



Original Article

TipB, a novel cell wall hydrolase, is required for efficient conjugative transfer of pXO16 from *Bacillus thuringiensis* sv. *israelensis*

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ABSTRACT

pXO16, a large plasmid from *Bacillus thuringiensis* serovar *israelensis*, exhibits unique features. Not only is pXO16 able to transfer at high frequencies within few minutes, but it is also able to transfer among virtually all members of the *Bacillus cereus* group. Among the proteins encoded in the *tip* transfer locus of pXO16, TipB displays an atypical organization compared to known conjugative cell wall hydrolases with the large central soluble lytic transglycosylase (SLT) domain missing from the protein. We constructed a *tipB* deletion mutant which led to significant reduction in transfer efficiencies in both liquid and filter mating. The initial transfer frequencies could be restored when complementing *tipB* in *trans* thus showing the TipB implication in pXO16 conjugative transfer. Additionally, truncated versions of TipB were expressed in *Escherichia coli* to assess the protein lytic activity. When applied exogenously, TipB-2TM, in which the two N-terminal TM domains were removed, yielded a decrease of ca. 40% in optical density of *B. thuringiensis* sv. *israelensis*, a lytic activity that could partially be explained by the C-terminal CHAP-like domain. These results confirm TipB conjugative hydrolase function and provide new insights into pXO16 unique conjugative apparatus.

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1. Introduction

Acquisition of new genes among bacteria not only happens via parental (vertical) transmission but also via horizontal gene transfer (HGT) with conjugation as one of the main mechanisms. HGT provides access to a wide pool of genes, conferring to bacteria the potential of exhibiting diverse metabolic properties and of surviving various conditions, including resisting to antibiotic drugs [1]. Conjugation involves a relaxosome processing the conjugative plasmid prior to its transfer and a Type IV Secretion System (T4SS) mediating the transfer to the recipient cell [2]. T4SSs have been widely studied in Gram-negative bacteria with the VirB/D4 system from *Agrobacterium tumefaciens* being one of the best characterized [3]. In this system, the multi-protein complex spans the cell envelope and mediates conjugative plasmid transfer. Insertion of the T4SS complex into the bacterial envelope is thought to be facilitated by conjugative peptidoglycan (PG) hydrolases locally degrading the cell wall [4]. In Gram-negative bacteria, conjugative hydrolases are

usually not essential for conjugative transfer while their absence significantly reduces or abolishes conjugation in Gram-positive bacteria [3,5–7].

pXO16 is a conjugative plasmid of the entomopathogen *Bacillus thuringiensis* sv. *israelensis*, a bacterium often used as biopesticide to control larvae of mosquitoes and blackflies, vectors of animal and human diseases. This 350-kb plasmid transfers very quickly (<5 min) and efficiently, with frequencies reaching about 100% in *B. thuringiensis* sv. *israelensis* matings [8,9]. Another peculiar feature of pXO16 is the formation of macroscopic cell aggregates when liquid cultures of donor and recipient cells are mixed for conjugative mating [10,11]. Aggregation phenotype has long been considered mandatory for transfer, thus restricting the number of potential recipients for pXO16 transfer [8]. However, when the 25-kb “aggregation” locus was deleted from pXO16 sequence, the transfer could still occur despite the absence of aggregate formation [12]. Furthermore, recent work on pXO16 transfer focused on solid mating instead of liquid mating by passing donor

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and recipient cells through a filter or spotting on plates. When using these artificially compacted cell partners, the plasmid host spectrum was extended to virtually all members of the *Bacillus cereus* group [13].

Even though phenotypic characterization of pXO16 conjugation has been widely addressed, less information was available on the genetics of its transfer. Recently however, its transfer apparatus was identified as a novel Type IV Secretion System (T4SS) encoded by the 24-kb *tip* (transfer of *israelensis*) region [12]. Within this region, one candidate gene, *tipB*, stands out for encoding a potential conjugative cell wall hydrolase. Preliminary bioinformatics analyses highlighted distant similarities between TipB sequence and domains of NlpC/P60 cell-wall peptidase family proteins, also found in the conjugative cell-wall hydrolases TcpG and CwIT from the *Clostridium perfringens* pCW3 and *Bacillus subtilis* ICEBs1 elements, respectively [6,12,14]. In this paper, we provide experimental information confirming the role of TipB as *bona fide* conjugative cell wall hydrolase and determining its role and importance in pXO16 conjugative transfer.

