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The resolution and regeneration of a cointegrate plasmid reveals a model for plasmid evolution mediated by conjugation and *oriT* site-specific recombination

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Summary

Cointegrate plasmids are useful models for the study of plasmid evolution if their evolutionary processes can be replicated under laboratory conditions. pBMB0228, a 17 706 bp native plasmid originally isolated from *Bacillus thuringiensis* strain YBT-1518, carries two nematicidal crystal protein genes, *cry6Aa* and *cry55Aa*. In this study, we show that pBMB0228 is in fact a cointegrate of two plasmids and contains two functional replication regions and two functional mobilization regions. Upon introduction into *B. thuringiensis* strain BMB171, pBMB0228 spontaneously resolves into two constituent plasmids via recombination at its *oriT1* and *oriT2* sites. The resolution does not require conjugation but can be promoted by conjugation. We further confirm that the resolution is mediated by *oriT* site-specific recombination requiring Mob02281 or Mob02282. Additionally, the two constituent plasmids of pBMB0228 are mobilizable, and can fuse back via *oriT* site-specific integration after entering into the same cell by conjugation. Our study confirms that native plasmid can reversibly interconvert between a cointegrate structure and its constituent plasmids. This study provides insight into the evolution of cointegrate plasmids, linking plasmid evolution with conjugation and the *oriT* site-specific recombination function of relaxase.

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Introduction

Plasmids are extrachromosomal DNA elements found in many species of bacteria, archaea and even eukarya (Bouma and Lenski, 1988; Ma *et al*., 2012). As key vectors of horizontal gene transfer, plasmids often introduce genes associated with antibiotic resistance, virulence, detoxification and other important processes (Trefault *et al*., 2004; Shintani *et al*., 2011; Svara and Rankin, 2011). Therefore, an understanding of plasmid evolution is critical for understanding the acquisition of these important adaptive traits.

Cointegrate plasmid formation is a special type of plasmid evolution, in which one plasmid acquires traits for host adaptation by capturing a second plasmid carrying potentially beneficial genes. Many plasmids containing two or more functional replicons have been reported (Imanaka *et al*., 1984; Banerjee *et al*., 1992; Oskam *et al*., 1992; van Kranenburg and de Vos, 1998; Lee and O'Sullivan, 2006; Akhtar and Khan, 2012). If a native cointegrate plasmid has undergone a complicated history of recombination, it may no longer be possible for it to resolve into its constituent plasmids. If so, its precise evolutionary history is difficult to reconstruct. Inferences about evolutionary processes are possible based on sequence analysis of whole plasmids or on functional studies of characteristic elements, such as insertion sequences or transposons located on the flanking regions of gene clusters or functional modules (Berry *et al*., 2002; Sarno *et al*., 2002; Trefault *et al*., 2004). Native cointegrate plasmids which can replicate their evolutionary processes under laboratory conditions would be especially valuable for improving our understanding of plasmid evolution. To date, the formation processes of a few cointegrate plasmids have been determined, and homologous recombination and transposon integration have been shown to be related to these processes (Clewell *et al*., 1982; O'Sullivan *et al*., 2001). A few studies suggest that site-specific recombination at *oriT* sites may play a role in the evolution of plasmids (Francia and Clewell, 2002b; Draper *et al*., 2005). Analysis of native cointegrate plasmids could help elucidate this mechanism.

Horizontal transfer is an important characteristic of plasmids. Accordingly, plasmids can be classified into three categories: conjugative, mobilizable and non-mobilizable [for a review, see Smillie *et al*. (2010)]. Transmissible plasmids, whether conjugative and mobilizable, contain two common components, *oriT* and relaxase. The origin of transfer, *oriT*, is a specific DNA sequence required for the conjugation of a plasmid. Relaxase, which is termed a Mob protein in a mobilizable plasmid, catalyses the initial and final stages in conjugation. In the initial stage, relaxase cleaves the *nic* site of *oriT* and covalently attaches to the 5′ end of the transferred strand, producing a single-strand DNA (ssDNA)-relaxase complex that can be transferred. Upon completion of the transfer, relaxase catalyses the ligation of the transported DNA in the recipient cell to reconstitute the plasmid (Smillie *et al*., 2010). Compared with Gram-negative systems, the mechanism of conjugation in Gram-positive systems has not been examined closely, except for the enzymatic properties of a few relaxases (Guzman and Espinosa, 1997; de Antonio *et al*., 2004; Kopec *et al*., 2005). The relaxases of mobilizable plasmids from Gram-positive bacteria mainly belong to the pMV158 superfamily (Grohmann *et al*., 2003). pMV158-MobM protein is the best characterized relaxase in this category of plasmids, and most such relaxases show a high degree of similarity to MobM (Francia *et al*., 2004).

Besides this function in conjugation, it has been reported that certain relaxases can also catalyse site-specific recombination between two *oriT* copies, including NikB (R64), TrwC (R388) and MobA (R1162) (Meyer, 1989; Furuya and Komano, 2003; Cesar *et al*., 2006). Moreover, this site-specific recombination could mediate the formation of a cointegrate structure from two small staphylococcal plasmids, pE194 and pT181, which contain identical 24 bp core sequences in their *oriT* sites (Gennaro *et al*., 1987). From an evolutionary perspective, two plasmids with similar *nic* sites could have the potential to enter the same cell by conjugation and then to form a cointegrate structure by utilizing relaxase-mediated site-specific recombination. For example, the native plasmid pAMα1 encoding tetracycline resistance in *Enterococcus faecalis* was shown to be a cointegrate which was speculated to have been generated by *oriT* site-specific recombination (Francia and Clewell, 2002a). Although these authors demonstrated that recombination could mediate the amplification of tetracycline resistance under selective pressure, the formation process was not directly observed experimentally. Therefore, the role of relaxases in plasmid evolution should be more thoroughly investigated.

Bacillus thuringiensis is a Gram-positive, spore-forming bacterium that can be isolated from diverse environments including soil, fresh water, the phylloplane, stored products, gain dust, and dead and living insects (Yuan *et al*., 2007; Raymond *et al*., 2010). *B. thuringiensis*, which is characterized by the production of insecticidal crystal proteins (ICP), has been widely used as a biopesticide for controlling agricultural pests (Rosas-Garcia, 2009). Most ICP-coding genes are located on large plasmids (> 70 kb) (Berry *et al*., 2002; Yuan *et al*., 2010; He *et al*., 2011). So far, only two exceptions have been reported. One is the 6.7 kb plasmid pBMBt1 that harbours a *cry*-like gene, *cry14-4,* in *B. thuringiensis* subsp. *darmstadiensis* INTA Mo14-4 (Loeza-Lara *et al*., 2005). The putative gene product shares a low sequence identity with the crystal protein Cry15Aa (21.9%, coverage 34%). Apart from the coding sequence (CDS), detailed information about the crystal formation and the toxicity of Cry14-4 protein is lacking. Another is plasmid pBMB0228, isolated from the nematicidal *B. thuringiensis* strain YBT-1518. Two typical crystal protein genes, *cry55Aa* and *cry6Aa*, are found to be located on this 17.7 kb plasmid. The two crystal proteins can form rice-shaped crystals showing high toxicity to the root-knot nematode. Sequence alignment shows that pBMB0228 additionally contains two Rep-like protein genes and two Mob protein genes (Guo *et al*., 2008).

In this study, we show that pBMB0228 is a cointegrate plasmid which can spontaneously resolve into two constituent plasmids. Moreover, the resolution is reversible because the cointegrate plasmid can be regenerated from the two constituent plasmids. We further show that the mechanism of the resolution and regeneration of pBMB0228 is relaxase-mediated *oriT* site-specific recombination. This study provides new insights into plasmid evolution by reproducing the formation process of a cointegrate plasmid, linking both the conjugation and *oriT* site-specific recombination functions of relaxases with plasmid evolution.

