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**Insights into key steps of DNA transfer and the
host spectrum of the large conjugative plasmid
pXO16 from *Bacillus thuringiensis* sv. *israelensis***

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Not all those who wander are lost.

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Abstract

Horizontal gene transfer (HGT) plays a crucial role in shaping bacterial populations and communities. Among others, conjugation is a key mechanism of HGT and participates in the transfer of genetic material, mainly plasmids, between mating partners that are in direct contact with one another. As plasmids carry important functions among the species of the *Bacillus cereus* group, their horizontal displacements among the group members are scrutinised. One of the most studied species of the *B. cereus* group is *Bacillus thuringiensis*, well known for its entomocidal toxins and used worldwide as a biopesticide, notably the serovar *israelensis*, mainly toxic against mosquito larvae. *B. thuringiensis* sv. *israelensis* features a large pool of plasmids, including the unique conjugative plasmid pXO16. pXO16 can transfer itself at extremely high frequencies and can efficiently trigger the transfer of virtually any co-resident plasmid or host chromosomal locus. At the molecular level, the mechanism underlying pXO16 conjugation remains rather poorly described. This work therefore aimed at providing new understanding about pXO16 transfer, both regarding its molecular bases and the extent of its transferability and its influence across the *B. cereus* group.

This work first focuses on elucidating key steps and major components of the conjugative apparatus of pXO16. Conjugation implies the action of two main machineries, namely a relaxosome and a transferosome. The transferosome of pXO16 is thought to work as a T4SS (Type IV Secretion System), with some specificities. Here, the importance and activity of key T4SS components are investigated, primarily the conjugative cell wall hydrolase, and the uniqueness of pXO16 relaxosome is reported for the first time. The second part of this work investigates the transfer of pXO16 among the *B. cereus* group to assess the potential impact of its highly efficient mobilisation properties. While a wide host spectrum is uncovered, the natural distribution of pXO16 is restricted to *B. thuringiensis* sv. *israelensis*. Evaluating the impact of the plasmid on its *B. cereus* hosts thus points towards an intimate relationship between pXO16 and its natural *israelensis* host, opening up new fields of investigation.

Preamble

This PhD thesis was supported by the FNRS (National Foundation for Scientific Research) and was conducted in the Food and Environmental Microbiology laboratory (MIAE) within the Earth and Life Institute (ELI) at the Université Catholique de Louvain (UCLouvain).

The present work investigates the conjugative properties of the large plasmid pXO16 from *B. thuringiensis* sv. *israelensis*, with a dedicated focus on major elements of the conjugative machinery and on the transferability of the plasmid throughout the *B. cereus* group. *B. cereus* group species are characterised by large numbers of plasmids, including toxin-encoding plasmids. These plasmids are subjected to horizontal dissemination, mostly through conjugation. The large plasmid pXO16 exhibits highly efficient conjugation properties and presents a unique transfer apparatus. To better grasp the extend of pXO16 conjugation, it is necessary to decipher the molecular bases underlying its transfer, as well as to evaluate the transmission of the plasmid among *B. cereus* species and its impact on its hosts.

The first part of this manuscript gives an overview of the *B. cereus* group and the taxonomic controversy surrounding its description (Chapter 1). The introductory section also presents the overall mechanism of conjugation (Chapter 2) and the conjugative plasmid pXO16 (Chapter 3). Chapter 4 reviews the current knowledge on conjugation within the *B. cereus* group, including data gathered during this thesis, and forms an independent manuscript. The result part is divided into two main sections, comprising three published manuscripts and one monograph. Chapter 6 focuses on key elements of the transfer apparatus of pXO16, especially its relaxosome and its peptidoglycan hydrolase. On the other hand, the range of potential hosts of pXO16 and the impact of the plasmid on these hosts are evaluated in Chapter 7. The general discussion, conclusion and perspectives of this work are gathered in a third part (Chapters 8 and 9). At last, the appendices present a collaborative work on *Bacillus cytotoxicus* (Appendix A) and a list of scientific communications (Appendix B).

Included manuscripts

Published papers

- Hinnekens, P., Koné, K. M., Fayad, N., Leprince, A., & Mahillon, J. (2019). pXO16, the large conjugative plasmid from *Bacillus thuringiensis* serovar *israelensis* displays an extended host spectrum. *Plasmid*, 102, 46-50.
- Hinnekens, P., Leprince, A., & Mahillon, J. (2021). TipB, a novel cell wall hydrolase, is required for efficient conjugative transfer of pXO16 from *Bacillus thuringiensis* sv. *israelensis*. *Research in Microbiology*, 172(6), 103866.
- Koné, K.M., Hinnekens, P., Jovanovic, J., Rajkovic, A., & Mahillon, J. (2021). New insights into the potential cytotoxic role of *Bacillus cytotoxicus* cytotoxin K-1. *Toxins*, 13(10), 698.
- Hinnekens, P., & Mahillon, J. (2022). Conjugation-mediated transfer of pXO16, a large plasmid from *Bacillus thuringiensis* sv. *israelensis*, across the *Bacillus cereus* group and its impact on host phenotype. *Plasmid*, 122, 102639.
- Hinnekens, P., Fayad, N., Gillis, A., & Mahillon, J. (2022). Conjugation across *Bacillus cereus* and kin: a review. *Frontiers in Microbiology*, *In press*.

Monograph

- Hinnekens, P., Jaumain, R., Gillis, A., & Mahillon J. Investigating candidate elements for the relaxosome of pXO16, the conjugative plasmid of *Bacillus thuringiensis* sv. *israelensis*. Monograph.

List of abbreviations

aa	amino acid
AAD	All-Alpha Domain
AFM	Atomic Force Microscopy
<i>agr</i>	aggregation region
ANI	Average Nucleotide Identity
BAM	Bacteriology Analytical Manual
<i>Bti</i>	<i>Bacillus thuringiensis</i> sv. <i>israelensis</i>
CC	Coiled-Coil
CDS	Coding DNA Sequence
CHAP	Cysteine, Histidine-dependent Aminohydrolases/ Peptidases
CP	Coupling Protein
Cry	Delta-endotoxin/Crystal protein
Cyt	Cytotoxin
CytK	Cytotoxin K
dDDH	digital DNA-DNA Hybridization
dsDNA	double-stranded DNA
ssDNA	single-stranded DNA
Dtr	DNA-transfer replication
EF	Oedema Factor
Hbl	Hemolysin BL
HGT	Horizontal Gene Transfer
HUH	Histidine-hydrophobic residue-Histidine
ICE	Integrative and Conjugative Element
IHF	Integrated Host Factor
I-layer	Inner layer
IM	Inner Membrane
IMC	Inner Membrane Complex
kb	kilobase pairs
KO	KnockOut
LF	Lethal Factor
MGE	Mobile Genetic Element

MLS	Macrolide, Lincosamide and Streptogramin
MPF	Mating Pair Formation
NBD	Nucleotide-Binding Domain
Nhe	Nonhemolytic enterotoxin
O-layer	Outer layer
OM	Outer Membrane
OMCC	Outer Membrane Core Complex
<i>oriT</i>	origin of transfer
PA	Protective Antigen
PG	Peptidoglycan
RCR	Rolling-Circle Replication
RE	Restriction Endonucleases
RM	Restriction-Modification
SIP	Secreted Insecticidal Protein
S-layer	Surface layer
SLT	Soluble Lytic Transglycosylase
<i>s.l.</i>	<i>sensu lato</i>
<i>s.s.</i>	<i>sensu stricto</i>
SSB	Single-Strand Binding
sv.	serovar
T/D	Transconjugant/Donor
T4SS	Type 4 Secretion System
cT4SS	conjugative Type 4 Secretion System
pT4SS	pathogenic Type 4 Secretion System
T4ASS	type A T4SS
T4BSS	type B T4SS
<i>tip</i>	transfer of <i>israelensis</i> plasmid
TMD	TransMembrane Domain
<i>tra</i>	transfer genes
TR	Tyrosine Recombinase
T-strand/T-DNA	Transfer strand/Transferred DNA
VIP	Vegetative Insecticidal Protein
WGS	Whole genome sequencing

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Part I

Introduction and Objectives

The *Bacillus cereus* group

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Bacillus cereus sensu lato (s.l.) refers to a group of Gram-positive, ubiquitous and sporulating bacteria very closely related, yet displaying a large ecological diversity. They exhibit various lifestyles as some live symbiotically near plant roots or in insect and mammal guts, some are saprophytic soil bacteria and others are pathogens to insects or mammals (Kolstø *et al.*, 2009; Ceuppens *et al.*, 2013; Ramarao *et al.*, 2015). Three major species form the core of the *Bacillus cereus* group: the opportunistic pathogen *Bacillus cereus sensu stricto* (s.s.), the etiological agent of anthrax *Bacillus anthracis* and the entomopathogen *Bacillus thuringiensis*. Other species belong to the *B. cereus* group, including the rhizoid growing *Bacillus mycoides* and *Bacillus pseudomycoides*, the psychotrophic *Bacillus weihenstephanensis* and the thermotolerant *Bacillus cytotoxicus*. Over the years, more species, such as *Bacillus toyonensis*, *Bacillus wiedmannii* and *Bacillus luti*, were proposed as species of the group based on numerous genetic analyses and taxonomic determinants.

B. cereus s.l. is at the very centre of taxonomic conflicts because the different "species" are hardly differentiable based on genetic criteria and because the major members of the group are of health, environmental or commercial interests. Therefore, the classification of the multiple members in different species cannot be restricted to taxonomic criteria, but must consider historical, economical and clinical aspects. Furthermore, *B. cereus s.l.* bacteria carry complex genomes and plasmids in various sizes and numbers, providing a large pool of candidate genes for horizontal gene transfer (HGT) (Kolstø *et al.*, 2009; Yuan *et al.*, 2010). Those plasmids often encode toxins that may show competitive advantages in microbial communities across *B. cereus s.l.* multiple habitats (Ceuppens *et al.*, 2013). Also, plasmid-encoded toxins have long been the basis of species discrimination among *B. cereus* group members. The important potential of gene exchange via HGT across the group might then further blur the lines of species differentiation among *B. cereus s.l.*

Understanding the characteristics usually associated with the main members of the group and their economic, health or environmental applications, as well as considering historical accounts and scientific advances in regards to the different members description and taxonomic classification are essential to further grasp the extend of the classification issue regarding the *B. cereus* group.

1.1 Major members of the *Bacillus cereus* group

The three main members of the *B. cereus* group, *i.e.* *B. cereus s.s.*, *B. anthracis* and *B. thuringiensis*, have been of upmost interest because of their marked impacts on human society. Despite sharing highly similar genetic features, they cover a wide range of ecological and biological conditions and niches and present various pathogenic spectra (Jensen *et al.*, 2003; Vilas-Bôas *et al.*, 2007; Ehling-Schulz *et al.*, 2019). In this section are presented the main characteristics usually associated with each of the major members of the group. However, the plasmid-borne pathogenic properties of these species are directly involved in their differentiation and make them highly inclined to HGT.

1.1.1 *Bacillus cereus sensu stricto*

Generally recognized as non-pathogenic, some strains of *B. cereus* s.s. are pathogenic and have been associated with food poisoning. In 2018, *B. cereus* was reported to contribute to 1.4 to 12% of foodborne outbreaks worldwide (Grutsch *et al.*, 2018). However, these statistics certainly suffer from underestimations as *B. cereus*-induced illnesses are usually mild, exempting patients from medical consultations and hence limiting case reports (Ramarao and Sanchis, 2013). Also, *B. cereus* food poisoning resembles *Staphylococcus aureus* intoxication and *Clostridium perfringens* or viral infection (Ravindran and Neelakantan, 2019).

Pathogenic *B. cereus* can cause two types of syndromes: the emetic and the diarrhoeal syndromes (Dietrich *et al.*, 2021; Jovanovic *et al.*, 2021). The emetic syndrome is a food intoxication characterised by a short incubation time (1-5 hours) and caused by an emetic toxin, the cereulide (Ceuppens *et al.*, 2013; Ramarao and Sanchis, 2013). The cereulide is a dodecadepsipeptide encoded by a cluster of seven *ces* genes, which are localised on the 270-kb plasmid pCER270 (or pCERE01) (Hotton *et al.*, 2005; Rasko *et al.*, 2007; Hu *et al.*, 2009). Interestingly, pCER270 is related to the *B. anthracis* virulence plasmid pXO1, but lacks the pathogenesis island encoding the anthrax factors (Hu *et al.*, 2009) (see Chapter 4).

Contrary to the emetic syndrome, the diarrhoeal syndrome breaks out 8-16 hours after ingesting food contaminated by live bacteria or spores (Ramarao and Sanchis, 2013; Jovanovic *et al.*, 2021). The best known putative enterotoxins causing *B. cereus*-associated diarrhoeal syndrome are the nonhemolytic enterotoxin (Nhe), the hemolysin BL (Hbl) and the cytotoxin K (CytK). Two variants of CytK-encoding genes have been reported: *cytK-1*, specific to *B. cytotoxicus*, and *cytK-2*, present in some strains of *B. cereus* s.s. (Guinebretière *et al.*, 2013). Both variants of CytK share up to 89% of protein sequence identity (Stevens *et al.*, 2019). Besides the cereulide and enterotoxins, plethora of other putative virulence factors are produced by *B. cereus* (e.g. phospholipases, haemolysins, proteases, or degradative enzymes) (Kolstø *et al.*, 2009; Dietrich *et al.*, 2021).

1.1.2 *Bacillus anthracis*

B. anthracis is known as the causative agent of anthrax, a disease infecting primarily wild and domesticated herbivores, but all vertebrates are at risk (Kolstø *et al.*, 2009). Anthrax is the oldest known disease with evidence dated back to biblical times in the 5th and 6th plagues of Egypt (Exodus, chapter 9). *B. anthracis* was the first bacterium used for preparing attenuated bacteria-based vaccine by Pasteur in 1881 and one organism used for developing Koch's postulates in 1884, both fundamental foundations of modern science. Nowadays, however, *B. anthracis* is more of a concern because of its potential as a biological weapon (Inglesby *et al.*, 1999; Helgason *et al.*, 2000).

B. anthracis distinguishes from the rest of the *B. cereus* group by unique specificities, such as the absence of motility and haemolysis and the production of a capsule (Pilo and Frey, 2018). *B. anthracis* primarily thrives in the soil as dormant spores highly resistant to extreme environmental conditions. Spores can be ingested by herbivores, where they germinate into vegetative forms and express their virulence factors, leading to host death. Given their poor survival rate in the environment, vegetative cells quickly sporulate upon release from a host (Inglesby *et al.*, 1999; Mock and Fouet, 2001).

Two main virulence factors cause anthrax: a tripartite toxin and an antiphagocytic capsule (Baillie and Read, 2001). The tripartite toxin is formed by the protective antigen (PA), the lethal factor (LF) and the oedema factor (EF). These AB-like toxins act in binary combinations forming two anthrax toxins, the oedema toxin (EF + PA) and the lethal toxin (LF + PA) (Okinaka *et al.*, 1999; Pilo and Frey, 2018). The genetic determinants of PA, EF and LF are all extra-chromosomally encoded in the 181-kb plasmid pXO1. pXO1 also encodes the master regulator AtxA (anthrax toxin activator), known for controlling the expression of genes located both on *B. anthracis* chromosome and on the virulence plasmids (Fouet, 2010). A second plasmid, pXO2 (96 kb), encodes the genes involved in the capsule synthesis, the genes associated with the capsule degradation and some regulatory genes (Okinaka *et al.*, 1999; Mock and Fouet, 2001; Jensen *et al.*, 2003).

1.1.3 *Bacillus thuringiensis*

In 1901, Ishiwata described *Bacillus sotto*, a bacterium causing damage to the Japanese silk industry by infecting *Bombyx mori* (Beegle and Yamamoto, 1992). It was isolated for the second time in 1915 by Berliner from a dead moth larvae in Thuringia, Germany, and named *B. thuringiensis*. In 1938, the first *B. thuringiensis*-based biopesticide was commercialised in France. Since the late 20th century, *B. thuringiensis*-based preparations are commercialised as biopesticide to fight against insect pests. Since 1996, *B. thuringiensis* crystal toxin genes have also been inserted into plant cultivars to create genetically modified crops with an enhanced resistance to lepidopteran and/or coleopteran insect pests (Jouzani *et al.*, 2017).

B. thuringiensis owes its entomocidal properties to the production of crystal proteins, accumulating as parasporal crystals during sporulation (Fig. 1.1). These crystals are large proteinaceous aggregate inclusions of delta-endotoxins (Cry proteins) and cytotoxins (Cyt proteins) and they represent up to 25% of sporulating *B. thuringiensis* dry weight (Schnepf *et al.*, 1998; Palma *et al.*, 2014). Most of the Cry toxins present a first N-terminal domain involved in the pore formation, a domain responsible for binding of the receptor and a third C-terminal domain implicated in membrane insertion and receptor recognition (Xu *et al.*, 2014). All *B. thuringiensis* toxins are classified according to the Crickmore nomenclature, which accounts for protein sequence identity, and are collected in the Bacterial Pesticidal Protein Database (<https://www.bpprc-db.org/>) (Crickmore *et al.*, 2021).

After ingestion by a target insect larvae, the toxin crystal is dissolved in the alkaline environment of the larvae midgut, releasing the Cry and Cyt proteins. As crystals are formed by oligomers of polypeptide protoxin subunits, protoxins are first processed by midgut proteases to activate the toxin proteins. Two models were proposed for the subsequent action of *B. thuringiensis* toxins. The sequential binding model suggests that the formation of pores in the cell membrane by oligomerisation of the toxins leads to osmotic imbalance and further cell death. On the other hand, the signalling pathway model proposes that the *B. thuringiensis* toxins trigger ion



Figure 1.1: *B. thuringiensis* spore and parasporal crystal. Spore (S) and parasporal crystal (C) of *B. thuringiensis* INTATA24-6 under electron microscopy ; black bar = 0.2 μm (Sauka *et al.*, 2010).

channel opening, successively activating apoptosis (de Almeida Melo *et al.*, 2016). The entomocidal spectrum of *B. thuringiensis* varies depending on the serovar. Overall, 82 serovars have been described based on H (flagellar) antigen classification (Lecadet *et al.*, 1999).

B. thuringiensis also produces toxins at other stages of its life cycle. For example, Vegetative Insecticidal Proteins (VIPs) and Secreted Insecticidal Proteins (SIPs) are produced during the bacterial vegetative growth phase and are toxic against specific groups of insects. Besides entomocidal activity against a wide range of insect pest orders, *B. thuringiensis* also shows toxicity against other organisms, like nematodes, mites and ticks, and produces different molecules with multiple properties. *B. thuringiensis* can for instance promote plant-growth and show antagonist effects against plant and animal pathogenic microorganisms (Jouzani *et al.*, 2017). It can also produce parasporines, crystal proteins exhibiting anticancer activities, antifungal components, such as chitinases, and bacteriocins (Palma *et al.*, 2014; Jouzani *et al.*, 2017).

Similarly to the anthrax toxin, the characteristic *B. thuringiensis* toxins, *i.e.* Cry and Cyt toxins, are encoded by genes located on large, potentially transferable plasmids (Palma *et al.*, 2014). Even more than for other strains of *B. cereus s.l.*, plasmids are key elements for *B. thuringiensis* ecology. Each strain of *B. thuringiensis* indeed carries an average of 6.4 plasmids, which corresponds to 11.2% of the strain genome, *i.e.* a percentage more than 2.5 times higher than for *B. cereus s.s.* (Fayad *et al.*, 2019).

