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## Identification of a toxin coding fragment in pBSSB1, a linear plasmid from *Salmonella enterica* serovar Typhi that can stabilize a multicopy plasmid

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### ABSTRACT

**Objective:** To identify the region conferring stability to pBSSB2 (a linear plasmid, pBSSB1, containing a kanamycin cassette), which is unique to Indonesian isolates of *Salmonella enterica* serovar Typhi. **Methods:** The open reading frame (ORF) 009 was identified as a toxin coding gene in the plasmid through introduction of translational termination codons in the ORF. **Results:** The stability function was located in a fragment that spanned nucleotides 5 766 to 6 828 in the linear plasmid genetic map. Ectopic expression of ORF009 in pBAD18 vector indicated ORF009 codes for a toxin. This fragment could stabilize plasmid pUC18 previously destabilized through mutation of the *pcnB* (plasmid copy number control) gene that codes for polyA polymerase. Majority of the cells expressing ORF009 were non-viable according to phase contrast microscopy. **Conclusions:** This study demonstrated that a linear plasmid fragment that carries a gene encoding a toxin possibly conferred stability to the parent plasmid. It was able to stabilize a multicopy plasmid of *Escherichia coli*.

## 1. Introduction

*Salmonella enterica* serovars Typhi can express several flagellar antigens[1–9]. Indonesian isolates of *Salmonella* Typhi express the novel antigen, H: z66 (z66), encoded by a linear plasmid, pBSSB1[6]. Plasmids are extrachromosomal elements and can replicate independently of the host chromosome, which are usually circular. Linear plasmids were first reported in *Streptomyces rochei* in 1979[10]. Linear plasmids of *Streptomyces* spp. range in size between 12 and 640 kb[11]. Linear plasmids possessing a different structure have also been reported in *Borrelia* spp[12]. The linear plasmid

pBSSB1 was first reported by Baker *et al.*[6]. It is 27 kb containing terminal inverted repeats of 1 230 bp and terminal proteins that make it resistant to 5' to 3' exonucleases[6]. Thirty-three open reading frames (ORFs) have been identified, of which ORF031 codes for the flagellar antigen, H: z66[6]. Previously, the protein Fis has been shown to affect the stability of pBSSB1[13]. However, the specific regions involved in multiplication and stable survival of the linear plasmid have not been identified. Here we describe a region of

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pBSSB1 with a putative toxin-antitoxin function that influences the stability of the plasmid and can also stabilise a circular multicopy plasmid of *Escherichia coli* (*E. coli*). We believe that the findings of this study will help to understand the mechanism of stability of linear plasmids. To our knowledge, this is the first report of the stabilization of a multicopy, circular plasmid by the stability function of a linear plasmid.

## 2. Materials and methods

### 2.1. Plasmids

The linear plasmid pBSSB2 (pBSSB1 with a kanamycin resistance cassette integrated at a location of 1 495 bp)[6] was provided by Gordon Dougan, Wellcome Trust Sanger Institute, Hinxton, UK. Plasmids pREG531 and pFH450[9] used in stability assays were obtained from Dr. Finbarr Hayes, University of Manchester. The expression vector pBAD18 was described elsewhere[14].

### 2.2. Bacterial strains

*E. coli* SP2[15] (*pcnB*,  $\Delta$ *lac*, *recA*) which is a plasmid copy number mutant and DNA polymerase I mutant BR825[16] deficient in DNA polymerase I was obtained from Finbarr Hayes. Strain BW25141 (*lacI<sup>r</sup>rrnB<sub>T14</sub>ΔlacZ<sub>WJ16</sub>ΔphoBR580 hsdR514ΔaraBAD<sub>AH33</sub>ΔrhaBAD<sub>LD78</sub>galU95 endA<sub>BT333</sub>uidA(ΔMlu I)::pir<sup>+</sup> recA1*) was described earlier[14].

