The Tubulin-Like RepX Protein Encoded by the pXO1 Plasmid Forms Polymers In Vivo in *Bacillus anthracis*[▽]

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Bacillus anthracis contains two megaplasms, pXO1 and pXO2, that are critical for its pathogenesis. Stable inheritance of pXO1 in *B. anthracis* is dependent upon the tubulin/FtsZ-like RepX protein encoded by this plasmid. Previously, we have shown that RepX undergoes GTP-dependent polymerization in vitro. However, the polymerization properties and localization pattern of RepX in vivo are not known. Here, we utilize a RepX-green fluorescent protein (GFP) fusion to show that RepX forms foci and three distinct forms of polymeric structures in *B. anthracis* in vivo, namely straight, curved, and helical filaments. Polymerization of RepX-GFP as well as the nature of polymers formed were dependent upon concentration of the protein inside the *B. anthracis* cells. RepX predominantly localized as polymers that were parallel to the length of the cell. RepX also formed polymers in *Escherichia coli* in the absence of other pXO1-encoded products, showing that in vivo polymerization is an inherent property of the protein and does not require either the pXO1 plasmid or proteins unique to *B. anthracis*. Overexpression of RepX did not affect the cell morphology of *B. anthracis* cells, whereas it drastically distorted the cell morphology of *E. coli* host cells. We discuss the significance of our observations in view of the plasmid-specific functions that have been proposed for RepX and related proteins encoded by several megaplasms found in members of the *Bacillus cereus* group of bacteria.

Bacillus anthracis is a potent bioterror agent that causes anthrax in humans (17, 28, 34). The spores of the bacilli harboring two megaplasms, pXO1 (181.6 kb) and pXO2 (96.2 kb), are the infectious form of the organism. Both of these plasmids are required for the full virulence of this organism as strains lacking pXO1 (such as Pasteur) or pXO2 (such as Sterne) are severely impaired in their virulence (15, 26, 27, 39, 43, 52, 55). The pXO1 plasmid encodes the anthrax toxin proteins termed the protective antigen, lethal factor, and edema factor as well as proteins that are involved in germination of the spores (16, 22, 27, 28). It also carries the genes *atxA* and *pagR* that regulate the expression of the anthrax toxin and other virulence genes (6, 7, 12, 28). The pXO2 plasmid (96.2 kb) carries genes that are required for capsule synthesis, which is critical for anthrax pathogenesis (28, 38). Plasmids with considerable homology to pXO1 have been found in other members of the *Bacillus cereus* group (*Bacillus cereus*, *Bacillus thuringiensis*, and *Bacillus mycoides*) (42). 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 lost (28, 34). Genetic exchange can occur among different *B. anthracis* strains and between *B. anthracis* and other members of the *B. cereus* group (28), making it possible that *B. cereus* group members could potentially evolve into *B. anthracis* after acquiring the virulence plasmids (46). Indeed, recently the *B. cereus* strain G9241 was identified, which causes an anthrax-like disease and contains the pBXO1 plasmid that is almost identical to pXO1 (23). Furthermore, *B. cereus* strain

10987 contains a pXO1-like plasmid, although it lacks the pathogenicity island carrying the anthrax toxin genes (23).

In spite of the central role played by pXO1-encoded proteins in anthrax virulence and pathogenesis, very little is known at present about the replication and partitioning of pXO1 and related plasmids. The factors responsible for the restricted host range of pXO1 are also not known. In our earlier work, we characterized RepX, which is a 48-kDa protein encoded by pXO1 and the sole plasmid-encoded protein essential for the maintenance of a mini pXO1 replicon in *B. anthracis* (51). RepX harbors limited homology at its amino terminus to the bacterial cell division protein FtsZ and has GTP binding and GTPase- and GTP-dependent polymerization activities (1, 8). Both FtsZ and RepX proteins belong to the tubulin family. Members of the tubulin family act as cytoskeletal proteins involved in cell division and DNA segregation in prokaryotic and eukaryotic cells (1, 8, 29, 32, 33, 40, 51). However, unlike FtsZ, RepX harbors a GTP-dependent DNA binding activity, although it binds only to DNA substrates nonspecifically (1). The presence of a tubulin signature motif is essential for GTP binding, GTPase, and polymerization activities of RepX, as a mutant in the tubulin signature motif (T125A) affected the activities of the protein described above and also severely impaired its ability to support pXO1 replication in vivo (1, 51). The ability of the protein to assemble into polymers in vitro led us to postulate that RepX may also play a role in the partitioning of the pXO1 plasmid.