Bacillus thuringiensis sv. *israelensis*

A novel bacterial strain with high mosquitocidal properties was isolated from dead mosquito larvae in a temporary pond in Israel in 1976, before being described as a new subspecies of *B. thuringiensis* and identified as the *israelensis* serovar of serotype H14 (Goldberg *et al.*, 1977; de Barjac, 1978). *B. thuringiensis* sv. *israelensis* (*Bti*) exhibits specific toxicity against dipteran vectors of parasitic and viral diseases. It is mostly toxic against *Culex*, *Aedes* and *Anopheles* mosquito species, vectors of diseases like malaria, yellow fever and dengue fever, and against blackflies, vectors of the onchocerciasis filarial worm, responsible for river blindness (Ben-Dov, 2014). Given the high threat of mosquito-borne diseases on the world's population, *Bti* has been used for years in large-scale public health programs (World Health Organization (WHO), 1999).

The entomocidal parasporal crystals of *Bti* are composed of four Cry (Cry4Aa, Cry4Ba, Cry10Aa and Cry11Aa) and three Cyt proteins (Cyt1Aa, Cyt2Ba and Cyt1Ca), toxic to different insect groups and capable of synergistic effect (Bravo *et al.*, 2007). The related toxin-encoding genes are encoded on the 128-kb toxin-carrying plasmid pBtoxis (Berry *et al.*, 2002). Alongside pBtoxis, strains of *Bti* typically carry eight extrachromosomal DNA molecules (Table 1.1) (Gillis *et al.*, 2018). A large proportion of *Bti* genome is composed by extrachromosomal molecules, as plasmid DNA represents 15-20% of the chromosome by size (Bolotin *et al.*, 2017). In natural environments, plasmid exchange, including transfer of insecticidal toxin-encoding plasmids, actively occurs between *israelensis* strains and are mainly caused by the large conjugative plasmid pXO16 (Bolotin *et al.*, 2017).

Table 1.1: Extrachromosomal molecules typically carried by strains of *Bti*.

Plasmid	Size (kb)	Main features	Reference
pTX14-1,2,3	5.4, 6.8, 7.6	Small, circular plasmids	Andrup <i>et al.</i> (2003)
pGIL01	15	Linear, tectivirus prophage	Verheust <i>et al.</i> (2003)
pBtic100	100	Two additional <i>cry</i> genes	Bolotin <i>et al.</i> (2017)
pBtoxis	128	Toxin encoding plasmid (four <i>cry</i> & three <i>cyt</i> genes)	Berry <i>et al.</i> (2002)
pBtic235	235	Cryptic prophage-like molecule	Gillis <i>et al.</i> (2017)
pXO16	350	Conjugative plasmid	Reddy <i>et al.</i> (1987)
pBtic360	360	Genome sequencing-based detection	Bolotin <i>et al.</i> (2017)

1.2 Other *Bacillus cereus* group members

Alongside the three core members of the *B. cereus* group stand other species of interest: *B. mycoides* and *B. pseudomycoides*, *B. weihenstephanensis* and *B. cytotoxicus*.

B. mycoides and *B. pseudomycoides* both exhibit easily identifiable rhizoid-like structures, due to the development of filaments extending from the initial inoculation point of a colony. *B. mycoides* is one of the first *B. cereus* species described back in 1886 (Flügge *et al.*, 1886). *B. pseudomycoides* was only proposed as a distinct species from *B. mycoides* over a century later, after investigating differences in the ratio of whole-cell fatty acid profiles within *B. mycoides* (Nakamura, 1998).

Although the majority of *B. cereus* species are mesophilic, *B. weihenstephanensis* and *B. cytotoxicus* display more specific thermal growth profiles, as psychrotolerant and thermotolerant species, respectively. *B. weihenstephanensis*, first described in 1998, produces chromosome-borne cold shock proteins that enable its growth at low temperatures (4-7°C) (Lechner *et al.*, 1998). Some strains of *B. weihenstephanensis* can produce the emetic cereulide toxin (Hoton *et al.*, 2009). Contrary to the *B. cereus* plasmid-borne cereulide,

the cereulide determinants of *B. weihenstephanensis* are either encoded on plasmids unrelated to *B. anthracis* pXO1 or on the strain chromosome (or very large plasmid) (Thorsen *et al.*, 2006; Hoton *et al.*, 2009).

B. cytotoxicus, first isolated in 1998 in France during a severe food poisoning outbreak, was described as a novel species in 2013 (Guinebretière *et al.*, 2013). It grows optimally at 30-37°C and up to 53°C (Guinebretière *et al.*, 2013), and it is characterised by CytK1, the most cytotoxic variant of the cytotoxin K, and by the absence of cereulide gene determinants (Lund *et al.*, 2000; Brillard and Lereclus, 2004; Koné *et al.*, 2019). The cytotoxicity of *B. cytotoxicus* varies from one strain to another, possibly due to an amino acid substitution next to a conserved residue in CytK1 sequence (Stevens *et al.*, 2019). Also, 6 distinct RAPD and 11 different plasmid content profiles were identified among *B. cytotoxicus* strains (Koné *et al.*, 2019). Overall, *B. cytotoxicus* isolates remain rare and were mainly recovered from potato flakes and other potato-containing foods, forming a strong genetic cluster (Koné *et al.*, 2019).

1.3 Taxonomic controversy

1.3.1 Timeline of the *B. cereus* group description: from 4 to 23 species

The history of *B. cereus* species description can be divided into three eras, a pre-genomic era, an era of genetic analyses and a whole-genome sequencing (WGS) era, as illustrated on Fig. 1.2. Four microorganisms form the original core of the *B. cereus* group and were described based on phenotypic features (pre-genomic era). First, *B. anthracis* was described in the late 19th century as the agent causing black coal-like (black coal = *antrakis* in Greek) skin lesions among anthrax patients. *B. mycoides* and *B. cereus* s.s. were respectively described by their root-like colonies (Flügge *et al.*, 1886) and by the waxy (*cera* in Latin) aspect of their colonies. At last, *B. thuringiensis* was isolated in 1915 from a dead flour moth in Thuringia, Germany, hence its name (Beegle and Yamamoto, 1992).

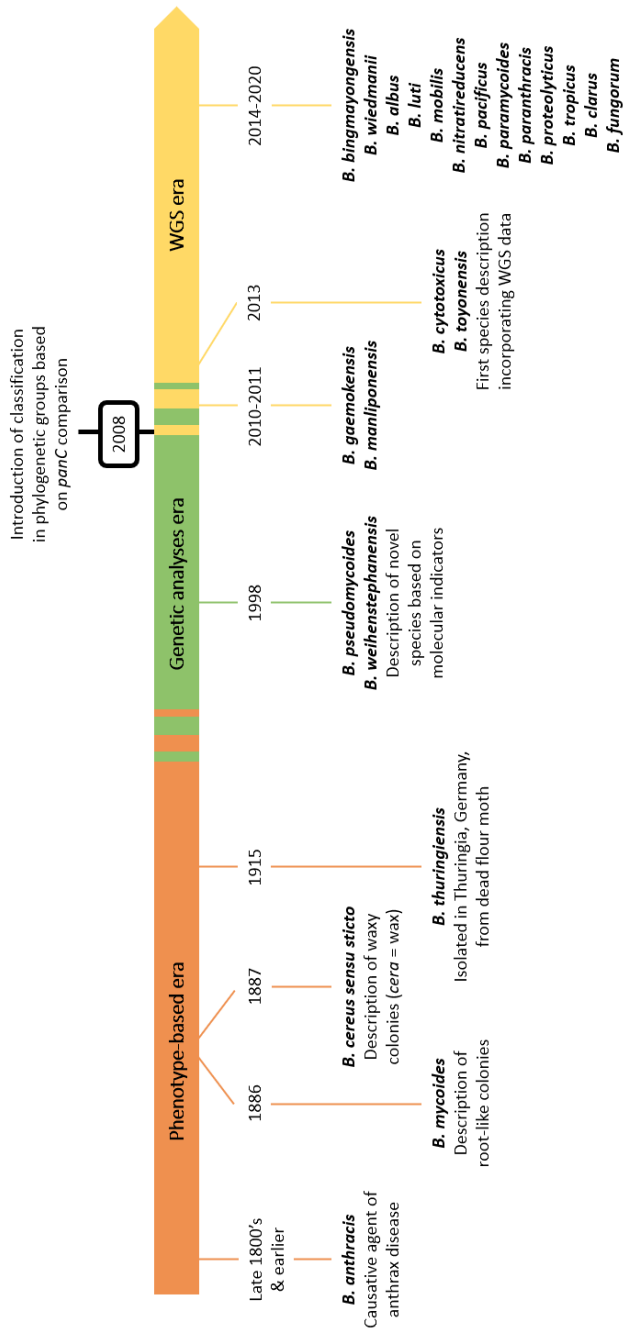


Figure 1.2: Timeline of the description of *B. cereus* species. From the four founding species described in the pre-genomic era, the *B. cereus* group progressively expanded. Novel species were successively added as *B. cereus* members as genetic and genomic analyses were developed and adapted to genome comparison. The three eras based on different identification approaches are coloured in red (phenotypic description), green (genetic approaches) and yellow (WGS data). Adapted from Carroll *et al.* (2021).

Since 1998, reporting novel *B. cereus* s.l. species relied on gene-sequencing based approaches and molecular indicators, such as DNA-DNA hybridization and 16S and 23S rDNA comparison. These new methods led to the description of *B. pseudomycoides*, *B. weihenstephanensis*, *B. gaemokensis* and *B. manliponensis* (Lechner *et al.*, 1998; Nakamura, 1998; Jung *et al.*, 2010, 2011). In 2013, WGS data were used for the first time to decipher two new species, *B. cytotoxicus* and *B. toyonensis* (Guinebretière *et al.*, 2013; Jiménez *et al.*, 2013). Since the beginning of the WGS era, novel species were regularly reported based on whole genome analyses. For example, the average nucleotide identity (ANI) value describes the degree of similarity between two genomes. If the ANI value obtained for two genomes exceeds the set genomospecies threshold (usually 95%), both bacterial strains are defined as the same species, or genomospecies, and *vice-versa*. The digital DNA-DNA hybridization (dDDH) metric relies on a typical cut-off value of 70% to delineate bacterial species. Over the years, several species have been outlined in the *B. cereus* group by applying different threshold for the ANI value (92-96) and the dDDH value, such as *B. bingmayongensis*, *B. wiedmannii*, *B. clarus*, *B. fungorum* and others (Liu *et al.*, 2014; Miller *et al.*, 2016; Liu *et al.*, 2017; Méndez Acevedo *et al.*, 2019; Liu *et al.*, 2020).

1.3.2 Classification dilemma: taxonomy vs. society interests

For decades, species of the *B. cereus* group were discriminated based on their phenotype, toxins, morphology or specific growth properties, such as the psychrotolerance of *B. weihenstephanensis* and the rhizoid-like growth of *B. mycoides*. Phenotypic observations are still in use for early identification of *B. cereus* s.l. strains, as described in the Bacteriological Analytical Manual (BAM) protocol (Tallent *et al.*, 2020). However, they only allow for the distinction between *B. anthracis*, *B. thuringiensis*, *B. cereus* s.s., *B. mycoides* and *B. weihenstephanensis*. Furthermore, many of these phenotypical characteristics actually rely on plasmids, which are susceptible to horizontal dissemination (Rasko *et al.*, 2005; Kolstø *et al.*, 2009). The emergence of genetic techniques, like DNA hybridisation, 16S rDNA comparison and multilocus sequence profile analyses, progressively highlighted the genomic proximity between *B. cereus* species. In that context, the concept of *B. cereus* s.l. was propagated to bring together the different species and emphasise their taxonomic relatedness

(Léonard *et al.*, 1997; Maughan and Van der Auwera, 2011).

At the heart of the classification controversy lie the three main members of the *B. cereus* group, *B. anthracis*, *B. cereus* s.s. and *B. thuringiensis*. These three species share high levels of similarity based on multiple genetic analyses (Vilas-Bôas *et al.*, 2007). As previously mentioned, deciphering these species from one another mainly relies on genes carried by plasmids. For example, Cry toxins-encoding plasmids usually characterise *B. thuringiensis*, and cry-less strains of *B. thuringiensis* can no longer be differentiated from non-emetic strains of *B. cereus* s.s. (Helgason *et al.*, 2000). To answer this confusion, it was proposed to consider *B. anthracis*, *B. cereus* s.s. and *B. thuringiensis* as a single species (Helgason *et al.*, 2000; Vilas-Bôas *et al.*, 2007). However, the potential pathogenic properties of *B. anthracis* and *B. cereus* s.s. and the biopesticide applications of *B. thuringiensis* counterbalanced such proposition to limit conflicts between microbiologists and non-microbiologists (Okinaka and Keim, 2016). To avoid such confusion, Stackebrandt and colleagues suggested in 2002 that "medical organisms with defined clinical symptoms may continue to bear names that may not necessarily agree with their genomic relatedness", according to the Rule 56a of the International Code of Nomenclature of Bacteria (Lapage *et al.*, 1992; Stackebrandt *et al.*, 2002).

In 2008, Guinebretière and colleagues proposed a novel classification approach based on Single Locus Sequence Typing (SLST). They suggested classifying *B. cereus* members in seven groups based on their *panC* profiles, which would provide a greater resolution than profiles of other loci, like the commonly used 16S rDNA. This classification also accounts more for ecological adaptations, such as thermal niches, a driving force in *B. cereus* global evolution. For example, *B. cytotoxicus* forms a specific thermophilic group (group 7), separate from other groups (Guinebretière *et al.*, 2008). The *panC* framework was revised in 2020 to propose eight distinct groups (I to VIII), instead of seven (Carroll *et al.*, 2020a). Even though such classification may acknowledge species ecology and genomic considerations, its applicability remains limited for industrial, health and economic considerations as previously distinct species could now be found within the same group (Fig. 1.3). In recent years, systematic analyses of WGS data led to multiple

interpretations of the classification of *B. cereus* s.l. For instance, it was argued that the *B. cereus* group could be divided into 30 different clusters based on dDDH values, introducing around 20 novel species in the group (Liu *et al.*, 2015). Also, *B. weihenstephanensis* was reclassified as one species with *B. mycoides* (Liu *et al.*, 2018).

A novel approach for the nomenclature of the *B. cereus* group was recently proposed, based on a genomospecies/subspecies/biovars framework (Carroll *et al.*, 2020b). In this framework, the ANI value threshold for the genomospecies definition was set at 92.5 to resolve overlapping genomospecies, yielding 18 distinct genomospecies within the *B. cereus* group (Fig. 1.3). These genomospecies are rather congruent with the adjusted *panC* classification framework mentioned hereinabove (Guinebretière *et al.*, 2008; Carroll *et al.*, 2020a). Besides taxonomic genomospecies, the proposed framework accounts for lineages of medical importance via two subspecies names as well as for phenotypes of clinical and/or industrial interests via biovar annotation. Thereby, the interpretation of the new terminology remains accessible for non-microbiologists, mainly through the subspecies and biovar terms (Carroll *et al.*, 2021). Briefly, subspecies termination connects clinically relevant strains with the anthrax disease (subsp. *anthracis*) or the emetic phenotype (subsp. *cereus*) based on lineage affiliation. For example, isolates sharing at least 99.9 ANI with the reference anthrax strain *B. anthracis* strain Ames (reclassified as *B. mosaicus* subsp. *anthracis* strain Ames) are assigned to the *anthracis* subspecies. In addition, three proposed biovars (Anthraxis, Emeticus and Thuringiensis) account for toxin production or toxin production machinery based on phenotypic evidences or on genomic predictions. The global classification of *B. cereus* main species according to the genomospecies/subspecies/biovars framework is summarized on Fig. 1.3. Even though it may solve many debates regarding *B. cereus* genomic and phenotypical features, this novel classification remains controversial and has not formally been adopted yet.

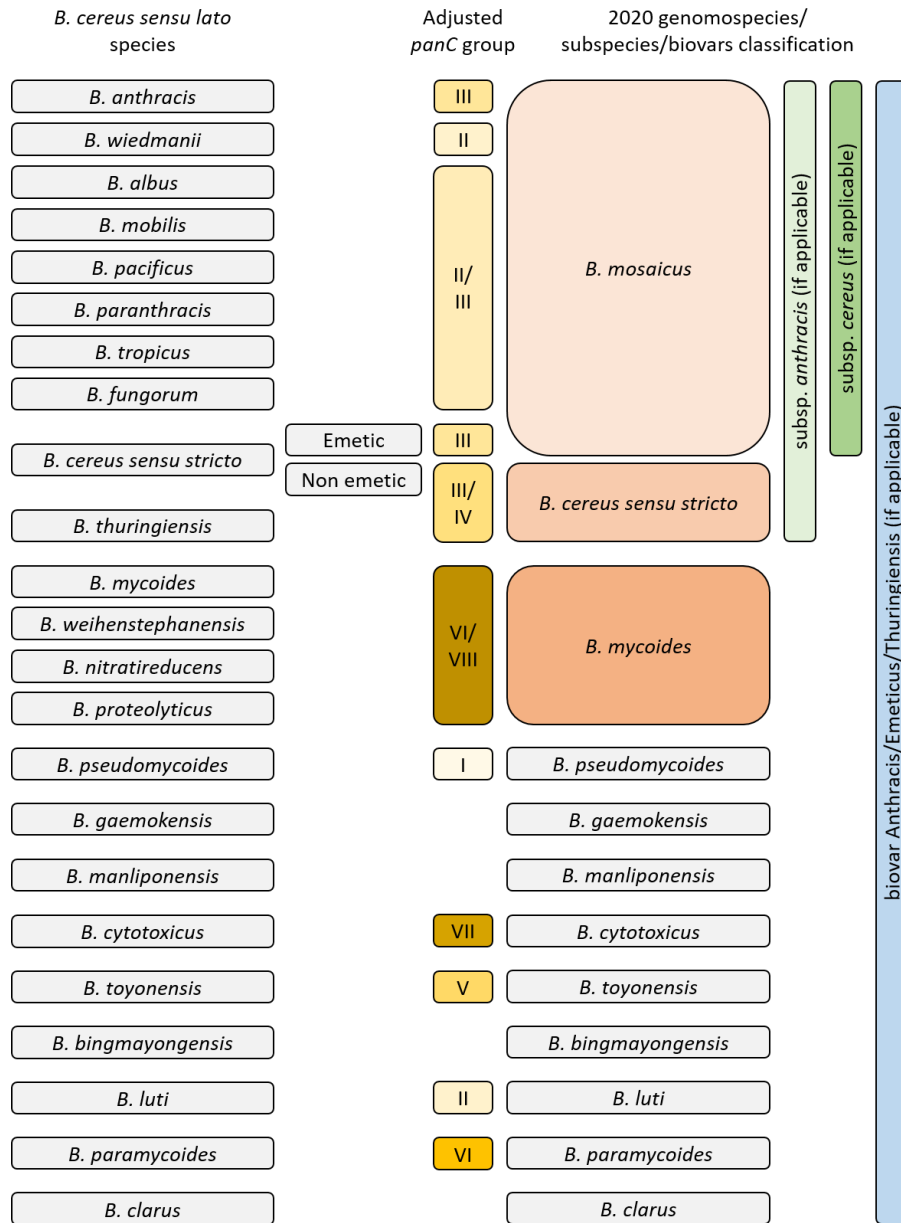


Figure 1.3: Overview of the *B. cereus* group taxonomy. The left column lists the 23 published and proposed species of *B. cereus s.l.* The central yellow column associates each species with a *panC* profile (adjusted groups I to VIII), as presented by Guinebrière *et al.* (2008) and updated by Carroll *et al.* (2020a). The right column presents the 2020 genomospecies/subspecies/biovars classification of the *B. cereus s.l.* species. The red colours indicate the new genomospecies, the green lines refer to the proposed subspecies and the blue line presents the current biovar collection. Adapted from Carroll *et al.* (2020b).