2. Materials and methods

2.1. Bacterial strains and growth conditions

All bacterial strains and plasmids used in this study are reported in Table 1. Bacteria were grown in Lysogeny Broth (LB) medium containing NaCl (5 g.L⁻¹), yeast extract (5 g.L⁻¹) and Tryptone (10 g.L⁻¹). LB was solidified with 1.4% (w/v) agar for agar plates. *Bacillus* strains were grown at 30 °C and *Escherichia coli* strains were grown at 37 °C (unless mentioned otherwise). When appropriate, antibiotics (Sigma) were added at the following

concentrations (μg.mL⁻¹): Sm, streptomycin (100); Nd, nalidixic acid (15); Kan, kanamycin (50); Amp, ampicillin (100); Ery, erythromycin (10).

2.2. General molecular techniques

PCR amplifications were performed using the Q5 High-Fidelity DNA polymerase (New England Biolabs™, Leiden, The Netherlands). Restriction enzymes were obtained from New England Biolabs™ and the T4 DNA Ligase from Promega (Leiden, The Netherlands). PCR and restriction products were purified and recovered by GeneElute™ PCR Clean-Up Kit (Sigma–Aldrich™, Overijse, Belgium). Constructions with pMAD vector were transformed into chemically competent *E. coli* NEB5α cells (New England Biolabs™) while these using the pET-30a vector were transformed into chemically competent *E. coli* 10β (New England Biolabs™). Transformants were checked by PCR and plasmids DNA were extracted using the GeneElute™ Plasmid MiniPrep Kit (Sigma–Aldrich™) to verify the constructions by sequencing (Macrogen, Amsterdam-Zuidoost, The Netherlands).

2.3. Deletion of *tipB* and complementation

2.3.1. Construction of *tipB* knockout mutant

A 1-kb fragment upstream and a 1-kb fragment downstream of the *tipB* gene were amplified by PCR from pXO16 using the *tipB*-up-F/R and *tipB*-down-F/R primers (Table 2), and the kanamycin resistance cassette (*kan*) was amplified from pDG783 using the *tipB*-Kan-F/R primers. PCR products were cloned into the pMAD thermo-sensitive shuttle vector via the HiFi DNA Assembly method (New England Biolabs™). The resulting vector

Table 1
Bacterial strains and plasmids used in this work.