Most low-copy-number plasmids rely on partitioning or segregation systems for their stable inheritance (9, 10, 21). Proteins that resemble the bacterial cytoskeleton constitute the functional core of such partitioning systems that have been termed segrosomes (21). A functional segrosome is composed of three plasmid-encoded components (20). These consist of a

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
<i>B. anthracis</i> UM23C1-1		T. Koehler
<i>B. anthracis</i> UT252		T. Koehler
<i>E. coli</i> DH5 α		Invitrogen
<i>E. coli</i> GM2163		NEB
<i>E. coli</i> C41(λ DE3)	Expression strain for toxic proteins from T7 promoter	Lucigen
<i>E. coli</i> Top 10		Invitrogen
Plasmids		
pMutin-GFP+	ColE1 <i>ori</i> , GFP fusion vector, Amp ^r , Em ^r	25
pKL2427	pMutin-GFP+ with <i>repX</i> and 786-bp-upstream sequence	This study
pKL2425	pMutin-GFP+ with the gene <i>repX</i>	This study
pWH1520	ColE1 <i>ori</i> , pBC16 <i>ori</i> for <i>Bacillus</i> , xylose-inducible expression vector, Amp ^r , Tc ^r	44
pKL2468	pWH1520 with RepX-GFP	This study
pKL2509	pWH1520 with RepX-GFP and 786-bp upstream sequence	This study
pET14b	T7 promoter, His tag, Amp ^r	Novagen
pKL2390	<i>repX</i> cloned into pET14b, Amp ^r	This study

cis-acting DNA sequence, which constitutes a minimal centromere, a DNA binding protein such as ParB (11, 47, 48) or ParR (35, 37), which binds to the centromere-like repeat sequences, and a polymerizing protein such as ParA, ParM, or ParF (2, 14, 21). The ParB-type proteins interact with ParA-type proteins and help to anchor the plasmid DNA to the partitioning machinery formed by the polymerizing protein (10, 20, 47, 49). The polymerizing protein generates the force that is required for pushing or transporting the plasmid to the opposite poles of the dividing cell. To date, with the exception of the TubZ protein that is encoded by the pBtoxis plasmid of *B. thuringiensis*, the polymerizing components of the segrosomes have been shown to fall into either the actin superfamily (typified by ParM) or the ParA superfamily (typified by ParA) (4, 10, 21, 24). Plasmid segregation utilizing the ParM protein of the R1 plasmid, which belongs to the actin family, is dependent upon the ability of the protein to form polymers (35, 37). The TubZ protein of pBtoxis has been shown to assemble into dynamic filaments and exhibits treadmilling in vivo (29). Mutations that affected the dynamic nature of TubZ filaments were shown to affect pBtoxis stability (29). It has been proposed that TubZ and related proteins could form a new family of tubulin-based cytoskeletal proteins that act as force-generating systems to accomplish plasmid partitioning (1, 8, 29, 32, 33, 40). Thus, the plasmid segrosomes characterized to date utilize polymerizing proteins that belong to three major families, namely actin family proteins, ParA-type ATPases, and tubulin-type GTPases.

Although RepX protein is essential for the maintenance of pXO1 in vivo (51) and also has been shown to exhibit GTP-dependent polymerization in vitro (1, 8), its vivo localization and polymerization properties are not known. Here we show that RepX protein expressed as a green fluorescent protein (GFP) fusion from an exogenous plasmid assembles into polymers in *B. anthracis* in vivo. RepX-GFP exhibited three distinct localization patterns, which probably represent different stages of polymerization of the protein in vivo. By expressing the fusion RepX-GFP protein in *B. anthracis* cells harboring the native pXO1 plasmid, we show that the presence of pXO1 did not influence the localization of RepX-GFP in vivo. The pres-

ence as well as the nature of RepX-GFP polymers inside *B. anthracis* cells were dependent upon the concentration of the fusion protein within the cell.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *B. anthracis* strains were grown at 37°C in LB medium. Bacterial strains and plasmids used in this study are listed in Table 1. UT252, a *B. anthracis* strain containing a pXO1 derivative with a spectinomycin resistance gene (obtained from T. Koehler), was grown at 37°C in LB medium supplemented with 100 μ g/ml spectinomycin. UT252 derivatives containing the pKL2509 and pKL2468 plasmids containing the tetracycline resistance gene were grown in the presence of both spectinomycin and 5 μ g/ml tetracycline.

