Original Article

Isolation of the *Bacillus thuringiensis* plasmid carrying Bacthuricin F4 coding genes and evidence of its conjugative transfer

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Abstract

Introduction: Conjugation is an excellent natural mode of DNA transfer *in vivo* between bacteria, particularly when these conjugative elements carry technological traits such as bacteriocin encoding genes. In the present work, the bacteriocinogenic plasmid pIBF4 from *Bacillus thuringiensis* responsible of Bacthuricin F4 synthesis was isolated and characterized.

Methodology: To isolate pIBF4, the total plasmid DNA from a non-bacteriocin transposant carrying the mini-Tn10 spectinomycin selective marker was extracted and used to transform *Escherichia coli* strain Top10. PIBF4 was extracted from the obtained transformant and then subjected to restriction enzyme analysis. Plasmid curing experiments were conducted to test the stability of pIBF4 at a stringent temperature of 42°C. Conjugative behavior of pIBF4 was assessed by mating experiments using the non-bacteriocin transposant mutant as a donor strain and several *Bacillus thuringiensis* strains as recipients.

Results: The pIBF4 plasmid was isolated and had a molecular weight of 19.1 kb. Ninety-five percent of cells retained the pIBF4 plasmid after 200 generations, demonstrating its high stability. PIBF4 was successfully transferred to *Bacillus thuringiensis* HD1CryB strain with a transfer frequency of 1×10^{-8} transconjugants per donor cell. The study of the recipient host range revealed that pIBF4 is specifically transferable to *Bacillus thuringiensis* strains with variable transfer frequencies depending on the recipient host strain.

Conclusion: Our results show that pIBF4 is a 19.1 kb highly stable plasmid transferable by conjugation to *Bacillus thuringiensis* strains with deferent transfer frequencies.

Introduction

The widespread use of antibiotics in medicine, animal feeding, and agriculture is the most important cause of bacterial resistance to antibiotics. Such resistance is particularly due to the horizontal transfer of antibiotic resistance genes between bacteria [1]. Conjugative transfer of plasmids encoding the synthesis of several products such as bacteriocins has been well studied by Rauch *et al.* [2]. The investigation of mechanisms and conditions allowing plasmid transfer and expression of plasmid-encoded genes in host cells have been reported [3,4,5]. *B. thuringiensis* is the most known insecticidal bacterium worldwide for the control of various plant pests. This bacterium produces many virulent factors such as chitinase [6,7] vegetative insecticidal proteins [8], and bacteriocins [9,10]. Bacteriocins are extracellularly released peptides or proteins affecting growth of closely related bacteria and several pathogenic species

[9]. Genetic determinants responsible for bacteriocin activity are usually grouped on one or two operons encoding structural proteins involved in its synthesis and secretion as well as immunity proteins protecting the producer from its own bacteriocin [11]. Bacteriocin genes could be located on plasmids such as bacteriocin 32 produced by *Enterococcus* strains [12] on chromosomes such as mesarcidin [13], or on conjugative transposons, as in the case of lacticin 481 [14]. *B. thuringiensis* strain BUPM4 is known for its ability to produce a bacteriocin, called Bacthuricin F4 (BF4), which could be potentially used for the control of harmful related *Bacillus* species in agricultural products [9]. The search for genes impaired in BF4 synthesis has been carried out in our laboratory via the insertional transposon mutagenesis approach, which is based on the capability of the mini-Tn10 to insert into different sites in *B. thuringiensis* chromosomes or plasmids [9]. A non-bacteriocin producing mutant

(MB1) was obtained by insertion mutagenesis, in which the mini-Tn10 transposon had targeted a plasmidic gene involved in BF4 synthesis (ORF1) (accession number FJ499396) [10]. In this study, we aimed to isolate and characterize the *B. thuringiensis* plasmid carrying the genes involved in BF4 synthesis from this transposant MB1. In fact, the mini-Tn10 spectinomycin resistance gene was used as an important selective marker necessary for the isolation of this plasmid. The conjugative transfer capabilities of this plasmid and its recipient host range were also examined in mating experiments using the insertional non-producer MB1 mutant as a donor strain.

