A new tubRZ operon involved in the maintenance of the *Bacillus sphaericus* mosquitocidal plasmid pBsph

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pBsph is a mosquitocidal plasmid first identified from *Bacillus sphaericus*, encoding binary toxins (Bin toxins) that are highly toxic to mosquito larvae. This plasmid plays an important role in the maintenance and evolution of the bin genes in *B. sphaericus*. However, little is known about its replication and partitioning. Here, we identified a 2.4 kb minimal replicon of pBsph plasmid that contained an operon encoding TubR-Bs and TubZ-Bs, each of which was shown to be required for plasmid replication. TubR-Bs was shown to be a transcriptional repressor of tubRZ-Bs genes and could bind cooperatively to the replication origin of eleven 12 bp degenerate repeats in three blocks, and this binding was essential for plasmid replication. TubZ-Bs exhibited GTPase activities and interacted with TubR-Bs:DNA complex to form a ternary nucleoprotein apparatus. Electron and fluorescence microscopy revealed that TubZ-Bs assembled filaments both in vitro and in vivo, and two point mutations in TubZ-Bs (T114A and Y260A) that severely impaired the GTPase and polymerization activities were found to be defective for plasmid maintenance. Further investigation demonstrated that overproduction of TubZ-Bs-GFP or its mutant forms significantly reduced the stability of pBsph. Taken together, these results suggested that TubR-Bs and TubZ-Bs are involved in the replication and probably in the partitioning of pBsph plasmid, increasing our understanding of the genetic particularity of TubZ systems.

INTRODUCTION

Little is known regarding the factors and mechanisms in bacteria that correlate plasmid replication and segregation (Yamaichi *et al.*, 2011). Replication initiation proteins encoded by plasmids usually bind to DNA repeats (iterons), resulting in melting of the nearby origin of replication that allows plasmid replication to initiate. Partition proteins encoded by most low-copy-number plasmids are essential for plasmid stability and are always recruited into the formation of a ternary partition (par) system, which is composed of a centromere-like DNA site, a centromere-binding protein and a cytomotive NTPase (Barilla, 2010). Based on the phylogeny and type of NTPase involved, the bacterial par systems have been generally classified into three types (Gerdes *et al.*, 2010). Type I systems contain ATPase (ParA) with a deviant Walker-box motif and actively segregate both bacterial plasmids and chromosomes by ParAs patterning on the nucleoid (Vecchiarelli *et al.*, 2013). The most well-understood type II systems encode actin-like ATPase (ParM), which assembles into dynamic anti-parallel filaments and pushes the plasmids to opposite cell poles (Gayathri *et al.*, 2012). Type III systems are recently characterized par systems that encode tubulin/FtsZ-like GTPase (TubZ) (Larsen *et al.*, 2007).

The TubZ system was first demonstrated to play a role in plasmid replication (Tang *et al.*, 2006) and then suggested to be a filament-forming GTPase involved in plasmid partition (Larsen *et al.*, 2007). Thus far, TubZ homologues have only been found on large plasmids replicating in *Bacillus cereus* group strains (Hoshino & Hayashi, 2012; Tang *et al.*, 2007; Tinsley & Khan, 2006) and bacteriophages from *Clostridium* and *Pseudomonas* (Aylett *et al.*, 2013; Kraemer *et al.*, 2012; Oliva *et al.*, 2012). One well-characterized TubZ system is the one encoded on the *Bacillus thuringiensis* pBtOxis plasmid, which encodes a GTPase called TubZ and centromere-binding protein called TubR. Both are required for plasmid maintenance (Tang *et al.*, 2006). Tang *et al.* (2007) showed...
that TubR bound to a cis sequence of four iterons located upstream of the genes, which was sufficient for initiation of plasmid replication. Later studies revealed that tubC was actually composed of seven iterons divided into two clusters (Aylett & Lowe, 2012). TubR contains a winged-helix–turn–helix motif and binds to tubC to form a flexible DNA–protein filament, which is suitable for capturing polymerizing protein TubZ to produce a stabilized triple partition complex (Aylett & Lowe, 2012; Ni et al., 2010). Previous results have also demonstrated that TubZ assembles into dynamic filaments that exhibit directional growth and translocate in the cell by treadmilling, elongating at one end while retracting at the opposite end (Larsen et al., 2007). This cytoskeletal behaviour is believed to play an important role in plasmid partition and stability.

Another important example is the Bacillus anthracis pXO1 plasmid, which encodes TubZ-Ba (RepX), the sole plasmid-encoded protein required for maintenance of a minireplicon (Tinsley & Khan, 2006). TubZ-Ba harbours non-specific DNA-binding activities and could assemble filaments both in vivo and in vitro (Akhtar et al., 2009; Anand et al., 2008), leading to the postulation that TubZ-Ba may play a role in both replication and partitioning of plasmid pXO1. The genetic particularity of the TubZ system, involved in both replication and partitioning, has made this system quite different from the type I and II systems, and the specific roles of TubZ and TubR in plasmid replication and partitioning are still controversial and need to be further investigated.

Bacillus sphaericus is a Gram-positive, aerobic, mesophilic and spore-forming bacterium that is commonly isolated from soil. Some strains produce active insecticidal proteins against mosquito larvae, and thus have been widely used as biocontrol agents for disease-transmitting mosquitoes (Berry, 2012). Since the initial discovery of B. sphaericus mosquitocidal activity, studies have shown the binary toxins (Bin toxins) to be major mosquitocidal factors (Baumann et al., 1988; Hindley & Berry, 1987). Later analyses revealed that the Bin toxins from B. sphaericus C3–41 were encoded by both the chromosome and the large plasmid pBsph, and that this plasmid also contains genes involved in the germination of spores and limitation of foreign DNA acquisition (Hu et al., 2008).