Strains/Plasmids	Main features	Sources
<i>E. coli</i> strains		
<i>E. coli</i> NEB5α	Chemically competent	New England Biolabs™
<i>E. coli</i> 10β	Chemically competent	New England Biolabs™
<i>dam</i> / <i>dcm</i> ⁻ <i>E. coli</i> C2925	K12-derivative deficient for adenine and cytosine methyl-transferases, chemically competent	New England Biolabs™
<i>E. coli</i> BL21(DE3)	Suitable for high level protein expression using a T7 RNA polymerase-IPTG induction system	Novagen
<i>E. coli</i> BL21(DE3) (pET-30::TipB)	<i>E. coli</i> BL21(DE3)-derivative containing pET-30::TipB (Kan ^R)	This study
<i>E. coli</i> BL21(DE3) (pET-30::TipB-2TM)	<i>E. coli</i> BL21(DE3)-derivative containing pET-30::TipB-2TM (Kan ^R)	This study
<i>E. coli</i> BL21(DE3) (pET-30::CTR _{TipB})	<i>E. coli</i> BL21(DE3)-derivative containing pET-30::CTR _{TipB} (Kan ^R)	This study
<i>E. coli</i> BL21(DE3) (pET-30::CHAP _{TipB})	<i>E. coli</i> BL21(DE3)-derivative containing pET-30::CHAP _{TipB} (Kan ^R)	This study
<i>B. thuringiensis</i> sv. <i>israelensis</i> strains		
GSX002	Plasmid-less derivative of strain GBJ002 (Nd ^R)	[15]
GSX002(pXO16)	GSX002-derivative containing wild-type pXO16	This study
GBJ001	Sm ^R spontaneous mutant of reference strain 4Q7 cured of its plasmids (except pBtic235)	[11]
GSX002(pXO16, pMAD::KOTipBkan)	GSX002-derivative containing wild-type pXO16 and pMAD::KOTipBkan (Kan ^R , Ery ^R)	This study
GSX002(pXO16Δ <i>tipB</i>)	GSX002-derivative containing pXO16Δ <i>tipB</i> (Kan ^R)	This study
GSX002(pXO16Δ <i>tipB</i> , pHT304-18Z:: <i>tipB</i> _compl)	GSX002-derivative containing pXO16Δ <i>tipB</i> and pHT304-18Z:: <i>tipB</i> _comp (Kan ^R , Ery ^R)	This study
Plasmids		
pET-30a	<i>E. coli</i> recombinant expression vector, containing 6xHis-tag coding sequence at 5'/3' of cloning region; Kan ^R	Novagen
pET-30::TipB	Derivative of pET-30, containing TipB coding sequence	This study
pET-30::TipB-2TM	Derivative of pET-30, containing TipB-2TM coding sequence	This study
pET-30::CTR _{TipB}	Derivative of pET-30, containing CTR _{TipB} coding sequence	This study
pET-30::CHAP _{TipB}	Derivative of pET-30, containing CHAP _{TipB} coding sequence	This study
pDG783	Plasmid carrying a kanamycin resistance cassette	[16]
pMAD	Gram+/Gram- shuttle vector containing a thermo-sensitive Gram+ replicon; Ery ^R , Amp ^R	[17]
pMAD::KOTipBkan	Derivative of pMAD containing antibiotic resistance cassette with upstream and downstream regions of <i>tipB</i> , Kan ^R	This study
pXO16Δ <i>tipB</i>	Derivative of pXO16, carrying the replacement of <i>tipB</i> gene by Kan ^R cassette	This study
pHT304-18Z	Shuttle vector, Ery ^R	[18]
pHT304-18Z:: <i>tipB</i> _compl	Derivative of pHT304-18Z containing the <i>tipB</i> gene with upstream and downstream regions	This study

Table 2
Primer pairs used in this work.

Target	Primer name	Sequence (5' → 3')	Reference
tipB-up	tipB-up-F	agatctatcgatgcacgaagggtattccaactatgaag	This work
	tipB-up-R	gtacatccgctccttttagtctctagattg	
tipB-down	tipB-down-F	ctatcacctcctgataaaacgtaacgaagtataaaac	This work
	tipB-down-R	cggatccatgatgacgtcgacttttgccttttaacgagttg	
KanR cassette	tipB-Kan-F	actaaaaggagcggtgtagcttctcagaaaag	This work
	tipB-Kan-R	gttttatcaggagggtataggaagattatac	
tipB_compl	tipB-comp-F	gagctgcagaaggagggtaaaccatttgattgc	This work
	tipB-comp-R	ctcggatccataaagatgacctccttctacttctaa	
tipB	tipB-F	tatccatggctattgctctcgtggtgtttta	This work
	tipB-R	tatggatccttattgattacattaccatacgaatt	
tipB-2TM	tipB-2TM-F	tatccatggctgcttctgtacaaactagac	This work
	tipB-2TM-R	tatggatccttattgattacattaccatacgaatt	
CTR domain	tipB-2TM-F	tatccatggctgcttctgtacaaactagac	This work
	CTR-tipB-R	ttagatccctatcatgccctcatatagtc	
CHAP domain	CHAP-tipB-F	tatccatggctttaaaccgctttactaacagg	This work
	CHAP-tipB-R	tatggatccatccgaaacatactccc	

was first transformed into *dam⁻/dcm⁻* *E. coli* C2925 cells and then electroporated into GSX002(pXO16) cells as previously described by Mahillon and Lereclus [19]. pXO16Δ*tipB* derivatives were obtained from Kan^REry^R transformants by successive cultures at non-permissive temperature and selection of Kan^REry^S colonies. Since pMAD also contains a β-galactosidase cassette, pXO16Δ*tipB* derivatives were also selected based on blue-white screening from growth on LB with X-gal. Replacement of the *tipB* gene by the *kan* gene was verified by PCR.

