 ori would be necessary for proper initiation of replication. Not all initiator proteins show binding specificity to linear substrates. Some initiators have higher affinity for specific sequences when certain DNA topology such as supercoiling is present. For example, DnaA shows increased affinity for specific substrates when the DNA is negatively supercoiled (Fuller and Kornberg, 1983), and the replication initiator protein O of λ phage requires supercoiling for proper origin unwinding (Schnos et al., 1988). Additionally, the binding specificity of the origin recognition complex (ORC) in higher organisms has been difficult to characterize and appears to require DNA topology (Remus et al., 2004) and ATP for specific binding

(Bell, 2002). Different results have been obtained with ORCs purified as recombinant proteins vs. native proteins (Bell, 2002), and possibly RepX may display different DNA binding properties depending upon the purification strategy. Some initiator proteins appear to require other host factors for increased affinity for specific ori sequences. For example, the replication initiator protein RepB of pAL5000 binds specifically to the ori DNA only when cell extracts are present (Basu et al., 2002). Possibly, RepX may require host proteins such as chaperones that allow for RepX remodeling for specific binding to ori DNA. Some evidence exists that chaperones are involved in activating both monomeric and dimeric replication initiator proteins by allowing proper protein folding (Chattoraj, 2000). Although, the recombinant RepX protein is active for NTPase activity, RepX could require host chaperone proteins for origin binding activity. Additional host protein requirements could also serve to limit the host range of pXO1. Consistent with this possibility, we have been unable to demonstrate replication of the mini-pXO1 plasmid in a host other than *B. anthracis*. Another parameter that can affect initiator protein activity is its genetic context; although RepB of pAL5000 can bind specifically to DNA when uncoupled from its operon, translational coupling in the context of its operon seems to yield higher amounts of protein capable of specific ori binding (Basu et al., 2004). It appears that *repX* is part of an operon based on the size of the detectable *repX* transcript in Northern blots, and therefore, separation of *repX* from its operon may affect its binding activity. In conclusion, a host of parameters that currently remain untested could affect RepX ori binding.

7.6 FUTURE GOALS FOR THE PXO2 REPLICON

Further Characterization of Binding Properties of the RepS Protein of pXO2. Though our studies have revealed that RepS binds specifically to the ds ori of pXO2, its ability to induce changes in DNA topology similar to RepE of the pAMβ1 replicons is currently unknown. Potassium permanganate footprinting could provide this data both in vitro and in vivo by revealing what bases were distorted or bent in the presence of RepS. Additionally, it is unknown if DNA topology such as supercoiling affects RepS binding; supercoiled, linear, bubble, and RNA-DNA hybrid substrates could be tested for RepS binding. RepS could bind primarily in the major or minor groove of DNA, and various footprinting reagents such as DMS and DNaseI could be used to compare the efficiency of protection. Interaction of RepS with various replication proteins such as RNA polymerase, RNaseH, and DNA polymerase I could be tested using pulldown assays of epitope tagged RepS in the presence or absence of DNA. The domains necessary for the interactions of RepS with the ori and other cellular proteins could also be identified.

Further Characterization of the Replication Properties of pXO2. Although the in vitro, in vivo, and sequence data all provide evidence that supports the classification of pXO2 as a member of the pAMβ1 family, identifying the mode of replication would be beneficial to confirm this classification. Identification of the start-site of replication and RNA primers in the ori region would also strengthen this conclusion. The necessity of RepS for proper primer formation could be tested. In pAMβ1, RepE but not replication,

is required for proper primer formation (Bruand and Ehrlich, 1998). It would be interesting to characterize how RepS might be involved in proper primer formation.