Methodology

Bacterial strains and growth conditions

The bacterial strains used in this study and their relevant characteristics are listed in Table 1. *B. thuringiensis* strains BUPM19, BUPM103, *B. cereus* strain V1, and *B. subtilis* strain V26 are spontaneous streptomycin (Str) resistant mutants isolated through this study, and used as recipient strains in the mating experiments. *Escherichia coli* strain Top10, used for the isolation of the plasmid carrying BF4 coding genes, was transformed as described by Sambrook *et al.* [15]. A single *E. coli* colony was inoculated in 3 mL LB medium and grown overnight at 200 rpm at 37°C. Three milliliters of the preculture were transferred into 50 mL LB, and cells were grown until OD ˜0.4. Bacterial cells were transferred to a sterile ice-cold 50 mL polyprophylene tube and maintained on ice for 10 minutes. After centrifugation at 4,000 rpm for 10 minutes at 4°C, the pellet was resuspended by swirling in 10 mL of ice-cold CaCl2 solution (0.1M) and incubated on ice for 45 minutes. The pellet obtained after a second centrifugation at 4,000 rpm for 10 minutes at 4°C was resuspended in 2 mL of ice-cold 0.1 mM $CaCl_2$. Next, 200 µL of the obtained competent cells were subjected to a heat shock in the presence of the plasmid DNA to transfer (90 minutes at 42°C, then cells were moved rapidly onto ice for 2 minutes). Finally, 800 µL of LB medium were added and cells were incubated at 37°C for 45 minutes, and then plated onto LB selective agar medium.

PIC333 is a temperature-sensitive vector [16] used by Kamoun *et al.* [10] in the mini-Tn10 mutagenesis to yield the BF4 non-bacteriocin producing (MB1) strain used in this study. The mini-Tn10 transposon targeting the plasmid carrying genes involved in BF4 synthesis is a 2.2 kb element composed of two ends of Tn10, the spectinomycin resistance gene (Spc), and

the replication region of the plasmid pBR322, and lacks the gene encoding the transposase of Tn10 [16]. *B. thuringiensis, B. cereus*, and *B. subtilis* strains were grown at 30°C in LB medium supplemented with appropriate antibiotics. Transconjugants were cultured in LB medium supplied with two specific antibiotics, streptomycin (Str) and erythromycin (Erm) at final concentrations of, respectively, 100 and 50 µg/mL. Spectinomycin (Spc) was used at concentrations of 180 µg/mL for selection of *B. thuringiensis* transformants and 50 µg/mL for selection of *E. coli* Top10 transformants.

Plasmid DNA extraction and PCR amplification

Plasmid DNA was extracted using the alkaline lysis method [15]. This method is based on the fact that chromosomal DNA could be hydrolyzed on an alkaline environment more easily than the circular plasmid DNA could. After neutralization by the potassium acetate, a complex of denaturized protein, RNA with high molecular weight and re-natured DNA was formed, allowing for the easy extraction of plasmid DNA in the soluble fraction. More experimental details are described by Sambrook *et al.* [15]. Extracted plasmids were resolved in a 0.7% (w/v) horizontal agarose gel. To prove transfer of the plasmid carrying the BF4 coding genes, plasmid DNA extracts were subjected to PCR amplification using specific mini-Tn10 primers E1inv: 5'-GCATTAATGAATCGGCCAACG-3'; E3inv: 5'-GTGGGTAAACCGTGAATAT CG-3'and specific BF4 encoding region primers O1: 5'-CATGACTCACGACGCCAGT-3'; and O2: 5'-AGCCGTTAAACCCAGTGAAT-3'. PCR was performed using the following steps: one initial cycle of 94°C for 3 minutes, followed by 30 cycles of 45 seconds at 94°C, 45 seconds at 54°C, and 1 minute 30 seconds at 72°C. PCR products were analyzed by electrophoresis in horizontal 1% agarose gel.