In spite of the role that pBsph plays in pathogenicity, little is known about how this plasmid is maintained. In this study, we characterized a minireplicon from plasmid pBsph that bore an operon coding for two proteins, TubR-Bs and TubZ-Bs, both of which were essential for plasmid replication. We also demonstrated that TubZ-Bs assembled filaments both in vitro and in vivo and was recruited into the formation of a ternary complex with TubR-Bs:DNA, suggesting that TubR-Bs and TubZ-Bs may also be involved in the partitioning of pBsph plasmid. To our knowledge, this is the first extensive investigation on the molecular mechanism of TubZ-encoded plasmid replication and partitioning in non-B. cereus group strains. We believe this information is of importance for further understanding the evolution and inheritance of Bin toxins in B. sphaericus.

### METHODS

**Plasmids, oligonucleotides, strains and DNA manipulation.** Oligonucleotides and plasmids used in this study are listed in Tables S1 and S2 (available in the online Supplementary Material). Escherichia coli JM109 was used for plasmid construction and BL21(DE3) for recombinant protein overproduction. Bacillus species strains used in this study are listed in Table 1, and transformation was performed essentially as described previously (Wu et al., 2012). Bacterial strains were grown in Luria–Bertani (LB) medium at 30 or 37 °C. Antibiotics were added at final concentrations as follows: 100 μg ampicillin ml⁻¹ and 50 μg kanamycin ml⁻¹ for E. coli; 100 μg kanamycin ml⁻¹ for B. cereus group strains; 10 μg kanamycin ml⁻¹, 5 μg erythromycin ml⁻¹ and 5 μg tetracycline ml⁻¹ for B. sphaericus.

### Table 1. Bacillus species strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant characteristics</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. thuringiensis</td>
<td>Acrystalliferous mutant of B. thuringiensis subsp. kurstaki</td>
<td>Huang et al. (2006)</td>
</tr>
<tr>
<td>BMB171</td>
<td></td>
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<tr>
<td>B. cereus</td>
<td>ATCC 33018R</td>
<td>Hu et al. (2005)</td>
</tr>
<tr>
<td>B. weihenstephanensis</td>
<td>KBAB4</td>
<td>Réjasse et al. (2012)</td>
</tr>
<tr>
<td>B. sphaericus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C3–41</td>
<td>Wild-type strain of B. sphaericus bearing native pBsph</td>
<td>Hu et al. (2008)</td>
</tr>
<tr>
<td>NRS1693</td>
<td>Wild-type strain of B. sphaericus lacking pBsph</td>
<td>Ge et al. (2011)</td>
</tr>
<tr>
<td>NRS1694</td>
<td>B. sphaericus NRS1693 introduced with plasmid pBU4-tubZ-gfp</td>
<td>This work</td>
</tr>
<tr>
<td>C3–41ΔP₂bin</td>
<td>C3–41 with the kanamycin resistance gene integrated into the bin operon of plasmid pBsph</td>
<td>This work</td>
</tr>
<tr>
<td>G725</td>
<td>pBsph-cured derivative of B. sphaericus C3–41</td>
<td>This work</td>
</tr>
<tr>
<td>BH101</td>
<td>G725 with the tubRZ-Bs operon integrated into the bin operon of chromosome</td>
<td>This work</td>
</tr>
<tr>
<td>BH102</td>
<td>BH101 with all of tubR-Bs deleted from the chromosome</td>
<td>This work</td>
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<tr>
<td>BH103</td>
<td>BH101 introduced with plasmid pCtubR</td>
<td>This work</td>
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<tr>
<td>BH104</td>
<td>BH102 introduced with plasmid pCtubR</td>
<td>This work</td>
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Random mutagenesis. The mutation Y260A in tubZ-Bs was introduced by error-prone PCR using Taq DNA polymerase (Transgen). The region encoding amino acids 215–425 was amplified by PCR using primers #6 and #8. The PCR fragments were purified, digested with Nhel–KpnI and cloned into pXK15 to generate plasmid pXK15-tubZ(Y260A) (Table S2). The mutation was confirmed by DNA sequence analysis.

Construction of B. sphaericus mutants. B. sphaericus mutants were generated by homologous recombination according to the method described by Poncet et al. (1997). Briefly, the upstream and downstream DNA regions of the sequences to be deleted were amplified from the B. sphaericus C3–41 genome by PCR, and cloned into the temperature-sensitive suicide plasmid pRN5101 (Table 1), in which a kanamycin resistance gene (kan) was cloned between the homologous flanks. Each mutation was introduced into the B. sphaericus chromosome or the plasmid pBsph by double crossover events and confirmed by PCR analysis. Plasmid pRN-MbinAB was used to generate a selection marker for pBsph by replacing a 1834 bp fragment of the pBsph-borne bin operon with kan, resulting in the mutant C3–41Δbin (Table 1). pRN-Bin-RZ and pRN-Bin-Z were used for the integration of tubRZ-Bs and tubZ-Bs (tubRZ-Bs with tubR-Bs deleted) into the chromosome of G725. This was accomplished by replacing a 1834 bp fragment of the chromosomal bin operon with kan and tubRZ-Bs operon (or tubZ-Bs), yielding the mutants BH101 and BH102 (Table 1).

Quantitative real-time PCR (qPCR). The copy number of pBsph and its derivatives in B. sphaericus was measured by qPCR using the SYBR Premix EX Taq Mixture (Takara). The adenylate kinase gene (adk, Bspsh_4593), a single copy gene in the B. sphaericus chromosome (Ge et al., 2011), was used as the reference gene. Two reporter plasmids, pBK-Nadk and pXK10-Bin-Cadk (Table S2), were constructed to generate the standard curves of adk, tubZ-Bs and beta-lactamase gene (bla) by using the primer pairs listed in Table S1. Total DNA was purified from 1 ml of exponential-phase cultures using an Omega bacterial DNA kit and added to the 25 µl-scale qPCR mixtures. For determining the relative mRNA levels of tubZ-Bs, quantitative reverse-transcriptase PCR (qRT-PCR) was performed using a One-step SYBR PrimeScript PLUS RT-PCR kit (Takara). Total RNA was extracted from B. sphaericus cells grown to an OD600 of ~0.8 with TRIzol reagent (Invitrogen), and 2 µl of DNA-free RNA sample was added to the 18 µl reaction mixtures. The rpoB gene (Bspsh_4630), which could be used as an internal standard in Bacillus (Ho et al., 2011), was used as a reference gene to normalize the data. For each DNA or RNA sample, qPCR was assayed in technical triplicate, and for each reaction, the calculated Ct value was normalized to the Ct of adk or rpoB amplified from the corresponding sample. The relative placmid copy number (PCN) or mRNA level of tubZ-Bs was calculated using the 2-ΔACT comparative method (Lee et al., 2006). The results were obtained from the analyses of at least three independent samples.