Copy number of wt pXO2 and the minireplicon should be determined. The effect of the countertranscript on *repS* mRNA and protein levels and on pXO2 copy number should also be tested. It would also be possible to test for the interaction of the CT with *repS* mRNA. Possibly elements encoded by pXO1 could affect copy number of pXO2 since transcriptional regulators such as atxA, which are involved in modulating capsule gene expression, exist on pXO1, (Koehler, 2002). The copy number of pXO2 could be assessed in the presence or absence of pXO1. Stability studies in the presence and absence of RepB, ORF40, and the iterons could be done to assess the involvement of this locus in partitioning of pXO2.

7.7 FUTURE GOALS FOR THE pXO1 REPLICON

Our studies have demonstrated the necessity of RepX and a sequence containing a stem-loop structure downstream of RepX for pXO1 replication. We have also shown that RepX GTPase activity is required for replication and that RepX can bind to DNA non-specifically.

To fully understand RepX function in pXO1 replication, further structural and biochemical analysis is necessary. The involvement and necessity of the C-terminal portion of RepX in pXO1 replication, DNA binding, and protein interactions could be tested using deletion mutagenesis. Pull-down assays using epitope tagged wt and mutant forms of RepX could be done to test for its ability to interact with host replication machinery. Location of RepX in the cell using GFP fusion proteins should also be studied which could give insights into its function as a potential partitioning protein. Recently studies using stop-flow measurements revealed that RepX polymerizes in a GTP dependent manner. Further analysis of the polymerization of RepX will be done including EM studies.

The DNA binding properties of RepX should also be studied. Importantly, the conditions necessary for specific interaction of RepX with its ori should be elucidated. The effects of DNA topology, presence of host proteins, the genetic context of RepX, and nucleotide requirements should be tested for their effects on the ability of RepX to specifically bind ori DNA.

8.0 BIBLIOGRAPHY

Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. (1990). Basic local alignment search tool. J Mol Biol *215*, 403–410.

Anand, S. P., Mitra, P., Naqvi, A., and Khan, S. A. (2004). Bacillus anthracis and Bacillus cereus PcrA helicases can support DNA unwinding and in vitro rolling-circle replication of plasmid pT181 of Staphylococcus aureus. J Bacteriol *186*, 2195-2199.

Andrup, L., Jensen, G. B., Wilcks, A., Smidt, L., Hoflack, L., and Mahillon, J. (2003). The patchwork nature of rolling-circle plasmids: comparison of six plasmids from two distinct Bacillus thuringiensis serotypes. Plasmid *49*, 205-232.

Atlas, R. M. (2002). Responding to the threat of bioterrorism: a microbial ecology perspective--the case of anthrax. Int Microbiol *5*, 161-167.

Basu, A., Chatterjee, S., and Das Gupta, S. K. (2004). Translational coupling to an upstream gene promotes folding of the mycobacterial plasmid pAL5000 replication protein RepB and thereby its origin binding activity. J Bacteriol *186*, 335-342.

Basu, A., Chawla-Sarkar, M., Chakrabarti, S., and Das Gupta, S. K. (2002). Origin binding activity of the Mycobacterial plasmid pAL5000 replication protein RepB is stimulated through interactions with host factors and coupled expression of repA. J Bacteriol *184*, 2204-2214.

Battisti, L., Green, B. D., and Thorne, C. B. (1985). Mating system for transfer of plasmids among Bacillus anthracis, Bacillus cereus, and Bacillus thuringiensis. J Bacteriol *162*, 543-550.

Bell, S. P. (2002). The origin recognition complex: from simple origins to complex functions. Genes Dev *16*, 659-672.

Berry, C., O'Neil, S., Ben-Dov, E., Jones, A. F., Murphy, L., Quail, M. A., Holden, M. T., Harris, D., Zaritsky, A., and Parkhill, J. (2002). Complete sequence and organization of pBtoxis, the toxin-coding plasmid of Bacillus thuringiensis subsp. israelensis. Appl Environ Microbiol *68*, 5082-5095.

Bigot, S., Corre, J., Louarn, J. M., Cornet, F., and Barre, F. X. (2004). FtsK activities in Xer recombination, DNA mobilization and cell division involve overlapping and separate domains of the protein. Mol Microbiol *54*, 876-886.