Mating experiments

The mating experiments were carried out using the methods of Gammon *et al.* [17] with minor modifications. A single colony of a donor or recipient strain, from LB agar plates supplemented with the appropriate antibiotic, was used to inoculate 3 mL LB medium and grown for 6 hours. The donor strain was cultured in LB medium supplemented with spectinomycin (180 µg/mL) (Sigma-Aldrich, St. Louis, USA). Recipient strains were cultured in LB media with erythromycin (50 µg/mL) or streptomycin (100 µg/mL), depending on the strain used as

mentioned in Table 1. From each resulting culture, a fresh 3 mL broth (supplemented with appropriate antibiotics) was inoculated and incubated overnight at 30°C. These were then subcultured in 10 mL of fresh LB medium (without antibiotics) and re-incubated at 30°C under shaking conditions for a further 6 hours. Next, 200 µL of donor and recipient strains were mixed, and 200 µL of the obtained mixture were spotted onto LB agar plates. Following overnight incubation at 30°C, the bacterial colonies were scraped off the plates and inoculated in 3 mL LB broth. After 4 hours of incubation, serial dilutions were plated onto LB agar medium selective for donor cells, recipients, and transconjugants. Plates were incubated for 12 hours at 30°C. Transfer frequencies are expressed as the number of transconjugants per donor cells.

Plasmid curing experiment

The non-bacteriocin producer mutant (MB1) was subjected to plasmid curing procedures to study the stability of the plasmid encoding the BF4 synthesis. For this purpose, MB1 was cultured in 2 mL LB medium at 42°C and transferred daily into fresh LB media until 200 generations of *B. thuringiensis* were grown. Serial dilutions of cured strains with LB medium were made and plated onto LB agar. After an overnight incubation at 30°C, the colonies were replica plated onto LB agar and LB agar supplied with spectinomycin (180 µg/mL). The percentage of the colonies retaining their resistance to spectinomycin was calculated to determine the stability of the BF4 coding plasmid carrying the spectinomycin resistance gene.

Results

Isolation of the *B. thuringiensis* plasmid involved in BF4 synthesis

The total plasmid DNA from MB1 was extracted and used to transform *E. coli* strain Top10. A spectinomycin-resistant transformant named EpIBF was obtained. The latter allowed the easy extraction of the plasmid encoding BF4 synthesis named pIBF4::mini-Tn10. The presence of both the mini-Tn10 and the flanking BF4 coding region was checked by PCR amplification using the isolated plasmid as template (data not shown). The pIBF4 DNA restriction enzyme analysis using *Eco*RI and *Bam*HI allowed the establishment of the pIBF4::miniTn10 restriction map shown in Figure 1.

Study of the segregational stability of pIBF4

The non-bacteriocin producer MB1 mutant was subjected to a plasmid curing experiment. The study of the segregational stability of the plasmid pIBF4::mini-Tn10 was conducted at 42°C, without selection pressure. In this study, the fact that pIBF4::mini-Tn10 carries the spectinomycin resistance gene was exploited in order to determine its stability. It was concluded that 95% of cells retained their resistance to spectinomycin and thereby the pIBF4::mini-Tn10 plasmid after 200 generations, demonstrating its high stability.

Evidence of the conjugative transfer of the pIBF4 plasmid

To test whether pIBF4::mini-Tn10 was transferable by conjugation, mating experiments were performed using the non-bacteriocin producer MB1 mutant as a donor strain. The first recipient strain used for the mating experiments was the artificially plasmid-cured *B. thuringiensis* strain named HD1CryB, which harbored a chromosomal streptomycin selective marker. The transfer frequency was estimated to be 1×10^{-8} transconjugants per donor cell. The transconjugant plasmid DNA(s) analysis revealed the presence of pIBF4::mini-Tn10 transferred from MB1 donor strain to HD1CryB (Figure 2). These results were confirmed by positive PCR amplification of the mini-Tn10 and the BF4 coding region of plasmid DNA(s) from transconjugants and donor cells, but not from the recipient used as a negative control (data not shown). Such results demonstrate that pIBF4 was actually transferred from MB1 to HD1CryB.

Recipient host range and transfer frequencies

In order to investigate the host range of the plasmid pIBF4::mini-Tn10, its transferability to several *Bacillus* recipient strains was investigated. Streptomycin- and erythromycin-resistant strains of *B. thuringiensis* and other species were used as recipient strains (Table 1). Mating, strain phenotypes, and transfer frequencies are summarized in Table 2. Based on these results, it appears that the plasmid pIBF4::mini-Tn10 is specifically transferable to *B. thuringiensis* strains with variable transfer frequencies, depending on the recipient host strain. This result was confirmed by PCR amplification using specific primers corresponding to the mini-Tn10 and the BF4 coding region.