Gene transfer and bacterial conjugation

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Bacteria are well known for displaying extraordinary variations in their metabolic properties, cellular structures and lifestyles, which rely on the wide diversity of their genetic content. Genomes are passed through generations, by a vertical parent-offspring transmission. However, to face challenging conditions and adapt to ever-changing environments, bacteria can diversify their

gene content through different mechanisms. Bacterial genomes can be altered by point mutations, which are raw material for natural selection at the gene level (Taylor *et al.*, 2022), and by genetic rearrangements. On the other hand, bacteria can acquire DNA material from other organisms through HGT. HGT can be defined as "the non-genealogical transmission of genetic material from one organism to another", in opposition to the vertical parental inheritance (Goldenfeld and Woese, 2007). HGT plays a key role in bacterial genome plasticity, and it is well accepted that a large proportion of bacterial genomes actually results from horizontal gene flow (Thomas and Nielsen, 2005; Bello-López *et al.*, 2019). A main threat regarding the horizontal transmission of genetic material concerns the spread of antibiotic resistance. Many studies have highlighted the correlation between HGT and the dissemination of antibiotic resistance genes (von Wintersdorff *et al.*, 2016; Schwarz *et al.*, 2017; Bello-López *et al.*, 2019; Álvarez-Rodríguez *et al.*, 2020). However, the scope of HGT extends far beyond the problematic of antibiotic resistance, as its influence on bacterial evolution shaped current bacterial populations (Soucy *et al.*, 2015).

2.1 Horizontal gene transfer

Three main mechanisms are responsible for horizontal transmission of genetic material among bacteria: transformation, transduction and conjugation (developed hereunder) (Fig. 2.1) (Soucy *et al.*, 2015; von Wintersdorff *et al.*, 2016). A fourth mechanism, involving fusion of cells, also participates to HGT, although it seems less common and was less described than the other mechanisms (Johnson and Grossman, 2015). Transduction and conjugation are both mediated by mobile genetics elements (MGEs). MGEs encode the machinery for moving genetic material between different bacteria (intercellular transfer) or across different DNA molecules within a same bacterium (intracellular transfer) (Frost *et al.*, 2005). Typically, bacteriophages, conjugative and mobilisable plasmids, genomic islands and integrated conjugative elements (ICEs) are MGEs involved in intercellular transfer, while MGEs like transposable elements and insertion sequences cause intracellular transfer.

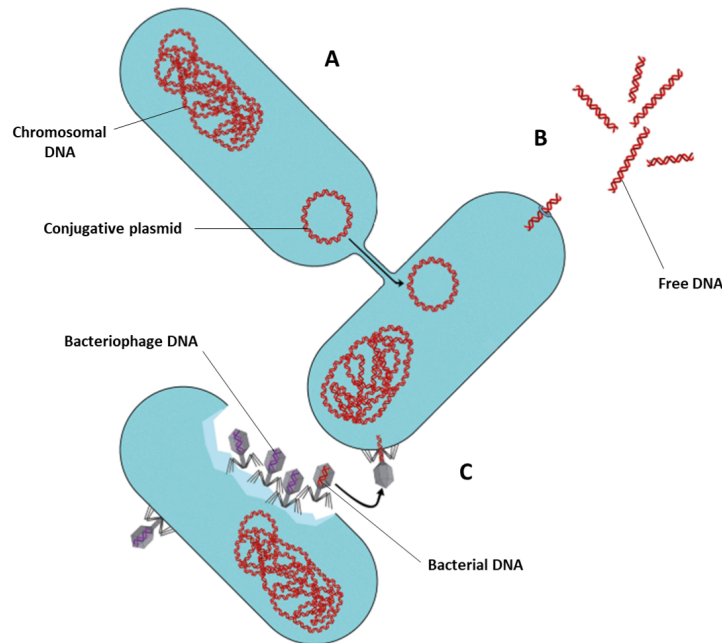


Figure 2.1: Main mechanisms of HGT. (A) Bacterial conjugation requires cell-to-cell contact between the mating partners, usually mediated by conjugative pili or adhesins. (B) Natural transformation is the uptake and integration of naked DNA from the environment by a naturally competent bacterial cell. (C) Transduction is mediated by phages, which can transfer bacterial DNA between successively infected cells. Adapted from von Wintersdorff *et al.* (2016).

2.1.1 Natural transformation

Natural transformation is a process enabling naturally competent bacteria to internalise DNA from their surroundings and integrate it into their genome by homologous recombination. First evidences of transformation date back to almost a century, when Griffith demonstrated the direct exchange of pathogenic traits between pneumococci (Griffith, 1928). Griffith's studies were also the first reports of alternative routes for bacteria to acquire genetic material, besides parental transmission (Schneider, 2021). Contrary to conjugation and transduction, transformation relies solely on the recipient bacterium. Transformation is evolutionarily conserved and the mechanisms

of DNA uptake and processing are shared by almost all transformable bacteria (Johnston *et al.*, 2014). DNA is transported across the bacterial cell envelope by a system reminiscent of type II secretion systems and type IV pili, and internalised as single-stranded DNA (ssDNA), an ideal substrate for homologous recombination. Even if free extracellular DNA is often heavily fragmented, transformation is also capable of mediating the transfer of large genomic segments (Blokesch, 2017). Although only a subset of relatively conserved genes is required for transformation *per se*, the competent state is tightly controlled by an impressive variety of regulatory pathways (Blokesch, 2016). Natural competence is genetically encoded and often limited to specific growth phases. For example, it is induced during early exponential phase in *Streptococcus pneumoniae* and during the stationary phase in *Bacillus subtilis*, but it is a constitutive property in *Neisseria* spp. (Johnston *et al.*, 2014). In some species, natural competence is induced in response to environmental stresses, such as the presence of antibiotics and DNA-damaging agents (Claverys *et al.*, 2006; Prudhomme *et al.*, 2006).

In some nutrient-limiting conditions, transforming DNA can serve a nutritional purpose, by providing new building blocks and bypassing the costly *de novo* synthesis. On the other hand, it can be used as genetic material upon homologous recombination, leading to DNA repair or to the acquisition of new genetic traits (Fig. 2.2) (Blokesch, 2016). Transformation was also proposed to play a role in genome maintenance by freeing bacterial genomes from selfish MGEs. Indeed, transformation only requires short fragments of highly homologous DNA because it is solely based on double homologous recombination (Croucher *et al.*, 2016). It was long thought that transformation relied on the release of DNA from dead bacteria in the vicinity of the competent recipient. Instead, recipients are thought to take an active part in making naked DNA available by preying on unrelated bacteria. Such "competence-dependent kin-discriminated killing" was for instance reported between *Vibrio cholerae* and *S. pneumoniae* (Veening and Blokesch, 2017).

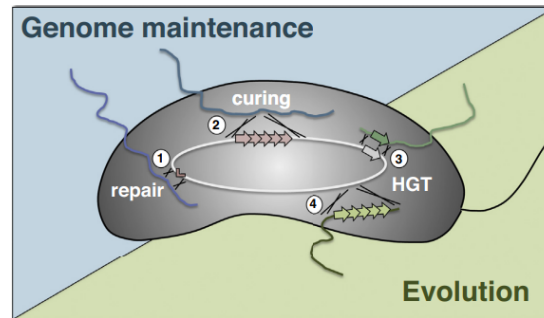


Figure 2.2: Natural transformation at the interface of genome maintenance and evolution. Transforming DNA can be integrated into the bacterial genome, leading to DNA repair (1) or curing the genome from MGEs (2), both contributing to genome maintenance. Alternatively, transforming DNA can provide new genes (3) or genomic islands (4) to the recipient bacteria, thereby participating in evolution (Blokesch, 2017).

2.1.2 Phage-mediated transduction

Considered as the most abundant biological entities on Earth, bacteriophages (or phages) are viruses infecting bacteria. Bacterial communities are almost all susceptible to at least one type of phages, which can play a key role in mediating HGT among their hosts. To propagate, phages hijack the replication machinery of their host for multiplying their genome (Campbell, 2003; Buckling and Brockhurst, 2012). When the viral genome is packaged for phage assembly prior to host lysis, some bacterial genetic material can also be encapsidated with the viral genome, resulting in transducing particles. These particles behave like typical phages, except that they will inject bacterial DNA material instead of their viral genome when they will infect a new host. The new bacterial DNA can be maintained in the new host by recombination into the chromosome or as a self-replicating plasmid (Chiang *et al.*, 2019). Transduction significantly differs from the other main mechanisms of HGT. Indeed, phages can survive in the environment overtime, unlike DNA necessary for transformation, and transduction does not rely on direct physical contact between bacterial cells, unlike conjugation (Touchon *et al.*, 2017). Nowadays, the extend to which transduction contributes to HGT among bacteria remains poorly known (Schneider, 2021).

Transduction was first reported by Zinder and Lederberg (1952), who actually documented a case of generalised transduction. Generalised transduction occurs when the phage encapsulates bacterial host DNA instead of viral DNA. It results from errors of phage terminases in discriminating the terminating sites during DNA packaging. Instead of recognising viral terminating sites, they wrongly recognise pseudo-sites on the host chromosome (Fig. 2.3). Any part of the bacterial chromosome is assumed to be susceptible to generalised transduction, hence the denomination (Chiang *et al.*, 2019). On the contrary, the range of genes susceptible to specialised transduction is limited to specific sets of genes. Specialised transducing particles are hybrid molecules of viral and host bacterial DNA encapsidated after an inaccurate excision of prophage DNA from the host chromosome. The viral DNA is not properly excised from the host chromosome and remains attached to some adjacent fragments (Fig. 2.3). Therefore, only genes surrounding the phage-insertion site can be transferred by specialised transduction. This type of transduction is also limited to lysogenic integrative phages as it requires a prophage state (Touchon *et al.*, 2017). A third type of transduction, called lateral transduction, was recently described as a natural, non-mistaken part of the life cycle of temperate phages of *S. aureus* (Fig. 2.3) (Chen *et al.*, 2018a). *S. aureus* prophages only excise from the bacterial host chromosome in late phases of their life cycle and the phage DNA is thus amplified for packaging while still embedded into its host chromosome. The DNA is encapsidated prior to excision, resulting in phage molecules packed with viral DNA and adjacent bacterial genes. With such mechanism, gene transfer frequencies are 1,000-fold greater than by generalised or specialised transduction (Chen *et al.*, 2018a).

2.1.3 Bacterial conjugation

Bacterial conjugation is a mode of unidirectional DNA transfer from one donor cell to one recipient cell, necessitating direct physical contact between the mating partners. Cell-to-cell contact is usually established via a conjugative pilus in Gram-negative bacteria and by other means, like adhesins, in Gram-positive bacteria. From the three main HGT mechanisms, conjugation was probably the most abundantly studied. Lederberg and Tatum (1946) were the first to determine that bacteria could exchange genetic information

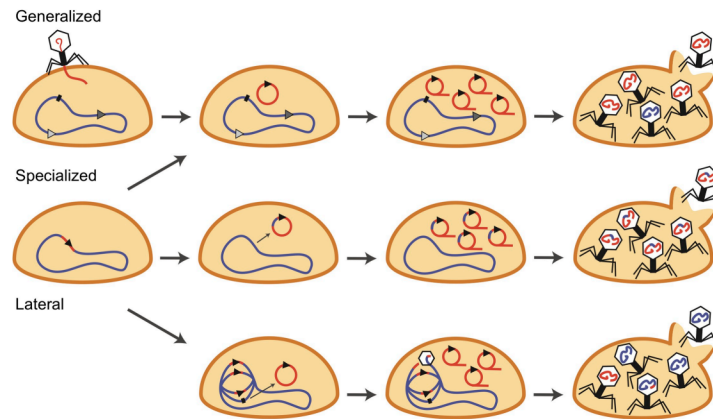


Figure 2.3: Three mechanisms of phage-mediated transduction. Generalised transduction (top) can induce the transfer of virtually any host chromosomal fragment, while specialised transduction (middle) is restricted to bacterial DNA flanking the virus-insertion site in the host chromosome. Lateral transduction (bottom) involves phage replication prior to its excision from the host chromosome (Chiang *et al.*, 2019).

through a form of sexual reproduction. Others later explained that the original observation of Lederberg and Tatum required a physical contact between the mating cells (Davis, 1950), that both partners should be of different mating types and that the DNA transfer was unidirectional (Wollman *et al.*, 1956). Since the discovery of the conjugation process, the identification and description of plethora of conjugative systems revealed that conjugation is universally conserved among Gram-negative and Gram-positive bacteria (Grohmann *et al.*, 2003; de la Cruz *et al.*, 2010). Conjugation was also observed among bacterial communities in all sorts of environments, showing through the ubiquity of conjugal DNA transfer (Davison, 1999). At the opposite of natural transformation, conjugation allows the transfer of genetic material through a structure protecting it from the environment (Schneider, 2021). Such protection of DNA offers a more efficient way of entering the recipient cell than transformation. Besides, compared to the restricted host range of transduction relying on phage-bacteria interactions, the host range of conjugation is wider (von Wintersdorff *et al.*, 2016).

The machinery utilised during conjugation to execute DNA transfer is usually encoded by conjugative plasmids or other MGEs, like ICEs (Smillie *et al.*, 2010; Wozniak and Waldor, 2010). Conjugative, or self-transmissible, plasmids can mediate their own transfer as they carry the full set of genes encoding the conjugative apparatus. Other plasmids, called non-self-transmissible plasmids, lack a part or the entirety of this conjugation gene set. However, some non-self-transmissible plasmids, referred to as mobilisable plasmids, retain the capacity to be transferred through a conjugation-related process, called mobilisation (see section 2.2.6) (Smillie *et al.*, 2010). Contrary to conjugative plasmids, ICEs primarily reside in the chromosome, but are capable of excising and transferring by conjugation upon induction. All ICEs are composed of functional modules, responsible for maintenance, dissemination and regulation (Burrus and Waldor, 2004). Despite their differences, conjugative plasmids and ICEs share conserved mechanisms of conjugative transfer (Guglielmini *et al.*, 2011).

The conjugation mechanism functions as a succession of replication and secretion of genetic material. DNA is first processed by a relaxase, and then transported to a type IV secretion system (T4SS) via a coupling protein (CP) (Llosa *et al.*, 2002). The molecular mechanism of conjugation and the different machineries involved in the conjugation process are detailed in the following section (section 2.2), by describing the current models for Gram-negative bacteria. A further section (section 2.3) is devoted to different conjugative systems encountered in Gram-positive bacteria.

2.2 DNA transfer by conjugation

The different steps and machineries involved in conjugal DNA transfer were mostly studied for conjugative plasmids of Gram-negative origin, especially for the F plasmid and the R388 plasmid from *Escherichia coli* and for the tumour-inducing plasmid (pTi) from *Agrobacterium tumefaciens*. Three large complexes mediate conjugal transfer in Gram-negative bacteria: a DNA-processing machinery - the relaxosome -, a membrane-embedded transport machinery - the T4SS -, and a conjugative pilus (Ilangoan *et al.*, 2015). Previously, the genes involved in conjugation were divided in two

distinct sets: the DNA-transfer replication (*dtr*) genes, in charge of the DNA processing, and the mating pair formation (*mpf*) genes responsible for mating junctions and pilus biogenesis (Cabezón *et al.*, 2015). It is nowadays more common to refer to the Dtr components as the relaxosome, and the MPF complex as the T4SS. Both complexes are linked by a type IV CP, recruiting the relaxosome to the T4SS (Waksman, 2019).

In this section, the overall process of conjugal DNA transfer is exposed by following the different steps of the life cycle of a typical conjugative plasmid during conjugation (Fig. 2.4). Then, the main conjugation-mediating machineries are described in more details.

2.2.1 Overview of the conjugative transfer process

The ability of a donor cell to enter the conjugation process resides in the expression of the transfer (*tra*) genes clustered on the conjugative plasmid. *tra* genes encode all the proteins involved in the production of the three conjugative machineries, *i.e.* the relaxosome, the T4SS and the conjugative pilus (Fig. 2.4, step 1). The expression of *tra* genes is tightly regulated by the activities of both plasmidial and chromosomal host factors, combined in complex regulatory circuits (Virolle *et al.*, 2020). For example, the *tra* genes of the F plasmid are placed under the control of a unique promoter, except for the two most upstream genes, *traJ* and *traM* (Ippen-Ihler *et al.*, 1972). *TraJ* is the primary activator of *tra* gene expression (Willetts, 1977) and is itself regulated at the post-transcriptional level by a fertility inhibition system, FinOP (Koraimann *et al.*, 1996). In addition, chromosome-encoded host factors also intervene in the regulation of *tra* gene expression (Wong *et al.*, 2012). In the case of the *A. tumefaciens* pTi plasmid, the regulation of the expression of the *vir* transfer genes relies on quorum-sensing. Such regulation coordinates the ability of the plasmid to conjugate with the host state or the density of bacteria in infected plant tissues (Dessaix and Faure, 2018).

Conjugation-mediated transfer is initiated in the donor cell by the assembly of the first machinery, the relaxosome, in charge of plasmid processing prior to transfer (Fig. 2.4, step 2). Upon binding to the plasmid origin

of transfer (*oriT*), the main relaxosome protein, the relaxase, distorts the DNA to expose the *nic* site, where it catalyses a site- and strand-specific nicking (Guasch *et al.*, 2003). The resulting ssDNA will further be transferred (T-strand). The nicking reaction requires a transesterase activity for the nicking itself, and a helicase activity to unwind the plasmid DNA (Lanka and Wilkins, 1995). These multiple functions can either be performed by multiple domains of the relaxase, or by a relaxase assisted by accessory proteins. The transesterification reaction generates a nucleoprotein complex formed by the relaxase bound to the 5'-end of the T-strand (Fig. 2.4, step 3). When the CP binds to the relaxosome complex, the relaxase becomes activated and switches to a translocation mode (Lang *et al.*, 2011). For translocation, the activated relaxase is thought to pilot the T-strand through the conjugative pore in an unfolded conformation (de la Cruz *et al.*, 2010; Zechner *et al.*, 2017). Processing of the T-strand is thus spatiotemporally linked to its translocation through the conjugation apparatus (Li and Christie, 2018). The circular ssDNA plasmid remaining in the donor cell is further converted into double-stranded DNA (dsDNA) by rolling circle replication (RCR), while the T-strand is transferred to the recipient cell (Fig. 2.4, step 5). In the donor cell, the 3'-OH resulting from the relaxase-catalysed transesterification nicking serves as primer for the host DNA polymerase in charge of strand elongation. The RCR is terminated by a second transesterification reaction, releasing the newly synthesised strand and resulting in a dsDNA conjugative plasmid in the donor cell (Virolle *et al.*, 2020).

Once transferred into the recipient cell, the relaxase protein is proposed to pull the T-strand, in addition to the CP pushing through the conjugation pore (Fig. 2.4, step a) (de la Cruz *et al.*, 2010). In the case of the F plasmid, genes of the leading region are the first to be transferred to the recipient cell, and they are thought to be expressed rapidly, even before the conversion of the T-strand into dsDNA. To protect ssDNA from enzymatic degradation in the recipient cell, the T-strand is coated and protected by the host single-strand binding (SSB) protein, a universally conserved protein with non-specific ssDNA binding activity (Virolle *et al.*, 2020). When the 3' end of the *oriT* is brought into the recipient, the relaxase catalyses a second transesterification reaction, called joining reaction, to recircularise the T-strand into a ssDNA

plasmid (Fig. 2.4, step b) (Garcillán-Barcia *et al.*, 2007). The circularised ssDNA plasmid can then be converted into dsDNA (Fig. 2.4, step c), although the ssDNA to dsDNA conversion remains poorly described to date. Besides their conjugation genes, conjugative plasmids may carry genes involved in a plethora of functions, such as virulence or resistance to antibiotics. The expression of such genes may convert the recipient cell into a transconjugant cell supplemented with new metabolic functions (Fig. 2.4, step d). The acquisition of new metabolic properties are key for bacterial thriving in changing and challenging environments, making conjugation a key player of bacterial genome evolution (Virolle *et al.*, 2020).