DNA manipulations. All DNA manipulations were done using standard protocols (45) using restriction enzymes and T4 DNA ligase (NEB), deoxynucleoside triphosphates and alkaline phosphatase (Roche), and a plasmid DNA purification kit (Promega). Plasmids were introduced into both *Escherichia coli* and *B. anthracis* by using the Bio-Rad Gene Pulser.

Plasmid constructions. The RepX-GFP plasmid, pKL2427, was constructed by amplifying a 2,090-bp DNA fragment (RepX open reading frame [ORF] plus 786-bp upstream sequence corresponding to pXO1 coordinates 59,752 to 57,662) from the pXO1 plasmid using the forward (5'-CGCGGTACCGGTAGTAGTCGGATGAGCG-3') and reverse (5'-CGCGGTACCGAATGATAATTCTTCTTTGTTG-3') primers containing KpnI linkers and cloning into the TOPO 2.1 vector, followed by transforming *E. coli*. The *repX* and upstream sequences were then excised by digesting the recombinant plasmid with KpnI and ligating the 2,090-bp fragment into KpnI-digested pMUTIN-GFP-positive plasmid (25). The resulting plasmid, pKL2427, contains GFP fused to the C terminus of the RepX ORF, and the fusion protein is expected to be expressed from the putative *repX* promoter. Since pKL2427 replicates in *E. coli* but not in *B. anthracis*, we amplified the *repX-gfp* sequence from the plasmid described above using forward (5'-CAAACAGTTCGGTGATTAGTCGGATGAGCG-3') and reverse (5'-CGCACTAGTGGTA CCATTATTTGTAGAGCTC-3') primers. The 2.8-kb amplified fragment with SpeI linkers was then ligated into the SpeI site of pWH1520, which is an *E. coli-Bacillus* sp. shuttle vector (44). The resulting pKL2509 plasmid was recovered by transforming *E. coli*. This plasmid replicates at a high copy number in *B. anthracis* and is expected to express RepX-GFP from the native *repX* promoter.

We also constructed a plasmid that replicates in *B. anthracis* and expresses RepX-GFP from the inducible xylose promoter. The *repX* ORF lacking its promoter (corresponding to pXO1 nucleotides 58,981 to 57,662) was amplified using pXO1 DNA as the template and forward (5'-CGCGGTACCGAGGAGGCTATACATATGGCAGGC-3') and reverse (5'-CGCGGTACCGAATGATAATTCTTCTTTGTTG-3') primers having KpnI linkers and ligated into the TOPO 2.1 vector. Following this, the recombinant TOPO 2.1 plasmid containing the *repX* ORF was digested with KpnI and the 1.3-kb *repX* fragment was ligated into KpnI-digested *E. coli* plasmid pMUTIN-GFP+ (25). The resulting plasmid,

pKL2425, was isolated by transforming *E. coli*. Using the plasmid described above as a template, the *repX-gfp* sequence was PCR amplified using forward (5'-CAAAGTAGTAGGGAGGCTATACATATGGCAGGC-3') and reverse (5'-CGCACTAGTGGTACCATTATTTGTAGAGCTC-3') primers. The PCR product was digested with SpeI and ligated into the SpeI site of pWH1520 which is an *E. coli-Bacillus* sp. shuttle vector (44). The ligation mixture was introduced into *E. coli*, and the recombinant plasmid pKL2468 that expresses the RepX-GFP fusion from the xylose-inducible promoter was isolated.