Figure 1. Restriction map of the plasmid pIBF4::miniTn10 carrying *B. thuringiensis* bacteriocin coding genes. pIBF4 and miniTn10 have sizes of 19.1 kb and 2.2 kb, respectively.
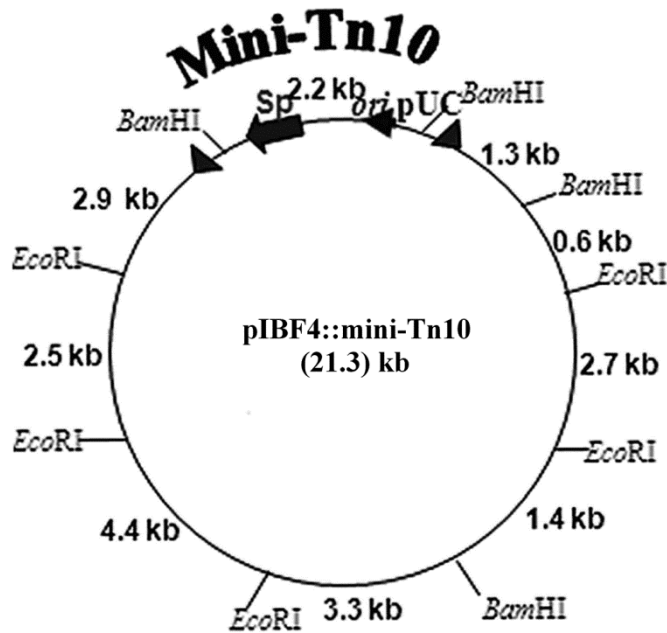
Figure 2. Electrophoresis of transconjugants DNA harboring pIBF4::mini-Tn10. Agarose gel electrophoresis demonstrating the conjugative transfer of pIBF4::mini-Tn10 from BUPM4 mutant MB1 to the cured strain HD1CryB. Lane 1: HD1CryB, recipient strain; lane 2: donor mutant MB1; lanes 3 and 4: transconjugants.
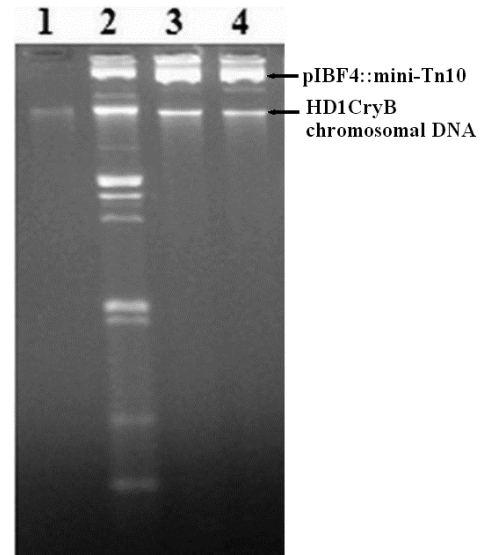

Table 1. Bacterial strains, plasmids, and relevant characteristics

Strains	Characteristics	References
B. thuringiensis (Bt)		
BUPM4	Wild-type bacteriocinogenic strain BF4 producer	[9]
MB1	Non-bacteriocinogenic derivative of BUPM4 (Spc^r)	[10]
HD1CryB	Plasmid-cured strain (Str^r)	[23]
BBP12	*Bt* subsp. *kurstaki* strain non-bacteriocin producing (Erm^r)	This study
BBP76	*Bt* subsp. *kurstaki* strain non-bacteriocin producing (Erm^r)	This study
BBP14	*Bt* subsp. *tenebrionis* strain non-bacteriocin producing (Erm^r)	This study
E. coli		
EpIBF	Transformant strain containing the pIBF4 plasmid	This study
Plasmid		
pIBF4	Plasmid carrying genes encoding BF4 synthesis from *B. thuringiensis*	This study

Table 2. Mating experiments and transfer frequencies. Values for conjugation frequency are means of two experiments.