Among others, a major question remaining about the conjugation process concerns the mechanism employed by the donor cell to recognise an appropriate recipient. Studies successfully demonstrated interdomain conjugal transfer by reporting the transfer of some broad-host range conjugative plasmids between bacteria and archaea or eukaryotes (Heinemann and Sprague, 1989; Waters, 2001; Dodsworth *et al.*, 2010), suggesting that no specific factors or active mechanisms are required in the recipient cell for conjugation. It was also proposed that the T4SS could act as a syringe, capable of injecting DNA through any kind of membrane, a conjugal transfer model called "shoot and pump" (Llosa *et al.*, 2002). These elements point towards a lack of requirements for specific factors on the recipient cell surface, but the question of genetic factors that could act as target receptors on the recipient cell remains unanswered so far.

2.2.2 The relaxosome: relaxase, *oriT* and accessory proteins

The relaxosome is a nucleoprotein complex composed of the plasmid *oriT*, the plasmid-encoded relaxase and plasmid- or host-encoded accessory proteins, and in charge of DNA processing prior to transfer (de la Cruz *et al.*, 2010).

The different relaxase families

The main constituent of the relaxosome is the relaxase, a usually large multidomain protein encoding an N-terminal relaxase (or transesterase) domain in charge of the transesterification at the *oriT* *nic* site. The C-terminal domain

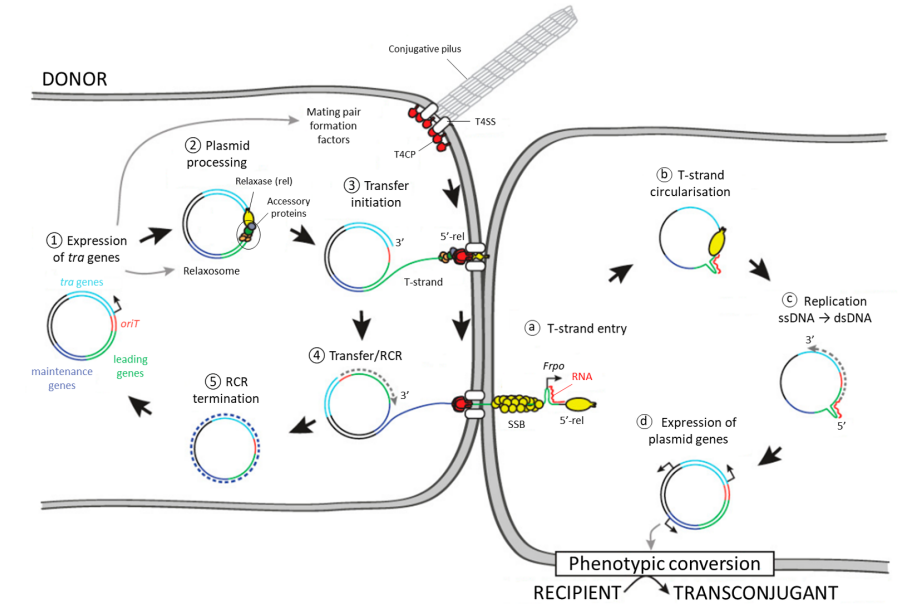


Figure 2.4: Life cycle of the conjugative F plasmid during conjugal transfer from a donor to a recipient cell. (1) The conjugal transfer is initiated by the expression of the *tra* genes, resulting in the formation of the T4SS, the conjugative pilus and the establishment of the mating pair. (2) Some *tra* genes encode the constituents of the relaxosome, in charge of the DNA processing prior to transfer. (3) The transfer of the T-strand is initiated upon recruitment of the relaxosome by the CP. (4)(5) The strand remaining in the donor cell is replicated by rolling-circle to form a novel circular dsDNA plasmid. (a) When the T-strand enters the recipient cell, it is coated by host SSB proteins. The single-stranded promoter *F_{rpo}* (F plasmid RNA polymerase), which forms a hairpin structure, is recognised by the host RNA polymerase. The RNA polymerase can then synthesise RNA primers, initiating the expression of the leading genes. (b) The T-strand is recircularised by the action of the relaxase. (c) The RNA-DNA duplex formed by the RNA primers on *F_{rpo}* initiates the replication of the complementary strand by the host DNA polymerase. (d) The expression of plasmidial genes can lead to the acquisition of new phenotypic properties by the newly formed transconjugant cell. Adapted from Virolle *et al.* (2020).

often encodes additional functions, like DNA helicase, DNA primase or other yet unknown activities (Garcillán-Barcia *et al.*, 2009). Relaxase proteins can be classified into distinct *MOB* families, the eight classical *MOB_F*, *MOB_H*, *MOB_C*, *MOB_Q*, *MOB_P*, *MOB_V*, *MOB_T* and *MOB_B* families (Garcillán-

Barcia *et al.*, 2009; Guglielmini *et al.*, 2011), and the recently suggested novel families *MOB_K* (Nowak *et al.*, 2021) and *MOB_L* (Ramachandran *et al.*, 2017) (Table 2.1).

Relaxases of the *MOB_F*, *MOB_Q*, *MOB_P*, *MOB_V* and *MOB_B* families belong to a larger family of endonucleases characterised by a conserved HUH (histidine-hydrophobic residue-histidine) motif. To catalyse ssDNA nicking and joining, HUH endonucleases use a tyrosine residue to create a 5'-phospho-tyrosine intermediate and a 3'-OH at the cleavage site (Chandler *et al.*, 2013). Beside the HUH motif, *MOB_F*, *MOB_Q*, *MOB_P* and *MOB_V* relaxases usually encode a third histidine residue, forming a classical 3H motif involved in metal coordination, as well as one (for *MOB_Q*, *MOB_P* and *MOB_V* relaxases) or two (for *MOB_F* relaxases) catalytic tyrosine residues (Zechner *et al.*, 2017). These relaxases are thought to recognise a hairpin formed by an inverted repeat next to the *nic* site of the *oriT*. In the recipient cell, both sections of the inverted repeat also form a hairpin structure that can be recognised by the relaxase to recircularise the T-strand. The 3'-OH resulting from the transesterification reaction can serve in both the initiation and the termination steps of the RCR. In the case of *MOB_F* relaxases, the RCR is initiated in the donor cell and terminated in the recipient cell by a distinct tyrosine residue (Chandler *et al.*, 2013). *MOB_F* and *MOB_Q* relaxases also systematically contain a C-terminal helicase domain, involved in dsDNA unwinding. The *MOB_Q* family overlaps with the *MOB_P* family, which represents the largest relaxase family described to date (Garcillán-Barcia *et al.*, 2009).

The mechanism used by *MOB_H*, *MOB_C* and *MOB_T* remains so far unknown, but is probably different from relaxases of the HUH type (Zechner *et al.*, 2017). *MOB_H* relaxases are characterised by an alternative 3H motif and an HD hydrolase motif (Garcillán-Barcia *et al.*, 2009). Similarly to the HUH relaxases, *MOB_C* relaxases are characterised by a triad of acidic residues (different from the 3H motif), also involved in metal cation coordination, and present a C-terminal domain belonging to a family of restriction endonucleases (RE). Globally, the DNA processing and catalytic mechanism performed by RE relaxases are similar to those performed by HUH relaxases (Francia

et al., 2013). Relaxases of the *MOB_T* family are related to RCR initiator proteins (Zechner *et al.*, 2017). They notably include the relaxases of the Gram-positive conjugative transposons Tn916 and ICEBs1. Interestingly, the relaxase of these conjugative transposons functions as a replicative relaxase and the *oriT* also serves as the replication origin (Lee *et al.*, 2012; Wright and Grossman, 2016). At last, the novel *MOB_L* family was proposed when describing the relaxase of pLS20 from *B. subtilis* and might be specific to Firmicutes bacteria (Ramachandran *et al.*, 2017). A well-described atypical relaxase, associated with the family of tyrosine recombinases (TR), is that of the clostridial plasmid pCW3 (Wisniewski *et al.*, 2016; Wisniewski and Rood, 2017). This relaxase remained unclassified until Nowak *et al.* (2021) proposed its classification with the TR-like relaxase of the pIGRK plasmid from *Klebsiella pneumoniae* in the *MOB_K* group.

The *oriT* and accessory proteins

The *oriT* is a plasmid-borne sequence found in conjugative and mobilisable plasmids. Its size varies from about 350 bp in the case of *MOB_F* and *MOB_P* plasmids to a compact locus of 38 bp in *MOB_Q* plasmids (de la Cruz *et al.*, 2010). Within the *oriT* is the specific site, *nic*, where the DNA is cleaved by the relaxase (Lanka and Wilkins, 1995; Guasch *et al.*, 2003). Inverted repeats typically flank the *oriT* and can form hairpin structures extruding from the dsDNA and involved in *oriT* recognition and binding (Waksman, 2019). The *oriT* region also contains the docking sites for the relaxase and the accessory proteins. In the case of the F-plasmid, two plasmid-encoded accessory proteins, TraM and TraY, and the host genome-encoded IHF (integrated host factor) form the relaxosome with the relaxase TraI and the *oriT*. Two TraI molecules are loaded on the *oriT*, one on either side of the *nic* site (Ilangoan *et al.*, 2017). Assembly of the relaxosome on the *oriT* distorts DNA and disrupts the supercoiled dsDNA *oriT*. Studies demonstrated that the relaxosome proteins interact with each other (Zechner *et al.*, 2017), that the presence of accessory proteins stimulates the activity of the relaxase (Nelson *et al.*, 1995; Ragonese *et al.*, 2007), and that the relaxosome is recruited to the transfer apparatus by interacting with the CP (Llosa and Alkorta, 2017).

Table 2.1: Main characteristics of the ten MOB families of relaxases. HUH: histidine-hydrophobic residue-histidine; RE: restriction endonuclease; TR: tyrosine recombinase; *Rep_trans*: RCR initiation proteins; HEN: variant motif of HUH superfamily; HD: HD hydrolase; 3H: 3H motif; 3H*: alternative 3H motif; n/d: not determined. Adapted from Guzmán-Herrador and Llosa (2019).

MOB family	Related superfamily	Characteristic motif	Catalytic site	Type relaxase (source plasmid)
<i>MOB_F</i>	HUH endonuclease	HUH, 3H	Tyr x2	TraI (F plasmid), TrwC (R388)
<i>MOB_Q</i>	HUH endonuclease	HUH, 3H	Tyr	TraA (pIP501)
<i>MOB_P</i>	HUH endonuclease	HUH, 3H	Tyr	TraI (RP4), VirD2 (pTi)
<i>MOB_V</i>	HUH endonuclease	HUH, 3H	His	MobM (pMV158)
<i>MOB_B</i>	HUH endonuclease	HUH	n/d	Specific to bacterioidetes
<i>MOB_H</i>	n/d	3H*, HD	Tyr	TraI from <i>Nesseria gonorrhoeae</i> GGI
<i>MOB_C</i>	RE	Triad of acidic residues	Tyr	TraX (pAD1)
<i>MOB_T</i>	<i>Rep-trans</i>	n/d	n/d	Orf20_Tn916 (Tn916), NicK (ICEBs1)
<i>MOB_K</i>	TR	n/d	Tyr	TcpM (pCW3), MobK (pIGRK)
<i>MOB_L</i>	HUH	HUH	Tyr	RelLS20 (pLS20)

2.2.3 The coupling protein

A CP is present in all conjugative systems and plays a crucial role in connecting the DNA substrate and the T4SS, hence its name. The complete role of the CP remains unclear, but its role in substrate recruitment and presentation to the T4SS is generally consented. In addition, the CP is most probably involved in energising the substrate transport due to its ATPase activity (Llosa and Alkorta, 2017). CPs are characterised by three or four structural domains, participating in the protein "coupling" functions (Gomis-Rüth and Coll, 2006). The N-terminal transmembrane domains (TMDs) anchor the

protein in the inner membrane, while the C-proximal domains are involved in substrate docking and energisation of DNA transfer (Li and Christie, 2018). Those C-proximal moieties are composed of two domains, an all-alpha domain (AAD) and a nucleotide-binding domain (NBD). The AAD, at the cytoplasmic side, binds to the relaxase and recruits it - along with the covalently bound T-strand - to the T4SS. The NBD is a signature domain among CPs as it is common to all CPs (Llosa and Alkorta, 2017). Some CPs, like the *A. tumefaciens* VirD4, possess an additional variable C-terminal domain typically enriched in acidic residues (Alvarez-Martinez and Christie, 2009).

At the interface between the DNA-processing and the transfer machineries, the CP of multiple conjugative plasmids was shown to interact with relaxosome components, as well as with the other ATPases of the T4SS (Llosa and Alkorta, 2017). At the functional level, a single CP can interact with different T4SS, while the interaction between a relaxosome and its cognate CP is highly specific (Llosa *et al.*, 2003).

2.2.4 The transfer apparatus

The conjugal DNA transfer is mediated by a T4SS. T4SS are not restricted to DNA transfer (conjugative T4SS, or cT4SS), as they are also involved in the secretion of virulent proteins (pathogenic T4SS, or pT4SS) (De Paz *et al.*, 2005). Some T4SS, like that employed by the pTi plasmid from *A. tumefaciens*, have a dual nature, being both involved in conjugation and pathogenesis (Li and Christie, 2018). This section focuses on the description of cT4SS, hereunder mentioned as T4SS. The classical nomenclature of T4SS components is based on the Vir system of the pTi plasmid from *A. tumefaciens*, which widely serves as model for Gram-negative T4SS in conjugation. The Vir T4SS is composed by 12 proteins (VirB1-11 and the CP VirD4) and belongs to the type A T4SS (T4ASS). A second type of T4SS, T4BSS, found in the Gram-negative bacteria *Coxiella* and *Legionella*, is much more complex, as it involves around 25 proteins, among which few are related to Vir proteins (Voth *et al.*, 2012). T4BSS are not detailed in this work, and the T4(A)SS structure and DNA transfer apparatus is detailed based on the VirB/D4 model.

Structure of the T4SS

As mentioned hereinabove, Gram-negative T4SS are constituted of 12 sub-units, VirB1 to VirB11 and VirD4, which can be grouped into four functional or spatial categories: the outer membrane core complex (OMCC), the inner membrane complex (IMC), the cytoplasmic ATPases and the pilus-assembly components (Grohmann *et al.*, 2018). The overall structure of a conjugative T4SS and the relative organisation of the Vir proteins are schematically represented on Fig. 2.5.

The OMCC, composed by the VirB7, VirB9 and VirB10 proteins, spans both membranes of the bacterial cell wall by forming a channel in the outer membrane (OM) and inserting into the inner membrane (IM). 14 copies of each of the proteins are organised in two distinct layers in the OMCC. VirB7 and the C-terminal domains of VirB9 and 10 form the outer (O-) layer of the OMCC, while the N-terminal moieties of VirB9 and 10 compose the inner (I-) layer (Fronzes *et al.*, 2009). VirB10 lines the interior of the O-layer and projects alpha-helices forming a channel through the OM. VirB7 and 9 wraps around VirB10 in a protective crown (Chandran *et al.*, 2009). VirB9 and 10 C-terminal domains contact their respective N-terminal domains in the I-layer by a long linker (Rivera-Calzada *et al.*, 2013; Sgro *et al.*, 2018).

Twelve copies of each of the VirB3, VirB4, VirB6 and VirB8 proteins assemble in the IMC with the 14 copies of the N-terminal domains of VirB10 inserted into the IM (Ilangovan *et al.*, 2015). The IMC forms two symmetrical barrel-like structures, with the two lower tiers of each barrel protruding in the cytoplasm and the upper tier inserting into the IM (Low *et al.*, 2014). The cytoplasmic part of the IMC is actually composed of the VirB4 ATPase, arranged as trimers of VirB4 dimers (Walldén *et al.*, 2012). The IMC is connected to the OMCC by a slightly flexible section, called the stalk, which is structured with pillars of unknown origin (Low *et al.*, 2014; Trokter *et al.*, 2014). At the base of the T4SS are localised the ATPases. As stated previously, VirB4 forms two hexameric (trimers of dimers) barrels at the base of the IMC barrel-like structures. The two other ATPases, VirD4 and VirB11, also function as hexamers (Gomis-Rüth *et al.*, 2001; Savvides *et al.*, 2003). Both

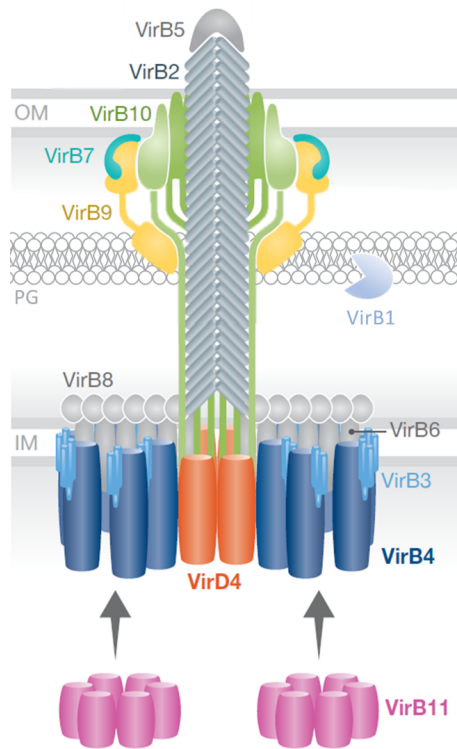


Figure 2.5: Schematic representation of the architecture of a conjugative T4SS. The OMCC is composed of the proteins VirB7, VirB9 and VirB10, while the IMC comprises the proteins VirB3, VirB6 and VirB8. The ATPases VirB4 and VirB11 and the CP VirD4 interact at the cytosolic side of the IMC to energise the T4SS machinery. The pilus is formed by VirB2 and VirB5 and the PG hydrolase VirB1 facilitates the insertion of the T4SS into the cell envelope. OM: outer membrane; IM: inner membrane; PG: peptidoglycan layer. Adapted from Waksman (2019).

VirB4 and 11 are required for pilus biogenesis, while the CP VirD4 replaces VirB11 for substrate translocation (Grohmann *et al.*, 2018). The translocated substrate was suggested to pass unfolded through one of the ATPases as the IMC forms no central channel. Work from Trokter and Waksman (2018) reinforced this hypothesis by demonstrating that unfolding of the relaxase is required for transfer.

Three proteins are associated with the pilus-assembly components, the VirB1 transglycosylase, the VirB2 pilin and the pilus-tip VirB5 (Grohmann *et al.*, 2018). The N-terminal domain of VirB1 is characterised by a muraminidase activity and provides local degradation of the cell wall, allowing insertion of the T4SS. On the other hand, its C-terminal domain is secreted outside of the cell, and is thought to be involved in pilus biogenesis by interacting with VirB2 and 5 (Baron *et al.*, 1997; Zupan *et al.*, 2007). The major pilin VirB2 is the main component of the pilus, while the minor pilin VirB5 is localised at the tip of the pilus and was proposed to be responsible for contact with the recipient cell (Aly and Baron, 2007).