2.3.2. Complementation of tipB knockout

The complementation of *tipB* knockout was performed as previously described by Makart et al. [12]. Briefly, a fragment covering *tipB* gene with its upstream and downstream intergenic regions was amplified by PCR from pXO16 sequence using the tipB-comp-F/R primers and ligated into the *Pst*I/*Bam*HI sites of pHT304-18Z shuttle vector. The resulting vector pHT304-18Z::tipB_compl placed *tipB* under the control of the constitutive promoter. After transformation into *dam⁻/dcm⁻* *E. coli* C2925, the construction was electroporated into GSX002(pXO16Δ*tipB*) as described by Mahillon and Lereclus [19].

2.4. Mating assays

Plasmid transfers in liquid media were performed as previously described by Timmerly et al. [20] with the modifications indicated in Hinnekens et al. [13] and plasmid transfers in filter matings were performed as described in Hinnekens et al. [13]. Briefly, donor and recipient cells were streaked separately on selective LB plates and incubated overnight at 30 °C. Separate pre-cultures from initial plates were grown in 10 ml liquid medium and incubated overnight at 30 °C without shaking. For liquid mating, equal amounts of mating partner cells from pre-cultures were mixed in 10 ml of LB medium. For filter mating, equal amounts of conjugative partner cells were successively passed through a 0.20 μm filter, which was then collected and placed on non-selective LB plates. In both liquid and filter conditions, mating experiments were performed during a 4 h contact and were repeated at least three times, independently. Each mating experiment included a plating control of donor and recipient cells on selective media to assess the presence of potential spontaneous mutants. The transfer frequencies were calculated as the ratio of transconjugants per donor cells (T/D) at the end of the conjugation time.

2.5. Construction of TipB and variants for protein expression

To further express TipB and its variants, the *tipB* gene and truncated versions were cloned into the pET-30a vector (Fig. 1). The fragments were amplified by PCR using the appropriate primer pair (Table 2) and each fragment was ligated into the *Nco*I/*Bam*HI sites of pET30a to generate pET-30::TipB, pET-30::TipB-2TM, pET-30::CTR_{TipB} and pET-30::CHAP_{TipB}. Finally, the constructions were transformed into the expression strain *E. coli* BL21(DE3).

2.6. Protein expression and purification

LB broth (Kan) was inoculated with BL21(DE3) containing the different constructions and incubated O/N with an agitation of 180 rpm. O/N cultures were then diluted 1:10 in fresh LB broth (Kan) containing 2% (w/v) of glucose and further incubated until the optical density (OD, 600 nm) reached 0.5–0.8. Protein expression was then induced using 0.5 mM of Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma–Aldrich™) and the cultures were incubated at 22 °C for 24 h. Following expression, cells were collected by centrifugation (21,000g for 15 min at 4 °C) and stored at –20 °C. To lyse the cells, the pellet was thawed on ice for 30 min and resuspended in lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole pH 8) supplemented with a protease inhibitor cocktail (1x, SIGMAFAT™ Protease Inhibitor Cocktail Tablet, EDTA-Free, Sigma), 0.5 mg/ml lysozyme (Sigma–Aldrich™) and 0.1% of Triton-X100 (Sigma–Aldrich™). After incubation for 30 min at 10 °C, three Units/ml of Benzonase Nuclease (Sigma–Aldrich™) were added to reduce viscosity (15 min incubation on ice). The lysate was centrifuged (21,000g for 30 min at 4 °C) and the supernatant (i.e. soluble proteins) filtrated on 0.45 μm (VWR). Recombinant proteins were purified on Ni-NTA affinity columns (Qiagen) according to the manufacturer's recommendations. Proteins were concentrated and their buffer exchanged for 25 mM Tris–HCl pH 8 using Amicon Ultra centrifugal filter unit (Cut-off 10 kDa, Merck). Purified proteins were loaded on an SDS-page gel to assess purity. Their concentration was assessed via a Bradford assay [21].