Results

Plasmid pBMB0228 contains two functional replication regions and two functional mobilization regions

In our previous study, two crystal protein genes, *cry55Aa* and *cry6Aa*, were found to be located on plasmid pBMB0228 (Guo *et al*., 2008). Now, we report the complete nucleotide sequence of pBMB0228. The plasmid is 17 706 bp with a G + C content of 29.5%. Sequence alignment reveals pBMB0228 contains two replication regions, two mobilization regions and two crystal protein genes (Table S1).

The replication regions of pBMB0228, annotated as rep0228 and repL0228, each contains typical replication features of rolling circle plasmids, including a replication protein gene and a double-strand origin (*dso*). However, only one putative single-strand origin (*sso*), located between the two replication regions, is apparent. To

assess the functionality of these two regions, five recombinant plasmids carrying putative replication elements were produced and transformed into *B. thuringiensis* BMB171. The results indicated that both rep0228 and repL0228 were functional in *B. thuringiensis* (Fig. S1). The pBMB0253 construct including both replication regions was found to be 100% segregationally stable (Fig. S1). The pBMB0252 and pBMB0214 constructs including only rep0228 were also highly stable (100% and 82% respectively), whereas the pBMB0251 and pBMB0220 constructs including only repL0228 were not (10% and 16% respectively) (Fig. S1).

The plasmid pBMB0228 also contains two mobilization regions, each composed of a *mob* gene and an *oriT* site overlapping the *mob* promoter. The two Mob proteins, Mob02281 and Mob02282, show 86% identity with each other and respectively show 78% and 80% identity with Mob14-4 of pBMBt1 from *B. thuringiensis* (Loeza-Lara *et al*., 2005). Both putative relaxases reveal three motifs that are conserved among members of the pMV158 superfamily (Fig. S2). To test the mobilization ability of the two regions, recombinant plasmids pHTMob02281 and pHTMob02282 which respectively contained *mob02281* with *oriT1* and *mob02282* with *oriT2* were constructed, and the pAW63 conjugative system was used. This system mobilizes non-conjugative plasmids via a Mob-dependent mechanism (Wilcks *et al*., 1998). With the help of conjugative plasmid pAW63::Tn5401, plasmid pHTMob02281 and pHTMob02282 were transferred from donor strain 4Q7 to recipient strain BMB171R by conjugation followed by plating on appropriate selective media. No transfer of vector plasmid pHT304 could be detected under the same conditions. The mobilization efficiency of plasmids pHTMob02281 and pHTMob02282 reached 3.4×10^{-5} and 4.3×10^{-6} respectively. This efficiency is similar to that observed for pBMBt1, pGI2 and pTX14-3 from other *B. thuringiensis* strains (Hoflack *et al*., 1999; Loeza-Lara *et al*., 2005). The mobilization efficiency of plasmid pHTMob02281 was one order of magnitude higher than that of pHTMob02282, which suggested that mob02281 mobilization region was more efficient in this plasmid, at least in this system.

These data show that the two replication regions and the two mobilization regions of pBMB0228 are functional.

Plasmid pBMB0228::Erm can resolve into two plasmids at oriT1 *and* oriT2 *sites*

Plasmid pBMB0228::Erm was obtained by inserting an erythromycin-resistant gene into the *R2* gene (Yu *et al*., 2008) (Table S1). We transformed pBMB0228::Erm by electroporation into *B. thuringiensis* BMB171, a plasmid-cured strain that is widely used for gene function and regulation studies (He *et al*., 2010). Interestingly, after growing the recombinant strain BMB171 (pBMB0228::Erm) for about 60 generations, we found an unusual plasmid profile in which two smaller plasmids appeared under the normal band (Fig. 1, lane 1). Because sequence analysis showed pBMB0228 appeared to be a cointegrate of two plasmids, we further examined this phenomenon. We isolated four individual colonies from this 60 generation culture and extracted their plasmid DNA. Three of them contained a single normal band (designated L colony, Fig. 1, lanes 3–5), and one only contained two smaller bands (designated S colony, Fig. 1, lane 2). If we cultured the L colony for an additional 60 generations, the two smaller plasmids also could appear (Fig. S3, lanes 1 and 2). In contrast, the two smaller plasmids were not observed when the same operation was repeated with strain YBT-1518 (Fig. S3, lanes 3 and 4).

Sequence analysis of the two smaller plasmids revealed that they were resolved from pBMB0228::Erm by recombination at the core regions of *oriT1* and *oriT2* without any change in nucleotide sequence (Figs 2 and 3A and B). The two *oriT* regions are 352 bp and 370 bp in length, respectively, and exhibit 84% sequence identity with each other. Both contained several inverted repeats with highly similar predicted secondary structures (Fig. 3C). Sequence analysis revealed the resolution junctions of pBMB0228::Erm contain *nic* sites (Fig. S2A) which are similar to those found in other members of pMV158 superfamily (Guzman and Espinosa, 1997; Szpirer *et al*., 2001; Francia *et al*., 2004). In addition, we also detected the resolution of pBMB0228::Erm by conjugation. The results showed all the transconjugants contained the resolved plasmid pBMB02281::Erm (Fig. S4). In summary, these data suggested that pBMB0228::Erm

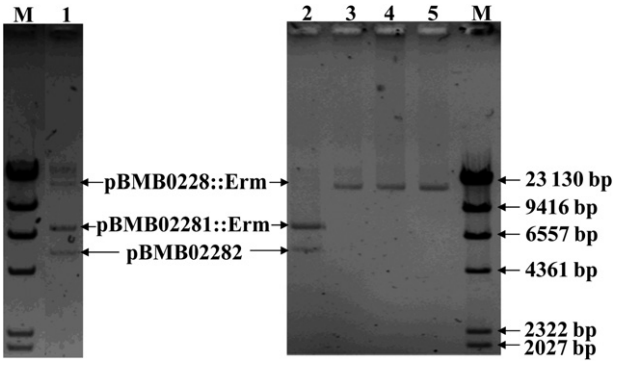

Fig. 1. Agarose gel electrophoresis of plasmids. Lane M, λDNA/HindIII marker. Lane 1, plasmid profiles of the recombinant strain BMB171 (pBMB0228::Erm) after growing for 60 generations. Lanes 2–5, four single colonies isolated from the 60 generation culture of Lane 1. Lane 2, BMB171 contains two smaller plasmids pBMB02281::Erm and pBMB02282. Lanes 3–5, BMB171 contains single pBMB0228::Erm.

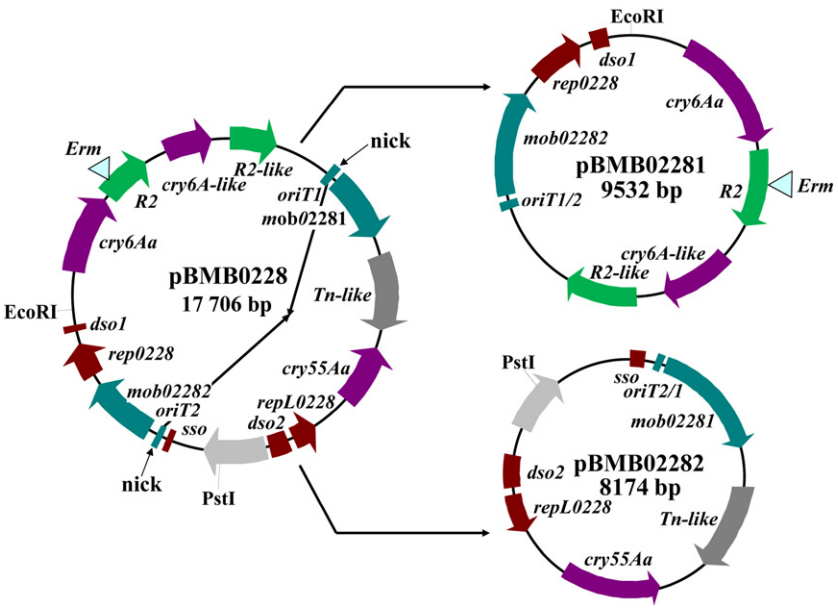

Fig. 2. The resolution of pBMB0228 into two plasmids. Putative *mob* genes, *rep* genes, crystal protein genes, double-strand origin (*dso*), single-strand origin (*sso*) and origin of transfer (*oriT*) are indicated. The orientations of arrows indicate the direction of transcription. Same colours indicate these elements have the same or similar function. 'Nick' indicates the resolution sites within *oriT* sequences.

could resolve into two plasmids by recombining at the *oriT1* and *oriT2* sites, and that while this event did not require conjugation, it could be promoted by conjugation.