Bourgogne, A., Drysdale, M., Hilsenbeck, S. G., Peterson, S. N., and Koehler, T. M. (2003). Global effects of virulence gene regulators in a Bacillus anthracis strain with both virulence plasmids. Infect Immun *71*, 2736-2743.

Brantl, S. (1994). The copR gene product of plasmid pIP501 acts as a transcriptional repressor at the essential repR promoter. Mol Microbiol *14*, 473-483.

Brantl, S. (2002). Antisense RNAs in plasmids: control of replication and maintenance. Plasmid *48*, 165-173.

Brantl, S., and Behnke, D. (1992a). The amount of RepR protein determines the copy number of plasmid pIP501 in Bacillus subtilis. J Bacteriol *174*, 5475-5478.

Brantl, S., and Behnke, D. (1992b). Characterization of the minimal origin required for replication of the streptococcal plasmid pIP501 in Bacillus subtilis. Mol Microbiol *6*, 3501-3510.

Brantl, S., Behnke, D., and Alonso, J. C. (1990). Molecular analysis of the replication region of the conjugative Streptococcus agalactiae plasmid pIP501 in Bacillus subtilis. Comparison with plasmids pAM beta 1 and pSM19035. Nucleic Acids Res *18*, 4783-4790.

Brantl, S., and Wagner, E. G. (1996). An unusually long-lived antisense RNA in plasmid copy number control: in vivo RNAs encoded by the streptococcal plasmid pIP501. J Mol Biol *255*, 275-288.

Brantl, S., and Wagner, E. G. (1997). Dual function of the copR gene product of plasmid pIP501. J Bacteriol *179*, 7016-7024.

Bruand, C., and Ehrlich, S. D. (1998). Transcription-driven DNA replication of plasmid pAMbeta1 in Bacillus subtilis. Mol Microbiol *30*, 135-145.

Bruand, C., Ehrlich, S. D., and Janniere, L. (1991). Unidirectional theta replication of the structurally stable Enterococcus faecalis plasmid pAM beta 1. Embo J *10*, 2171-2177.

Bruand, C., Ehrlich, S. D., and Janniere, L. (1995). Primosome assembly site in Bacillus subtilis. Embo J *14*, 2642-2650.

Bruand, C., Le Chatelier, E., Ehrlich, S. D., and Janniere, L. (1993). A fourth class of theta-replicating plasmids: the pAM beta 1 family from gram-positive bacteria. Proc Natl Acad Sci U S A *90*, 11668-11672.

Carballido-Lopez, R., and Errington, J. (2003). A dynamic bacterial cytoskeleton. Trends Cell Biol *13*, 577-583.

Chang, T. L., Kramer, M. G., Ansari, R. A., and Khan, S. A. (2000). Role of individual monomers of a dimeric initiator protein in the initiation and termination of plasmid rolling circle replication. J Biol Chem *275*, 13529-13534.

Chattoraj, D. K. (2000). Control of plasmid DNA replication by iterons: no longer paradoxical. Mol Microbiol *37*, 467-476.

Chen, Y., Tenover, F. C., and Koehler, T. M. (2004). Beta-lactamase gene expression in a penicillin-resistant Bacillus anthracis strain. Antimicrob Agents Chemother *48*, 4873-4877.

Coker, P. R., Smith, K. L., Fellows, P. F., Rybachuck, G., Kousoulas, K. G., and Hugh-Jones, M. E. (2003). Bacillus anthracis virulence in Guinea pigs vaccinated with anthrax vaccine adsorbed is linked to plasmid quantities and clonality. J Clin Microbiol *41*, 1212-1218.