2.7. Activity of TipB variants against *B. thuringiensis* sv. israelensis

The hydrolase activity of TipB variants was assessed via a turbidity reduction assay, as described by Bantwal et al. [14] and by

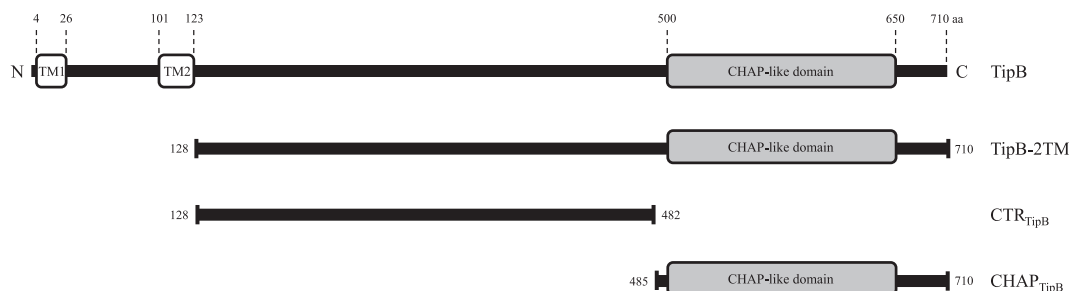


Fig. 1. Structure of TipB and truncated variants. The predicted domains were depicted based on bioinformatics analyses. The CHAP-like domain was delimited based on structural homologies retrieved from HHpred analyses. Each truncated variant is represented with its corresponding amino acid (aa) location. TM: transmembrane domain; CHAP, C-terminal cysteine, histidine-dependent amidohydrolases/peptidase. Numbers refer to the aa position.

DeWitt and Grossman [6], with some modifications. Briefly, a 4 h culture ($OD_{600} \sim 0.8\text{--}0.9$) of *B. thuringiensis* sv. *israelensis* GBJ001 was centrifuged (10,000g for 5 min) and resuspended in a Tris Buffer (50 mM Tris–HCl pH 8) to reach an OD_{595} between 0.8 and 1. Bacteria and purified proteins at various concentrations were mixed in a 96-well plate which was incubated for 1 h at 30 °C in a Multiskan™ FC Microplate spectrophotometer (Thermo Fisher Scientific, Waltham, USA) where the OD_{595} was monitored. Results were expressed as inhibition percentage after subtracting the reduction due to the protein buffer. Experiments were performed in triplicate.

2.8. Bioinformatics

The bioinformatics analyses were done using InterPro [22], HHpred [23], Phyre2 [24] and I-Tasser [25] software for domain predictions and TMHMM server v. 2.0 for transmembrane domain prediction. pXO16 sequence from *B. thuringiensis* HD789 can be found using the accession number CP003764.1.

3. Results

3.1. TipB as a cell wall-hydrolase candidate

Conjugative PG hydrolases from Gram-positive conjugation systems display a common organization with an N-terminal transmembrane (TM) domain, a central soluble lytic transglycosylase (SLT) domain and a C-terminal cysteine, histidine-dependent amidohydrolases/peptidases (CHAP) domain [26]. However, compared to other conjugative PG hydrolases, the organization of TipB appears to be quite unique (Fig. 1). A first interesting feature is TipB size of 711 amino acids (aa). Indeed, even though large conjugative PG hydrolases have been identified previously (e.g. PrgK from pCF10 871 aa [7]), the usual size ranges from 200 aa (e.g. VirB1 239 aa [27]) to 400 aa (e.g. TraG from pIP501 369 aa [28]). Beside TipB size, a CHAP-like region, characterized by an NlpC/P60 endopeptidase domain, was highlighted in the C-terminal region of the protein (residues 496–671 according to InterPro predictions) and two TM domains were identified by TMHMM predictions at the N-terminal part (residues 4–26 and 101–123). TipB protein sequence was also submitted to structure prediction (i.e. HHpred, Phyre2 and I-Tasser) that confirmed a CHAP-like domain in the C-terminal region (aa ~500–650) displaying structural similarities with other PG hydrolases and endopeptidases (data not shown). Furthermore, the overall predicted topology of TipB (based on TMHMM predictions) is reminiscent of that of other conjugative PG hydrolases, with the C-terminal part of the protein, including the CHAP-like domain, facing towards the extracellular medium. Although a CHAP-like region is present in C-ter and two

TM domains were identified at the N-terminal part, no SLT domain seems to be present in the large central part of the protein. The large central part of TipB was submitted to structure predictions, but no homologies were retrieved. This could either suggests that *tipB* encodes an atypical SLT domain that has not been identified yet or that no SLT-like domain is encoded, reinforcing the uniqueness of TipB compared to other described conjugative PG hydrolases.