Conjugative pili

Conjugative T4SS are functionally complex as they form pilus biogenesis machines as well as DNA and protein transporters. Two types of pili have been reported in Gram-negative conjugative systems: the F-like and the IncP-like pili (Bergé *et al.*, 2017). The F-like pili are long (2-20 μm) and flexible structures, able to dynamically extend and retract (Clarke *et al.*, 2008). In opposition, IncP-like pili are shorter (1 μm) and more rigid structures. Both types of pili exhibit a similar structure, as they are formed by repeating units of the VirB2 major pilin. VirB2 is first expressed as a pro-pilin, with a signal peptide for insertion into the IM. After maturation, the pilin subunits accumulate in the IM until their recruitment for the assembly of the pilus (Hospenthal *et al.*, 2017). The integration of the minor pilin VirB5 into the pilus remains poorly known to date.

From pilus biogenesis to substrate translocation

The dynamic structure of T4SS, especially the ATPase subunits, allows switching between two temporally distinct mode: pilus assembly and DNA/protein secretion. The pilus is assembled when the VirB4 ATPase extracts the VirB2 pilins from the IM and hypothetically deposits them on an undefined structure, which would serve as a prepilus. The VirB11 ATPase is also involved in pilus biogenesis, by interacting with and probably modulating the activity of VirB4. Interaction studies of Low *et al.* (2014) and Redzej *et al.* (2017) suggest that stacking of VirB11 against VirB4 (Fig. 2.5) might activate VirB4

by converting inactive trimers of dimers into functional hexamers. The model presented hereafter was recently reviewed by Waksman (2019).

After assembly of the pilus, a pre-initiation complex is formed if a relaxosome is recruited to the T4SS. The pre-initiation complex is thought to be activated when a conjugative junction is tightly established with a recipient cell, further leading to the activation of the relaxosome. Then, the DNA substrate is nicked at the *oriT* and is pumped through the T4SS by the helicase domain. It is mostly hypothesised that the relaxase bound to the T-strand is first transferred to the recipient cell, followed by the T-strand. Such model implies a switch of T4SS function from a protein transport machinery to a ssDNA-transport system. Very little is known about the protein-transport system, except that unfolding of the relaxase is required (Trokter and Waksman, 2018). Furthermore, many questions remain concerning the T-DNA pathway across the bacterial cell envelope.

A first model proposes that DNA sequentially contacts VirD4, VirB11, 6 and 8 in the IMC, VirB9 in the OMCC and VirB2 in the pilus (Cascales and Christie, 2004). In this model, VirB4 does not interact directly with the T-strand. However, this model is not supported by any architectural data available so far. In a second model, based on the architectural stacking of VirB11 with VirB4, VirD4 is absent and VirB4 would interact with the T-strand (Chetrit *et al.*, 2018). Another major uncertainty about ssDNA transport concerns the subsequent mechanism of transfer through the recipient cell envelope. More information is available about the termination of conjugative transfer in the recipient cell (see section 2.2.1).

2.2.5 Control of conjugative transmission

Both plasmids and host bacteria developed different strategies to prevent from conjugation (Getino and de la Cruz, 2018). Bacteria can protect themselves from invading genomes by their restriction-modification (RM) systems. Defense by RM systems was more abundantly reported against bacteriophages (Samson *et al.*, 2013), but some cases were reported for conjugation impairment (Elhai *et al.*, 1997; McMahon *et al.*, 2009; Roer *et al.*, 2015). In brief,

RM systems consist in two opposing enzymatic activities: methyltransferases methylate host DNA at a define residue, protecting it from the action of endonucleases, that cleave unmethylated DNA (Vasu and Nagaraja, 2013). Besides the RM innate immunity, bacteria also developed the CRISPR-Cas adaptive immunity. These combine a first immunisation step consisting of the acquisition of spacer sequences of invading genomes, followed by an immunity phase during which the Cas nucleases specifically target the invading DNA via the acquired spacer sequences (Marraffini, 2015).

Control of conjugation by plasmids was first reported when *E. coli* cells harbouring the F plasmid were noticed to be poor recipients of the same plasmid (Lederberg *et al.*, 1952). This can be explained by two independent mechanisms interfering with the conjugation and replication mechanisms, and respectively called exclusion and incompatibility (Novick, 1969). The exclusion mechanism prevents DNA from entering a cell containing an identical plasmid. The F plasmid, for instance, encodes both a surface and an entry exclusion system. The former involves an OM protein and reduces the ability to form stable mating pairs. Entry exclusion, on the other hand, involves an IM protein and reduces the efficiency of DNA transfer after the establishment of a stable mating junction (Getino and de la Cruz, 2018). The incompatibility mechanism is linked to replication. Plasmids with identical or similar replicon cannot coexist in a cell and are experimentally grouped in the same incompatibility group. Although they are distinct mechanisms, exclusion and incompatibility are often associated as plasmids showing exclusion towards one another are usually classified in the same incompatibility group (Novick, 1969). These control systems contribute to balancing the advantages of conjugal DNA transfer (*e.g.* acquisition of new traits) with the burden of plasmid maintenance, or the so-called plasmid paradox (Carroll and Wong, 2018).

2.2.6 Mobilisation, a conjugation-dependent mechanism

Horizontal transfer of plasmids can occur by conjugation for conjugative plasmids, *i.e.* encoding both the relaxosome and the transferosome machineries, or by mobilisation for mobilisable plasmids, *i.e.* lacking at least the T4SS genes (Smillie *et al.*, 2010; Ramsay and Firth, 2017). Mobilisable

plasmids can indeed exploit the transfer apparatus of a co-resident conjugative plasmid to trigger their own transfer (Fig. 2.6) (Fernández-López *et al.*, 2014). Usually, mobilisable plasmids encode a relaxase and its cognate *oriT*. After DNA processing at the *oriT*, the relaxase of the mobilisable plasmid can be recruited to the T4SS by the CP of the conjugative plasmid (Fig. 2.6 (C)). Some mobilisable plasmids encode their own CP. In this case, transfer will only occur if the CP of the mobilisable plasmid is compatible with the co-resident T4SS (Fig. 2.6 (D)). In both cases, the *oriT* is nicked and bound by its cognate relaxase, thus the mobilisation mechanism is referred to as "relaxase-*in cis* mobilisation" (Smillie *et al.*, 2010; Ramsay and Firth, 2017).

Besides conjugative and mobilisable plasmids, a third type of plasmids, called non-mobilisable, do not encode any of the conjugation machineries. Among these non-mobilisable plasmids, some were shown to carry a locus that could be recognised by a compatible conjugative relaxase. The latter can thus nick and bind to the *oriT*-mimicking sequence, prior to recruitment by the CP to the mating channel (Fig. 2.6 (B)). In this scenario, the *oriT* mimic is bound by a relaxase encoded by a different plasmid, and the mechanism is therefore called "relaxase-*in trans* mobilisation" (Ramsay *et al.*, 2016; Ramsay and Firth, 2017). The transfer frequencies observed for *in trans* mobilisation are usually lower than for conjugation or *in cis* mobilisation. This might be explained by stronger *cis* affinity of relaxases (O'Brien *et al.*, 2015).

One last mechanism of mobilisation is often associated with the conjugative transfer of ICEBs1-like elements. Some non-mobilisable plasmids replicate by rolling-circle and encode a replicative relaxase. Such relaxase is involved in both replication and conjugation-like mechanisms. Indeed, a replicative relaxase can directly interact with a compatible co-resident T4SS and trigger conjugal transfer (Lee *et al.*, 2012).

2.3 Conjugation in Gram-positive bacteria

Although conjugation was mostly studied among Gram-negative bacteria, substantial work was performed to elucidate the mechanisms of conjugal DNA transfer between Gram-positive bacteria (Grohmann *et al.*, 2003;

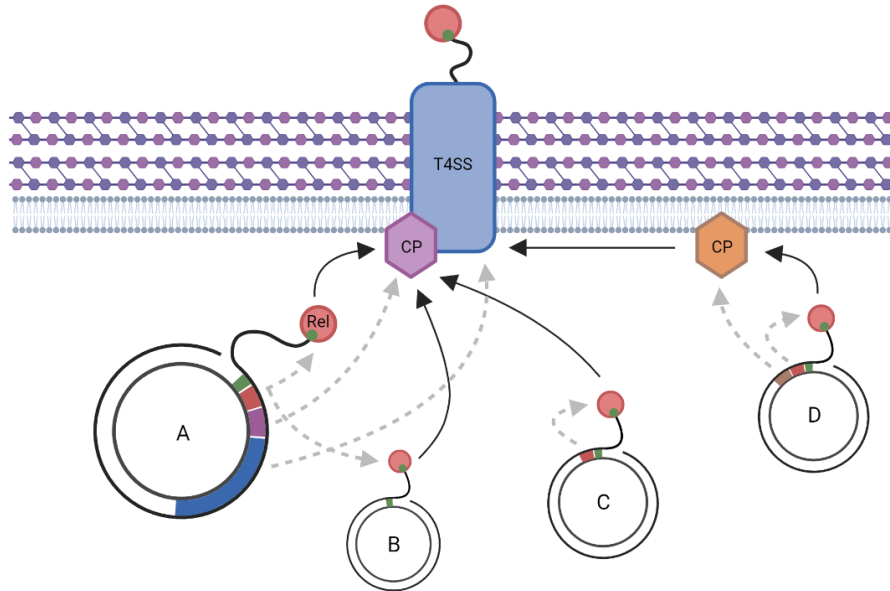


Figure 2.6: Plasmid transfer by conjugation-related mechanisms. (A) Conjugative plasmids carry genes coding for a T4SS (blue), a coupling protein (CP, purple or brown) and a relaxase (Rel, red), as well as an *oriT* sequence (green). (B) Some non-mobilisable plasmids possess a sequence mimicking the *oriT* of a conjugative plasmid, which can induce "*in trans* mobilisation" upon recognition by a compatible relaxase. (C) Mobilisable plasmids usually encode a relaxase and its cognate *oriT*. The mobilisable relaxase can be recruited by the CP of a co-resident conjugative plasmid, triggering "*in cis* mobilisation". (D) Some mobilisable plasmids encode their own CP in addition to a relaxase and an *oriT*. Full black arrows illustrate the interactions between the relaxase and the CP, or between the mobilisable plasmid-encoded CP and the T4SS. Dotted gray arrows indicate the genes encoding the different transfer elements. Adapted from Ramsay *et al.* (2016); Ramsay and Firth (2017), using Biorender.com

Goessweiner-Mohr *et al.*, 2013a; Grohmann *et al.*, 2017). Most Gram-positive conjugative systems studied to date depend on the canonical VirB/D4-like T4SS. Another type of transfer system was developed by *Streptomyces* spp. (Thoma and Muth, 2015). This system is T4SS-independent and relies on a single translocase to propagate dsDNA across mycelia. As the dsDNA transfer was so far only described for *Streptomyces*, this section rather focuses on the more widespread ssDNA transfer mechanism encoded by Gram-positive

MGEs. With increasing knowledge on such mechanism, some thoroughly studied conjugative systems have emerged as prototypes for Gram-positive conjugation.

The main steps of DNA transfer are conserved between Gram-positive and Gram-negative bacteria: DNA is first processed in the donor cell by a relaxase protein at the *oriT*, then recruited to a T4SS-like by a CP, transferred and replicated in the recipient cell, while the strand remaining in the donor cell is also converted into dsDNA (Grohmann *et al.*, 2018). The DNA processing mechanism prior to transfer and the respective roles of the relaxase and the *oriT* are particularly conserved (Grohmann *et al.*, 2003). Despite the significant divergence in the architecture of the cell wall of Gram-negative and Gram-positive bacteria, functional homologues to VirB1, 4, 6, 8 and VirD4, as well as VirB3 in some cases, were identified in most T4SS from Gram-positive conjugative plasmids (Table 2.2). Unsurprisingly since Gram-positive bacteria lack an OM, no homologues to the OMCC components (VirB7, 9 and 10) were retrieved from any Gram-positive T4SS so far (Walldén *et al.*, 2010). Gram-positive T4SS also usually lack a VirB11 homologue and pilus-associated proteins, as the target cell is attached through adhesins (or other means) instead of a conjugative pilus. Compared to their Gram-negative counterparts, Gram-positive T4SS exhibit an overall lower level of complexity, and are therefore often depicted as minimised-T4SS (Goessweiner-Mohr *et al.*, 2013a).

The main models for conjugation in Gram-positive bacteria are encoded by plasmids or ICEs of *Enterococcus*, *Streptococcus*, *Staphylococcus*, *Bacillus* and *Clostridium*. The conjugation genes of these models are usually organised in operons (Fig. 2.7). The main components of these conjugative systems are listed in Table 2.2 and some of their specificities are detailed in the following sections.

Table 2.2: Main constituents of the model plasmids and ICEs for conjugation in Gram-positive bacteria. Each model encodes the main components of T4SS as described for Gram-negative conjugation, except for the OMCC. VirB4-like: VirB4-like motor ATPase; VirB6/8/3-like: channel components; -: not known.

Function	pIP501	pCF10	pCW3	ICEBs1
Relaxase	TraA	PcfG	TcpM	NicK
<i>oriT</i>	Upstream <i>tra</i>	Between <i>pcfE</i> and <i>pcfF</i>	Between <i>tcpM</i> and <i>tcpK</i>	Spanning 5'-end of <i>nicK</i>
VirB4-like	TraE	PrgJ	TcpF	ConE
CP	TraJ (+ TraI)	PcfC	TcpA	ConQ
PG hydrolase	TraG	PrgK	TcpG	CwlT
VirB6-like	TraL	PrgH	TcpH	ConG
VirB8-like	TraM + TraH	PrgD + PrgL	TcpC	ConB
VirB3-like	-	-	TcpD	ConD

2.3.1 Model conjugative plasmids in streptococci, enterococci and staphylococci

T4SS-like conjugative plasmids have been reported from diverse streptococci, staphylococci and enterococci, including pathogenic strains of these genera. The best studied T4SS-like are those of pIP501 and pCF10. pIP501 belongs to the Inc18 (incompatibility group 18) family of plasmids, whose representatives provide resistance to vancomycin and the macrolide, lincosamide and streptogramin (MLS) group of antibiotics. Inc18 plasmids are well known for disseminating resistance to MLS and vancomycin throughout enterococci, participating in the global rise of pathogenic multi-resistant bacteria and nosocomial infections (Palmer *et al.*, 2010; Kohler *et al.*, 2018b). First isolated from *Streptococcus agalactiae* (Horodniceanu *et al.*, 1979), pIP501 displays the broadest host spectrum known so far among Gram-positive conjugative plasmids, and successfully transfers to Gram-positive bacteria, including *Enterococcus* spp., *Staphylococcus* spp., *Listeria* spp. and *Streptomyces* spp., as well as to the Gram-negative *E. coli* (Kurenbach *et al.*, 2003). pCF10 is a pheromone-responsive plasmid from *Enterococcus faecalis* (Dunny *et al.*, 1982). It is a major model for sex-pheromone regulation of conjugative plasmids.

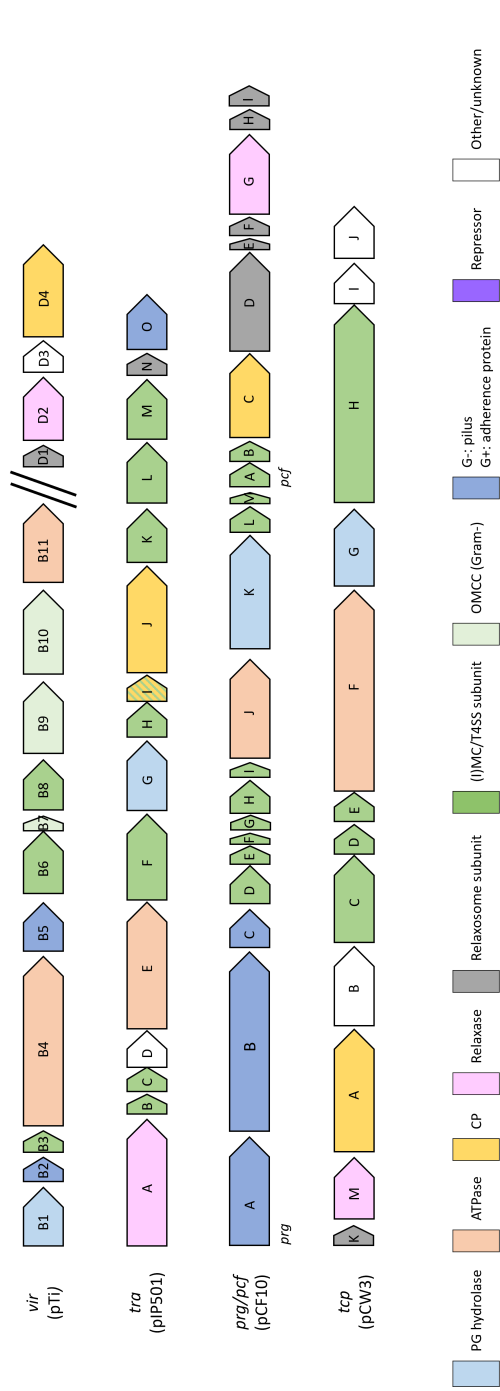


Figure 2.7: Genetic organisation of the conjugative loci of different Gram-positive conjugative plasmids and the canonical VirB/D4 system. Genetic organisation of the *tra* locus from pIP501, *prg/pcf* locus from pCF10 and *tcp* from pCW3 are compared to the *vir* system of pTi. Arrows of the same colour represent genes encoding functionally homologous proteins. (I)MC: (inner-) membrane complex; OMCC: outer-membrane core complex. Adapted from Goessweiner-Mohr *et al.* (2013a).

Both plasmids share strong similarities in terms of protein components of their conjugative systems (Fig. 2.7) (Bhatty *et al.*, 2013). Conjugation proteins are encoded in a single 14-kb operon, *tra*, for pIP501 (Kurenbach *et al.*, 2002; Alvarez-Martinez and Christie, 2009; Arends *et al.*, 2013; Goessweiner-Mohr *et al.*, 2013a,b, 2014; Fercher *et al.*, 2016), whereas for pCF10, the transfer genes are encoded in the Prg/Pcf cassette of the *prgQ* operon (Staddon *et al.*, 2004, 2006; Chen *et al.*, 2008; Li *et al.*, 2012; Bhatty *et al.*, 2013; Goessweiner-Mohr *et al.*, 2013a; Sterling *et al.*, 2020; Jäger *et al.*, 2022) (Fig. 2.7 and Table 2.2). The conjugative system of both pIP501 and pCF10 relies on adhesins, respectively TraO and PrgA, B and C to establish a mating contact between donor and recipient cells, in place of the Gram-negative conjugative pilus (Goessweiner-Mohr *et al.*, 2013a; Bhatty *et al.*, 2013).

Regulation of pIP501 conjugation

The signal preceding plasmid processing by the relaxase TraA prior to conjugal transfer has not been identified yet (Kohler *et al.*, 2019). On the other hand, TraA was shown to negatively regulate the transcription of the other *tra* genes by binding to the *tra* promoter P_{tra} (Kurenbach *et al.*, 2006). Additionally, a small cytosolic protein, TraN, was shown to bind to its cognate site upstream P_{tra} and the *oriT*, thereby further repressing pIP501 conjugation (Kohler *et al.*, 2018a). TraN can also bind to a second promoter, P_{traNO} , probably fine-tuning the expression of the TraO adhesin (Fig. 2.8).