The resolution of pBMB0228 requires Mob02281 or Mob02282

In mobilizable plasmids, relaxases called Mob proteins can recognize the origin of transfer (*oriT*) to initiate mobilization (Smillie *et al*., 2010). To determine if the two Mob proteins were involved in the resolution of pBMB0228, three derivatives (pBMBM1, pBMBM2 and pBMBM12) were constructed and transformed into BMB171 by electroporation. Plasmids pBMBM1 and pBMBM2 were *mob02281* or *mob02282* gene disruptions of pBMB0228, respectively, while pBMBM12 was disrupted in both genes. After allowing cultures containing these plasmids to grow for 60 generations, we observed plasmid resolution in pBMBM1 and pBMBM2 and in the pBMB0228::Erm control, but did not observe resolution in pBMBM12 (Fig. 4). To compare the resolution frequency of these mutant plasmids, the plasmid DNAs were extracted from these 60 generation cultures and retransformed into strain BMB171 by electroporation. Antibiotic-resistant transformants were examined for the presence of the cointegrate or of a resolved plasmid (Fig. S5). From each transformation experiment, we arbitrarily isolated 48 independent transformants, extracted plasmid DNA from each and used the DNA as a template for PCR amplification using primer pairs directed at the replication regions of pBMB02281 (primers rep1-rep2) and pBMB02282 (primers repL1-repL2). A plasmid testing positive for only one of the replication regions was considered resolved, while a plasmid testing positive for both regions was considered a cointegrate (Fig. S5C and D). The results showed that none of 48 transformants from the double knockout BMB171 (pBMBM12) contained the resolved constituent plasmids. However, 16 of 48 single colonies from BMB171 (pBMBM1), 24 of 48 single colonies from BMB171 (pBMBM2) and 12 of 48 single colonies from BMB171 (pBMB0228::Erm) contained the resolved plasmids (Table S2). These results suggested that either Mob02281 or Mob02282 was required to catalyse the resolution of pBMB0228.

The resolution of pBMB0228 is mediated by Mob involved oriT *site-specific recombination*

To demonstrate that Mob proteins and *oriT* sequences were sufficient for the resolution of pBMB0228, a recombination detection cassette was constructed. This cassette contained a spectinomycin resistance gene flanked by the two *oriT* regions in the same orientation, all inserted between the promoter and CDS of a *gfp* gene (Fig. 5A). Plasmid pBMBT3 and pBMBT10 were generated by ligating the recombination detection cassette into vector pHT315 in opposite orientations (Fig. 5B). Recombination at the two *oriT* sites should simultaneously eliminate the spectinomycin resistance gene and join the *gfp* gene with its promoter, allowing its expression (Fig. 5A). Both constructs were tested for their ability to support site-specific recombination in the presence of four plasmids: pBMBmob1 containing *mob02281*, pBMBmob2 containing *mob02282*, pBMBmob12 containing both *mob02281* and *mob02282*, or pEMB0603 containing neither.

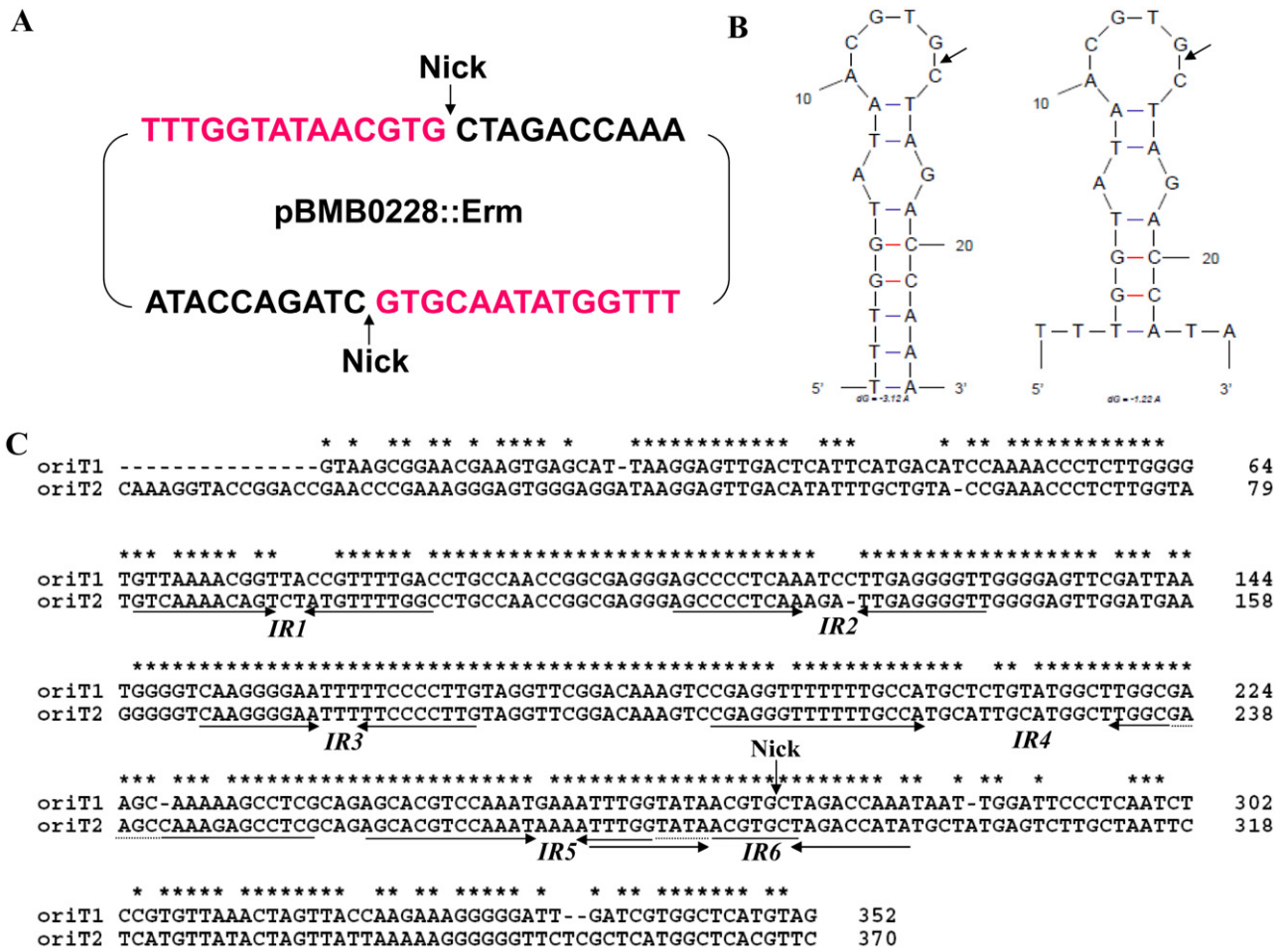

Fig. 3. A. Nucleotide sequences and B. the secondary structure analysis of the resolution sites of pBMB0228. C. Sequence alignments of nucleotide sequences of *oriT1* and *oriT2* regions in pBMB0228 tested in the mobilization and recombination experiments. Asterisks indicate conserved nucleotides. Number and horizontal arrows indicate inverted repeats. 'Nick' indicates the sites in the *oriT* sequences where the nicking occurs, corresponding to the nick site of pMV158 involved in mobilization (Fig. S2A).