Dai, Z., Sirard, J. C., Mock, M., and Koehler, T. M. (1995). The atxA gene product activates transcription of the anthrax toxin genes and is essential for virulence. Mol Microbiol *16*, 1171-1181.

del Solar, G., and Espinosa, M. (2000). Plasmid copy number control: an ever-growing story. Mol Microbiol *37*, 492-500.

del Solar, G., Giraldo, R., Ruiz-Echevarria, M. J., Espinosa, M., and Diaz-Orejas, R. (1998). Replication and control of circular bacterial plasmids. Microbiol Mol Biol Rev *62*, 434-464.

Drysdale, M., Bourgogne, A., Hilsenbeck, S. G., and Koehler, T. M. (2004). atxA controls Bacillus anthracis capsule synthesis via acpA and a newly discovered regulator, acpB. J Bacteriol *186*, 307-315.

Dunny, G. M., Lee, L. N., and LeBlanc, D. J. (1991). Improved electroporation and cloning vector system for gram-positive bacteria. Appl Environ Microbiol *57*, 1194-1201.

Etienne-Toumelin, I., Sirard, J. C., Duflot, E., Mock, M., and Fouet, A. (1995). Characterization of the Bacillus anthracis S-layer: cloning and sequencing of the structural gene. J Bacteriol *177*, 614-620.

Fouet, A., and Mock, M. (2006). Regulatory networks for virulence and persistence of Bacillus anthracis. Curr Opin Microbiol *9*, 160-166.

Francia, M. V., Fujimoto, S., Tille, P., Weaver, K. E., and Clewell, D. B. (2004). Replication of Enterococcus faecalis pheromone-responding plasmid pAD1: location of the minimal replicon and oriV site and RepA involvement in initiation of replication. J Bacteriol *186*, 5003-5016.

Fuller, R. S., and Kornberg, A. (1983). Purified dnaA protein in initiation of replication at the Escherichia coli chromosomal origin of replication. Proc Natl Acad Sci U S A *80*, 5817-5821.

Gerdes, K., Moller-Jensen, J., and Bugge Jensen, R. (2000). Plasmid and chromosome partitioning: surprises from phylogeny. Mol Microbiol *37*, 455-466.

Goehring, N. W., and Beckwith, J. (2005). Diverse paths to midcell: assembly of the bacterial cell division machinery. Curr Biol *15*, R514-526.

Green, B. D., Battisti, L., Koehler, T. M., Thorne, C. B., and Ivins, B. E. (1985). Demonstration of a capsule plasmid in Bacillus anthracis. Infect Immun *49*, 291-297.

Green, B. D., Battisti, L., and Thorne, C. B. (1989). Involvement of Tn4430 in transfer of Bacillus anthracis plasmids mediated by Bacillus thuringiensis plasmid pXO12. J Bacteriol *171*, 104-113.

Guidi-Rontani, C., Pereira, Y., Ruffie, S., Sirard, J. C., Weber-Levy, M., and Mock, M. (1999). Identification and characterization of a germination operon on the virulence plasmid pXO1 of Bacillus anthracis. Mol Microbiol *33*, 407-414.

Guignot, J., Mock, M., and Fouet, A. (1997). AtxA activates the transcription of genes harbored by both Bacillus anthracis virulence plasmids. FEMS Microbiol Lett *147*, 203-207.

Hallin, P. F., and Ussery, D. W. (2004). CBS Genome Atlas Database: a dynamic storage for bioinformatic results and sequence data. Bioinformatics *20*, 3682-3686.

Han, C. S., Xie, G., Challacombe, J. F., Altherr, M. R., Bhotika, S. S., Bruce, D., Campbell, C. S., Campbell, M. L., Chen, J., Chertkov, O.*, et al.* (2006). Pathogenomic sequence analysis of Bacillus cereus and Bacillus thuringiensis isolates closely related to Bacillus anthracis. J Bacteriol *188*, 3382-3390.

Hanna, P. (1998). Anthrax pathogenesis and host response. Curr Top Microbiol Immunol *225*, 13-35.