Plasmid stability and incompatibility assays. B. sphaericus transformants carrying the pBsph replicons were grown overnight at 30 °C in LB medium supplemented with kanamycin. At generation zero, cells were removed from kanamycin-containing medium by centrifugation, resuspended and transferred to 5 ml fresh LB without antibiotics to reach a total of 10 generations. Every five generations, cultures were diluted and plated on LB plates without antibiotics. The fraction of the plasmid-containing cells was determined by replica-picking 100 randomly chosen colonies from LB plates without antibiotics to LB plates containing kanamycin. For the incompatibility test, B. sphaericus C3–41Δbin harbouring pRN5101 or its derivatives was grown overnight with erythromycin at 30 °C. Cultures of 5 µl were then transferred every 12 h (~10 generations) to 5 ml fresh LB with kanamycin but lacking erythromycin to reach a total of 40 generations. The destabilization effect was expressed as the percentage of kanamycin-resistant and erythromycin-sensitive colonies. The percentage of plasmid loss (L) per generation was calculated from the formula L = 1 – (Pf/Pi)1/K, where Pi is the fraction of cells bearing the plasmid initially and Pf is the plasmid-carrying fraction after g generations of non-selective growth. For measuring the stability of pBsph when TubZ-Bs-GFP or its mutant proteins were expressed in trans, B. sphaericus C3–41 transformants harbouring pBu4 or its derivatives were first grown for 24 h on LB plates with tetracycline, and a single colony was then inoculated in 5 ml LB with tetracycline and grown for ~17 generations to an OD600 of ~1.5. The cultures were diluted and plated onto LB plates with tetracycline. The resulting colonies were used for PCR using primer pairs #61/#62 to amplify a region of orfo01 from pBsph and primer pairs #63/#64 to amplify a region of ccpp (Bspsh_4200) from the chromosome. In all assays, means and standard deviations were obtained from the analyses of at least two independent transformants.

Protein overproduction and purification. Plasmids pET28b-TubZ, pET28b-TubR, pET28a-TubZ (T114A) and pET28b-TubZ(Y260A) were constructed (Table S2) for overproduction of the six-histidine-tagged TubZ-Bs, TubR-Bs, TubZ-Bs T114A and TubZ-Bs Y260A proteins (Fig. S1), respectively. Following the transformation into E. coli BL21 (DE3), protein expression was induced with 1 mM IPTG and incubated overnight at 30 °C (16 °C for TubR-Bs). The purified proteins were dialysed with stock buffer [20 mM HEPES-NaOH (pH 7.5), 10 % glycerol]. Protein concentrations were determined using the BCA protein assay kit (Pierce) with the BSA standards.

Western blotting. TubZ-Bs antisera was prepared according to Zhang et al. (2013), and the Cpca antisera was prepared previously (Li et al., 2012). Western blot analysis was performed as described previously (Wu et al., 2012), and the protein bands were quantified using the AlphaEaseFC software.

Electrophoretic mobility shift assay (EMSA). The biotin-labelled DNA fragments were amplified from plasmid pBK using the primer pairs listed in Table S1. DNA fragment (2.5 nM) and various amounts of proteins (final concentrations, 0.1–2.0 µM) were mixed in 20 µl EMSA binding buffer [20 mM HEPES (pH 7.5), 30 mM KCl, 5 mM MgCl2, 1 µg poly[dI:dC], 1 mM DTT, 1 mM EDTA, 0.4 µg BSA and 5 % glycerol] and incubated for 30 min at room temperature. For protein–protein interaction experiments, the TubR-Bs protein (1 µM) was first incubated for 30 min with DNA prior to adding the TubZ-Bs protein (0.5–4.0 µM). Reaction mixtures were electrophoresed on a 6 % native polyacrylamide gel in 0.5 × Tris-borate-EDTA buffer at 100 V on ice. DNA was transferred by electroblotting to positively charged nylon membranes and immobilized by UV cross-linking. Detection of biotin-ended DNA was performed using the Chemiluminescent Nucleic Acid Detection Module (Pierce).

DNase I footprinting. A 466 bp DNA fragment containing the promoter region of the tubRZ-Bs operon was amplified from plasmid pXK with unlabelled primer #71 and 5’-biotinylated primer #72. Footprinting reactions were performed as described by Zampini et al. (2009). Briefly, reactions containing biotin-labelled DNA (5 nM) and TubR-Bs (0.1–2.0 µM) were mixed in 50 µl EMSA binding buffer and incubated for 30 min at room temperature. Each reaction was treated with 40 µU Dnase I (Promega) for 1 min at room temperature. Reaction mixtures were extracted with phenol/chloroform (1:1), and the DNA was precipitated with absolute ethanol and resuspended in formamide loading buffer. Separation of DNA fragments on a 6 % polyacrylamide gel containing 8 M urea and DNA sequencing were performed using the Accupower & Top DNA Sequencing kit (Bioneer) using plasmid pXK as template and the same 5’-biotinylated primer.
**Primer extension analysis.** Total RNA was extracted from exponential-phase *B. sphaericus* C3-41 cells with TRizol reagent (Invitrogen). The cDNA was synthesized with biotin-labelled primer #73 using the PrimeScript reverse transcriptase (Takara). The reaction mixture was treated as described above, and the dissolved DNA sample was separated on a 6% sequencing gel containing 8 M urea. DNA sequencing reactions were performed using plasmid pXK as template and the same 5'-biotinylated primer.