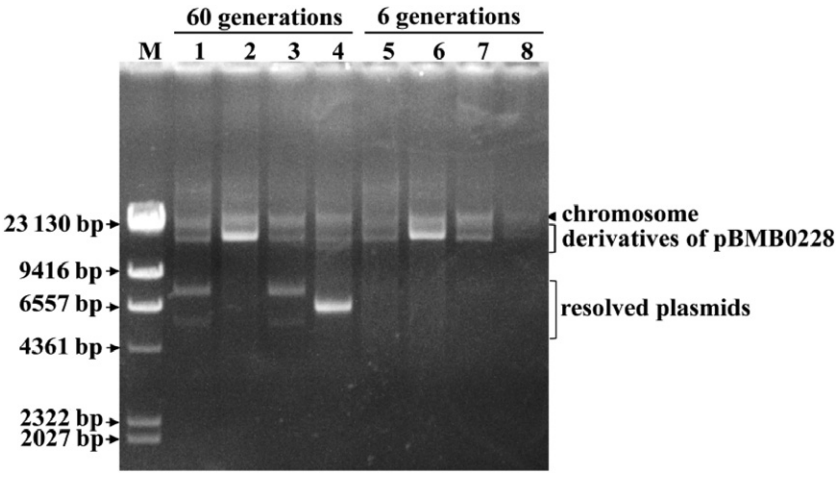

Fig. 4. The resolution of pBMB0228 requires Mob02281 or Mob02282. Plasmid extraction of derivative plasmids of pBMB0228 in strain BMB171 after growing for 60 generations (lanes 1–4) and 6 generations (lanes 5–8). Lane M, λDNA/HindIII marker; Lane 1, BMB171 containing plasmid pBMB0228::Erm and its resolved plasmids of 10 746 bp and 8174 bp; Lane 2, BMB171 containing plasmid pBMBM12; Lane 3, BMB171 containing plasmid pBMBM2 and its resolved plasmids of 10 819 bp and 8174 bp; Lanes 4, BMB171 containing plasmid pBMBM1, and its resolved plasmids of 9532 bp and 9283 bp; Lane 5, BMB171 containing plasmid pBMB0228::Erm, 18 920 bp; Lane 6, BMB171 containing plasmid pBMBM12, 20 102 bp; Lane 7, BMB171 containing plasmid pBMBM2, 18 993 bp; Lanes 8, BMB171 containing plasmid pBMBM1, 18 815 bp.

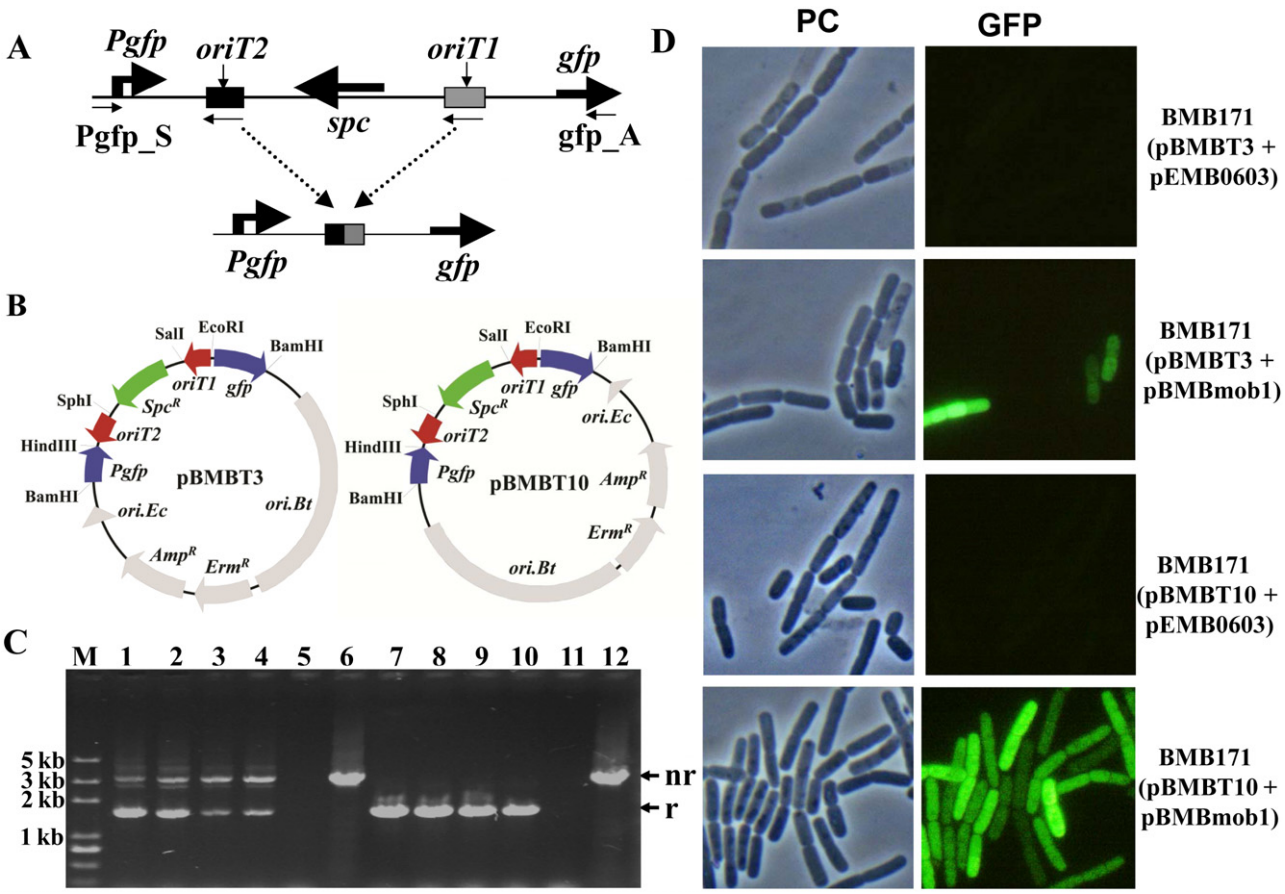

Fig. 5. Site-specific recombination at *oriT1* and *oriT2* sites of pBMB0228.
A. Structure of the recombination cassette and the resulting product after recombination at *oriT1* and *oriT2*. Arrows indicate the direction of transcription; *spc*, spectinomycin resistance gene; *Pgfp*, the promoter of kanamycin resistance gene; *gfp*, gene encoding green fluorescent protein; Arrows marked Pgfp_S and gfp_A indicate the sites for the primers used in PCR detection.
B. Structures of the two substrate plasmids containing the same recombination cassette. The recombination cassette was inserted by a single BamHI site with different directions into pHT315, generating pBMBT3 and pBMBT10. Arrows indicate the direction of transcription of the *erm* and *amp* genes. The origin of replication in *E. coli* (*ori*.Ec, *ori* from pBR322) indicates the direction of replication of pBR322. The origin of replication in *B. thuringiensis* (*ori*.Bt, *ori* from pHT1030) is shown as a box that indicates the orientation of the replication of pHT1030 is not known (Arantes and Lereclus, 1991).
C. PCR analysis of pBMBT3 and pBMBT10 recombined plasmids. Lane M, *Trans*2K Plus DNA Marker. Lanes 1–12, products of PCR reactions with primer pair Pgfp_S and gfp_A as shown in Figure 4A. Lanes 1–4, four different colonies obtained from BMB171 (pBMBT3 + pBMBmob1). Lanes 7–10, four different colonies obtained from BMB171 (pBMBT10 + pBMBmob1). Lanes 5 and 11, negative control BMB171 (pBMBmob1), Lanes 6 and 12, positive control BMB171 (pBMBT3) and BMB171 (pBMBT10). nr, non-recombined cassette, 3.2 kb. r, recombined cassette, 1.6 kb.
D. Observation of the derivatives of strain BMB171 containing either pBMBT3 or pBMBT10, with pBMBmob1 or vector plasmid pEMB0603. PC, phase contrast; GFP, fluorescence microscopy.