Regulation of pCF10 by pheromone balance

The *prgQ* operon of pCF10 is tightly controlled to ensure an appropriate expression of the Prg/Pcf proteins. The major transcriptional regulator of *prgQ*, PrgX, binds to the *prgQ* promoter, P_Q , and constitutively represses the transcription of the operon in non-induced cells (Kozłowicz *et al.*, 2004). Two peptides can bind to binding sites in the C-terminal domain of PrgX: cCF10, an inducer pheromone encoded by chromosomal genes (production by donor and recipient cells), and iCF10, an inhibitor encoded by genes of the *prgQ* operon (production by donor cells only) (Nakayama *et al.*, 1994; Dunny, 2007; Kohler *et al.*, 2019; Sterling *et al.*, 2020). The balance between cCF10 and iCF10 defines the induction or the repression of P_Q and the transcription of the *prgQ* locus

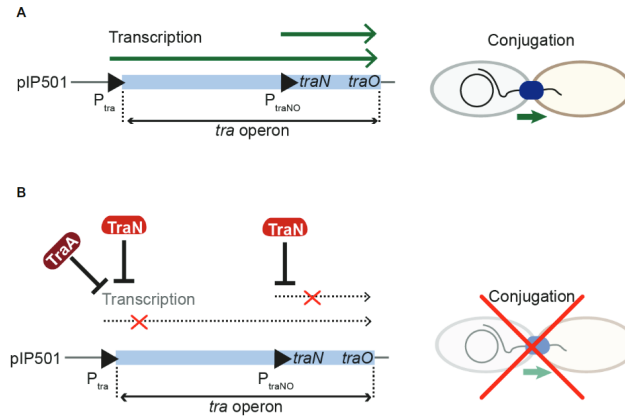


Figure 2.8: Regulation mechanism of pIP501 *tra* operon. The transcription of *tra* genes is modulated by two promoters, P_{tra} and P_{traNO} . (A) Transcription of *tra* genes leads to the production of the conjugation machinery, and TraA is expressed and can regulate the levels of *tra* gene transduction by binding to P_{tra} . (B) Both TraA and TraN can regulate the levels of *tra* genes transduction by binding to P_{tra} and TraN can further repress P_{traNO} . Such binding creates a feedback loop down-regulating the expression of the Tra proteins and inhibiting conjugation (Kohler *et al.*, 2019).

(Fig. 2.9). Binding of cCF10 on its cognate binding site on PrgX induces conformational changes that free P_Q for RNA polymerase access and subsequent transcription of the *prgQ* genes (Shi *et al.*, 2005). Enhanced transcription from P_Q increases the levels of iCF10, which competes with cCF10 for binding on PrgX. Contrary to cCF10, binding of iCF10 is thought not to alter the structural conformation of PrgX, which naturally prevents the RNA polymerase from accessing P_Q (Shi *et al.*, 2005). Binding of iCF10 therefore results in transcriptional repression of the *prgQ* operon (Kozłowicz *et al.*, 2006). In addition to iCF10-mediated conjugation inhibition, self-induction of pCF10-carrying cells is prevented by the plasmid-encoded membrane protein PrgY. The extracellular domain of this protein was proposed to interact with cCF10, reducing the import of inducing molecules into the cell (Chandler *et al.*, 2005).

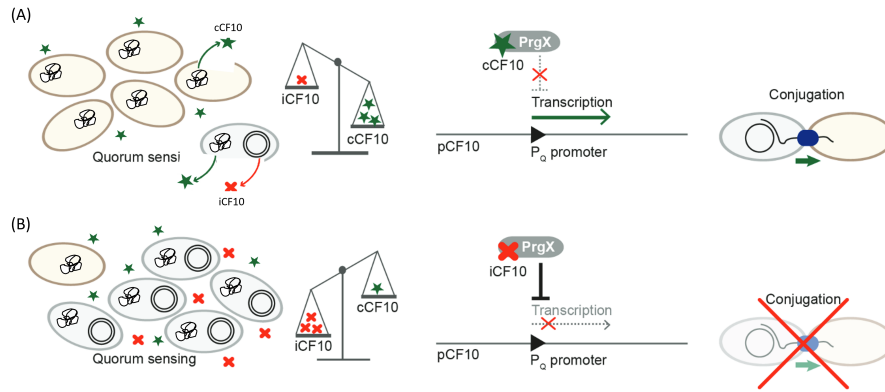


Figure 2.9: Regulation mechanism of pCF10 conjugation by the competing peptides ccF10 and iCF10. (A) When a majority of neighbouring cells lack pCF10, the relative ratio ccF10/icF10 increases. More cCF10 peptides are imported into donor cells and bind to PrgX, which interferes with the repression of P_Q promoter and leads to the transcription of *prgQ* genes. Conjugation is then activated and pCF10 transfer takes place. (B) When the majority of the bacterial population carries pCF10, the ratio cCF10/iCF10 decreases, leading to an increased import of iCF10. Binding of iCF10 on PrgX represses the P_Q promoter, thus inhibiting the expression of the *prgQ* locus and the conjugation mechanism. Adapted from Kohler *et al.* (2019).

2.3.2 Model conjugative plasmid in *Clostridia*

In the human and animal pathogen *Clostridium perfringens*, many disease-related toxins are encoded on pCW3-like plasmids, forming the Tcp plasmid family. Many Tcp plasmids were experimentally shown to be conjugative, and the conjugation system was extensively studied for the archetype pCW3 (Rood *et al.*, 1978; Bannam *et al.*, 2011; Wisniewski and Rood, 2017). The conjugation locus of pCW3, *tcp*, contains the 11 genes *tcpA* to *tcpJ* and *tcpK* and *tcpM* (Fig. 2.7), which encode the T4SS-like apparatus of pCW3 (Table 2.2) (Bannam *et al.*, 2006; Parsons *et al.*, 2007; Teng *et al.*, 2008; Bantwal *et al.*, 2012; Porter *et al.*, 2012; Goessweiner-Mohr *et al.*, 2013a; Wisniewski *et al.*, 2015, 2016; Traore *et al.*, 2018).

Upstream of the *tcp* locus is located the *cna* region, highly conserved among pCW3-like plasmids and spanning the genes from *cnaC* to *dam* (Fig. 2.10) (Revitt-Mills *et al.*, 2021). *cnaC* encodes a putative adhesin that might be

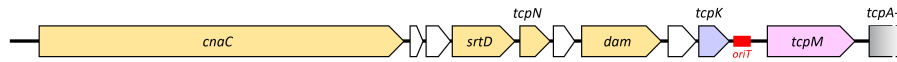


Figure 2.10: Genetic organisation of the *cna* region, located upstream of the *tcp* locus. The *cna* region encodes the genes from *canC* to *dam*. Arrows in pale yellow represents the *cnaC*, *srtD*, *tcpN* and *dam* genes, important for pCW3 conjugal transfer. The pale purple arrow represents the accessory protein TcpK, while the pale pink arrow represents the relaxase TcpM (as shown in Fig. 2.7). The *oriT* is represented by the red box between *tcpK* and *tcpM*. Blank arrows represent genes encoding unknown functions. Adapted from Traore *et al.* (2018) and Revitt-Mills *et al.* (2021).

involved in the formation of intimate contact between donor and recipient cells (Bannam *et al.*, 2006). The second predicted protein SrtD is a putative sortase, potentially anchoring CnaC to the cell surface (Bannam *et al.*, 2006; Bhatta *et al.*, 2013). The last predicted protein Dam, a putative methyl-transferase, could be involved in regulation of pCW3 conjugation through methylation (Bannam *et al.*, 2006; Revitt-Mills *et al.*, 2021). These three proteins, together with TcpN, were shown to enhance the conjugal transfer of pCW3, although their exact roles remain to be elucidated (Revitt-Mills *et al.*, 2021).

2.3.3 Model conjugative elements in *Bacilli*

Two conjugative elements are often presented as models for conjugation among *Bacilli*: the conjugative plasmid pLS20 and the conjugative transposon ICEBs1, both originating from *B. subtilis*. Conjugation is also at the heart of gene transfers in the *B. cereus* group. The importance of conjugation and the conjugative plasmids of this bacterial group are described in the next chapters (see Chapter 3 and Chapter 4).

The *B. subtilis* conjugative plasmid pLS20

pLS20 is a well-known conjugative plasmid originating from *B. subtilis* (Tanaka and Koshikawa, 1977; Koehler and Thorne, 1987). Mostly the regulatory pathways controlling pLS20 conjugal transfer were studied, but recent

data provided insights into its transfer apparatus. pLS20 sequence presents a modular structure and includes a conjugation module that occupies more than half of the plasmid sequence. The conjugation module carries genes involved in the regulation of conjugation, genes involved in mating pair formation, the genes coding for the transfer apparatus and for the relaxosome and genes responsible for ssDNA/dsDNA conversion (Val-Calvo *et al.*, 2021). The T4SS-encoding locus includes genes encoding homologues to the channel components VirB6 and 8, the ATPases VirB4, 11 and D4 and a PG hydrolase. About fifteen additional genes are clustered with the *virB/D* homologues and are therefore probably involved in pLS20 transfer complex. The relaxase of pLS20, RelLS20, shares only low levels of similarities with other known relaxases and was proposed to constitute a novel family of relaxases (see Table 2.1) (Ramachandran *et al.*, 2017). The *oriT* was delineated in the intergenic region preceding two genes encoding auxiliary proteins, Aux1 and Aux2, recently demonstrated as accessory proteins of pLS20 relaxosome (Ramachandran *et al.*, 2017; Crespo *et al.*, 2022).

The details about the regulation of pLS20 regulation will not be explained here. Briefly, the conjugation is repressed by default, and only activated during a brief time window at the end of the exponential growth phase. Inter-cellular signalling intervenes to detect favourable conditions for conjugation. Contrary to pCF10 pheromone-mediated regulation, the system employed by pLS20 does not activate conjugation, but rather tightly regulates it (Singh *et al.*, 2013). The second major layer of regulation consists in different regulators acting on the main promoter of the conjugation operon to strictly repress gene expression, all the while allowing quick activation (Meijer *et al.*, 2021).

The *B. subtilis* conjugative transposon ICEBs1

Unlike conjugative plasmids, ICEs integrate into their host chromosome, where they are replicated and vertically transmitted by cell division. Specific conditions can induce the excision of ICEs, which then form plasmid-like structures, subsequently transmissible by conjugation. Upon arrival in the recipient cell, ICEs are recircularised into dsDNA plasmid-like molecules before recombining into the chromosome (Johnson and Grossman, 2015). The T4S apparatus of ICEBs1 contains the typical Gram-positive T4SS components

(Table 2.2) (Lee and Grossman, 2007; Fukushima *et al.*, 2008; Berkmen *et al.*, 2010; Lee *et al.*, 2012; DeWitt and Grossman, 2014; Leonetti *et al.*, 2015; Auchtung *et al.*, 2016).

In absence of induction, the expression of ICEBs1 genes is tightly repressed by binding of an immunity repressor (ImmR) onto the promoter. ImmR also regulates its own transcription and that of an activator gene, ImmA, by binding onto the respective promoters. The two main conditions promoting the excision and transfer of ICEBs1 are the activation of the SOS response and high concentrations of potential recipient cells lacking ICEBs1 (Auchtung *et al.*, 2005). Both pathways lead to the activation of ImmA, that cleaves ImmR, therefore allowing ICEBs1 gene expression (Bose *et al.*, 2008). In the recipient cell, newly-acquired ICEBs1 prevents from the acquisition of another copy of ICEBs1 by immunity functions. ImmR expression was shown to drastically reduce ICEBs1 transfer, but other yet unknown factors probably also play a role in immunity (Auchtung *et al.*, 2007, 2016).

The pXO16 story

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The intriguing conjugative system of the *Bti* plasmid pXO16 was first mentioned by Reddy *et al.* (1987). At the time, a conjugation-like event was indirectly witnessed, involving the transfer of a mobilisable plasmid, pBC16, triggered by a large plasmid, named pXO16. Mobilisation occurred between a donor strain of *Bti* and recipient strains of *B. cereus* and *B. anthracis*. Further analyses indicated that pXO16 was not involved in the synthesis of Cry toxins and that no specific characteristic, other than self-transmissibility, could be attributed to the plasmid (Reddy *et al.*, 1987). Although the conjugative properties of pXO16 were thoroughly investigated by different research teams, conjugation still remains the only feature attributed to pXO16. Much attention was nonetheless given to the phenotypic description of pXO16, such as the plasmid-induced aggregation phenotype, as well as to the genetics underlying its powerful conjugation mechanism.

3.1 Aggregation-mediated conjugation

Andrup *et al.* (1993) studied in more details the mobilisation of small plasmids between strains of *Bti* and noticed the formation of macroscopic cell aggregates when mixing donor and recipient cells in broth medium during matings (Fig. 3.1). Aggregation-mediated mechanisms had previously been reported for other conjugative systems. The most studied example is the

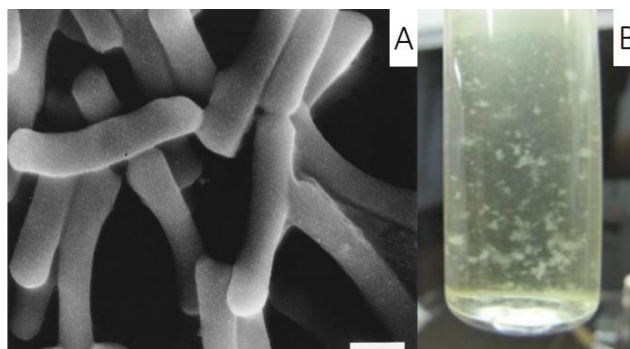


Figure 3.1: Aggregates formed by donor and recipient cells of *Bti* in liquid mating mixture. (A) Scanning electron micrograph of a mating mixture of donor and recipient cells; white bar = 1 μm (Andrup *et al.*, 1996). (B) Macroscopic aggregates observed in a liquid mating mixture of donor and recipient cells.

conjugative system of *E. faecalis*, which involves clumping of donor cells under the influence of a recipient-borne sex pheromone (Dunny *et al.*, 1978). Cell aggregates also mediate plasmid transfer in conjugative systems of some lactic bacteria, such as *Lactococcus lactis* and *Lactobacillus plantarum*. In *L. lactis*, the aggregation-inducing factor is chromosomally encoded (Walsh and McKay, 1981; Van Der Lelie *et al.*, 1991). On the contrary, the aggregation-mediated conjugation system of *L. plantarum* is neither plasmid-encoded nor plasmid-specific and facilitates the formation of mating pairs for various conjugative plasmids (Reniero *et al.*, 1992).

For the aggregation phenomenon of *Bti*, strains were classified based on two phenotypes, Agr^+ and Agr^- (Andrup *et al.*, 1993). Strains of both phenotypes could form aggregates when mixed together, but not independently, and the Agr^+ phenotype could be unidirectionally transferred to cells of the Agr^- alongside plasmid mobilisation. The presence of pXO16 was then connected with the Agr^+ phenotype, suggesting that pXO16 was actually encoding the genetics underlying aggregation (Jensen *et al.*, 1995). The role of cell surface components was further established in the aggregation-mediated conjugative transfer, especially surface-layer (S-layer)-like proteins (Wiwat *et al.*, 1995). Indeed, treating conjugative cells with anti-S layer proteins

antibodies inhibited the transfer of pXO16 between mating partners. Yet, little information was given on the exact nature of these S-layer proteins. More recently, S-layer proteins were explained to form the outermost layer of many micro-organisms, in direct contact with the external environment and often involved in surface interactions, like aggregation (Gerbino *et al.*, 2015).

The host spectrum of pXO16 was initially explored by following the transmission of the aggregation phenotype (Jensen *et al.*, 1996). Agr^+ cells, chosen as donors of the pXO16-encoded *Agr* phenotype, appeared limited to strains of *Bti*. Only Agr^- strains, *i.e.* strains able to form aggregates with the donor Agr^+ strain but not with other Agr^- strains, were considered as potential recipients of pXO16. These Agr^- strains encompassed strains of eleven *B. thuringiensis* serovars and three *B. cereus* strains (Jensen *et al.*, 1996). Strains unable to form aggregates with the donor strain were ruled out of the host spectrum of the plasmid, because aggregation was taken as a prerequisite for pXO16 conjugative transfer. Transconjugants were defined as such when they could become donors and form aggregates with other Agr^- recipient strains (Jensen *et al.*, 1996). pXO16 was demonstrated to transfer to seven subspecies of *B. thuringiensis* and two strains of *B. cereus* out of the 14 Agr^- strains considered, and to reach 100% of transfer efficiency between strains of *Bti* within a few hours (Jensen *et al.*, 1996). Besides, pXO16 was shown to successfully induce the transfer of both mobilisable and non-mobilisable co-resident plasmids (Andrup *et al.*, 1996).

3.2 Kinetics of transfer and DNA capture properties of pXO16

Andrup *et al.* (1998) published a thorough study of pXO16 kinetics of transfer, further detailed by Timmery *et al.* (2009). Several key characteristics of pXO16 transfer can be retrieved for these studies. (i) pXO16 transfers at high-speed, with a transfer rate of *ca.* 1 kb/s, resulting in a total transfer of the plasmid in less than 5 minutes. (ii) Inside the aggregates gathering thousands of cells, mating pairs are formed by one donor and one recipient cell. (iii) pXO16 is most likely a low copy number-plasmid as one donor cell can only

transfer the plasmid to one recipient cell at a time. (iv) Primary transfer of pXO16 can be detected within 10 min and peaks at 40-50 min after mating onset. (v) A same donor cell requires a 10-min recovery period between two successive transfer events. (vi) Secondary transfer can happen, *i.e.* newly formed transconjugants can turn into donors, after a 40-min maturation time.

The original description of pXO16-mediated plasmid transfer actually reported the displacement of the mobilisable plasmid pBC16 by a mobilisation process (Reddy *et al.*, 1987). More insights into the plasmid capture properties of pXO16 were gathered by studying plasmid transfer under the light of three related mechanisms: conjugation, mobilisation and retromobilisation (Timmery *et al.*, 2009). Retromobilisation can be defined as the "capture of DNA from a recipient by a donor cell harbouring a conjugative plasmid" (Timmery *et al.*, 2009). The presence of *mob* genes, *i.e.* genes encoding a relaxase or other relaxosome components, and *oriT*-like sequences on plasmids positively affected their mobilisation or retromobilisation by pXO16. Indeed, mobilisation efficiency was greater for *mob*⁺ (or mobilisable) plasmids than for *mob*⁻ (or non-mobilisable) plasmids. Mobilisation frequencies were also always higher than frequencies of retromobilisation events. Furthermore, pXO16-mediated retromobilisation was defined as following a successive, two-step model, meaning that two distinct transfer events take place. First, the conjugative plasmid is transferred from the donor to the recipient, and then the small plasmid is transmitted from the recipient to the donor cell (Timmery *et al.*, 2009). Recent studies reported that pXO16 could also trigger the displacement of chromosomal loci between mating partners (Makart *et al.*, 2017). Chromosomal fragments of at least 791 kb could be transferred between strains of *Bti*, which corresponds to twice the size of the plasmid itself (Makart *et al.*, 2018).

pXO16 transfer and capture capacities were also evaluated under different ecological and food-related conditions. Successful transfer was reported under different temperature conditions and in several environmental substrates, such as river water, dead dipteran larvae, and the intestine of gnotobiotic rats (Thomas *et al.*, 2001; Wilcks *et al.*, 2008). pXO16-mediated conjugation and mobilisation mechanisms were also apprehended in liquid and semi-liquid food

products, especially dairy products (cow milk and rice pudding) and plant-based drinks (soy and rice-based drinks) (Van der Auwera *et al.*, 2007; Timmerly *et al.*, 2009). Conjugation, mobilisation and retromobilisation in milk and soy-based drink were reported at frequencies similar to frequencies obtained in LB broth, while frequencies were on average 10-fold lower in rice-based drink (Timmerly *et al.*, 2009). Both conjugation and mobilisation also occurred in rice pudding (Van der Auwera *et al.*, 2007). Biparental matings, implicating one donor and one recipient cell, are great laboratory tools, but in natural environments, bacteria usually live in more complex organisations, like biofilms, where they encounter multiple potential partners at a time. Therefore, pXO16 transfer capacities were also assessed under triparental mating settings, with one donor cell carrying the conjugative plasmid, one helper cell containing a small non-self-transmissible plasmid, and one recipient cell. Overall, pXO16 transfer was as efficient in the more complex triparental matings as in simpler biparental settings (Timmerly *et al.*, 2009).