3.2. tipB is required for efficient conjugative transfer of pXO16

As mentioned hereinabove, bioinformatics analyses highlighted one putative conjugative PG hydrolase, TipB, encoded in the *tip* region. To further confirm whether *tipB* was involved in pXO16 transfer mechanism, a *tipB* derivative mutant (pXO16Δ*tipB*) was obtained through double homologous recombination, selecting for the kanamycin phenotype provided by the resistance cassette used for the gene replacement. The genotype of the double recombinant mutant was verified by multiple PCRs. The conjugative transfer capacities of the derivative plasmid were then assessed in liquid mating when using the derivative strain GSX002(pXO16Δ*tipB*) as donor. As shown in Fig. 2, the derivative mutant showed a reduction of pXO16Δ*tipB* transfer of almost 200 times compared to that of wildtype pXO16. However, if evaluated in filter mating, the conjugative transfer capacities of pXO16 were less dramatically affected by the mutation (Fig. 2). Indeed, the transfer efficiency of pXO16Δ*tipB* was only reduced by 5 times compared to that of wildtype pXO16.

The *tipB* derivative was then complemented *in trans* with the shuttle vector pHT304-18Z::*tipB*_compl containing the wildtype *tipB* gene along with its upstream and downstream intergenic regions. When used as donor in liquid matings, the complementation strain GSX002(pXO16Δ*tipB*, pHT304-18Z::*tipB*_compl) reached transfer frequencies of 1.5×10^{-1} T/D, demonstrating a significant rise compared to pXO16Δ*tipB* transfer and a return to levels close to that of wildtype pXO16 (Fig. 2). When the complementation strain was used as donor in filter matings, transfer frequencies of 1.6 T/D were reached, demonstrating a complete recovery of wildtype levels (Fig. 2). These results indicate that the cell wall hydrolase candidate TipB is involved in pXO16 conjugation and is required for reaching optimal transfer frequencies.

3.3. TipB has a cell wall hydrolase activity

To demonstrate the lytic effect of TipB, we planned on assaying lytic activity of exogenously added purified TipB onto *B. thuringiensis* cells. For purification purpose, we first attempted to express the full-length TipB protein in the heterologous *E. coli* host. However, the OD monitoring of the *E. coli* cultures expressing TipB showed that the induction of the full-length protein significantly

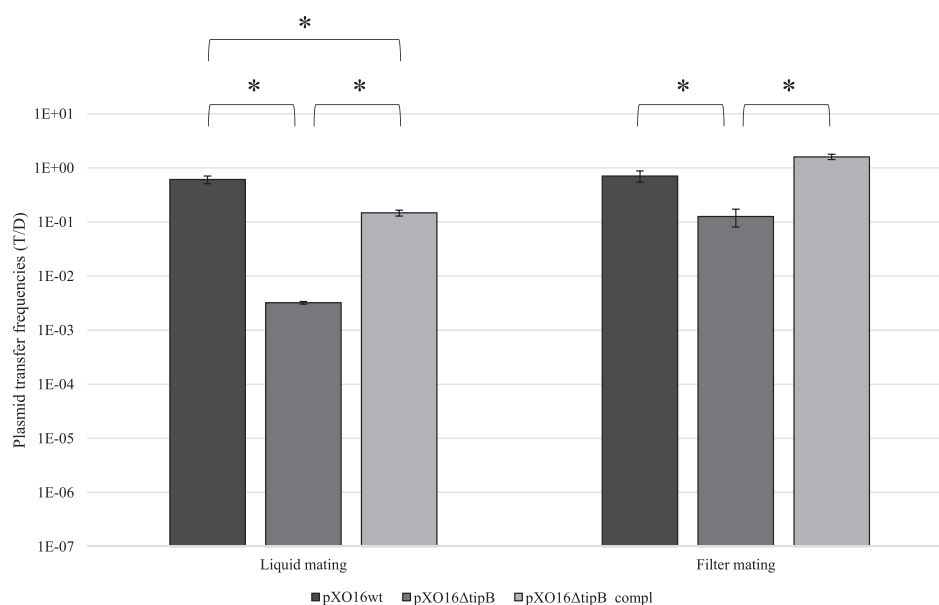


Fig. 2. Conjugative transfer frequencies of pXO16wt, pXO16ΔtipB and pXO16ΔtipB_compl (the complemented pXO16ΔtipB) in liquid and filter matings. Transfer frequencies are expressed as transconjugants per donor cells (T/D). Each frequency corresponds to the mean $\pm$ SEM out of three replicates. A star indicates that the differences between frequencies (under the same bracket) were significant, following an unpaired Student's t-test ($p < .05$). Otherwise, the differences were not significant.