Antibody production and immunoblot analysis. To obtain anti-RepX antiserum, RepX protein was purified by overexpressing it as a His₆ fusion in *E. coli*. The *repX* ORF (amino acid positions 2 to 435) was PCR amplified using forward (5'-CCGGATCCGGCAGGCAATTTTCTGAAATCG-3') and reverse (5'-CCGGATCCTAGAATGATAATTTCTTCTTTCTTG-3') primers that contained BamHI linkers. The amplified PCR product was digested with BamHI and ligated into BamHI-digested pET14b(+) vector (Novagen). The resulting pKL2390 plasmid was recovered by transforming *E. coli* C41(ΔDE3). This plasmid is expected to express RepX with a His₆ tag at its N terminus under the control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible T7 promoter. His-RepX protein was overexpressed by treating *E. coli* C41(ΔDE3) cells with 1 mM IPTG for 4 h, and the protein was affinity purified using Ni-nitrilotriacetic acid agarose affinity chromatography. His-RepX bound to the column was eluted with 0.25 M imidazole, and the protein was approximately 99% pure as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The purified protein was used to raise anti-RepX polyclonal antibodies in rabbits, and the antibodies were affinity purified (Pacific Immunology, Ramona, CA). For immunoblot analysis, 15 μg of total protein from each sample of *B. anthracis* (lysed with three passes through a French press at 18,000 lb/in²) or *E. coli* was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to the polyvinylidene difluoride membrane (Immobilon-P; Millipore). The membrane was blocked with 5% nonfat dry milk and probed with primary anti-RepX antibody (1:400,000 dilution) followed by goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology) and developed with the enhanced chemiluminescence reagent (GE Healthcare).

Microscopy. *B. anthracis* strains were grown overnight at 37°C in LB medium with 100 μg/ml spectinomycin and 5 μg/ml tetracycline and subsequently used to freshly inoculate LB medium containing the antibiotics described above. Cells were grown for 4 to 5 h either without induction (expression from the native *repX* promoter) or after induction with xylose (for expression from the xylose-inducible promoter), pelleted, and washed twice with 1× Hank's balanced salt solution buffer (Invitrogen). *E. coli* DH5α harboring a plasmid expressing RepX-GFP was grown at 37°C in LB medium supplemented with 100 μg/ml ampicillin. Live cells (2 to 3 μl) were mounted on microscope slides having a thin layer of 1% LB agarose solution and covered with poly-lysine-coated coverslip. An Olympus FluoView 1000 confocal microscope, with a 100× UPLSAPO 1.40 numerical-aperture oil-immersion objective equipped with an Olympus photomultiplier detector, was used for acquiring images. A GFP filter was used, which was excited by an Ar⁺ laser emitting light at 488 nm. Images were acquired and analyzed by using the FluoView computer software. The images are presented without deconvolution processing.

RESULTS

Expression of RepX-GFP fusion protein in *B. anthracis*. The RepX-GFP fusion protein was expressed in *Bacillus anthracis* under two different conditions. In one, it was expressed from pWH1520, a multicopy replicative vector that harbors the pBC16 *ori* that has been shown to be functional in *Bacillus cereus* (44). In this construct, the expression of RepX-GFP is under the control of a 0.78-kb sequence derived from the upstream region of RepX ORF in pXO1, which contains the *repX* promoter (unpublished data). Western blotting with affinity-purified polyclonal anti-RepX antibodies showed that the expression level of RepX-GFP from this construct was approximately fivefold higher than that of the native RepX expressed from wild-type (wt) pXO1 (Fig. 1A). This is due to the high copy number of the rolling circle-replicating pWH1520 plasmid compared to the low-copy-number pXO1 plasmid. In the second approach, we obtained regulated ex-

FIG. 1. Expression of native RepX and RepX-GFP in *B. anthracis*. (A) Western blot analysis of RepX and RepX-GFP in cell lysates from *B. anthracis* (Ba) containing either the wt pXO1 plasmid encoding RepX or the multicopy pKL2509 plasmid constitutively expressing RepX-GFP. (B) Xylose (Xyl) concentration-dependent expression of RepX-GFP from pKL2468 in *B. anthracis* cells. Total protein from plasmid-free (Ba) and pXO1-containing *B. anthracis* cells was used as the control.

pression of RepX using pKL2468, in which expression of RepX-GFP was controlled by a xylose-inducible promoter (44). Expression of RepX-GFP from the pKL2468 vector was found to be dependent upon xylose concentration (Fig. 1B). Our results showed that at 0.03% xylose, expression of RepX-GFP is similar to that of native RepX in *B. anthracis* cells harboring the pXO1 plasmid (Fig. 1B).