We observed that recombination occurred if the substrate plasmid contained tandem *oriT* sites and the helper plasmid contained either one or both *mob* genes. In contrast, recombination was not observed if the substrate plasmid contained only a single *oriT* sequence (see Table 1, experiments 1–2 and 6) or if the helper plasmid failed to contain a *mob* gene (see Table 1, experiments 3 and 4–6; experiments 7 and 8–10). However, there was no obvious difference in recombination if *mob02281* and *mob02282* occurred singly or together in the helper plasmid (see Table 1, experiments 4–6 and 8–10). These data suggested that the resolution of pBMB0228 was mediated by Mob-mediated recombination between tandem *oriT* sites in the cointegrate.

It is worth noting that recombination frequency was substantially higher with pBMBT10 as substrate plasmid than with pBMBT3 (see Table 1, experiments 4–6 and 8–10). This phenomenon was evidenced both by the PCR analysis and by the observation of fluorescent BMB171 (pBMBT3 + pBMBmob1) and BMB171 (pBMBT10 + pBMBmob1) cells under phase contrast and fluorescence microscopy (Fig. 5C and D).

Table 1. Site-specific recombination at the two *oriT* sites of pBMB0228.

Experiments	Plasmids	OriT1	OriT2	Mob02281	Mob02282	Recombination frequency (% Spc^S colonies)
1	pBMBT1 + pBMBmob12	+		+	+	0
2	pBMBT2 + pBMBmob12		+	+	+	0
3	pBMBT10 + pEMB0603	+	+			0
4	pBMBT10 + pBMBmob1	+	+	+		97%
5	pBMBT10 + pBMBmob2	+	+		+	93%
6	pBMBT10 + pBMBmob12	+	+	+	+	82%
7	pBMBT3 + pEMB0603	+	+			0
8	pBMBT3 + pBMBmob1	+	+	+		2%
9	pBMBT3 + pBMBmob2	+	+		+	6%
10	pBMBT3 + pBMBmob12	+	+	+	+	10%

Recombinant strain of BMB171 containing the indicated plasmids were plated on selective media. The resulting colonies were grown with Erm and Kan for 60 generations and plated on Erm and Kan plates, and the resulting single colonies were transferred onto Spc-containing plates. Plasmids pBMBmob1, pBMBmob2 and pBMBmob12 were used as helper plasmid coding for mobilization protein; pEMB0603 was used as the negative control. Recombination frequency was estimated as the number of Spc-sensitive colonies. Each experiment was performed in triplicate.

The two constituent plasmids can regenerate pBMB0228

After observing that pBMB0228 could resolve into two independent plasmids, we next asked whether the two plasmids could fuse back together. To facilitate the experiments, plasmid pBMB02281::Erm was recovered from the resolution plasmid of pBMB0228::Erm, and pBMB02282::Spc was constructed by inserting a spectinomycin resistance gene into the PstI site of pBMB02282. We found both pBMB02281::Erm and pBMB02282::Spc were mobilizable (Table S3), so it seemed possible that conjugation would provide a means to place both plasmids in the same cell. Matings were carried out using helper plasmid pAW63::Tn5401 and donor strain 4Q7 carrying either pBMB02281::Erm or pBMB02282::Spc, together with recipient strains already carrying one or the other of these plasmids (Table S3). We did not observe fusion plasmids directly when the plasmid DNA from transconjugants was extracted and visualized by gel electrophoresis (Fig. S6). This negative result might be due to a low frequency of cointegration in the absence of selective pressure. For this reason, we attempted to detect fusion events via PCR using primer pairs T1S-M2A and T2S-M1A, which flanked the two predicted junctions in the cointegrate pBMB0228 (Fig. 6A). This approach indeed detected amplification products corresponding to the two junctions in four single transconjugants of each recipient (Fig. 6B and C). Such products were not observed if either the donor or recipient alone was used as template. Sequence analysis of all obtained PCR products in Fig. 6B and C showed 100% identify with that of the 1680 bp and 370 bp junctions of pBMB0228 respectively. However, similar PCR products could not be observed in transconjugants with primer pairs T1S-T2S or M1A-M2A, indicating the fusion event is likely orientation dependent.

Cointegrate plasmids can be formed by oriT *site-specific integration in the recipient*

To confirm that the plasmid fusion we observed was mediated by *oriT* site-specific cointegration, another conjugation system was constructed (Fig. 7A). Donor strain 4Q7 contained three plasmids: pBMBD1, pBMBKmob1 and pAW63::Tn5401. Plasmid pBMBD1 contained the *oriT1* of pBMB0228 and a promoterless spectinomycin resistance gene; non-mobilizable plasmid pBMBKmob1 contained the CDS of *mob02281* gene and the promoter of the kanamycin gene; and pAW63::Tn5401 provided the T4SS (type IV secretion system). Recipient strain BMB171R contained one plasmid, pBMBD2, carrying the *oriT2* of pBMB0228 and the promoter of the kanamycin resistance gene. In pBMBD1, the spectinomycin resistance CDS lies upstream of *oriT1*. The expression of this gene could be activated after integration into the recipient plasmid pBMBD2, which carries a strong promoter downstream of *oriT2*. In this strategy, integration events could be selected by plating cells directly onto spectinomycin-selective medium after mating. Spc-resistant colonies were obtained only when the *oriT*-containing pBMBD1 was mobilized into a recipient strain carrying the *oriT*-containing pBMBD2 (Table 2, lines 1 and 2). The plasmids from Spc-resistant transconjugants were harvested and transformed into *Escherichia coli*. DNA from Spc-resistant *E. coli* colonies was extracted and subjected to PCR and restriction analysis as shown in Figure 7B. The amplification products were then sequenced. Results confirmed the Spc-resistant transconjugants were created by integration events at the *oriT* sites. Selection of resistance to erythromycin, kanamycin and rifampicin provides an estimate of pBMBD1 transfer frequency in this system. The frequency of cointegration is ≈ 1 in 600 transconjugants

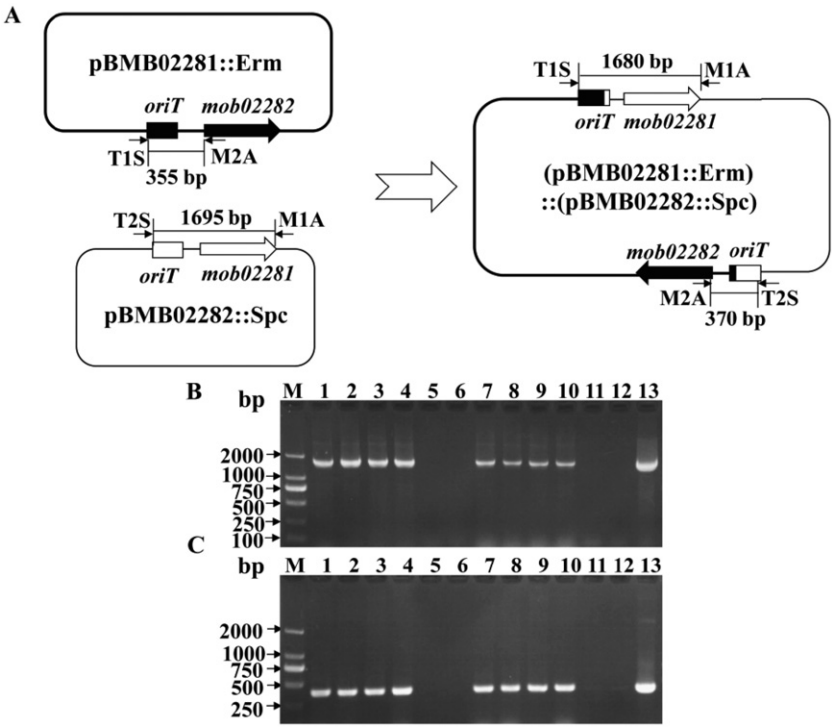

Fig. 6. The fusion of pBMB02281::Erm and pBMB02282::Spc within recipient cell.
A. Experimental design for detection of the fusion plasmid in transconjugants.
B. PCR detection of the junction sites of the fusion plasmid with primer pair T1S-M1A.
C. PCR detection of the junction sites of the fusion plasmid with primer pair T2S-M2A. The templates (lanes 1–12) were shown in Figure S7. Lanes 1–4, four independent transconjugants arising from mating 4Q7 (pBMB02281::Erm + pAW63::Tn5401) with BMB171R (pBMB02282::Spc); Lane 5, donor 4Q7 (pBMB02281::Erm + pAW63::Tn5401); Lane 6, recipient BMB171R (pBMB02282::Spc); Lanes 7–10, four independent transconjugants arising from mating 4Q7 (pBMB02282::Spc + pAW63::Tn5401) with BMB171R (pBMB02281::Erm); Lane 11, donor 4Q7 (pBMB02282::Spc + pAW63::Tn5401). Lane 12, recipient BMB171R (pBMB02281::Erm); Lane 13, Positive PCR control for the two junctions, using pBMB0228 from wild strain YBT-1518 as template. Lane M, *Trans*2K DNA Marker.