Helgason, E., Okstad, O. A., Caugant, D. A., Johansen, H. A., Fouet, A., Mock, M., Hegna, I., and Kolsto (2000). Bacillus anthracis, Bacillus cereus, and Bacillus thuringiensis--one species on the basis of genetic evidence. Appl Environ Microbiol *66*, 2627-2630.

Hoffmaster, A. R., and Koehler, T. M. (1999). Control of virulence gene expression in Bacillus anthracis. J Appl Microbiol *87*, 279-281.

Hoffmaster, A. R., Ravel, J., Rasko, D. A., Chapman, G. D., Chute, M. D., Marston, C. K., De, B. K., Sacchi, C. T., Fitzgerald, C., Mayer, L. W.*, et al.* (2004). Identification of anthrax toxin genes in a Bacillus cereus associated with an illness resembling inhalation anthrax. Proc Natl Acad Sci U S A *101*, 8449-8454.

Ito, M., and Nagane, M. (2001). Improvement of the electro-transformation efficiency of facultatively alkaliphilic Bacillus pseudofirmus OF4 by high osmolarity and glycine treatment. Biosci Biotechnol Biochem *65*, 2773-2775.

Janniere, L., Bidnenko, V., McGovern, S., Ehrlich, S. D., and Petit, M. A. (1997). Replication terminus for DNA polymerase I during initiation of pAM beta 1 replication: role of the plasmid-encoded resolution system. Mol Microbiol *23*, 525-535.

Jensen, G. B., Fisker, N., Sparso, T., and Andrup, L. (2005). The possibility of discriminating within the Bacillus cereus group using gyrB sequencing and PCR-RFLP. Int J Food Microbiol *104*, 113-120.

Khan, S. A. (1997). Rolling-circle replication of bacterial plasmids. Microbiol Mol Biol Rev *61*, 442-455.

Khan, S. A., Carleton, S. M., and Novick, R. P. (1981). Replication of plasmid pT181 DNA in vitro: requirement for a plasmid-encoded product. Proc Natl Acad Sci U S A *78*, 4902-4906.

Koehler, T. M. (2002). Bacillus anthracis genetics and virulence gene regulation. Curr Top Microbiol Immunol *271*, 143-164.

Koehler, T. M., Dai, Z., and Kaufman-Yarbray, M. (1994). Regulation of the Bacillus anthracis protective antigen gene: CO2 and a trans-acting element activate transcription from one of two promoters. J Bacteriol *176*, 586-595.

Koepsel, R. R., Murray, R. W., Rosenblum, W. D., and Khan, S. A. (1985). Purification of pT181-encoded repC protein required for the initiation of plasmid replication. J Biol Chem *260*, 8571-8577.

Kurenbach, B., Bohn, C., Prabhu, J., Abudukerim, M., Szewzyk, U., and Grohmann, E. (2003). Intergeneric transfer of the Enterococcus faecalis plasmid pIP501 to Escherichia coli and Streptomyces lividans and sequence analysis of its tra region. Plasmid *50*, 86-93.

Lambert, C., Leonard, N., De Bolle, X., and Depiereux, E. (2002). ESyPred3D: Prediction of proteins 3D structures. Bioinformatics *18*, 1250-1256.

Le Chatelier, E., Ehrlich, S. D., and Janniere, L. (1993). Biochemical and genetic analysis of the unidirectional theta replication of the S. agalactiae plasmid pIP501. Plasmid *29*, 50-56.

Le Chatelier, E., Ehrlich, S. D., and Janniere, L. (1994). The pAM beta 1 CopF repressor regulates plasmid copy number by controlling transcription of the repE gene. Mol Microbiol *14*, 463-471.

Le Chatelier, E., Ehrlich, S. D., and Janniere, L. (1996). Countertranscript-driven attenuation system of the pAM beta 1 repE gene. Mol Microbiol *20*, 1099-1112.