slowed down the bacterial growth compared to the uninduced control (data not shown). We hypothesized that the presence of the two TM domains may lead to the insertion of the protein into *E. coli* internal membrane and lead to cell toxicity hampering TipB production and subsequent purification. The two TM domains of TipB were therefore removed and the truncated version, TipB-2TM (Fig. 1), was expressed in, and purified from *E. coli*.

Addition of purified TipB-2TM at a final concentration of $\sim 3 \mu\text{M}$ (i.e. 200 $\mu\text{g/ml}$) to exponentially growing *B. thuringiensis* GBJ001 cultures resulted in a 40% reduction of OD compared to control samples after one hour of incubation (Fig. 3). Similarly, the CHAP-like domain alone (CHAP_{TipB}) was also expressed, purified and its lytic activity assessed using equimolar concentrations compared to TipB-2TM. When a 3 μM concentration (i.e. 100 $\mu\text{g/ml}$) was tested,

CHAP_{TipB} was only able to achieve $\sim 20\%$ of reduction in OD which represents half of the lytic activity observed for TipB-2TM. The differences in lytic activity between TipB-2TM and CHAP_{TipB} could indicate that the large central part of TipB may hold a second lytic domain or be involved in the activity of the CHAP-like domain by a stabilizing or regulating effect. Unfortunately, purification of the central part of TipB (CTR_{TipB}) has remained so far unsuccessful due to its insolubility, preventing from further investigation on its potential role in TipB activity.

4. Discussion

Since its first description in 1987 [29], pXO16 conjugation has been widely studied phenotypically. However, little information on

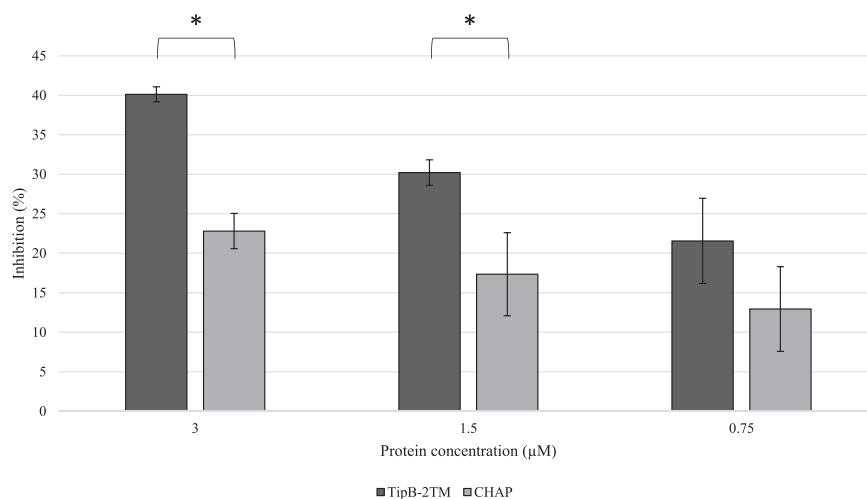


Fig. 3. Bacteriolytic activity of TipB-2TM and CHAP_{TipB} on *B. thuringiensis* GBJ001. The reduction in OD induced by TipB-2TM and CHAP_{TipB} on suspensions of GBJ001 was monitored during 1 h at 30 °C. Equimolar concentrations of each protein were tested. The experiment was done in triplicate. A star indicates that the differences between inhibition percentages obtained for the same concentration (under the same bracket) were significant, following an unpaired Student's t-test ($p < .05$). Otherwise, the differences were not significant.

the genetics underlying pXO16 transfer was available, until the recent description of its transfer apparatus encoded in the *tip* locus [12]. This transfer apparatus was identified as a novel T4SS and is therefore expected to contain the key elements of T4SS, such as ATPases, a coupling protein, channel proteins and a conjugative PG hydrolase [30]. Amongst the 16 *tip* genes, one candidate gene, *tipB*, was identified as pXO16 putative conjugative hydrolase. In the present paper, we provide experimental data supporting the role of TipB as the cell wall hydrolase in pXO16 conjugation apparatus.