### 2.3. Polymerase chain reaction (PCR)

Oligonucleotide primers are listed in Table 1. Long amplicons were generated by using the Expand Long Template PCR system (www.roche-applied-science.com). Reaction conditions and reaction

mixture compositions were prepared according to the manual of the manufacturer.

### 2.4. DNA digestion and purification

DNA digestion and ligation were carried out according to standard protocols. The Qiagen PCR purification kit was used to purify PCR products and digested DNA following the manufacturer's instructions. DNA was purified from the agarose gel by using the gel extraction kit (QIAquick, UK).

### 2.5. Construction of a library of overlapping fragments of pBSSB2

Overlapping 4-6 kb regions of pBSSB2 were amplified using PCR primer pairs designated RP2-RP10 (Table 1). *EcoR* I and *BamH* I restriction sites were added as primer tails to allow cloning of fragments. Amplified regions were cloned into pUC18 by using *EcoR* I and *BamH* I recognition sites.

### 2.6. Electroporation

The appropriate *E. coli* strain was inoculated in ten mL of LB-broth and incubated at 37 °C overnight in a shaker incubator. Cells were collected by centrifugation for 10 min (MSE Harrier) at 4000 rpm and washed using ice-cold sterile distilled water. The centrifugation and cell washing step was repeated thrice. Cells were suspended in 200 μL of ice-cold sterile distilled water. DNA was added to the competent cells (50 μL) and mixed briefly by gentle pipetting. Electroporation was carried out under conditions of pulse at 1.6 kV, 200 Ohms in a Gene Pulser unit (Bio-Rad). Next, 450 μL of SOC (Life technologies, UK) medium was quickly added to the cells, followed by a recovery step of 1 h at 37 °C at 120 rpm. Finally, cells were spread on appropriate medium and incubated overnight at 37 °C.

**Table 1**

Oligonucleotides used in the present study.

Fragment	Forward primer (5' to 3')	Reverse primer (5' to 3')	Amplicon size (bp)	
Oligonucleotides used for library construction	RP2	AGTCGAATTCCTGTTCTGAACAGCCTGTAA	AGTCGGATCCTCACCATCAGGGTTTATTCCA	4 005
	RP3	TAACGAATTCAGCAATTGGTACCCTTCTCG	TGCCGGATCCGAAAGCTATCCGCACGAAAAG	4 596
	RP4	TTTTGAATTCGGGATTTGTTGACAGGCATT	TGATGGATCCTTTTAACGGCGGCTTATCTG	4 521
	RP5	TGTCGAATTCCTGTGGAACAATGGCAAAAA	ACATGGATCCACTGCATTTCCGAGCAGGTCT	4 561
	RP6	CAATGAATTCGGCACTGTTAGCCGTAATCA	CGGCGGATCCTTCTGCCGTCTTACCTTGCT	4 046
	RP7	CTGGGAATTCGCTTGCCTTATCGTTATCA	TATGGATCCCCCTTTCATTTGGGTTTCCCT	4 582
	RP8	CTTGAATTCATGAACAACCGGAAGGTGAG	TGTCGGATCCTTCCAGAACCCAGGAGAATG	5 597
	RP9	CTTAGAATTCACCCAATGAAAGGGAAAGG	GTAGGGATCCCAACCACGATACCCGCTACT	4 555
	RP10	CCAAGAATTCCTGCGGATCAAGACAGTA	AGAGGGATCCAGAGCCTTACAGACGGCAAA	4 727
	Oligonucleotides used to generate deletion derivatives of RP4	RP4 Del 1	AAATTTGAATTCGGGATTTGTTGACAGGCATT	ATCGATGGATTGCTTTCACC
RP4 Del 2		AGCCGTCTGTGAGCGATT	ATCGATGGATTGCTTTCACC	1 438
RP4 Del 3		GTCAGTTAAGCTGTGGCTATC	ATCGATGGATTGCTTTCACC	963
RP4 Del 4		AGCCGTCTGTGAGCGATT	AATTAATTAAGGATCCTGGGGTTCGTGTTTTACTT	1 822
RP4 Del A		ATATATATATGAATTCGGCTGATTTGACGGCATAAC	AATTAATTAAGGATCCTGGGGTTCGTGTTTTACTT	1 149
RP4 Del B		ATATATATATGAATTCAGTCTTCTGCAAGTCTTTCACT	AATTAATTAAGGATCCTGGGGTTCGTGTTTTACTT	1 068
RP4 Del 3,4 Fwd		AAATTTGAATTCCTCCAGTAAAGGGCTTATG	AATTAATTAAGGATCCTGGGGTTCGTGTTTTACTT	995