RepX-GFP polymerizes in vivo into long filaments in *B. anthracis*. Localization of RepX-GFP protein in *B. anthracis* cells was monitored by confocal microscopy. In *B. anthracis* cells expressing RepX-GFP from its native promoter, RepX-GFP formed long polymers (Fig. 2A). As mentioned above, the expression of RepX from the multicopy pKL2509 construct results in fivefold-higher levels of the RepX-GFP fusion protein than the native RepX protein encoded by the pXO1 plasmid (Fig. 1A). Under these conditions, RepX-GFP polymers were of various lengths, probably representing polymerized RepX in various stages of filament growth (Fig. 2A). The polymers were either linear or curved (Fig. 2A). Comparison of the fluorescence signal with phase contrast images obtained for the same field showed that the longest polymers encompassed almost the full length of the cell (Fig. 2A and B) and that the curvature of some of the filaments was often the result of filaments reaching the cell poles. In addition to forming long filaments, RepX-GFP also appeared to form foci (Fig. 2C). A comparison of the fluorescence signal with phase contrast images showed that the foci were randomly distributed without any clear association with specific subcellular locations such as the mid-cell or cell poles (Fig. 2C and D). A count of several fields containing a total of 600 cells showed that more than 90% contained filaments while approximately 2 to 5% of cells contained foci (data not shown). Moreover, cells exhibiting the RepX-GFP foci generally lacked long filaments, suggesting that the foci may represent the initiation stage of RepX polymerization. Even though the predominant form of RepX-GFP polymers corresponded to relatively straight or curved filaments (Fig. 2A), we also observed rare cells in which they formed helical filaments (Fig. 2E).

Efficiency of RepX polymerization is dependent upon protein concentration. In vitro polymerization of RepX occurs only beyond a critical concentration of the protein (1, 8). Therefore, we wished to determine whether polymerization of RepX in vivo also required the protein to be present beyond a

FIG. 2. RepX-GFP assembles into polymers in *B. anthracis*. Fluorescence microscopic (A) and corresponding phase contrast images (B) of *B. anthracis* expressing RepX-GFP from pKL2509 showing straight (arrowheads) and curved (arrows) long polymers. Fluorescence microscopic (C) and corresponding phase contrast images (D) of *B. anthracis* harboring pKL2509 showing foci and long polymers. RepX-GFP foci at a higher magnification are shown in the inset of panel C. Fluorescence microscopic image of *B. anthracis* harboring pKL2509 (E), showing helical filaments assembled from RepX-GFP.

critical concentration. Since expression of RepX from its native promoter on the multicopy pKL2509 plasmid results in higher levels of protein than its native levels in *B. anthracis*, we utilized the pKL2468 plasmid expressing RepX-GFP from the xylose-inducible promoter to study RepX polymerization in vivo at different protein concentrations. *B. anthracis* cells harboring pKL2468 were grown in the presence of various concentrations of xylose, and RepX-GFP expression was monitored by Western blot analysis. At a low 0.01% xylose concentration, RepX-GFP was expressed to approximately 10 to 20% of the levels RepX expressed from the wt pXO1 plasmid (Fig. 1B and 3A). The levels of the RepX-GFP protein were similar whether or not the *B. anthracis* cells expressed the native RepX protein from the wt pXO1 plasmid (compare Fig. 1B and 3A). In the absence of the pXO1 plasmid, upon induction with 0.01% xylose most of the cells contained only diffused, cytoplasmic fluorescence from RepX-GFP (Fig. 3B). At 0.02% xylose, approximately 50% of RepX-GFP levels were obtained compared to those of RepX expressed from wt pXO1

(Fig. 1B and 3A). A count of several fields containing approximately 600 cells grown in the presence of 0.02% xylose showed that approximately 10 to 16% of cells contained RepX-GFP foci or short filaments (Fig. 3C). When the xylose concentration was increased to 0.03%, RepX-GFP was expressed to levels similar to that of native RepX expressed from wt pXO1. At this concentration, approximately 40 to 60% cells contained RepX-GFP filaments (Fig. 3D). RepX-GFP filaments were observed in approximately 80 to 90% of cells induced with 0.1% xylose, and a higher proportion consisted of longer filaments (Fig. 3E). Thus, our results show that the efficiency of RepX polymerization is dependent upon its concentration in vivo.