Detailed bioinformatic investigations revealed a unique organization for TipB as a conjugative cell wall hydrolase. In conjugative systems, PG hydrolases are commonly organized with an N-terminal TM domain, a central SLT domain and a C-terminal CHAP domain, the latter two being responsible for the enzymatic activity. Despite the lack of homology of TipB with known conjugative PG hydrolases, a conserved NlpC/P60 endopeptidase domain, reminiscent of a CHAP-like region, was identified in the C-terminal part of TipB, suggesting its hydrolase function. Additionally, two N-terminal TM domains were highlighted but no SLT domain was identified.

To assess the importance of *tipB* in pXO16 conjugative transfer, we built a *tipB* deletion mutant (pXO16Δ*tipB*) which decreased but not eliminated pXO16 transfer capacities. The reduction of transfer efficiency was more striking when using liquid mating compared to filter mating. This is in line with previous results depicting enhanced transfer efficiency when using aggregation-like mating [13]. The results obtained for TipB are consistent with those obtained for TcpG, the conjugative PG hydrolase of pCW3 from *C. perfringens*, whose deletion induced a 1000-fold reduction in conjugative transfer [14]. Similarly, studies of well-known Gram-negative conjugative systems (e.g. pTi and R1) showed a 10 to 100-fold transfer reduction but no transfer disruption when the conjugative cell wall hydrolase coding genes were deleted [3,5]. On the contrary, deletion of conjugative PG hydrolase coding genes in other Gram-positive conjugative systems such as pIP501, pCF10 and ICEBs1 led to the complete disruption of conjugative transfer [6,28,31]. DeWitt and Grossman [6] suggested that conjugative hydrolases may play a greater role in Gram-positive systems than in Gram-negative systems because of the thicker PG layer. Therefore, Gram-positive conjugative hydrolases would require higher hydrolytic capacities. This would be consistent with the presence of multiple hydrolytic domains found in hydrolases associated with Gram-positive conjugative elements (e.g. both CHAP and SLT domains from TraG (pIP501) display a PG-degrading activity) [28]. On the other hand, a redundancy of PG hydrolases, encoded on the bacterial host chromosome or on other mobile genetic elements, could (partly) compensate the loss of one hydrolase [4]. Previous works highlighted interaction of lytic transglycosylases from different secretion systems that can substitute each other [4,32,33]. Low specificity and genetic redundancy could then explain the sometimes-weak impact of a PG hydrolase gene disruption [34]. The analysis of pXO16 sequence outside the *tip* locus revealed that at least three CDS encode potential hydrolases that might substitute for TipB when disrupted. One of these CDS encode a putative peptidase M15 (accession number AND27619.1), while the other two are putative N-acetylmuramoyl-L-alanine amidases (accession numbers AND27673.1 and AND27674.1).

To further prove TipB hydrolase function, we recombinantly expressed truncated versions of the protein and assessed their lytic activity on *B. thuringiensis* sv. *israelensis*. A first version of the protein, lacking the N-terminal TM domains, exhibited a significant hydrolysing activity on bacterial suspensions. The recombinant protein containing the CHAP-like domain only revealed itself responsible for around half of the lytic activity. This may imply

that another so far unknown domain could hold some hydrolytic activity. Indeed, SLT domains are known for holding hydrolytic activity in other conjugative PG hydrolases, especially in Gram-positive conjugation systems [28]. However, the CHAP-like domain might also be less stable or less active on its own than within the whole protein, requiring for example the large uncharacterized central region of TipB. As we were unable to test this central region of TipB, further work should focus on recombinantly expressing this central region to evaluate its potential implication in TipB lytic activity.

Overall, this study brought to light the cell wall hydrolase function of TipB. The importance of *tipB* for pXO16 conjugative transfer was first highlighted by knockout and complementation approaches. The activity of the recombinant protein was then evaluated, and lysis of bacterial suspensions was demonstrated. These results confirm the previous hypothesis suggesting TipB as the conjugative cell wall hydrolase of pXO16 [12] and participate in providing new information about this unique conjugative apparatus.

Declaration of competing interest

The authors declare no conflict of interests.

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