## 2.7. Chemical transformation

Chemical transformation was used to introduce plasmid DNA into *E. coli* BR825 (DNA polymerase I mutant). DNA was added to 100  $\mu$ L of competent cells. Heat shock was applied at 42  $^{\circ}$ C for approximately 1 min, then cells were placed on ice for 2 min. Nine hundred  $\mu$ L of pre-warmed LB-broth was added. Transformed cells were allowed to recover for 3 h at 37  $^{\circ}$ C followed by further recovery for 18–24 h after adding 1 mL culture to 9 mL of LB-broth. Appropriate antibiotics were added to this 10 mL culture. The tube was incubated at 37  $^{\circ}$ C for 18–24 h in a static incubator. Following incubation, harvested cells were re-suspended in LB-broth (100  $\mu$ L). They were then spread on medium in the presence of the appropriate antibiotic and incubated at 37  $^{\circ}$ C for 18–24 h.

## 2.8. Stability assay

Stability was tested using the assay described earlier [17] in which strains were streaked twice to single colonies on non-selective agar before being tested for plasmid retention.

## 2.9. Phase contrast microscopy

Five hundred  $\mu$ L of boiling 1% (w/v) agarose in phosphate buffered saline was spread allowed to solidify on a microscope slide. Between 5 and 10  $\mu$ L of bacterial culture was spread on top of the agar after it had solidified. The slide was viewed immediately using an oil immersion objective and phase contrast illumination.

## 2.10. Site-directed mutagenesis

RP4 cloned in pUC18 was mutated by using pre-designed synthetic oligonucleotides following the method described elsewhere [18]. Stop codons were introduced in each ORF (ORF 007–009) by means of site directed mutagenesis [18] to determine its role in stability by using pre-designed oligonucleotides (Table 2).

**Table 2**

Oligonucleotides to insert stop codon in ORFs in RP4.

ORF to mutate	Mutant primer (5'-3')	Mutant complementary primer (5'-3')
ORF007	TCTCTATGCCATGACCGTTT	AAACGGTCATGGCATAGAGA
ORF008	AGGGGAGACACCCACTACCTA	TAGGTAGTGGGTGTCCTCCCT
ORF009	CGGCTTTCTACAACCTGGTTC	GAACCAGTTGTAGAAAGCCG

## 2.11. Toxicity assay

The expression vector pBAD18 [19] contains the arabinose promoter, P<sub>BAD</sub>, that is repressed by glucose and induced arabinose in the growth medium. ORF009 was cloned downstream of P<sub>BAD</sub> using *EcoRI* and *PstI* restriction sites and the resulting construct was coined pBAD18-ORF009. For the toxicity assay, cultures of *E. coli* XLI-Blue with pBAD18 or pBAD18-ORF009 were grown overnight

in LB-broth at 37  $^{\circ}$ C. Each overnight culture (250–300  $\mu$ L) was transferred to fresh LB-broth (50 mL) medium and allowed to reach an OD<sub>600</sub>  $\approx$  0.3. Each culture was then split into two. In one sub-culture cells were grown in LB-broth containing glucose (0.2%) while the other contained arabinose (0.2%) to induce ORF009 expression. The culture densities were measured 30 min, 60 min, 90 min and 150 min after the culture was split and samples were withdrawn simultaneously for estimation of viable counts on LB-agar plates without glucose or arabinose.