We also checked localization of RepX-GFP expressed from the xylose-inducible promoter in the pKL2468 plasmid in a strain of *B. anthracis* that also contained the wt pXO1 plasmid expressing native RepX. RepX-GFP formed foci at a 0.01% xylose concentration in these cells (Fig. 3F), in contrast to *B. anthracis* cells lacking pXO1 that required 0.02% xylose to form RepX-GFP foci (Fig. 3C). At a xylose concentration of 0.02%, approximately 60 to 70% of cells harbored foci as well as short filaments of RepX-GFP in pXO1-containing cells (Fig. 3G). This contrasted with cells lacking pXO1 which showed significant levels of short filaments (3 to 4 μ m) only in the presence of 0.03% xylose (Fig. 3D).

Similar results were obtained when we tested the amount of xylose required for the formation of long RepX-GFP polymers (5 μ m or longer) in *B. anthracis* cells lacking or containing wt pXO1. Long polymers were observed upon treatment of *B. anthracis* cells containing pXO1 and the RepX-GFP-expressing plasmid with 0.03% xylose, while such polymers were observed generally when cells expressing RepX-GFP alone were treated with 0.1% xylose (compare Fig. 3E and H). These results suggest that native RepX expressed from pXO1 may copolymerize with RepX-GFP in vivo, thereby reducing the levels of RepX-GFP required for polymer formation.

RepX-GFP polymerizes into long filaments in *E. coli* and causes filamentation of cells. We have previously shown that purified RepX protein can polymerize in vitro in the absence of any additional factors (1). To confirm if RepX-GFP polymerization was independent of a protein(s) unique to *B. anthracis*, we expressed RepX-GFP in *E. coli*. The pKL2509 plasmid expresses RepX-GFP in *E. coli* at levels that are similar to that of the native RepX expressed from pXO1 in *B. anthracis* (Fig. 4A). RepX-GFP expressed from pKL2509 formed polymers that were 2 to 3 μ m long (Fig. 4B). However, unlike *B. anthracis* cells, which often exhibited multiple RepX filaments (Fig. 2A), a vast majority (80 to 90%) of *E. coli* cells with visible filaments contained only a single filament (Fig. 4B). Our results suggest that polymerization is an intrinsic property of RepX and does not require an additional factor(s) that is unique to *B. anthracis* or that is encoded by the pXO1 plasmid. Overexpression of RepX in the homologous *B. anthracis* host does not alter cell morphology (Fig. 2A to D). In nature, pXO1 and related plasmids are found only in the *B. cereus* group of organisms. Therefore, we wished to check if overexpression of RepX-GFP has any effect on the heterologous host *E. coli*. The pKL2427 plasmid expresses approximately fourfold-higher levels of RepX-GFP in *E. coli* from the leaky Pspac promoter compared to the pKL2509 plasmid (Fig. 4A). Fluorescence

FIG. 3. RepX-GFP requires a critical concentration of the protein for assembly of polymers in *B. anthracis*. (A) Western blot analysis of cell lysates derived from a *B. anthracis* (Ba) strain containing both the wt pXO1 plasmid expressing native RepX and the pKL2468 plasmid expressing the RepX-GFP protein. Cells were treated with various concentrations of xylose (Xyl), and affinity-purified polyclonal anti-RepX antibodies were used for Western blot analysis. Shown also is fluorescence microscopy of *B. anthracis* harboring only the pKL2468 plasmid treated with 0.01% (B), 0.02% (C), 0.03% (D), and 0.10% (E) concentrations of xylose as well as fluorescence microscopic images of *B. anthracis* cells harboring both pKL2468 and wt pXO1 after growth in 0.01% (F), 0.02% (G), and 0.03% (H) xylose. RepX-GFP foci at a higher magnification are shown in the inset of panel C.

microscopy showed that overexpression of RepX-GFP from pKL2427 resulted in polymers that were frequently 10 to 15 μm long (Fig. 4D). Corresponding phase contrast images showed that the cell morphology of *E. coli* host was drastically altered, with extensive filamentation of many cells (Fig. 4E). Since *E. coli* and *B. anthracis* represent heterologous and homologous systems for RepX, respectively, these results suggest that while RepX expression does not appear to affect *B. anthracis*, it has a severe effect on *E. coli* cells.