**NTP hydrolysis.** The NTPase activity of TubZ-Bs, TubZ-Bs T114 and TubZ-Bs Y260A was measured using the EnzChek phosphate assay kit (Invitrogen) with 2-amino-6-mercapto-methylpurine riboside as the substrate, according to Chung et al. (2007).

**Fluorescence and electron microscopy.** For *in vivo* visualization of TubZ-Bs polymers, plasmids pBU4-tubZ-gfp, pBU4-tubZ(T114A)-gfp and pBU4-tubZ(Y260A)-gfp were constructed for overexpression of the TubZ-Bs-GFP, TubZ-Bs(T114A)-GFP and TubZ-Bs(Y260A)-GFP fusion proteins, respectively, from the native promoter of the *tubRZ*-Bs operon. Then, 3 μl of the exponential-phase *B. sphaericus* NRS1693 cells were spotted onto the glass slide and applied to the fluorescence microscope with a phase-contrast objective (100 × 1.30 Oil Ph3, Olympus). The 3D super-resolution images of individual structures were taken using a DeltaVision OMX fluorescence microscope supplied with a digital camera (Applied Precision).

For electron microscopy (EM), 5 μM of the TubZ-Bs or its mutant proteins were incubated with 2 mM GTP or 0.2 mM GTP for 5 min at 25 °C in 40 μl of polymerization buffer [50 mM HEPES-NaOH (pH 7.5), 100 mM KAc, 5 mM MgAc]. The reaction mixtures were immediately spotted onto a carbon-coated copper grid for 3 min, followed by negatively staining with 2% uranyl acetate for 2 min. The sample-absorbed grids were dried overnight and photographed using a Tecnai G² 20 transmission electron microscope at 200 kV.

**Sedimentation assay.** TubZ-Bs or TubZ-Bs T114A (5 μM) was assembled in polymerization buffer, followed by incubation with 2 mM NTP or 0.2 mM GTP for 3 min at 25 °C in a volume of 40 μl. The reaction mixtures were immediately centrifuged at 20 000 g for 30 min at 4 °C, and the pellets were resuspended in 40 μl of polymerization buffer. Aliquots (20 μl) were analysed by 12% SDS-PAGE with Coomassie brilliant blue staining, and protein bands were quantified using the AlphaEaseFC software.

**RESULTS**

**Identification of a pBsph minireplicon**

It was demonstrated previously that *B. sphaericus* C3-41 harbours a large plasmid pBsph of 177 642 bp with a mean G + C content of 33.1 mol% (Hu et al., 2008). Bioinformatic analyses revealed that ORF188 (Mr, 47.2 kDa) contained a tubulin signature motif (GGGTGTG) and shared 40% amino acid identity with pXO1-encoded TubZ-Ba and 23% identity with pBtoxis-encoded TubZ-Bt, while ORF189 (Mr, 12.3 kDa) contained a predicted winged-helix DNA-binding domain and shared 20% identity with pBtoxis-encoded TubR (hence designated TubZ-Bs and TubR-Bs, respectively). These results suggested that the *tubRZ*-Bs operon might be involved in the replication and partitioning of plasmid pBsph. Thus, a 5.0 kb DNA fragment encoding ORF001, TubR-Bs, TubZ-Bs and ORF187 was cloned into pUC19K to generate plasmid pBK (Fig. 1). Plasmid pBK could replicate in the pBsph-free *B. sphaericus* strain NRS1693 while pUC19K could not, suggesting involvement of a pBsph replicon.

To determine the minimal sequence required for replication, a series of deletion derivatives of pBK were constructed and transformed into *B. sphaericus* NRS1693. The results demonstrated that complete deletion of ORFO01 or ORF187 did not affect their replication, while the further truncated derivatives pHK and pXX8 did not generate transformants on the selection plates. Consequently, a 2.4 kb DNA fragment, which contained the intact *tubZ*-Bs and *tubR*-Bs, along with the 396 bp upstream sequence of *tubR*-Bs and...
311 bp downstream region of tubZ-Bs, was identified as a minimal replicon (minireplicon) of pBsph (Fig. 1).

No transformant was obtained when either tubZ-Bs or tubR-Bs of the minimal replicon was disrupted, or when codon 260 coding for tyrosine (Y260) (Fig. S2) in tubZ-Bs was mutated into a codon coding for cysteine, alanine, serine or phenylalanine. Furthermore, when the codon 114 coding for threonine in the tubulin signature motif (T114) (Fig. S2) of tubZ-Bs was mutated into a codon coding for alanine, the resulting plasmid pXK10-tubZ(T114A) (Table S2) produced only small transformants and had a dramatically reduced plasmid/chromosome ratio in the population (~0.5% of the wild-type plasmid). These results demonstrated that both TubZ-Bs and TubR-Bs, and the residue Y260 and T114 in TubZ-Bs, are required for plasmid replication.

**Replication characteristics of the pBsph replicons**

The pBsph replicons transformed *B. sphaericus* NRS1693 with an evidently higher efficacy than strain G725, a pBsph-cured derivative of *B. sphaericus* strain C3-41 (200 versus 5 c.f.u. DNA μg⁻¹). qPCR results revealed that the PCN in strain G725 ranged from 6.1 to 9.8 copies per chromosome, fivefold to sixfold lower than that in NRS1693 but notably higher than that of native pBsph in C3-41 (one or two copies per chromosome) (Fig. 1), indicating that the minimal replicons lacked some pBsph-encoded copy number determinants. Further studies revealed that the minireplicon (pXK10) could also transform *B. cereus*, *B. thuringiensis* and *Bacillus weihenstephanensis*, resulting in transformants at an efficacy of ~50 c.f.u. DNA μg⁻¹. Furthermore, when plasmid pXK10-Bin, which contained a 3.1 kb *bin* operon coding for the Bin toxins, was introduced into *B. thuringiensis* BMB171, the recombinant strain could synthesize Bin toxins (Fig. S3), indicating that plasmid pXK10 could be used as a shuttle expression vector for genetic manipulation of *B. sphaericus*.