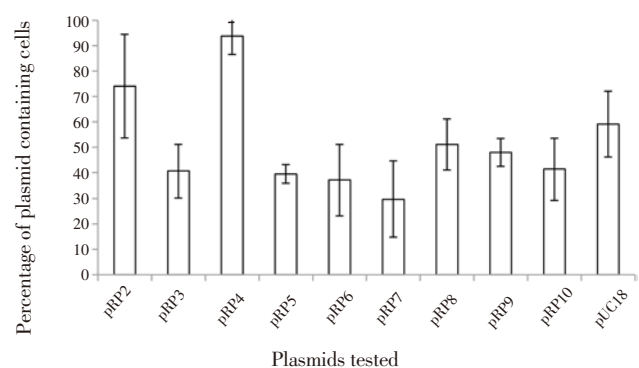
## 2.12. DNA sequencing

DNA sequencing was carried out by Geneservice Ltd. by applying BigDye Terminator and 3730 capillary sequencer (Applied Biosystems).

## 3. Results

### 3.1. Screening for stability functions of pBSSB2

A set of pUC18 derivatives containing overlapping 4–6 kb regions of pBSSB2 (pRP2–pRP10) were screened for increased stability in a *E. coli* *penB* (plasmid copy number control gene) mutant. In the negative control (pUC18, no insert), about 60% of cells retained the plasmid (Figure 1). With the exception of pRP2 and pRP4, all constructs were retained less efficiently than pUC18, presumably because of the increased metabolic load imposed by the 4–6 kb inserts. Construct pRP2 was retained, on average, by 74% of colonies while pRP4 was retained by 94%. The improvement in stability conferred by RP4 was confirmed in an independent stability assay using an unstable bi-replicon plasmid (data not shown).



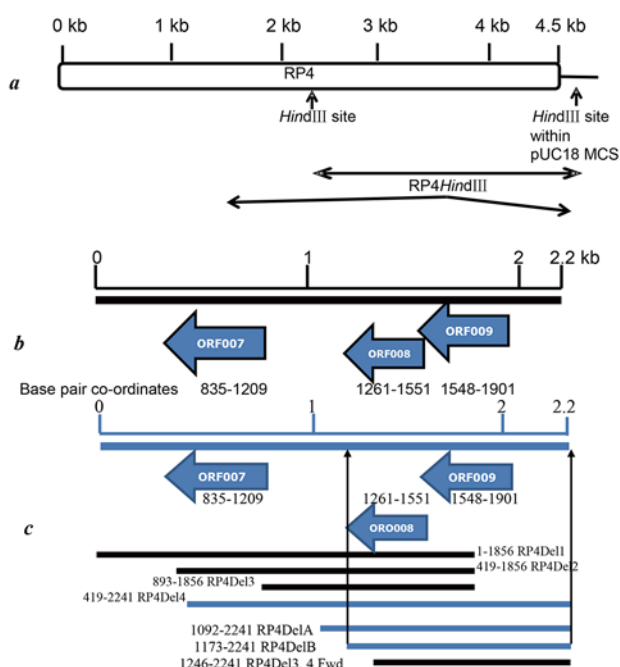
**Figure 1.** Stability of plasmids containing pBSSB2 fragments, RP2–RP10.

### 3.2. Deletion analysis of RP4 stability region

Fragment RP4 of pBSSB2 was approximately 4.5 kb (Figure 2a). The construct was digested with *HindIII* to leave approximately 2.2 kb of RP4. The resultant pRP4 derivative that contains three annotated ORFs was named pRP4 *HindIII* (Figure 2b). Plasmid

stability assays were carried out in a *penB* host to compare the stabilities of pRP4 *Hind* III, pRP4 and pUC18. It was found that fragment RP4 *Hind* III stabilized pUC18 as effectively as the full RP4 region.