Le Chatelier, E., Janniere, L., Ehrlich, S. D., and Canceill, D. (2001). The RepE initiator is a double-stranded and single-stranded DNA-binding protein that forms an atypical open complex at the onset of replication of plasmid pAMbeta 1 from Gram-positive bacteria. J Biol Chem *276*, 10234-10246.

Lee, K. N., Padmalayam, I., Baumstark, B., Baker, S. L., and Massung, R. F. (2003). Characterization of the ftsZ gene from Ehrlichia chaffeensis, Anaplasma phagocytophilum, and Rickettsia rickettsii, and use as a differential PCR target. DNA Cell Biol *22*, 179-186.

Lowe, J., and Amos, L. A. (1998). Crystal structure of the bacterial cell-division protein FtsZ. Nature *391*, 203-206.

Lu, C., Stricker, J., and Erickson, H. P. (1998). FtsZ from Escherichia coli, Azotobacter vinelandii, and Thermotoga maritima--quantitation, GTP hydrolysis, and assembly. Cell Motil Cytoskeleton *40*, 71-86.

Luna, V. A., King, D. S., Peak, K. K., Reeves, F., Heberlein-Larson, L., Veguilla, W., Heller, L., Duncan, K. E., Cannons, A. C., Amuso, P., and Cattani, J. (2006). Bacillus anthracis virulent plasmid pX02 genes found in large plasmids of two other Bacillus species. J Clin Microbiol *44*, 2367-2377.

M. Zuker, D. H. M. a. D. H. T. (1999). Algorithms and Thermodynamics for RNA Secondary Structure Prediction: A Practical Guide in RNA Biochemistry and Biotechnology, Kluwer Academic Publishers).

Makino, S., Uchida, I., Terakado, N., Sasakawa, C., and Yoshikawa, M. (1989). Molecular characterization and protein analysis of the cap region, which is essential for encapsulation in Bacillus anthracis. J Bacteriol *171*, 722-730.

Marrero, R., and Welkos, S. L. (1995). The transformation frequency of plasmids into Bacillus anthracis is affected by adenine methylation. Gene *152*, 75-78.

Mignot, T., Mock, M., and Fouet, A. (2003). A plasmid-encoded regulator couples the synthesis of toxins and surface structures in Bacillus anthracis. Mol Microbiol *47*, 917-927.

Minden, J. S., and Marians, K. J. (1985). Replication of pBR322 DNA in vitro with purified proteins. Requirement for topoisomerase I in the maintenance of template specificity. J Biol Chem *260*, 9316-9325.

Mock, M., and Fouet, A. (2001). Anthrax. Annu Rev Microbiol *55*, 647-671.

Moller-Jensen, J., Jensen, R. B., and Gerdes, K. (2000). Plasmid and chromosome segregation in prokaryotes. Trends Microbiol *8*, 313-320.

Mukherjee, A., Dai, K., and Lutkenhaus, J. (1993). Escherichia coli cell division protein FtsZ is a guanine nucleotide binding protein. Proc Natl Acad Sci U S A *90*, 1053-1057.

Okinaka, R., Cloud, K., Hampton, O., Hoffmaster, A., Hill, K., Keim, P., Koehler, T., Lamke, G., Kumano, S., Manter, D.*, et al.* (1999a). Sequence, assembly and analysis of pX01 and pX02. J Appl Microbiol *87*, 261-262.

Okinaka, R. T., Cloud, K., Hampton, O., Hoffmaster, A. R., Hill, K. K., Keim, P., Koehler, T. M., Lamke, G., Kumano, S., Mahillon, J.*, et al.* (1999b). Sequence and organization of pXO1, the large Bacillus anthracis plasmid harboring the anthrax toxin genes. J Bacteriol *181*, 6509-6515.

Osborn, M. (1999). Database of Plasmid Replicons (DPR).