(Table 2, compare Spc^R transconjugants/donor and transfer frequency in line 1).

Conjugative plasmid pAW63::Tn5401 cannot mediate recombination at the *oriT* sites, which was confirmed by a recombination assay using pBMBT10 as a substrate plasmid and pAW63::Tn5401 as a helper plasmid (Fig. S7). Spc-resistant colonies were not obtained when plasmid pBMBKmob1 was not in the donor strain at the presence of pAW63::Tn5401 (Table 2, compare lines 1 and 3). These results confirmed that the Mob02281 provided two functions in the integration assays: firstly, it mobilized pBMBD1 into the recipient strain; secondly, it mediated recombination at the *oriT* sites of pBMBD1 and pBMBD2. In summary, the two *oriT*-containing plasmids

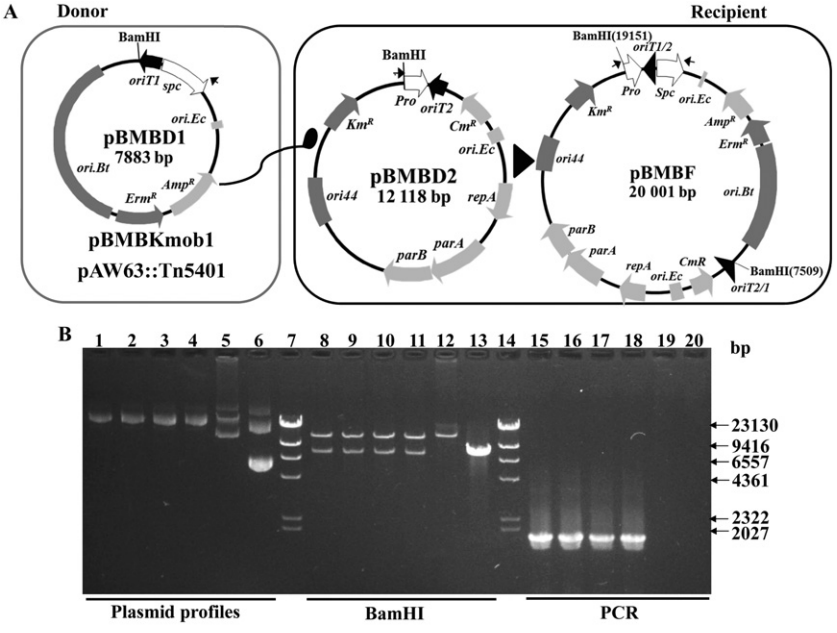

Fig. 7. Analysis of cointegrate plasmids.
A. Structure of the *oriT*-containing plasmids present in donor (pBMBD1), recipient (pBMBD2) and the resulting cointegrate plasmid (pBMBF). Arrows point in the direction of transcription. 'Pro' indicates the strong promoter used for expression of *spc* after recombination. Small inner arrows indicate the sites for the sequence-specific primers F-S and F-A used in PCR reactions. Relaxase is shown as ellipse leading the transferred DNA (wavy line) into recipient cells.
B. DNA analysis of cointegrate plasmids. Lanes 1–6, plasmid profiles. Lanes 7 and 14, λDNA/HindIII marker. Lanes 8–13, BamHI restriction digestion. Lanes 15–20, products of PCR reactions with F-S and F-A (see Table S5), 1796 bp. Lanes 1–4, 8–11 and 15–18, four different spc-resistant *E. coli* colonies obtained from four individual spc-resistant transconjugants in the integration assays. Lanes 5, 12 and 19, pBMBD2. Lanes 6, 13 and 20, pBMBD1. Other plasmids present in the matings (pBMBKmob1, pAW63::Tn5401) were also PCR-negative (not shown).

Table 2. Mob02281-mediated site-specific integration in recipient cells.

Plasmids in donor	Plasmids in recipient	Spc^R transconjugants/donor	Transfer frequency
pBMBD1 + pBMBKmob1 + pAW63::Tn5401	pBMBD2	1.1×10^{-6}	6.2×10^{-4}
pBMBD1 + pBMBKmob1 + pAW63::Tn5401	pEMB0603	$< 10^{-8}$	5.9×10^{-4}
pBMBD1 + pAW63::Tn5401	pBMBD2	$< 10^{-8}$	$< 8.5 \times 10^{-8}$

Conjugation from donor 4Q7 into recipient BMB171R was performed as described in *Experimental procedures*. Selective plating was done on plates containing Spc and Rif to select for the fusion plasmids. At the same time, selective plating was done on plates containing Erm, Kan and Rif to select for the transconjugants. Transfer frequency was estimated as number of Erm-, Kan- and Rif-resistant colonies per donor. Each experiment was performed in triplicate.

could fuse into a cointegrate by intermolecular *oriT* site-specific recombination.

Discussion

In our study, we observed that a native plasmid pBMB0228 from *B. thuringiensis* can spontaneously resolve into two constituent plasmids, each of which harbours one replication region, one mobilization module and one crystal protein gene. Although the existence of cointegrate plasmids is well known, the spontaneous resolution of a native cointegrate plasmid has, to our knowledge, not previously been directly observed. Moreover, the two plasmids also can fuse back into a single cointegrate after entering into the same cell. This study showed that a native plasmid can reversibly convert a cointegrate structure into its constituent plasmids. These results suggest that pBMB0228 is a valuable model for the study of the mechanism of plasmid evolution because it is possible to observe both its resolution and formation under laboratory conditions.

We further showed that both the resolution and regeneration of pBMB0228 is mediated by *oriT* site-specific recombination and that either Mob02281 or Mob02282 is required for this process. Sequence analysis reveals the resolution and integration events happen within the 24 bp core sequences of *oriT1* and *oriT2*, which contain *nic* sites that differ in only a single base. The two Mob proteins of pBMB0228 can specifically cleave supercoiled DNA containing *oriT1* or *oriT2* (P. Wang, C. Zhang, Y. Zhu, D. Peng, L. Ruan and M, Sun., unpublished), indicating that both Mobs can interact with the similar *nic* sites. Relaxases and *oriT* regions are necessary elements for the transfer of conjugative and mobilizable plasmids. Most relaxases are evolutionarily related and show common sequence motifs, often with highly similar sequences (Francia *et al*., 2004; Garcillan-Barcia *et al*., 2009). Moreover, some relaxases can mediate site-specific recombination between two tandemly cloned *oriT* copies, as has been previously described (Meyer, 1989; Francia and Clewell, 2002b; Furuya and Komano, 2003). Therefore, it is easy to envision that relaxases acting on *oriT* sites can first transfer two plasmids into one recipient cell and then mediate a site-specific recombination event to form a cointegrate structure. Our observation that cointegrate plasmid pBMBF can be generated from two *oriT*-containing plasmids, pBMBD1 and pBMBD2, after conjugation supports this model. The integration assay, based on strategy developed previously by Draper and colleagues (2005), also supports this viewpoint.