3.3 Genetic features underlying pXO16 conjugation

Despite detailed work on phenotypical features of pXO16 transfer, little was known about the genetics underlying the mechanism of pXO16 conjugation until its sequence was thoroughly analysed (Makart *et al.*, 2015). Bioinformatics analyses on the 434 CDS (coding DNA sequences) that cover 86.6% of the 350-kb sequence revealed functional annotations or relevant homologies for only 94 CDS. The other 340 CDS, *ca.* 80% of total CDS, had homologues lacking functional annotations or lacked significant homologues (Fig. 3.2). Among the few CDS with a functional annotation, none was related to any T4SS identified so far, suggesting that pXO16 conjugative system is rather unique (Makart *et al.*, 2015). Furthermore, no potential replication initiator was retrieved from sequence analyses. However, several CDS located within a same region displayed functional annotations or relevant homologues annotated with replication-related functions, such as primases, helicases, topoisomerases or polymerase subunits (replication region in Fig. 3.2).

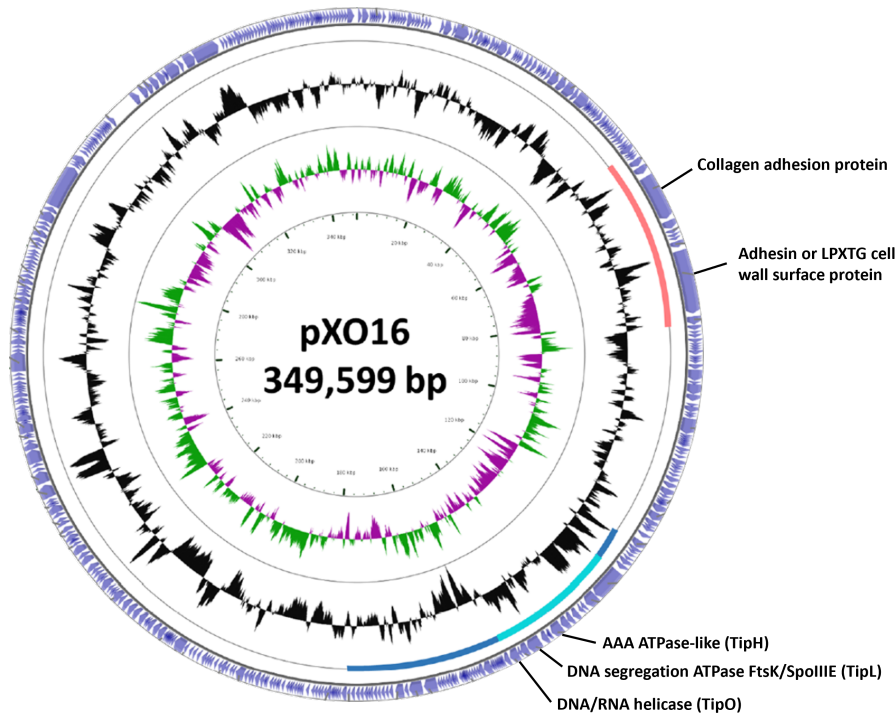


Figure 3.2: Circular map of pXO16. The blue block arrows in the outer circle indicate the predicted CDS in their direction of transcription, with or without functional annotation. The black circle represents the GC content, and the green/purple circle represents the GC skew. The pink and dark blue bars represent the aggregation and replication regions, respectively. The light blue bar inside the replication region illustrates the *tip* locus as delimited by Makart *et al.* (2018). Adapted from Gillis *et al.* (2018).

Based on detailed bioinformatics studies and on experimental data, a 25-kb locus involved in conjugation was delimited within the replication region (Makart *et al.*, 2018). This *transfer of israelensis plasmid (tip)* locus is composed by 16 CDS, *tipA* to *tipP* (Fig. 3.3). Two *tip* genes, *tipH* and *tipL* encode ATPases that were demonstrated as essential for pXO16 conjugation. TipH and TipL are considered as the putative motor ATPase and CP of pXO16 transfer apparatus, respectively (Makart *et al.*, 2018). TipA, TipE, TipI and TipJ are predicted as membrane proteins, among which TipA and TipE were proposed as T4SS channel components. TipB shares some similarities with NlpC/P60 family proteins and also contains two TMDs, and is therefore

a candidate for the PG hydrolase function (Makart *et al.*, 2018). Missing elements for a functional conjugative apparatus are the elements of the relaxosome, especially a relaxase and an *oriT*. *tipO* encodes a putative helicase that could act as an accessory protein in the early steps of pXO16 transfer. No potential relaxase was identified by bioinformatics analyses, although *tipP* was considered as a relaxase gene candidate due to its proximity with the putative accessory helicase gene *tipO* (Makart *et al.*, 2018).

The interest for pXO16-mediated aggregation got renewed when the genes encoding the aggregation proteins were localised within the 27-kb *agr* region of pXO16 sequence (aggregation region in Fig. 3.2) (Makart *et al.*, 2015). This region encodes 10 CDS, including two predicted cell surface proteins, one sortase and two large surface-exposed proteins of ~2,500 and ~3,500 amino acids (aa) predicted as putative adhesins with one collagen-binding B-type domain and one cell-wall anchoring LPXTG domain (Feuillie *et al.*, 2018). After over two decades of hypothesising that aggregation was a prerequisite for pXO16 transfer, an aggregation-defective mutant was constructed and a reduced, yet effective, transfer was demonstrated in absence of the aggregation proteins (Makart *et al.*, 2018). Although important, the aggregation mechanism was thus discovered as dispensable for pXO16 transfer. Based on this new assumption, some previously overlooked non-aggregating (neither Agr⁺ nor Agr⁻) *B. cereus* s.l. strains were reported to successfully act as recipients of pXO16 in liquid matings (Makart *et al.*, 2018).

Identifying the *agr* region and elaborating the *agr*-deficient pXO16 also opened up new possibilities for studying *Bti* aggregation mechanism. For example, the adhesion forces involved in the presence and absence of pXO16 were evaluated under atomic force microscopy (AFM) (Feuillie *et al.*, 2018). It was shown that pXO16 mediates strong adhesion forces (~2nN) between mating cell partners and that surface proteins encoded in the *agr* region mediate specific interactions between mating partners, resulting in strong adhesion (Feuillie *et al.*, 2018).

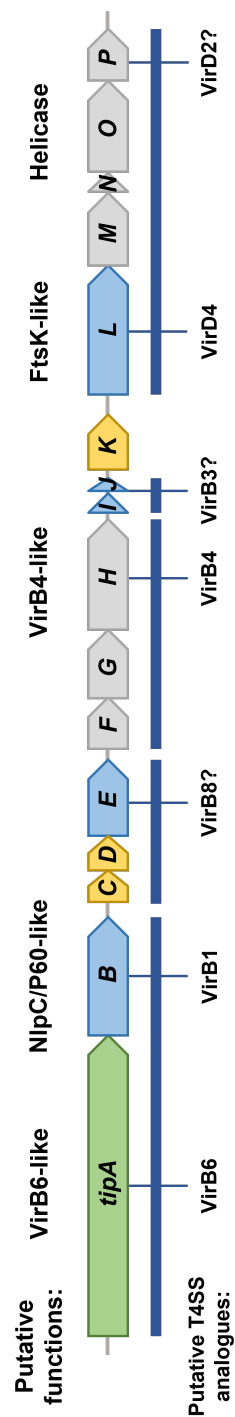


Figure 3.3: Genetic map of the *tip* locus. The 16 genes composing the *tip* locus are represented by arrows and alphabetically named from *tipA* to *tipP*. Probable functions of TipA, TipB, TipH, TipL and TipO are indicated above the corresponding genes. Putative Vir T4SS analogues are noted under the corresponding genes. Genes coloured in blue code for proteins with at least one predicted TMD, genes in yellow encode proteins with a predicted signal peptide and *tipA* is in green as a merge of blue and yellow, meaning that it codes for a protein with both a predicted TMD and a predicted signal peptide. Blue bars indicate the genes encoded on a same *in silico* predicted operon. Adapted from Makart *et al.* (2018).

Conjugation among *B. cereus* group members

Members of the *B. cereus* group carry a large number of plasmids, some of which are transmissible and/or toxin-encoding, that play a central role in the differentiation and specification of the group species. Horizontal transmission of plasmids within the *B. cereus* group occurs via conjugation and conjugation-related mechanisms. Plasmid transfer can raise concerns by moving about toxin genes and blurring the lines between the different species forming the *B. cereus* group. This section aims at collecting and reviewing data gathered about conjugation among *B. cereus* group members since the first report of a conjugation-like event within this bacterial group. It includes the study of various conjugative plasmids, of different plasmid families and of conjugation under various environmental conditions. It also presents a thorough analysis of conjugative plasmid candidates identified within *B. cereus s.l.* genomes available in public databases. Because this work reviews all data available to date, pXO16 and its conjugative properties are also included. Therefore, the section of the review concerning pXO16 might be redundant with the previous chapter of this manuscript (Chapter 3) and with further sections detailing data collected during this thesis (Chapters 6 and 7).

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Conjugation across *Bacillus cereus* and kin: a review

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Abstract

Horizontal gene transfer (HGT) is a major driving force in shaping bacterial communities. Key elements responsible for HGT are conjugation-like events and transmissible plasmids. Conjugative plasmids can promote their own transfer as well as that of co-resident plasmids. *Bacillus cereus* and relatives harbor a plethora of plasmids, including conjugative plasmids, which are at the heart of the group species differentiation and specification. Since the first report of a conjugation-like event between strains of *B. cereus sensu lato* (s.l.) forty years ago, many have studied the potential of plasmid displacement across the group, especially for plasmids encoding major toxins. Over the years, more than twenty plasmids from *B. cereus* isolates have been reported as conjugative. However, with the increasing number of genomic data available, *in silico* analyses indicate that more plasmids from *B. cereus* s.l. genomes present self-transfer potential. *B. cereus* s.l. bacteria occupy diverse environmental niches, which were mimicked in laboratory conditions to study conjugation-related mechanisms. Laboratory mating conditions remain nonetheless simplistic compared to the complex interactions occurring in natural environments. Given the health, economic and ecological importance of strains of *B. cereus* s.l., it is of prime importance to consider the impact of conjugation within this bacterial group.

Keywords: *Bacillus anthracis*; *Bacillus cereus*; conjugation; plasmid; T4SS; *Bacillus thuringiensis*; *Bacillus mycoides*; *Bacillus cytotoxicus*

1. Introduction

Genetic diversity is key for long-term survival of various organisms, be it micro-ones as bacteria or fungi, all the way to plants and animals, in continuously changing ecosystems. For bacteria, although their reproduction is mostly asexual by binary fission, they have developed the capacity to diversify their genetic content and to acquire DNA material from relatively distant organisms, by horizontal gene transfer (HGT) (Arnold *et al.*, 2021). Defined as "the non-genealogical transmission of genetic material from one organism to another" (Goldenfeld and Woese, 2007), HGT has a strong impact on bacterial genomes by increasing the potential of acquiring advantageous genes, resulting in better adaptation and colonization of new ecological niches (Soucy *et al.*, 2015; McInerney *et al.*, 2017). Diverse mechanisms drive HGT among prokaryotes, and the three major mechanisms are natural transformation, transduction and conjugation (von Wintersdorff *et al.*, 2016). While transformation consists in the capture of foreign DNA from the environment (Mell and Redfield, 2014) and transduction is mediated by bacteriophages (a.k.a. phages) (Colavecchio *et al.*, 2017), conjugation requires a physical contact between the cell partners prior to transfer of plasmid DNA (de la Cruz *et al.*, 2010). Aside from the three major mechanisms of HGT, several non-canonical mechanisms of DNA transfer have been identified, including the so-called "gene-transfer agents", transfer via membrane vesicles and intercellular nano-tubes (Arnold *et al.*, 2021). Yet, these non-canonical modes of HGT are beyond the scope of this review.

The mechanism of conjugation – or conjugative transfer – occurs in a stepwise process and requires a secretion system, consisting of a type IV secretion system (T4SS), and a replication machinery to obtain two copies of the conjugative element at the end of the transfer event (Cabezón *et al.*, 2015). During this process, a relaxase first recognizes the origin of transfer (*oriT*) of the conjugative element, nicks the *oriT* in a strand-specific manner and remains bound to the nicked strand. The resulting nucleoprotein complex is then recruited to the T4SS by a coupling protein (CP) and replication of the intact strand takes place in the donor cell, displacing the nicked strand for transfer and further replication into the recipient cell (de la Cruz *et al.*,

2010). T4SS are usually made of three ATPases, including one CP and two motor ATPases, one peptidoglycan hydrolase and several membrane proteins inserted into the donor cell envelope and forming the core of the T4SS complex (for a detailed description of T4SS, refer to Ilangovan *et al.*, 2015 and Waksman, 2019). Studies have mainly focused on the conjugative systems of Gram-negative bacteria, but the global mechanism of conjugative transfer is conserved among Gram-positive bacteria, despite structural differences in the T4S machinery (for a review on Gram-positive conjugation systems, refer to Goessweiner-Mohr *et al.*, 2013).

Conjugative plasmids are capable of self-transfer and encode all the genetic material necessary for a functional conjugative apparatus. Some plasmids, called mobilizable plasmids, lack the genes encoding for the T4SS, but usually encode an *oriT* and a relaxase. They can hijack the transfer apparatus of a co-resident plasmid to trigger their transfer (Fernández-López *et al.*, 2014). Other plasmids remain however non-transmissible and are often called non-mobilizable, as they lack all the elements necessary for conjugation-like movements, *i.e.* an *oriT*, a relaxase and a T4SS (Smillie *et al.*, 2010).

As suggested by its large diversity of plasmids and mobile genetic elements (MGEs), the *B. cereus* group is of prime interest when it comes to conjugation and conjugation-related plasmid transfer. The *B. cereus* group, or *B. cereus sensu lato* (*s.l.*), refers to a group of closely related bacterial species, yet displaying a large ecological diversity. *B. cereus* group members are ubiquitous sporulating Gram-positive bacteria, mainly studied because several members of the group are of health, environmental or commercial interests or concerns (Ramarao *et al.*, 2015). Within this review, we look back at the earlier work as well as at the data collected on *B. cereus* conjugation and conjugative plasmids described so far. We also examine the proportion of potential conjugative plasmids among the *B. cereus s.l.* genome sequences available to date. Overall, we aim at providing a full picture on conjugative plasmids residing within the plasmid-rich group of *B. cereus* and their implications in both laboratory and environmental-like conditions, as known to date.

2. *B. cereus* s.l., plasmid-borne toxins and horizontal gene transfer

Three main species form the core of the *B. cereus* group: the opportunistic pathogen *B. cereus sensu stricto* (s.s.), potentially associated with foodborne infections, the etiological agent of anthrax, *B. anthracis*, and the entomopathogen *B. thuringiensis*, well-known for its biopesticide application (Kolstø *et al.*, 2009; Ehling-Schulz *et al.*, 2019). In addition, the *B. cereus* group includes other species, like the rhizoid-growing *B. mycoides* and *B. pseudomycoides* (Flügge, 1886; Nakamura, 1998), the psychrotrophic *B. weihenstephanensis* (Lechner *et al.*, 1998; Hoton *et al.*, 2009) – recently reclassified as one species with *B. mycoides* (Liu *et al.*, 2018) – and the thermotolerant and cytotoxic *B. cytotoxicus* (Guinebretière *et al.*, 2013). Diverse classification methods have been proposed over the years (for a recent review, refer to Carroll *et al.*, 2021) in an effort to resolve the ongoing taxonomic conflicts surrounding *B. cereus* s.l. due to (i) an insufficient differentiation at the genomic level and (ii) whether or not plasmids and MGEs should be included in the phylogenetic analyses. Members of *B. cereus* s.l. carry a wide range of plasmids, some of which are susceptible candidates for horizontal dissemination (Kolstø *et al.*, 2009; Yuan *et al.*, 2010). Although many studies presented evidence that MGEs and HGT can confound the phylogenetic process (Bazinnet, 2017), there is no denying the importance of extra-chromosomal entities in defining a species' lifestyle, hence their importance in the phylogenetic process (Liu *et al.*, 2015; Patiño-Navarrete and Sanchis, 2017).

In fact, the three main members of the *B. cereus* group are discriminated based on specific toxins they secrete, which are often encoded on large transmissible plasmids, or on specific plasmids they carry. For instance, *B. anthracis* is characterized by two large plasmids, pXO1 and pXO2, responsible for the production of the bipartite anthrax toxins and the anthrax capsule, respectively (Baillie and Read, 2001). Both pXO1 and pXO2 encode genes reminiscent of conjugation systems, but they show no ability for self-transfer, although they were proven as mobilizable (Grynberg *et al.*, 2007). Other plasmids share the backbone of pXO1 and pXO2 and are therefore grouped into distinct plasmid families. pXO2-like plasmids are likely to depict self-

transmissible capacities as some of their representatives (*i.e.* pAW63 from *B. thuringiensis* sv. *kurstaki*, pBT9727 from *B. thuringiensis* sv. *konkukian* and pXO2 from *B. anthracis*) harbor a *tra* region with genes homologous to the main components of functional conjugative systems and T4SS. While both pAW63 and pBT9727 are effective conjugative plasmids, pXO2 has an interrupted *virD4*-like gene likely responsible for its inability to conjugate autonomously (Van der Auwera *et al.*, 2005; Hu *et al.*, 2009b) (see Section 4.1).

The pXO1-like plasmid family includes pCER270, a plasmid bearing the genetic determinants of the cereulide, a dodecadepsipeptide responsible for the emetic syndrome in *B. cereus*-mediated food infections (Hoton *et al.*, 2005; Rasko *et al.*, 2007). Other pXO1-like plasmids are pBMB67 from *B. thuringiensis* (Chao *et al.*, 2007), pER272 and pBC10987 from *B. cereus* strains (Rasko *et al.*, 2007; Mei *et al.*, 2014) and pBCXO1 from *B. cereus* G9241 and *B. cereus* biovar *anthracis* (Hoffmaster *et al.*, 2004; Brézillon *et al.*, 2015). These plasmids share a high degree of synteny with pXO1 but lack or diverge in their pathogenicity genetic determinants. Contrary to the pXO2-like plasmids, none of the pXO1-like plasmids harbor self-transfer features, although pBMB67 displays CDS (Coding DNA Sequence) homologous to the VirB/D4 model for T4SS and was therefore described as a putative conjugative plasmid (Chao *et al.*, 2007).

The main feature associated with *B. thuringiensis* is the production of entomopathogen delta-endotoxins (Cry toxins) in parasporal crystals, used worldwide in biopesticide preparations (Bravo *et al.*, 2007; Palma *et al.*, 2014). The genetic determinants of Cry toxins are plasmid-borne, encoded on *cry* plasmids, which are either transmissible upon mobilization, like pBtoxis from *B. thuringiensis* sv. *israelensis* (Berry *et al.*, 2002; Hu *et al.*, 2005), or conjugative themselves, like pHT73 from *B. thuringiensis* sv. *kurstaki* (Wilcks *et al.*, 1998). Establishing a link between the presence of plasmids and the production of Cry toxins was at the base of the discovery of conjugation-like events between strains of *B. thuringiensis* (see Section 3.2).