Mating (donor × recipient)	Selection markers for transconjugants	Transfer frequency (transconjugants / donor cfu)
MB1 × HD1CryB	Spc^r Str^r	$1.55 \times 10^{-8} \pm 0.77 \times 10^{-8}$
MB1 × BBP12	Spc^r Erm^r	$1.56 \times 10^{-6} \pm 1.95 \times 10^{-6}$
MB1 × BBP76	Spc^r Erm^r	$2.86 \times 10^{-7} \pm 1.49 \times 10^{-7}$
MB1 × BBP14	Spc^r Erm^r	$1.19 \times 10^{-4} \pm 1.44 \times 10^{-4}$
MB1 × BUPM103	Spc^r Str^r	$< 1 \times 10^{-8}$
MB1 × BUPM19	Spc^r Str^r	$< 1 \times 10^{-8}$
MB1 × *Bacillus cereus* V1	Spc^r Str^r	$< 1 \times 10^{-8}$
MB1 × *Bacillus subtilis* V26	Spc^r Str^r	$< 1 \times 10^{-8}$

Discussion

In a previously published work, we demonstrated the plasmid location of the genes encoding for the bacteriocin BF4 in *B. thuringiensis* BUPM4 strain via analysis of the insertional mutant MB1 [10]. The phenotype of a non-bacteriocin producer of MB1 is due to the insertion of the mini-Tn10 into the ORF1 (accession number FJ499396), preventing the synthesis of BF4 bacteriocin.

B. thuringiensis harbors many plasmids with various molecular weights. In this work, we exploited the fact that the mini-Tn10 contains an *E. coli* replication origin and a spectinomycin resistance gene for the isolation and purification of the plasmid harboring the ORF [1] that is required for BF4 synthesis. The transformation of *E. coli* with the total MB1 DNA plasmid was somewhat difficult in terms of transformation efficacy. This explains why only one single *E. coli* transformant in the specinomycin selective medium was obtained. The pIBF4::miniTn10 plasmid had a size of 21.3 kb. Since the size of the mini-Tn10 was 2.2 kb, we could conclude that the bacteriogenic plasmid pIBF4 encoding for BUPM4 Bacthuricin F4 has a size of 19.1 kb. This plasmid is highly stable since it could be maintained when the strain was cultured at a stringent temperature of 42°C.

The conjugative transfer of the pIBF4 plasmid has been evidenced by mating experiments when it was successfully transferred to the cured *B. thuringiensis* strain HD1CryB and other strains. The transfer frequency was higher when using the *B. thuringiensis* subsp. *tenebrionis* strain BBP14 ($1.19 \times 10\text{-}4 \pm 1.44 \times 10^{-4}$) than the two *B. thuringiensis* subsp. *kurstaki* BBP12 and BBP76 strains. Their corresponding transfer frequencies were, respectively, ($1.56 \times 10^{-6} \pm 1.95 \times 10^{-6}$) and ($2.86 \times 10^{-7} \pm 1.49 \times 10^{-7}$). The transfer frequency differences between tested strains reflected the dependence of the conjugative transfer frequency on recipient strains or species, as reported by Broadbent and Kondo [18]. According to these authors, the frequency of the nisin-sucrose plasmid transfer from ATCC11454 strain to *Lactococcus lactis* subsp. *cermoris* was about 10 times higher than that to *L. lactis* subsp. *lactis*. Nevertheless, the failure to detect transconjugants in the case of using BUPM103, BUPM19, *B. cereus*, and *B. subtilis* as recipient strains could be due to the absence of such events or to its occurrence with transfer frequencies below the detection of the threshold of our experimental system. This is probably related to mating conditions in which the cell's environment would be suitable for a gene transfer event as it occurs in nature. The pIBF4

conjugative transfer raised the question of whether this plasmid was transmissible on its own – meaning it retains the functionality of the transfer apparatus – or mobilized by other possible high molecular weight plasmids that were not detected. For example, plasmid pXO2 could be mobilized by pXO14, a conjugative plasmid from *B. thuringiensis* [19]. Indeed, the DNA sequencing of pIBF4, which is in progress, will provide more data about gene content and its implication in the conjugative process, as well as in bacteriocin BF4 synthesis and production. In fact, several reports have described the association between the bacteriocin genes and those involved in plasmid transfer [20,21]. The 33.333 bp plasmid from *Lactobacillus gasseri* LA39 was described to carry genes for production of and immunity to the circular bacteriocin gassericin, as well as genes for replication, plasmid maintenance, and conjugative transfer [22]. More investigations are also needed to determine the entire machinery involved in BF4 synthesis and production. It is likely that additional screening would yield more genes to better understand the synthesis and the regulation mechanisms of BF4.

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Conflict of interests: No conflict of interests is declared.