**Cooperative binding of TubR-Bs to eleven 12 bp degenerate repeats**

Sequence analyses revealed three sets of repeated sequences in the upstream region of tubR-Bs, and it was presumed that these repeats might be involved in protein binding. DNase I footprinting results showed that TubR-Bs protected three blocks (I, II and III) in the region from DNase I digestion (Fig. 2d). Further analyses demonstrated that the TubR-Bs binding sites were composed of eleven 12 bp degenerate repeats with the conserved sequences THATTACNGTAD (H=A/C/T, N=A/G/C/T, D=A/G/T) (Fig. S4b) and separated into three clusters by a 54 bp promoter region and a 59 bp AT-rich (70%) spacer bearing two putative *dnaA* boxes (Fig. S4a). In an attempt to determine the binding specificity of each set of repeats, five biotinylated DNA fragments (C1–C5; Fig. 2a) that carry one or more sets of repeats were dissected and incubated with TubR-Bs in EMSA reactions. Results showed that both the full-length repeated sequences (fragment C1) and the fragment lacking block I (fragment C2) were assembled into a single complex with TubR-Bs, and that the C2 fragment flanked on the left by an equivalent length non-specific DNA (ns-C2) showed a similar binding pattern with fragment C2 (Fig. 2e). In contrast, the fragments missing block II or III (C3, C4 and C5) could not generate shifted bands with TubR-Bs (Fig. 2f). Furthermore, using the same technique, no complex was observed when TubR-Bs or TubZ-Bs was incubated with the 311 bp downstream sequence of tubZ-Bs (Fig. 2g). Taken together, these results suggested that TubR-Bs is a DNA-binding protein that binds specifically and cooperatively to eleven 12 bp degenerate repeats.

**Replication origin of pBsph minireplicon**

As direct repeats (iterons) are characteristics of plasmid replication origin, attempts were made to test their ability to facilitate the replication of a plasmid in which TubR-Bs and TubZ-Bs were provided in trans. Thus, the fragments C1, C2, C3, C4 and C5 were cloned into pUC19K to generate plasmids pUCK1, pUCK2, pUCK3, pUCK4 and pUCK5, respectively (Fig. 2b). When these plasmids were electroporated into *B. sphaericus* G725 and NRS1693, no transformant was obtained under selection with kanamycin. However, when similarly introduced into pBsph-carrying *B. sphaericus* C3-41, a strain that could provide TubR-Bs and TubZ-Bs in trans, plasmids pUCK1 and pUCK2, but not pUCK3, pUCK4 or pUCK5, gave small transformants at efficiencies of ~10 c.f.u. DNA μg⁻¹. The copy number of pUCK1 and pUCK2 in C3-41 was ~1.5 copies per chromosome.

In all replication assays, the presence of the intact plasmids in *B. sphaericus* transformants was confirmed. Plasmids were extracted from recombinant strains and used to transform *E. coli*. These plasmids replicated in *E. coli* under ampicillin and kanamycin resistance. Restriction and PCR analyses demonstrated that they were identical to those used in autonomous replication assays in *B. sphaericus*. These results demonstrated that the direct repeats functioned as an origin of replication. By combining the results with the EMSA results, the inability of pUCK3, pUCK4 and pUCK5 to replicate in strain C3-41 could be due to the failure of TubR-Bs binding to C3, C4 and C5 fragments, indicating that TubR-Bs plays a critical role in pBsph replication.

To further investigate the function of direct repeats as the replication origin, fragments C1, C2, C3, C4 and C5 were also cloned into plasmid pRN5101 to test their ability to cause the iteron-mediated incompatibility (*inc*). The resulting plasmids pRN-C1, pRN-C2, pRN-C3, pRN-C4 and pRN-C5 were introduced into *B. sphaericus* C3-41Δ*Bin* (Table 1), a C3-41 mutant with insertion of the *kan* gene into pBsph. Under selection of the pBsph-encoded kanamycin resistance, pRN5101, encoding erythromycin resistance and replicating with a copy number of ~68 copies per chromosome, was stably maintained in the progeny cells
with a rate of 0.5% loss per generation (Fig. 2c). Insertion of the entire iteron sequence (C1 fragment) led to a significant stability defect with a loss rate of 3.5% per generation \( (P, 0.001) \). Furthermore, the plasmid harbouring fragment C2, which retained the reduced TubR-Bs binding affinity (Fig. 2e), showed a plasmid loss rate (2.0% loss per generation) somewhat lower than that of pRN-C1 \( (P, 0.05) \) but significantly higher than that of pRN5101 \( (P, 0.01) \). In contrast, plasmids pRN-C3, pRN-C4 and pRN-C5 loss rates (0.8, 0.6 and 0.6% per generation, respectively) were similar to that of pRN5101. These results suggested that the replication origin acts as an \textit{inc} determinant, and the different \textit{inc} activities may be due to the different TubR-Bs binding affinities to the competing plasmid.

**TubR-Bs is a transcriptional repressor of the \textit{tubRZ}-Bs genes**

Primer extension analysis revealed that the transcription start site was at position +204 upstream of the initiation.
codon of tubR-Bs (Fig. 3a), and putative −35 (TAATAA) and −10 (TTTTTT) hexamer boxes, which might be recognized by σA RNA polymerase, were identified with appropriate spacing (17 bp). Examination of the nucleotide sequence revealed that block I consists of four 12 bp repeats. Three of the repeats are in direct orientation, whereas one of the motifs is inverted (5′-CTACTGCAATAA-3′) (Fig. S4b). Furthermore, the putative −35 element for the promoter overlaps the block I repeats (Fig. S4a). This organization indicates that block I may serve as an operator implicated in regulation of tubRZ-Bs transcription.