The cointegrate plasmid pBMB0228, which appears to be a reversible association of pBMB02281 and pBMB02282, is a natural isolate that can be examined in light of this model. Another native plasmid that can be understood in this way is *E. faecalis* tetracycline resistance plasmid pAMα1, which may be generated by integrating pAMα1Δ1 into pAMα1Δ2 (Francia and Clewell, 2002a). We speculate that when tetracycline resistance plasmid pAMα1Δ1, which is not able to replicate in *E. faecalis*, was mobilized into an *E. faecalis* strain carrying the plasmid pAMα1Δ2, the latter plasmid captured the former via MobB/MobE-mediated recombination at the two similar *oriT* sites, acquiring its tetracycline resistance gene for host adaptation. Evidence to support our hypothesis comes from both experimental studies and analysis of plasmids isolated from the environment. Therefore, we posit that relaxase/*oriT* site-specific recombination after conjugation may play an important role in plasmid evolution.

Another interesting topic is how cointegrate plasmids maintain their segregational and structural stability. Specifically, how do cointegrate plasmids replicate, if both components are functional in the host strain, especially if both replicate by a rolling circle mechanism? Plasmid pBMB0228 provides an example, as it is composed of two apparent rolling circle plasmids, pBMB02281 and pBMB02282 (Fig. 2). Although our subcloning experiments revealed differences in segregational stability for these two replication regions (Fig. S1), the two resolved plasmids pBMB02281::Erm and pBMB02282::Spc are found to be highly stable (98% and 100% respectively) and compatible when coexisting in *B. thuringiensis* BMB171. It may be that the DNA fragments cloned in pBMB0251 and pBMB0220 are incomplete, and that other sequences found in pBMB02282 may be involved in the fully stable replication.

Recombination events at *oriT* sites result in the formation of a cointegrate, but these sites are not involved in its maintenance (Gennaro *et al*., 1987; Szpirer *et al*., 2001). Therefore, in populations of cells, there is a finite probability that the cointegrate structure will resolve into its constituents. Presumably beneficial traits for the host adaption provided by cointegrate plasmids will promote their maintenance, as in the case of tetracycline resistance with plasmid pAMα1 (Francia and Clewell, 2002a). In our study, the resolution of pBMB0228 was observed in *B. thuringiensis* BMB171 and other *B. thuringiensis* strains (not shown), but was never observed in wild strain YBT-1518. Therefore, there seems to be some control factors repressing the resolution of pBMB0228 in strain YBT-1518. Because each constituent plasmid harbours a nematicidal crystal gene, the formation of a cointegrate plasmid encoding multiple toxins might have been an important step in the evolution of the toxin-producing strain YBT-1518. Several studies have reported that site-specific recombination between two *oriT* sites can also cause DNA amplification, resulting in tandem copies of a gene or gene cluster, to increase their level of expression (Francia and Clewell, 2002a; Yanai *et al*., 2006; Murakami *et al*., 2011). By analogy, the integration of two crystal protein genes in pBMB0228 may have increased the nematicidal activity of strain YBT-1518. Future work will examine this question in detail.

It is worth noting that recombination frequency was substantially higher with pBMBT10 as substrate plasmid than with pBMBT3 (see Table 1, experiments 4–6 and 8–10, Fig. 5C and D). These substrate plasmids differ only in the orientation of their recombination cassette relative to the direction of replication. A similar effect has been previously reported for site-specific recombination catalysed by R64 NikB and R388 TrwC. In these studies, the authors proposed that different recombination frequencies resulted from the orientation of the two repeated *oriT* sequences relative to the direction of replication of the vector plasmid. They hypothesized that the presence of the *nic* site on the strand which would favour ssDNA exposure during replication would strongly promote recombination catalysed by relaxases (Furuya and Komano, 2003; Cesar *et al*., 2006).

In this study, we found a native cointegrate can resolve into two plasmids via *oriT* site-specific recombination during multiple generations of growth. In addition, we observed a high frequency of resolved plasmids among transconjugants. These results indicated this recombination was independent of conjugation but could be promoted by conjugation. This may be because the single-stranded state of the transfer strand during conjugation is a highly efficient substrate for Mob proteins. Work by Miyazaki and van der Meer (2011) showed site-specific recombination events catalysed by a relaxase coded within an integrative and conjugative element (ICE) containing two *oriT* sequences, both of which were active for conjugation, but only one of which was a target for recombination (Miyazaki and van der Meer, 2011). This may be one mechanism allowing the coexistence of dual *oriT* sequences within the ICE. In our study, when we used YBT-1518 (pBMB0228::Erm) as the donor strain, under conditions successfully used with BMB171 (pBMB0228::Erm) as donor (Fig. S4), we could not obtain transconjugants. This phenomenon may reflect a mechanism allowing coexistence of the two active *oriT* sequences in a native plasmid.

Several published studies showed that relaxase-mediated recombination requires an accessory protein, especially to act on double-stranded substrates. In R64, both NikA and NikB were required for intracellular recombination between two R64 *oriT* sequences on a plasmid vector; in a phage vector, recombination depended on the relaxase NikB, while the accessory nicking protein NikA promoted it (Furuya and Komano, 2003). In R388, the relaxase TrwC was required for site-specific recombination between two R388 *oriT* sequences in a plasmid vector, and the accessory protein TrwA increased its efficiency (Cesar *et al*., 2006). The intracellular recombination between two *oriT* sequences in a plasmid vector is highly related to conjugal DNA processing. These accessory proteins are the factors participating in the formation of relaxosomes. In this study, recombination between *oriT1* and *oriT2* required either Mob02281 or Mob02282. We have not detected similar predicted accessory proteins in the sequence of pBMB0228. Other factors participating in the recombination process can be the subject of future investigation.

In conclusion, the cointegrate plasmid pBMB0228 is not only a valuable model for the study of plasmid evolution, but also a useful tool for further analysis of the mechanism of *oriT* site-specific recombination.

Experimental procedures

Bacterial strains, plasmids, growth conditions and DNA manipulations

The strains and plasmids used in this study and their sources are listed in Table S4. Conditions and media used for growing strains of *E. coli* and *B. thuringiensis* have been described previously (Guo *et al*., 2008). Plasmids were extracted from *B. thuringiensis* following the procedure described by Andrup and colleagues (1993). Spectinomycin, tetracycline, erythromycin, kanamycin, ampicillin and rifampin were added to the media at final concentrations of 100, 10, 25, 50, 100 and 100 μg ml^{-1} respectively. All regular DNA manipulations were carried out following standard methods (Sambrook, 2001). PCR products were confirmed by DNA sequencing. Primers used for PCR amplification are listed in Table S5. For a detailed description on recombinant plasmids, see Appendix S1, which is supplied as supporting information.

Plasmid segregational stability test

The recombinant plasmids containing different replication regions were respectively transformed into *B. thuringiensis* BMB171 (Fig. S1 and Table S4), and were tested for survival and replication stability. The stability of these recombinant plasmids was tested under non-selective conditions at 28°C according to the method of Sanchis and colleagues (1997). Every 8 h of growth (about 10 generations) (Sanchis *et al*., 1997), bacterial suspensions were diluted 1000-fold in 10 ml of fresh Luria–Bertani (LB) liquid medium. This procedure was repeated thrice such that cultures had grown at least 40 generations after the initial inoculation. One hundred single colonies of each recombinant strain were transferred onto LB plates and LB plates with erythromycin. Plasmid stability was estimated as the number of resistant colonies after growing for about 40 generations. Each experiment was performed in triplicate.

Conjugation experiments

Conjugation experiments were conducted following the protocols described by Hu and colleagues (2004). To test the mobilization ability of the two mobilization regions of pBMB0228, triparental matings were performed by mixing donor strains 4Q7 carrying the targeted plasmids, helper strain AW48 carrying the conjugative plasmid pAW63::Tn5401 and recipient strain BMB171R. For fusion experiments of pBMB02281::Erm and pBMB02282::Spc, strains 4Q7 carrying pBMB02281::Erm or pBMB02282::Spc, and pAW63::Tn5401 were used as donors in matings, while strains BMB171R carrying pBMB02282::Spc or pBMB02281::Erm were used as recipients. In brief, overnight cultures of donor, recipient and helper strains were incubated separately at 28°C in LB medium with appropriate antibiotics. Equal quantities of donor, recipient and helper cells in logarithmic growth were mixed and incubated in 7 ml of prewarmed LB medium, without antibiotics, at 28°C with moderate shaking (180 r.p.m.). After mating for 3 h, appropriate dilutions were plated onto appropriate selective medium to determine the number of transconjugants. Controls of donors, recipients and helper strain grown separately were also tested. Transfer frequencies were calculated as the ratio of transconjugants per donor cells at the end of the experiment. Each experiment was performed in triplicate.