A series of subclones of RP4 *Hind* III was amplified by PCR, using primers listed in Table 1. Each PCR product was cloned in pUC18 with the aid of *Eco*R I and *Bam*H I restriction sites and amplified a subsection of RP4 *Hind* III. Plasmid stability was tested in the *penB* mutant. In all cases where the right end of RP4 *Hind* III had been removed, stability of the circular plasmid was reduced (Figure 2). From the left end deletions of 1 173 bp retained the stability function of RP4 *Hind* III in a circular derivative (Figure 2c). This defined a 1 068 bp fragment that conferred stability and contained ORFs 008 and 009 [Fragment bounded by arrows (Figure 2c)].



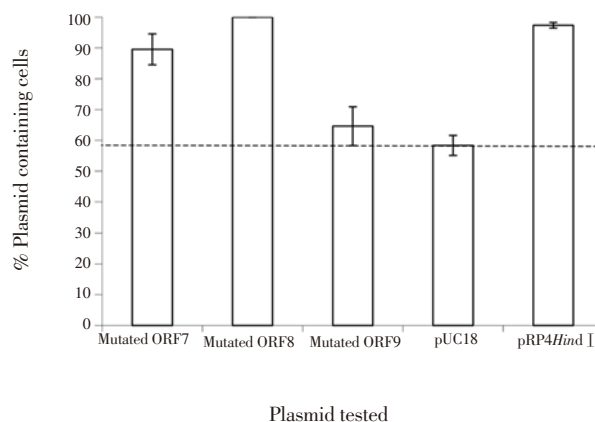
**Figure 2.** Map of pBSSB2 fragments RP4 and RP4*Hind* III (figure not drawn to scale).

a. *Hind* III sites within fragment RP4 and multiple cloning site of pUC18; b. RP4 *Hind* III generated after cutting RP4 with *Hind* III; c. Stabilization of pUC18 by deletion derivatives of RP4. pUC18 with blue fragments were stable and those with black fragments were unstable. Numbers represent position of nucleotide within RP4 *Hind* III.

### 3.3. Mutational analysis of RP4 stability region

RP4 *Hind* III contained three open reading frames: ORF007, ORF008 and ORF009 (Figure 2b). Mutated fragments were inserted into pUC18 with the help of *Eco*R I and *Bam*H I restriction sites. The presence of the desired mutation was confirmed by sequencing.

The effect of individual mutation in ORFs 007-009 in pRP4 *Hind* III derivatives was investigated in a *penB* mutant (Figure 3). The result showed that stability of the plasmid in which a translation termination codon was introduced in ORF009 was reduced, suggesting that only ORF009 was necessary for stabilization of the circular plasmid.