To examine whether TubR-Bs is an autoregulator of transcription, typical of replication initiator proteins, the tubRZ-Bs operon, along with its native promoter and the entire iteron sequences, was integrated into the chromosome of strain G725, and two mutant strains BH101 and BH102 were obtained (Table 1). RNA transcript levels of tubZ-Bs were determined by qRT-PCR, and normalized to the RNA levels of the housekeeping gene rpoB. Results showed that the RNA levels of tubZ-Bs were increased approximately 4.2-fold in the tubR-Bs mutant (BH102) compared with those in cells containing tubR-Bs (BH101). Furthermore, when the TubR-Bs protein was overexpressed from its native promoter using plasmid pCtubR (Table S2), the tubZ-Bs transcript levels in strains BH103 and BH104 (Table 1) were approximately 6.1-fold lower than those in strain BH101 (Fig. 3b). Western blot analysis revealed that protein levels of TubZ-Bs in the tubR-Bs mutant were about 3.8-fold higher than those in cells bearing tubR-Bs, whereas the overproduction of TubR-Bs had a significant negative role, resulting in an about 4.2-fold lower expression in strain BH103 and BH104 compared with that in BH101 (Fig. 3c). These results suggested that TubR-Bs is a transcriptional repressor of tubRZ-Bs expression.

**TubZ-Bs binds to the TubR-Bs : DNA complex and exhibits GTPase activity**

EMSAs showed that TubZ-Bs bound to the TubR-Bs : DNA complex, resulting in the formation of a supershifted band (Fig. 4a), while TubZ-Bs alone could not bind to DNA (Fig. 4b), indicating TubZ-Bs and TubR-Bs protein–protein interactions. Furthermore, when TubZ-Bs and TubR-Bs were incubated at a molar ratio of 3 : 2, the biotinylated DNA (fragment C2) was supershifted entirely (Fig. 4a).

The GTP hydrolytic activity assays showed that the wild-type (wt) TubZ-Bs protein exhibited significant GTPase activity and hydrolysed GTP at a rate of 0.9 GTP/TubZ-Bs min−1, while it hydrolysed ATP with a sevenfold lower efficiency (Fig. 4c). The GTPase activity was linearly proportional to TubZ-Bs concentration at a range from 0.5 to 6 μM, and the critical concentration obtained from the curve was approximately 0.3 μM (Fig. 4d). Further studies showed that the mutant protein TubZ-Bs T114A retained only 14.2 % of GTPase activity of wt TubZ-Bs and
hydrolysed ATP at very low levels (Fig. 4c), whereas the mutant protein TubZ-Bs Y260A was completely deprived of GTPase activity (data not shown).

**In vivo and in vitro structures of TubZ-Bs polymers**

We next wished to visualize the subcellular distribution and polymerization capacity of the wt and mutant TubZ-Bs proteins in vivo by using strains expressing TubZ-Bs-GFP fusions. Plasmid pBU4-tubZ-gfp (Table S2) was introduced into *B. sphaericus* NRS1693, resulting in strain NRS1694 (Table 1) permitting ectopic expression of TubZ-Bs-GFP. Plasmid pXK15-tubZ(Y260A), which was unable to replicate in strain NRS1693, was able to replicate in strain NRS1694 when TubZ-Bs-GFP was provided in trans. This result suggested that wt TubZ-Bs fused to GFP (TubZ-Bs-GFP) is functional. We then constructed NRS1693 derivative strains allowing, besides TubZ-Bs-GFP, conditional expression of TubZ-Bs(T114A)-GFP or TubZ-Bs(Y260A)-GFP. As shown in Fig. 5(a), both the wt TubZ-Bs-GFP and the mutant TubZ-Bs(T114A)-GFP were able to form filamentous structures, whereas the mutant TubZ-Bs(Y260A)-GFP displayed diffuse and cytoplasmic fluorescence within the cell and no obvious structure was observed. Furthermore, 3D super-resolution microscopy revealed that TubZ-Bs-GFP assembled into continuous filaments in *B. sphaericus* cells, which spanned the whole bacteria cell length (Fig. 5b).

Negative stain EM showed that TubZ-Bs assembled into short and somewhat twisted polymeric structures in the presence of GTP in vitro (Fig. 6a). Mean widths of the filaments were approximately 13 nm. These polymers seemed to be dynamic as most of the filaments disappeared or became shorter after incubation with GTP for 20 min (data not shown). When GTP;S, a non-hydrolysable GTP analogue, was incubated with TubZ-Bs, a large number of filaments were assembled similar to those assembled in GTP. Nevertheless, these filaments were longer and clustered into larger bundles (Fig. 6a). When the TubZ-Bs T114A protein was polymerized in the presence of GTP or GTP;S, the filaments were relatively fewer in number and somewhat irregular in shape. As expected, the TubZ-Bs Y260A protein could not form any obvious filamentous structures in the presence of GTP (Fig. S5).

Sedimentation assays showed that TubZ-Bs exhibited Mg²⁺-dependent polymerization activity in the presence of GTP. However, the TubZ-Bs T114A polymerization was not obviously enhanced by Mg²⁺, and even if the Mg²⁺ concentration was as high as 20 mM, only 5% of the TubZ-Bs T114A polymers was pelleted, much less than that of wt TubZ-Bs (Fig. 6b). When GTP;S was added, both TubZ-Bs and TubZ-Bs T114A exhibited higher assembly efficiency than that in GTP (Fig. 6b), indicating a critical role of GTP hydrolysis in TubZ-Bs polymerization.
**Fig. 5.** TubZ-Bs-GFP assembles filamentous structures in *B. sphaericus*. (a) Fluorescence micrographs of structures of TubZ-Bs-GFP and its mutant proteins TubZ-Bs(T114A)-GFP and TubZ-Bs(Y260A)-GFP. Images are to the same scale. (b) Super-resolution 3D fluorescence micrographs of TubZ-Bs-GTP polymers (left and middle) and TubZ-Bs(Y260A)-GFP (right). The red circles were drawn to scale to indicate the cell membrane. Images are to the same scale.