Pannucci, J., Okinaka, R. T., Sabin, R., and Kuske, C. R. (2002a). Bacillus anthracis pXO1 plasmid sequence conservation among closely related bacterial species. J Bacteriol *184*, 134-141.

Pannucci, J., Okinaka, R. T., Williams, E., Sabin, R., Ticknor, L. O., and Kuske, C. R. (2002b). DNA sequence conservation between the Bacillus anthracis pXO2 plasmid and genomic sequence from closely related bacteria. BMC Genomics *3*, 34.

Paulsson, J., and Chattoraj, D. K. (2006). Origin inactivation in bacterial DNA replication control. Mol Microbiol *61*, 9-15.

Perez-Casal, J., Caparon, M. G., and Scott, J. R. (1991). Mry, a trans-acting positive regulator of the M protein gene of Streptococcus pyogenes with similarity to the receptor proteins of two-component regulatory systems. J Bacteriol *173*, 2617-2624.

Quinn, C. P., and Dancer, B. N. (1990). Transformation of vegetative cells of Bacillus anthracis with plasmid DNA. J Gen Microbiol *136*, 1211-1215.

Rasko, D. A., Altherr, M. R., Han, C. S., and Ravel, J. (2005). Genomics of the Bacillus cereus group of organisms. FEMS Microbiol Rev *29*, 303-329.

Rasko, D. A., Ravel, J., Okstad, O. A., Helgason, E., Cer, R. Z., Jiang, L., Shores, K. A., Fouts, D. E., Tourasse, N. J., Angiuoli, S. V.*, et al.* (2004). The genome sequence of Bacillus cereus ATCC 10987 reveals metabolic adaptations and a large plasmid related to Bacillus anthracis pXO1. Nucleic Acids Res *32*, 977-988.

RayChaudhuri, D., and Park, J. T. (1992). Escherichia coli cell-division gene ftsZ encodes a novel GTP-binding protein. Nature *359*, 251-254.

Read, T. D., Salzberg, S. L., Pop, M., Shumway, M., Umayam, L., Jiang, L., Holtzapple, E., Busch, J. D., Smith, K. L., Schupp, J. M.*, et al.* (2002). Comparative genome

sequencing for discovery of novel polymorphisms in Bacillus anthracis. Science *296*, 2028-2033.

Reddy, A., Battisti, L., and Thorne, C. B. (1987). Identification of self-transmissible plasmids in four Bacillus thuringiensis subspecies. J Bacteriol *169*, 5263-5270.

Remus, D., Beall, E. L., and Botchan, M. R. (2004). DNA topology, not DNA sequence, is a critical determinant for Drosophila ORC-DNA binding. Embo J *23*, 897-907.

Rocha, E. P. (2004). The replication-related organization of bacterial genomes. Microbiology *150*, 1609-1627.

Romberg, L., and Levin, P. A. (2003). Assembly dynamics of the bacterial cell division protein FTSZ: poised at the edge of stability. Annu Rev Microbiol *57*, 125-154.

Rothfield, L., Justice, S., and Garcia-Lara, J. (1999). Bacterial cell division. Annu Rev Genet *33*, 423-448.

Saile, E., and Koehler, T. M. (2002). Control of anthrax toxin gene expression by the transition state regulator abrB. J Bacteriol *184*, 370-380.

Sambrook, J., E.F. Fritsch, and T. Maniatis. (1989). Molecular Cloning: a laboratory manual., 2nd edn (Cold Spring Harbor, N.Y., Cold Spring Harbor Laboratory Press).

Schaeffer, P. M., Headlam, M. J., and Dixon, N. E. (2005). Protein--protein interactions in the eubacterial replisome. IUBMB Life *57*, 5-12.

Schnos, M., Zahn, K., Inman, R. B., and Blattner, F. R. (1988). Initiation protein induced helix destabilization at the lambda origin: a prepriming step in DNA replication. Cell *52*, 385-395.