DISCUSSION

In this paper, we demonstrate that RepX forms various types of polymers in vivo in *B. anthracis*, including filaments at concentrations similar to those present in cells containing the wt pXO1 plasmid. RepX is a member of a growing subfamily of plasmid-encoded proteins that are related to tubulin and the bacterial cell division protein FtsZ (1, 8, 29, 32, 33, 40, 51). The members of this subfamily are encoded by megaplasmids found in the *B. cereus* group of organisms (23, 29, 40–42, 51). Previously, we showed that RepX is the only pXO1-encoded protein essential for plasmid replication, suggesting that it may represent the replication initiator protein of pXO1 (51). Subsequently, we also showed that purified RepX protein has GTP binding and GTPase- and GTP-dependent polymerization activities in vitro (1). In addition, RepX bound weakly and non-specifically to DNA in the presence of GTP, dGTP, and

GTPγS (1). A T125A mutant in the functional tubulin-signature motif (GGGTGTG) of RepX was found to be defective in its GTPase and polymerization activity in vitro and pXO1 replication activity in vivo (1, 51). These studies suggested that RepX may be a novel, bifunctional protein involved in both replication and partitioning of the pXO1 plasmid.

To provide further support for possible roles of RepX in pXO1 maintenance, we investigated whether RepX can form polymers in vivo in *B. anthracis* cells. We expressed RepX-GFP from both its native promoter in the multicopy pKL2509 plasmid (Fig. 2) as well as a xylose-inducible promoter in pKL2468 (Fig. 3). We then studied the polymerization of RepX-GFP in vivo in *B. anthracis* cells at different protein concentrations. Our studies show that the RepX-GFP fusion protein can assemble into straight, curved, and helical filaments in *B. anthracis* cells (Fig. 2). At very low expression levels (Fig. 3A), RepX-GFP gave a diffuse fluorescence pattern in *B. anthracis* cells (Fig. 3B). At slightly increased levels of expression (induction with 0.02% xylose), RepX-GFP formed distinct foci and short filaments that were physically well separated (Fig. 3C). Longer filaments of RepX-GFP appeared at levels of expression that were similar to that of the native RepX protein expressed from wt pXO1 plasmid (Fig. 3A and D), and their length increased from 1 to 2 μm to more than 5 μm when RepX-GFP levels increased approximately fivefold (upon constitutive expression from the pKL2509 plasmid or expression from the pKL2468 plasmid upon induction with 0.1% xylose) compared to RepX

FIG. 4. RepX-GFP assembles into long polymers in *E. coli* and affects host cell phenotype. (A) Western blot analysis of RepX-GFP in *B. anthracis* (Ba) or *E. coli* (Ec) cell lysates containing either the pKL2509 plasmid expressing RepX-GFP from the native *repX* promoter or from the leaky Pspac promoter in the pKL2427 plasmid. Cell lysates from *B. anthracis* strains either lacking any plasmid (Ba) or containing the pXO1 plasmid expressing the native RepX protein were used as controls. Shown also are fluorescence microscopic (B) and corresponding phase contrast (C) images of *E. coli* cells harboring pKL2509 that expresses RepX-GFP as well as fluorescence microscopic (D) and corresponding phase contrast (E) images of *E. coli* cells harboring the pKL2427 plasmid that overexpresses RepX-GFP.

levels expressed from the wt pXO1 plasmid (Fig. 2A and 3E). In general, the number of RepX-GFP foci per cell as well as the frequency of cells harboring foci and filaments increased at higher concentrations of the protein, suggesting that the nucleation frequency of RepX polymerization is correlated with the concentration of RepX inside the cells. The RepX foci and filaments of different morphology seen in vivo may represent

polymers at various stages of filament growth, similar to what has been observed for FtsZ and MreB proteins (5, 50, 53).