**Fig. 6.** *In vitro* assembly properties of TubZ-Bs. (a) Electron micrographs of TubZ-Bs polymers. TubZ-Bs (5 μM) was incubated with 2 mM GTP or 0.2 mM GTPγS at 25 °C for 5 min and visualized by negative stain EM at different magnifications. (b) 12% SDS-PAGE of sedimentation assays using 5 μM TubZ-Bs or TubZ-Bs T114A incubated with increasing concentrations (0, 5, 10, 15, 20 mM) of Mg2+ in the presence of 2 mM GTP, or incubated with 5 mM Mg2+ in the presence of 0.2 mM GTPγS (lane 1) or 2 mM ATP (lane 2). S, supernatant; P, pellet.
**TubZ-Bs is important for maintenance of pBsph**

The pBsph replicons were highly unstable in *B. sphaericus*, and even in the presence of kanamycin to select for the plasmid, only a fraction of cells actually contained a plasmid. Without selection pressure, the plasmids were lost rapidly, and the loss rates ranged from 13 to 22% per generation (Fig. 1). These results suggested that the *tubRZ*-Bs region was insufficient for stable maintenance of the replicons. We therefore examined whether TubZ-Bs was required for the maintenance of native pBsph in *B. sphaericus* C3-41, by perturbing its expression level. We expressed either the wt TubZ-Bs-GFP, or the mutants TubZ-Bs(T114A)-GFP and TubZ-Bs(Y260A)-GFP *in trans* from its native promoter in *B. sphaericus* C3-41. The presence of pBsph was detected by colony PCR, and results showed that overexpression of either TubZ-Bs-GFP or its mutant proteins from the native translation initiation region (TIR) (Vimberg *et al.*, 2007) resulted in a dramatic destabilization of the plasmid (>99% loss) (Fig. 7). However, when TubZ-Bs-GFP was expressed from a recombinant TIR (TIR of TubR-Bs) with relatively lower translational efficiency, 90% of the cells retained the plasmid pBsph, and the retention rate decreased to 36 and 46% when the mutant proteins were expressed. Western blotting with polyclonal anti-TubZ-Bs antibodies showed that no obvious protein band for TubZ-Bs was observed at high expression levels of these fusion proteins. However, when the GFP fusions were expressed at low levels, a single protein band for TubZ-Bs was observed and the relative protein levels of TubZ-Bs to TubZ-Bs-GFP were about 3.5- and 2.5-fold higher than that to TubZ-Bs(T114A)-GFP and TubZ-Bs(Y260A)-GFP, respectively. These results were generally in agreement with those measured by colony PCR. Taken together, our results suggested that proper expression levels of TubZ-Bs are important for the stability and/or replication of plasmid pBsph in *B. sphaericus*.

**DISCUSSION**

Thus far, there have been no reports on plasmids of *B. sphaericus* except for an 11 kb cryptic plasmid pLG in strain LP1-G (Wu *et al.*, 2007). In the present study, we demonstrated that a 2.4 kb DNA fragment in pBsph could replicate independently and constituted a minireplicon, replication of which required at least two *trans*-acting proteins (TubR-Bs and TubZ-Bs) and two adjacent DNA regions. TubR-Bs could bind specifically to eleven 12 bp degenerate repeats in the upstream region of *tubR*-Bs to initiate plasmid replication and regulate expression of the operon, while TubZ-Bs was recruited into the TubR-Bs: DNA complex, leading to the formation of a ternary nucleoprotein complex that was reminiscent of TubZRC nucleoprotein complex from plasmid pBtoxis in *B. thuringiensis* (Tang *et al.*, 2007). Sequence analyses revealed the presence of at least five clusters of direct/inverted repeats in the 311 bp downstream region of *tubZ*-Bs (Fig. S4a), and EMSA results showed that this region could not be bound by TubR-Bs or TubZ-Bs (Fig. 2g). A similar result was also found on pBtoxis plasmid, in which a downstream region of *tubZ*-Bt containing three sets of inverted repeats was important for plasmid replication (Tang *et al.*, 2006). How these downstream repeats function in plasmid replication remains unclear and merits further studies.

The copy number of the pBsph replicons in *B. sphaericus* NRS1693 was about sixfold higher than that in its native host (G725), indicating possible host regulation of initiation frequency. For many bacterial plasmids, plasmid replication requires the combined actions of chromosome-encoded DnaA protein and a plasmid-encoded initiator protein (Rep protein), expression of which determines the frequency of replication initiation (Betteridge *et al.*, 2004). The ability of pBsph replicons to transform *B. sphaericus, B. thuringiensis, B. cereus* and *B. weihenstephanensis* suggested that plasmids

![Fig. 7. Effects of overexpression of TubZ-Bs-GFP or its mutant proteins on the stability of pBsph. The TubZ-Bs-GFP and its mutants TubZ-Bs(T114A)-GFP and TubZ-Bs(Y260A)-GFP were expressed *in trans* in *B. sphaericus* C3-41 using either its native or the recombinant TIR, and an empty vector (pBU4) was used as a control. Cells were cultured as outlined in Methods, and the presence of pBsph was examined by colony PCR and Western blotting. Means were obtained from three independent experiments with SDs <10%. Western blot analysis was performed using affinity-purified polyclonal anti-TubZ-Bs with lyase prepared from equal amounts of cells (except for cells with pBU4 control). Protein levels of TubZ-Bs relative to TubZ-Bs-GFP or its mutant forms are shown below.](http://mic.sgmjournals.org)
derived from the replicons may have value in genetic manipulation of *B. sphaericus* strains, and the differences in transformation efficiencies could be due to different restriction/modification systems and/or possibly result from distinct host-encoded functions between species.