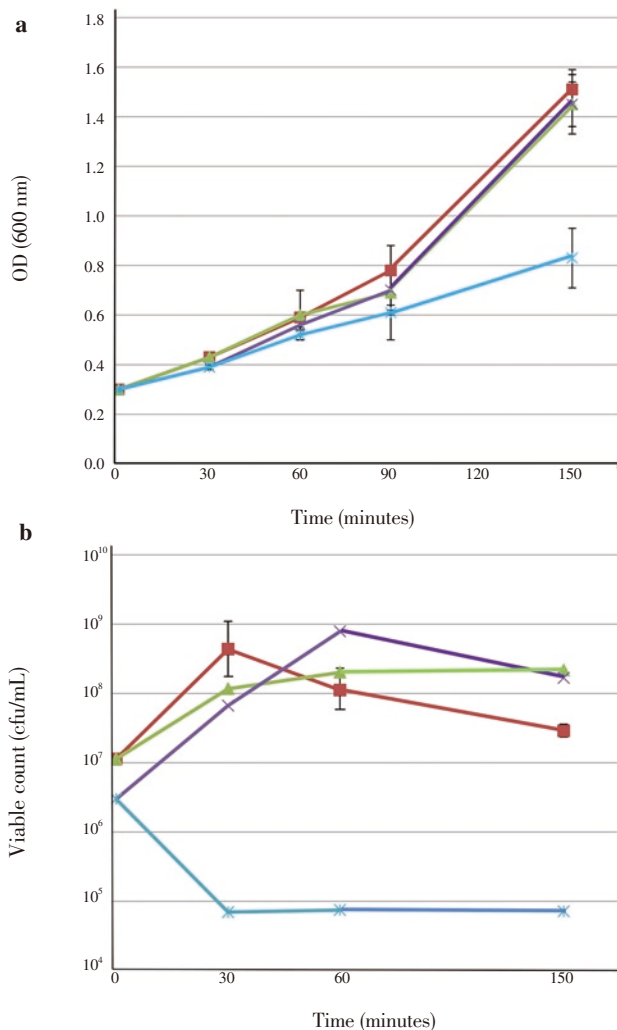
**Figure 3.** Effect of point mutation of ORFs 007-009 on stability of pUC18 carrying fragment RP4*Hind* III.

### 3.4. Characterization of the RP4 stability function

The RP4 stability region seems more likely to encode post-segregational killing than active partition since only one ORF is needed. Active partitioning systems normally have two ORFs that code for ATPase and DNA-binding proteins. On the other hand, in a toxin-antitoxin system, while the toxin is normally a protein, the antitoxin can either be protein or RNA. We therefore explored the possibility that ORF009 is the toxin counterpart of a post-segregational killing system.

In presence of pBAD18 only, a similar pattern of growth ( $OD_{600}$ ) was observed in the presence of glucose and arabinose. Cells containing pBAD18-ORF009 exhibited reduced growth when arabinose was present but grew normally in the presence of glucose. Cells containing pBAD18 or pBAD18-ORF009 exhibited different viable counts in the presence of glucose and arabinose. In the presence of arabinose, viable counts for cells containing pBAD18-ORF009 fell by  $10^4$ -fold after 30 min. Thus the expression of ORF009 resulted in the death, though not lysis, of 99.99% of the cells when compared with cells containing functional ORF009 (Figures 4a & b).

To observe whether expression of ORF009 produced changes in cell morphology, *E. coli* XLI-Blue with pBAD18 or pBAD18-ORF009 grown in LB-broth supplemented with either glucose or arabinose were observed by phase contrast microscopy. *E. coli* XLI-Blue transformed with pBAD18 showed no change in cell morphology when grown for 30 min in LB-broth containing glucose or arabinose. However, after induction with arabinose for 30 min, cells became elongated and appeared to be 'empty' or 'ghost' cells (data not shown). Besides, A psi BLAST search (<http://www.sbg.bio.ic.ac.uk/~phyre/>) indicated a similarity of ORF009 to RelE like proteins (E-value  $2e-27$ , 41.5% identity).



**Figure 4.** Growth of cultures containing pBAD18 or pBAD18-ORF009 in presence of glucose (0.2%) or arabinose (0.2%) measured in terms of OD<sub>600</sub> (a) and viable counts (b).

The red and the purple lines correspond to cells containing pBAD18 and pBAD18-ORF009, respectively, grown in LB-broth containing glucose. Green and blue lines represent cells containing pBAD18 and pBAD18-ORF009, respectively, grown in LB-broth with arabinose. All assays were performed in triplicate and results are expressed as mean±SD.