Previously, we reported concentration-dependent polymerization of RepX in vitro (1). Results presented here show that both the number and nature of RepX polymers are also dependent on the concentration of the protein in vivo. Polymerization of RepX as well as the length of polymers in vivo was not dependent on the presence of pXO1 (Fig. 2 and 3C and D). Although RepX-GFP formed foci and filaments at lower concentrations in the cells that also expressed wt RepX from the pXO1 plasmid (Fig. 3C to H), this could be attributed to the presence of native RepX in addition to RepX-GFP in the observed foci and polymers. This would be expected to effectively lower the concentration of RepX-GFP required for polymer formation.

RepX belongs to a novel class of tubulin-related cytoskeletal proteins that are encoded by the megaplasmids found in the *Bacillus cereus* group of organisms (23, 29, 40–42, 51). These proteins have been shown to play a role in both replication (51) and partitioning (29) of their cognate parental plasmids. Two members of this family, namely TubZ and RepX, have been shown earlier to form polymers in vitro (1, 8). The in vivo polymerization properties of TubZ, which is encoded by the pBtoxis plasmid found in *B. thuringiensis*, have been reported (29). The polymers formed by RepX-GFP in vivo generally resembled the TubZ-GFP filaments. Thus, RepX-GFP filaments either had a linear or curved appearance based on their subcellular location with the curvature appearing to result from the growth of the filaments close to the cell poles (Fig. 2B). Similar to RepX, TubZ was also reported to form long polymers in the absence of its cognate plasmid pBtoxis (29).

Polymerizing proteins form a force-generating system that drives replicated copies of low-copy-number plasmids to opposite poles of a cell (20, 21, 35, 36, 40). These proteins include the ATPases of the actin family (4, 24, 35, 37) or ATPases of the ParA family (3, 10, 18, 30, 31, 54). The more recently discovered GTPases of the tubulin family are also thought to bring about active partitioning of low-copy-number plasmids that encode these proteins by a mechanism that requires polymerization of these proteins (1, 8, 29, 33, 40, 51). Functional complexes formed by the polymerizing protein, the cognate plasmid and factors that sequester the polymerizing protein to the cognate plasmid are known as segrosomes (20, 21). In segrosomes, the interaction of the polymerizing protein with the cognate plasmid is thought to drive the active partitioning of the plasmids to daughter cells (20, 21). Polymers of ParM, an actin family member involved in partitioning of the low-copy-number R1 plasmid found in *E. coli*, are stabilized at their ends by cognate plasmid-protein complexes ParR and ParC (13, 37). Similarly, nucleation and stabilization of the polymers of SopA encoded by the *E. coli* F plasmid that belongs to the ParA family require the cognate F plasmid and SopB protein in vivo (31). Our results show that RepX does not require the cognate pXO1 plasmid or any plasmid-encoded factors for the formation of long polymers. Together with results that have been published for TubZ (29), it is possible that the polymers formed by the tubulin family plasmid partitioning proteins may be stabilized by a different mechanism.

RepX harbors significant amino acid sequence homology to the tubulin family cell division protein, FtsZ. In *B. subtilis* cells

treated with the FtsZ-specific inhibitor PC190723, FtsZ-GFP is present only as foci, suggesting an inhibition during the initial stages of polymer growth in vivo (19). The observation that foci are the first discernible structures assembled from RepX-GFP (Fig. 3C) suggests that assembly of RepX also initiates as foci.

RepX-GFP formed long filaments in *E. coli* cells (Fig. 4). This rules out the requirement of any *B. anthracis*-specific or pXO1-encoded factors for RepX polymerization. Similar results have been reported for TubZ (29). Overexpression of RepX-GFP drastically altered the cell morphology of the heterologous *E. coli* host and caused inhibition of growth (data not shown), whereas the homologous *B. anthracis* hosts remained unaffected (Fig. 2B and D and 4E). These results raise the intriguing possibility that RepX has evolutionarily adapted for functional sequestration of the plasmid-encoded polymerizing complex in the homologous system—a factor that could restrict the host range of pXO1 plasmid to the *B. cereus* group of organisms in nature. Future studies will be directed toward identifying the potential role of RepX in replication and partitioning of the pXO1 plasmid utilizing RepX mutants that are defective in its polymerization or DNA binding activities.

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