Schuch, R., Nelson, D., and Fischetti, V. A. (2002). A bacteriolytic agent that detects and kills Bacillus anthracis. Nature *418*, 884-889.

Schwarz, F. V., Perreten, V., and Teuber, M. (2001). Sequence of the 50-kb conjugative multiresistance plasmid pRE25 from Enterococcus faecalis RE25. Plasmid *46*, 170-187.

Spencer, R. C. (2003). Bacillus anthracis. J Clin Pathol *56*, 182-187.

Tang, M., Bideshi, D. K., Park, H. W., and Federici, B. A. (2006). A Mini-Replicon of pBtoxis from Bacillus thuringiensis subsp. israelensis. Appl Environ Microbiol.

Tinsley, E., and Khan, S. A. (2006). A novel FtsZ-like protein is involved in replication of the anthrax toxin-encoding pXO1 plasmid in Bacillus anthracis. J Bacteriol *188*, 2829-2835.

Tinsley, E., Naqvi, A., Bourgogne, A., Koehler, T. M., and Khan, S. A. (2004). Isolation of a minireplicon of the virulence plasmid pXO2 of Bacillus anthracis and characterization of the plasmid-encoded RepS replication protein. J Bacteriol *186*, 2717-2723.

Toth, J., and Biggin, M. D. (2000). The specificity of protein-DNA crosslinking by formaldehyde: in vitro and in drosophila embryos. Nucleic Acids Res *28*, e4.

Uchida, I., Hashimoto, K., and Terakado, N. (1986). Virulence and immunogenicity in experimental animals of Bacillus anthracis strains harbouring or lacking 110 MDa and 60 MDa plasmids. J Gen Microbiol *132*, 557-559.

Uchida, I., Makino, S., Sasakawa, C., Yoshikawa, M., Sugimoto, C., and Terakado, N. (1993). Identification of a novel gene, dep, associated with depolymerization of the capsular polymer in Bacillus anthracis. Mol Microbiol *9*, 487-496.

Uchida, I., Makino, S., Sekizaki, T., and Terakado, N. (1997). Cross-talk to the genes for Bacillus anthracis capsule synthesis by atxA, the gene encoding the trans-activator of anthrax toxin synthesis. Mol Microbiol *23*, 1229-1240.

Van der Auwera, G. A., Andrup, L., and Mahillon, J. (2005). Conjugative plasmid pAW63 brings new insights into the genesis of the Bacillus anthracis virulence plasmid pXO2 and of the Bacillus thuringiensis plasmid pBT9727. BMC Genomics *6*, 103.

Vietri, N. J., Marrero, R., Hoover, T. A., and Welkos, S. L. (1995). Identification and characterization of a trans-activator involved in the regulation of encapsulation by Bacillus anthracis. Gene *152*, 1-9.

Weaver, K. E., Clewell, D. B., and An, F. (1993). Identification, characterization, and nucleotide sequence of a region of Enterococcus faecalis pheromone-responsive plasmid pAD1 capable of autonomous replication. J Bacteriol *175*, 1900-1909.

Weiss, D. S. (2004). Bacterial cell division and the septal ring. Mol Microbiol *54*, 588-597.

Welkos, S. L., Vietri, N. J., and Gibbs, P. H. (1993). Non-toxigenic derivatives of the Ames strain of Bacillus anthracis are fully virulent for mice: role of plasmid pX02 and chromosome in strain-dependent virulence. Microb Pathog *14*, 381-388.

Wilcks, A., Smidt, L., Okstad, O. A., Kolsto, A. B., Mahillon, J., and Andrup, L. (1999). Replication mechanism and sequence analysis of the replicon of pAW63, a conjugative plasmid from Bacillus thuringiensis. J Bacteriol *181*, 3193-3200.

Worning, P., Jensen, L. J., Hallin, P. F., Staerfeldt, H. H., and Ussery, D. W. (2006). Origin of replication in circular prokaryotic chromosomes. Environ Microbiol *8*, 353-361.