#### 4. Discussion

The annotation of the sequence of linear plasmid pBSSB1 failed to identify replication or stability functions. A previous study reports that the protein Fis is required to stabilize the linear plasmid, however the study does not continue to identify the mechanism of stability function[13]. Here we have taken a functional approach to identify potential stability functions. In this approach, a SP2 mutant was used in which mutations in *penB* decreased the copy number of ColE1-like plasmids, causing instability[20]. The gene carries information for a poly (A) polymerase, which polyadenylates the replication repressor RNAi[21,22]. In the absence of polyadenylation, RNAi has a longer half-life and consequently the copy number and stability of ColE1-like plasmids is reduced. By cloning fragments of

pBSSB2 into such unstable, circular plasmids, a 1 062 bp fragment covering nucleotides 5 766 to 6 828 in the pBSSB2 genetic map was found to stabilise these plasmids. Only one ORF within this region (ORF009) was required to confer the stability phenotype.

Since a partitioning system usually required two proteins, one an ATPase and the other a DNA-binding protein, it was hypothesized that the stability function was a post-segregational killing system through expression of toxin-antitoxin. This hypothesis was tested by ectopic expression of the putative toxin ORF009. It was found that the great majority of cells expressing ORF009 were non-viable. These cells appeared to be empty when viewed under a phase contrast microscope. Cells with condensed poles with a central clearing as a result of the effect of a toxin have been termed as ‘ghost cells’[23].

ORF009 was found to be similar to RelE like proteins according to psi BLAST search. In *E. coli*, chromosomal DNA codes for a *relEB* toxin-antitoxin locus. The RelE protein toxin which inhibits translation by cleavage of mRNA can be inhibited by binding of the RelB protein antitoxin[24]. A candidate RNA anti-toxin was identified on the opposite strand to ORF009. However, further studies will be required to determine whether the candidate promoter is functional and whether the putative transcript does indeed function as an anti-toxin.

A toxin-antitoxin stability determinant has been found previously in a linear plasmid, pCLP, of *Mycobacterium celatum*[25]. The pCLP system shows similarity to PemI-PemK of plasmid R100 found in *E. coli*[26]. The pem operon of *Mycobacterium celatum* expresses 98 and 84 amino acid peptides that function as PemK (toxin) and PemI (antitoxin), respectively. Upon transformation of *Mycobacterium smegmatis* with the shuttle vector pMIP12 carrying the 373 bp *pemK* gene, it was found that transformants were unable to grow on selective medium. A point mutation added into the *pemK* gene was able to restore growth.

A toxin-antitoxin system is a secondary stability function that kills plasmid-free cells formed when the active partitioning system fails. Sequencing and annotation of the pBSSB2 genome (deposited at the National Centre for Biotechnology Information; accession number AM419040) suggests that ORF017 codes for a nucleotide binding protein that carries structural similarity to ATPase-like ParA protein. A sequence homology to other bacterial plasmid partitioning systems was found including 63% and 51% homology to *Vibrio caribbenthicus* and *Corynebacterium glutamicum*, respectively[6]. In the present study we did not investigate the function of the ParA homolog in pBSSB2 and neighbouring sequences. However, our data suggest that fragment RP2, which contains ORF017, when inserted into pUC18, may give some improvement in stability in a *penB* mutant. Similar to the *Mycobacterium celatum* pCLP linear plasmid ParA homolog[25], fragment RP2 is also located immediately adjacent to the RP7 replicon region. Further studies are required to determine whether RP2 contains a partitioning function in pBSSB2.

The linear plasmid, pBSSB2, from *Salmonella enterica* serovar

Typhi, harbours a toxin-antitoxin function which is presumed to play a role in plasmid stability. The toxin component is encoded by ORF009. Ectopic expression of the toxin is able to kill host cells. The antitoxin remains unidentified in this study. The identified toxin-antitoxin fragment was demonstrated to be able to stabilize a destabilized multicopy plasmid (pUC18) of *E. coli*. This study demonstrates that as with circular plasmids, linear plasmids harbour toxin-antitoxin functions for segregational stability. This constitutes the first report of the stabilization capacity of a linear plasmid function in a circular plasmid.

### Conflict of interest statement

We declare no conflict of interest.

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