TubZ-Bs shares sequence homology with two other plasmid-encoded cytoskeletal factors, pBtoxis-encoded TubZ-Bt and pXO1-encoded TubZ-Ba, which are involved in the replication and partitioning of plasmids from *B. cereus* group of bacteria, and therefore have been generally classified as type III plasmid partition systems. As expected, TubZ-Bs exhibited similar characteristics to TubZ-Bt and TubZ-Ba both in vivo and in vitro (Akhtar et al., 2009; Larsen et al., 2007), including significant GTP hydrolysis and GTP-dependent polymerization activities. Two residues in TubZ-Bs (T114 and Y260) were found to be important for its GTPase and polymerization activities, and their mutations (T114A and Y260A) resulted in a dramatic decrease in colony sizes, possibly as a result of a partitioning defect. Similar mutations in the tubulin signature motif in TubZ-Ba (T125A) and *Clostridium botulinum* phage TubZ (T100A) also affect GTPase activity and polymerization (Anand et al., 2008; Oliva et al., 2012). The sequence alignment of TubZ-related proteins revealed that the residue Y260 localizes in the C-terminal domain helix H9 (Fig. S2) that is crucial for the subunit–subunit interactions (Aylett et al., 2010), indicating that residue Y260 may play a role in the monomer–monomer association during TubZ-Bs assembly. Previous studies have shown that overexpression of TubZ-Bt or its mutant form has a negative effect on the segregational stability of pBtoxis plasmid in *B. thuringiensis* (Larsen et al., 2007). Here, we showed that overexpression of TubZ-Bs destabilized the plasmid pBsph, and faster plasmid loss was observed when its mutant proteins were overproduced, possibly as a result of a negative impact on the partitioning of pBsph plasmid. Overexpression of TubZ-Bs may also affect replication, indirectly leading to plasmid loss in time. Our results, along with previous ones, suggested that polymerizing protein TubZs is required for the replication and partitioning of plasmids in bacterial population.

TubR-Bs displays remarkably low sequence similarity to TubR-Bt (20% identity) and harbours a unique DNA-binding domain (winged-helix). The sequence and structure divergence may contribute to the binding specificity. For example, the TubR-Bt binding sites (tubC) are composed of two clusters of 12 bp repeats and TubR-Bt binds to each block independently (Aylett & Lowe, 2012), whereas the binding region of TubR-Bs harboured three blocks of 12 bp degenerate repeats, and a single block was insufficient for TubR-Bs binding. Although block I was not bound by TubR-Bs independently (Fig. 2f), it was required for maximal protein binding affinity (Fig. 2e) and plasmid incompatibility (Fig. 2c). Furthermore, block I contains an imperfect inverted repeat and overlaps the putative ~35 element of the tubRZ-Bs promoter (Fig. S4a), suggesting that block I may function as an operon of the operon. In many plasmid replicons, such as P1, F and pPS10, Rep proteins harbour the functions of both dimeric transcriptional repressor and monomeric initiator of replication (Gasset-Rosa et al., 2008). In the case of pBtoxis plasmid, TubR-Bt is a negative regulator of the operon and binds to four 12 bp direct repeats in *tubC* to initiate replication of pBtoxis (Larsen et al., 2007; Tang et al., 2007). Here we showed that eleven 12 bp degenerate repeats could function as a replication origin of pBsph, and plasmids carrying these repeats were destabilized in a strain containing pBsph, probably as a result of iteron-mediated incompatibility. Even in the presence of selection, only a fraction of cells actually contained a plasmid, and the recombinant strains showed a similar vegetative growth rate to the wt strain (Fig. S6). These observations raise the possibility that the origin region is incomplete or the native plasmid may contain another origin of replication, as for the pXO1 plasmid, in which two separate regions could support replication of pXO1 miniplasmids (Akhtar & Khan, 2012). TubR-Bs was shown to be a transcriptional autorepressor and could bind to the replication origin. This binding was required for replication initiation, suggesting a role of TubR-Bs in plasmid replication.

TubR-Bs may also play a crucial role in plasmid partitioning as it shared many properties with the components of other plasmid par systems, including DNA-binding activity and the ability to bridge together the partitioning protein TubZ-Bs and plasmid DNA. In other par systems, DNA segregation is carried out by a cytoskeletal protein and a cognate DNA-binding protein that anchors the replicated plasmids to the growing filaments, resulting in the dynamic relocation of the polymers in the cell and driving plasmids apart (Gerdes et al., 2010). In the TubZ system, the cytomotive protein TubZ is recruited into formation of a three-component complex, which is proposed to constitute a type III plasmid par system (Ni et al., 2010), although there is no direct evidence for the partitioning roles of tubC and TubR. Indeed, the TubZ system is highly unstable in both *B. thuringiensis* (Tang et al., 2006) and *B. sphaericus* (Fig. 1), indicating that the system is insufficient or missing some components for plasmid partition. This leads to the possibility that the particular genetics has made the TubZ system require other regulator(s) to stabilize the plasmid. More studies are needed to elucidate the specific roles of TubR and TubZ in plasmid replication and partitioning, and to identify other control elements involved in plasmid stabilization.

Although the replication and partitioning activities of TubZ-Bs and TubR-Bs are highly intertwined and difficult to separate definitively, our results demonstrated that both of them are required for the maintenance of plasmid pBsph in *B. sphaericus*, similar to those found on plasmid pBtoxis. The similar maintenance mode may indicate a relevant evolutionary relationship among these plasmids, leading to the suggestion that *B. sphaericus* may acquire plasmid pBsph by horizontal gene transfer events from other bacteria (e.g. *B. thuringiensis*) or phage invasion in the mosquito breeding...
habitat. The combination of a *B. sphaericus* Bin-like protein (Cry49Aa) and a *B. thuringiensis* three-domain Cry protein (Cry48Aa) in some *B. sphaericus* strains provides strong evidence in favour of this suggestion. When *B. sphaericus* obtained pBsp, this plasmid protected the bacterium against invading foreign genetic materials by introducing three sets of restriction-modification systems and maximized toxicity by increasing the copy number of the bin genes in bacterial cells, giving it the potential to transfer and replicate the toxic genes in nature as well as in the mosquito breeding habitat.

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