Recombination assays

To test the *oriT* site-specific recombination, substrate plasmid pBMBT3 and pBMBT10 were respectively introduced in BMB171 containing a helper plasmid. The resulting colonies were grown with Erm and Kan for 60 generations and plated in Erm and Kan plates by dilution, and the resulting single colonies were transferred onto Spc-containing plates. For growing 60 generations, bacterial suspensions were diluted 1000-fold in 10 ml of fresh LB liquid medium every 8 h of growth at 28°C. This procedure was repeated five times after the initial inoculation. pBMBmob1, pBMBmob2 and pBMBmob12 were used as helper plasmids, while pEMB0603 was used as the negative control. Recombination frequency was estimated as the number of Spc-sensitive colonies and combined with observations of the number of fluorescent cells under phase contrast and fluorescence microscopy. Each experiment was performed in triplicate.

Integration assays

Matings were carried out by using *B. thuringiensis* strain 4Q7 carrying targeted plasmids as donor and the recipient strain harbouring a plasmid with or without the *oriT2* of pBMB0228. pBMBD1, containing the *oriT1* of pBMB0228, was mobilized from donor containing helper plasmids for conjugative mobilization into recipient strain. Integration events were selected by plating cells directly onto spectinomycin-selective medium after mating. Spc-resistant colonies were harvested and respectively transformed into *E. coli* cell. DNA from Spc-resistant *E. coli* colonies was extracted and subjected to further analysis.

Nucleotide and protein sequence analysis

Open reading frame (ORF) prediction was performed using the CLC Free Workbench 4 programme (CLC bio, Aarhus, Denmark). DNA and protein sequence homology searches were performed using the BLAST algorithm (Altschul *et al*., 1990) against the GenBank database. The two *oriT* sequences of pBMB0228 were analysed using the software package RNAstructure 5.2 (Mathews lab, Rochester, NY, USA).

Transformation of E. coli *and* B. thuringiensis

Transformation of *E. coli* was carried out using $CaCl_2$-treated competent cells, as described by Sambrook and Russell (2001). Transformation of *B. thuringiensis* strain was performed by electroporation with the Bio-Rad gene pulser set (BIO-RAD, Hercules, CA, USA) as previously described (Shao and Yu, 2004).

Microscopic observation

For phase contrast microscopy and fluorescence microscopy, all *B. thuringiensis* strains were cultivated in LB liquid medium at 28°C. The morphology of the strains was observed with an Olympus photomicroscope (OLYMPUS, Tokyo, Japan).

Nucleotide sequence accession number

The sequence and annotation for the full-length 17 706 bp plasmid pBMB0228 (Fig. 2) has been deposited in the GenBank database under accession number CP002486.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Map of the replication region of pBMB0228. Relevant restriction sites and ORFs are shown. Putative *rep* genes, double-strand origins (*dso*) and single-strand origin (*sso*) are indicated. Five derivatives consisting of fragments subcloned from pBMB0249 are shown, together with their segregational stabilities in *B. thuringiensis* strain BMB171. The orientation of arrows indicates the direction of transcription.

Fig. S2. Comparison of Mob regions from different plasmids of Gram-positive bacteria.

A. Comparison of *oriT* from plasmids of the pMV158 superfamily. Arrow indicates the nick site.

B. Motifs conserved among Mob proteins from the pMV158 superfamily. The motifs HxxR (motif I), NY(D/E)L (motif II) and HxDExxPHuH (motif III) are shown. Conserved amino acids at a given residue are indicated by identical colour shadings.

Fig. S3. Plasmid extraction of L colony of BMB171 (pBMB0228::Erm) (lane 1 and 2) and YBT1518 (pBMB0228::Erm) (lane 3 and 4) after growing for 6 generations (lane 1 and 3) and 60 generations (lane 2 and 4); Lane M, λDNA/HindIII markers.

Fig. S4. Resolution of pBMB0228::Erm during conjugation. Lane 1, donor strain BMB171 (containing single pBMB0228::

Erm); lane 2, BMB171 (containing pBMB0228::Erm, pBMB02281::Erm and pBMB02282) used as reference with pBMB02281::Erm; lane 3, recipient strain *B. cereus* UW85R; lanes 4–7, four independent transconjugants with *B. cereus* UW85R as recipient; lanes 8–11, four independent transconjugants with *B. thuringiensis* BMB171R as recipient.

Fig. S5. The examination of transformants for the presence of the cointegrate or of a resolved plasmid containing the selective marker. Total plasmid extracted from 60 generation cultures and retransformed into BMB71, with selection for antibiotic resistance.

A. Overall experimental design used to compare the resolution frequency of these mutant plasmids.

B. Transfer the single colonies of BMB171 (pBMBM12) from Spc- or Kan-resistant plates on Spc- and Kan- resistant plates to determine whether pBMBM12 can resolve into two constituent plasmids.

C. Plasmids extracted from individual transformants: C, cointegrates; R, resolved plasmids.

D. PCR analysis of these transformants using primer pair repL1-repL2 (a and c) and primer pair rep1-rep2 (b and d). The 60 generation cultures were BMB171(pBMB0228::Erm) (lanes 1–11), BMB171(pBMBM12) (lanes 12–23), BMB171(pBMBM2) (lanes 24–35) and BMB171(pBMBM1) (lanes 36–46). Lanes numbered in black indicate transformants that contain the cointegrate. Lanes numbered in red indicate transformants that contain a resolved plasmid with the selective marker. Lane M1, λDNA/HindIII markers. Lane M2, *Trans*2K Plus DNA Marker. Lanes marked ck are a positive control for PCR, with YBT-1518 plasmid DNA as template.

Fig. S6. Plasmid profiles of donor, recipient and transconjugant strains. Lanes 1–4, four independent transconjugants from mating 4Q7 (pBMB02281::Erm + pAW63::Tn5401) with BMB171R (pBMB02282::Spc). Lane 5, donor strain 4Q7 (pBMB02281::Erm + pAW63::Tn5401). Lane 6, recipient strain BMB171R (pBMB02282::Spc). Lanes 7–10, four independent transconjugants from mating 4Q7 (pBMB02282::Spc + pAW63::Tn5401) with BMB171R (pBMB02281::Erm). Lane 11, donor strain 4Q7 (pBMB02282::Spc + pAW63::Tn5401). Lane 12, recipient strain BMB171R (pBMB02281::Erm). Lane 13, AW48 used to indicate the conjugative plasmid pAW63::Tn5401. pBMB171 is a endogenous plasmid in BMB171R which was not cured (He *et al*., 2010).

Fig. S7. pAW63::Tn5401 cannot mediate the *oriT* site-specific recombination.

A. Plating single colonies of BMB171 (pBMBT10 + pBMBmob1) and BMB171 (pBMBT10 + pAW63::Tn5401) onto Spc-containing plates after growing for 60 generations.

B. Observation of the derivatives of strain BMB171 containing pBMBT10, with pBMBmob1 or pAW63::Tn5401. PC, phase contrast; GFP, fluorescence microscopy. Recombination assays were performed as described in *Experimental procedures*. Recombination frequency was estimated as the number of Spc-sensitive colonies and combined with observations of the number of fluorescent cells under phase contrast and fluorescence microscopy. Each experiment was performed in triplicate.

Table S1. Open reading frames identified in pBMB0228.

Table S2. The resolution of pBMB0228 was mediated by Mob02281 or Mob02282.

Table S3. Mobilization efficiency of pBMB02281::Erm and pBMB02282::Spc into recipient cells.

Table S4. Bacterial strains and plasmids used in this study.

Table S5. Primers used in this study.

Appendix S1. Plasmid construction.