Besides toxin plasmids, *B. cereus* *s.l.* harbors a large pool of plasmids and other mobile elements. While plasmids typically promote intercellular

genetic moves, other MGEs, like insertion sequences (IS) and transposable elements (Tn), are usually responsible for intracellular mobility, *i.e.* moving from one location to another within the same genome. Transposable elements are for instance more prevalent in toxin plasmids of *B. thuringiensis*, namely about 30% of the total "*thuringiensis*" plasmid pool, as they often flank *cry* genes (Fiedoruk *et al.*, 2017; Fayad *et al.*, 2019, 2021a). In natural environments, *B. cereus s.l.* isolates also present a flexible pool of extrachromosomal molecules, with HGT evidenced between closely related isolates (Hu *et al.*, 2009a). HGT within the *B. cereus* group is not restricted to plasmids. Some chromosomally-encoded enterotoxin operons (*hbl* and *cytK*) of *B. cereus* were for example shown to have spread by HGT amongst different taxonomic clusters (Böhm *et al.*, 2015). Although the cereulide genetic determinants (*ces*) are located on non-self-transmissible plasmids or in the chromosome, they are also prone to horizontal dissemination because they are typically flanked by MGEs, like *Tnces* in emetic strains of *B. weihenstephanensis* (Mei *et al.*, 2014).

With their multiple combinations of plasmids and other MGEs, strains of *B. cereus s.l.* are provided with a strong potential for adaptability. Indeed, HGT is recognized as a major route for acquiring new potentially beneficial genetic material, enabling bacteria to adapt and migrate to new environmental niches (McInerney *et al.*, 2017), and the *B. cereus* group makes no exception. However, acquiring foreign DNA can sometimes be harmful to bacteria thriving in some habitats. Therefore, bacteria developed protection systems preventing from such invasion, such as the antiviral CRISPR-Cas system (Barrangou and Marraffini, 2014). In staphylococci, the immune and protective function of the CRISPR-Cas system acts as barriers limiting the acquisition of MGEs by HGT (Marraffini and Sontheimer, 2008). Interestingly, CRISPR-Cas systems are less prevalent and mostly inactivated in *B. cereus s.l.* (Zheng *et al.*, 2020). In addition, strains of *B. cereus* with functional CRISPR-Cas systems showed a restricted ecological niche, a poorer pool of MGEs and an impaired adaptation to harsh environments. On an evolutionary point of view, acquisition of MGEs and ecological adaptability were apparently favored by *B. cereus s.l.*, rather than protective CRISPR-Cas systems (Zheng *et al.*, 2020), further illustrating the importance of HGT within this specific bacterial group.

3. Historical accounts

Up until the discovery of conjugative transfer among the *B. cereus* group in the early 1980's, phage-mediated transduction was the main technique available for studying *B. cereus s.l.* at a molecular level (Thorne, 1978). Most early molecular studies focused on *B. thuringiensis* due to its entomocidal Cry toxins and their common application as biopesticide (for a review, see Palma *et al.*, 2014). The historical milestones described in this section are illustrated on Fig. 1.

3.1. *B. cereus s.l.* in the pre-conjugation era

Transducing phages were mostly used for genetic experiments in *B. cereus s.l.* during the 60's and 70's, before the discovery of conjugative plasmids and their application as molecular tools (Fig. 1). The first evidence of phage-mediated transduction in *B. cereus s.l.* dates back to 1968, when a transducing phage, designated CP51, was isolated from a soil sample along with its host bacterium *B. cereus* strain 569 (Thorne, 1968). Phage CP51 was later shown to successfully mediate the transfer of the plasmidial vectors pBC16, conferring resistance to tetracycline (Tet^R) and pC194, conferring resistance to chloramphenicol (Cm^R), to specific strains of *B. cereus*, *B. anthracis* and *B. thuringiensis* (Ruhfel *et al.*, 1984) and therefore became a tool for genetic manipulations in *B. cereus s.l.* Other CP-phages mediating generalized transduction in *B. cereus s.l.* were also described, such as CP53, stable at lower temperatures (Yelton and Thorne, 1970), CP54, displaying a broader host spectrum (Thorne, 1978) and CP54-Ber, isolated from CP-54 lysates and infecting *B. thuringiensis* sv. *berliner* strain 1715 (Lecadet *et al.*, 1980). Many non CP-phages were also described for their potential in generalized transduction in the *B. cereus* group (for extensive reviews, refer to Gillis and Mahillon, 2014 or Sorokin, 2012).

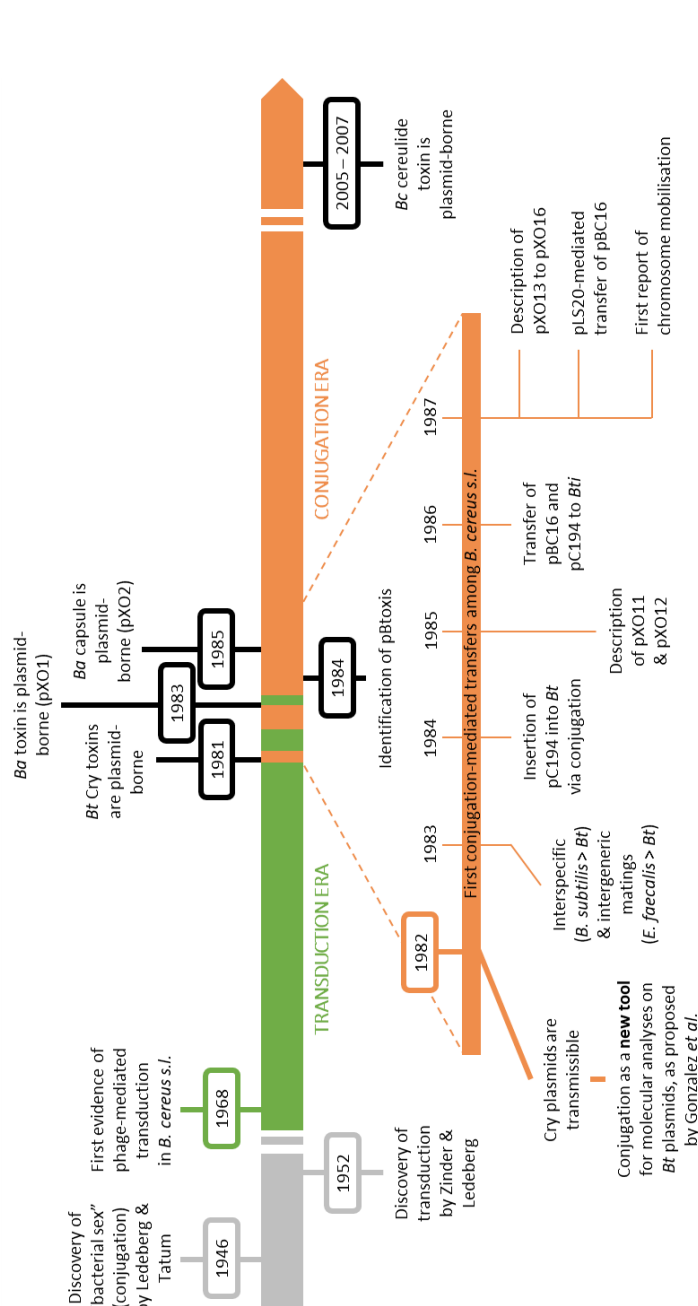


Figure 1: Timeline displaying milestones in *B. cereus* s.l. molecular research. In the 1960's and 1970's, molecular research in *B. cereus* s.l. was mostly conducted using phage-mediated transduction ("transduction era" in green). Since the discovery of conjugative transfer in *B. cereus* s.l., plasmid-mediated conjugation became the prevalent technique ("conjugation era" in orange). Plasmid-borne toxins play a key role in *B. cereus* s.l. taxonomy and were mostly identified alongside conjugation (in black). The historical discovery of conjugation and transduction are depicted in grey. *Ba*: *B. anthracis*; *Bt*: *B. thuringiensis*; *Bc*: *B. cereus*; *Bti*: *B. thuringiensis* sv. *israelensis*.

Transducing phages of *B. cereus s.l.* are grouped into three categories, based on their size and host range (Sorokin, 2012). A restricted host spectrum characterizes smaller phages. Medium-size phages show rather poor transduction efficiency, although they include CP51, the most-predominantly used phage for transduction experiments in *B. cereus s.l.* (Thorne, 1978). Large phages exhibit broader host spectra and were good candidates for early genome mapping experiments. For instance, the genome of *B. thuringiensis* strain 4042B was mapped using the phages TP13 and TP18 (Barsomian *et al.*, 1984). Given its insecticidal properties, *B. thuringiensis* was the main focus of most transduction-based studies in the *B. cereus* group (Sorokin, 2012).

3.2. First hints of conjugation and premises of a new transfer tool

Forty years ago, González and co-workers reported for the first time a conjugation-like process between strains of *B. thuringiensis* during growth in mixed cultures, while studying the genetic relationship between the presence of plasmids and the production of delta-endotoxin crystals. Notably, the plasmid-borne origin of *B. thuringiensis* delta-endotoxins was shown, along with the transmissible nature of the "cry plasmids" (González *et al.*, 1981; 1982). The transfer of a cry plasmid was also observed between strains of *B. thuringiensis* and *B. cereus s.s.* This conjugation-like event consisted in the transfer of a cry plasmid accompanied by the transfer of a cryptic conjugative plasmid, namely a mobilization event (González *et al.*, 1981; 1982). It is important to note that Cry toxins, and therefore cry plasmids, were, and still often are, considered a major feature distinguishing *B. thuringiensis* from other *B. cereus s.l.* strains. Thus, the transmissibility of cry plasmids reinforced the running hypothesis that both species were closely related (González *et al.*, 1982) and further blurred the lines of species distinction among members of the *B. cereus* group.

One of the obstacles in the study of the genetics of *B. thuringiensis* has long been the lack of effective genetic transformation tools. The newly described conjugation-based system provided researchers with a potential new "transformation" system for studying *B. thuringiensis* plasmids (Fig. 1) (González

and Carlton, 1982). The examples mentioned hereinafter rely on mobilization events, rather than on conjugative transfers *per se*. Conjugative plasmids are usually naturally cryptic and transfers were followed by assessing the transmissibility of detectable plasmids, like *cry* plasmids or small plasmids encoding an antibiotic resistance marker.

The newly proposed transfer tool was used to transfer the *cry* genes of *B. thuringiensis* sv. *berliner* 1715, cloned firstly into *B. subtilis*, to an acrystalliferous strain of *B. thuringiensis* sv. *kurstaki* and an "*israelensis*" strain. The acrystalliferous strain could then produce toxin crystals and the "*israelensis*" strain could therefore produce two types of delta-endotoxins, broadening its range of susceptible target insects (Klier *et al.*, 1983). Besides interspecies matings between *B. subtilis* and *B. thuringiensis*, pAM β 1 was successfully exchanged in intergeneric matings between *Streptococcus* (*Enterococcus*) *faecalis* and *B. thuringiensis* (Lereclus *et al.*, 1983, 1985). The conjugation-mediated transfer tool also served to identify a large 75MDa transmissible plasmid – later named pBtoxis – responsible for producing Cry toxins in the mosquito-cidal *B. thuringiensis* sv. *israelensis* (González and Carlton, 1984). The Cm^R plasmid pC194 and the Tet^R plasmid pBC16 were also successfully introduced into *B. thuringiensis* strains of *B. thuringiensis* by mobilization (Fischer *et al.*, 1984; Loprasert *et al.*, 1986). Other interspecies matings were later performed between *B. subtilis* and *B. cereus* s.l., where the transfer of pBC16 was mediated by the *B. subtilis* conjugative plasmid pLS20 (Koehler and Thorne, 1987).

In parallel, the transfer features of the large plasmids pXO11, pXO12, pXO13, pXO14, pXO15 and pXO16 originating from different serovars of *B. thuringiensis* were investigated by following the mobilization of pBC16 (Battisti *et al.*, 1985; Reddy *et al.*, 1987). All six plasmids could successfully be transferred to *B. cereus* s.s. and *B. anthracis*, except for pXO15, which was only transferred to *B. cereus* s.s. Because pXO12 carries Cry toxin determinants, its conjugation-mediated transfer resulted in transciipients with "hybrid" phenotypes, as *B. cereus* s.s. and *B. anthracis* transciipients could produce Cry toxins besides their characteristic "*cereus*" and "*anthracis*" toxins (Battisti *et al.*, 1985). On the contrary, no function other than conjugation could characterize the five other plasmids (Reddy *et al.*, 1987). With their ability to transfer a

large range of plasmids from their respective donor strains, plasmids pXO11 to pXO16 were useful exchange tools for analyzing genetic determinants on plasmids of *B. anthracis*, *B. cereus* and *B. thuringiensis* (Battisti *et al.*, 1985). For instance, both pXO12 and pXO14 successfully triggered the transfer of the "anthracis" plasmids pXO1 and pXO2 to plasmid-cured *B. anthracis* and *B. cereus*, while pXO16 was only capable of transferring pXO2, and pXO13 could mobilize none (Battisti *et al.*, 1985; Reddy *et al.*, 1987). In the case of pXO12-mediated mobilization of pXO1 and pXO2, the specific mobilization process is called conduction. Conduction usually calls for recombination events either between homologous or site-specific sequences, or mediated by transposable elements on one of the plasmids involved (Andrup, 1998). Here, cointegrates of pXO12 and pXO1 (or pXO2) were observed, and the recombination events were most likely mediated by the pXO12-encoded transposon Tn4430 (Green *et al.*, 1989). In opposition to conduction, pXO12 mediated the transfer of the small plasmid pBC16 by donation, a process whereby both plasmids remain physically distinct and do not associate (Green *et al.*, 1989; Andrup, 1998).

Chromosomal fragments were also discovered to transfer laterally at low frequencies alongside the transfer of a conjugative plasmid, by a mobilization-like mechanism (Aronson and Beckman, 1987). Chromosome mobilization was first noticed between strains of *B. thuringiensis* and *B. cereus* s.s., invariably accompanied by the transfer of at least one plasmid from the donor strain. The transfer efficiency of chromosomal fragments was reduced compared to the transfer efficiency of plasmids. The efficiency also varied for different chromosomal markers, suggesting that the localization of the marker could impact the transfer efficacy (Aronson and Beckman, 1987). Then, a study focusing on plasmid and chromosome transfers between *B. thuringiensis* sv. *israelensis* and various *B. thuringiensis* serovars showed that transfer frequencies varied depending on the recipient strain and on the transferred plasmid (Wiwat *et al.*, 1990). While transfer frequencies of chromosomal fragments using broth mating technique were very low, it was suggested that filter mating might improve such frequencies (Wiwat *et al.*, 1990). With current knowledge, the multiple mobilization events witnessed in *B. thuringiensis* sv. *israelensis* can most likely be attributable to pXO16. With that, researchers' observations of chromosome transfer and suggestion about

improving transfer frequencies by filter mating were later confirmed (Makart *et al.*, 2017, 2018; Hinnekens *et al.*, 2019) (see Section 4.2).

Circling back to toxin-carrying plasmids, establishing the connection between plasmids and *cry* genes in *B. thuringiensis* opened the possibility about the anthrax toxin genes being plasmid-borne (Mikesell *et al.*, 1983). The genetic determinants of both the protective antigen, *i.e.* one of the three proteins forming the anthrax bipartite toxins, and the anthrax capsule were indeed demonstrated to be encoded on two distinct large plasmids, pXO1 (Vodkin and Leppla, 1983) and pXO2 (Green *et al.*, 1985), respectively (Fig. 1). Isolating and characterizing strains of *B. anthracis* carrying both plasmids led to the conclusion that both pXO1 and pXO2 are involved in the pathogenicity and the virulence of *B. anthracis* (Uchida *et al.*, 1985, 1986).

4. Conjugative plasmids of *B. cereus* s.l.

Over the years, several plasmids from strains of *B. cereus* s.l. were characterized as conjugative, based either on experimental transfer evidence or on strong bioinformatics predictions (Table 1). Although recent works keep focusing on the importance of plasmids in the *B. cereus* group, especially for *B. thuringiensis* and its Cry toxins (Bolotin *et al.*, 2017; Gillis *et al.*, 2018; Fayad *et al.*, 2019, 2021a; Zheng *et al.*, 2020), few studies have investigated conjugative transfer *per se*. Furthermore, reported transfer events often consist in mobilization events without incriminating the implicated conjugative plasmid (Fguira *et al.*, 2014). Therefore, only 20 conjugative plasmids of *B. cereus* s.l. have been reported to date, but the conjugative capacities of eight of them lack experimental proofs (Table 1).

The first plasmids described as conjugative, *i.e.* pXO11 to pXO15, were presented in Section 3.2. In this section, the more recently reported *B. cereus* s.l. conjugative elements have been split into three groups for readily description: *i*) the conjugative plasmids sharing the *tra* region of pAW63 and the pXO2-like plasmids, *ii*) the unique conjugative system of pXO16, and *iii*) other major conjugative plasmids, including pHT73 and plasmids sharing an *ori44*-like replicon.

Table 1: Conjugative plasmids from *B. cereus* s.l. List of *B. cereus* s.l. plasmids reported as conjugative – or potentially conjugative – and their main characteristics.

Plasmid	Original host	Size (kb)	GenBank accession no.	Features	Type replicon	Proof of transfer	Source
pXOI1, pXOI3- pXOI5	<i>B. thuringiensis</i>	-	-	First reported conjugative plasmids from <i>B. thuringiensis</i>	-	Yes	(Battisti <i>et al.</i> , 1985; Reddy <i>et al.</i> , 1987)
pXOI2	<i>B. thuringiensis</i> sv. <i>thuringiensis</i> 4042A	112.5	-	Cry plasmid; one of the first reported conjugative plasmids from <i>B. thuringiensis</i>	-	Yes	(Battisti <i>et al.</i> , 1985)
pGBI30	<i>B. cereus</i> 5	-	-	Mercury resistance	-	Yes	(Belliveau and Trevors, 1990)
pXOI6	<i>B. thuringiensis</i> sv. <i>israelensis</i>	349.6	CP003764.1	Aggregation phenotype; novel T4SS	-	Yes	(Reddy <i>et al.</i> , 1987)
pAW63	<i>B. thuringiensis</i> sv. <i>kurstaki</i>	71.8	DQ025752.1	<i>tra</i> region with homologs to the VirB/D4 model, pXO2-like family	pAM/ β 1 family	Yes	(Wilcks <i>et al.</i> , 1998)
pBT9727	<i>B. thuringiensis</i> sv. <i>konkukian</i>	77.1	NC_006578.1	Instability in heterologous genomic background, pXO2-like family	pAM/ β 1 family	Yes	(Van der Auwera <i>et al.</i> , 2008)
pHT73	<i>B. thuringiensis</i> sv. <i>kurstaki</i> HD73	77.3	NC_020249.1	<i>cry</i> plasmid (<i>cry1Ac</i> gene), <i>tra</i> region	<i>ori44</i>	Yes	(Wilcks <i>et al.</i> , 1998)
pLUSID1	<i>B. thuringiensis</i> HER140	368.2	NZ_CP050184.1	Putative conjugative region around <i>fisZ</i> -like gene	-	No	(Lechuga <i>et al.</i> , 2020)

Continued on next page

Plasmid	Original host	Size (kb)	GenBank accession no.	Features	Type replicon	Proof of transfer	Source
pFR55	<i>B. thuringiensis</i> INTA-FR7-4	55.7	EU362919.1	<i>tra</i> region with homologs to the VirB/D4 model & similar to pAW63 <i>tra</i> region	<i>ori44</i>	No	(Amadio <i>et al.</i> , 2009)
pBMB67	<i>B. thuringiensis</i> YBT-1520	67.1	NC_009841.1	Modular organization, including one module with homology to VirB/D4 model	-	No	(Chao <i>et al.</i> , 2007)
pIS56-63	<i>B. thuringiensis</i> <i>sv. israelensis</i>	63.8	NC_020392.1	<i>cry</i> plasmid (<i>cry1Ab21</i> gene), <i>tra</i> region almost identical to pHT73 <i>tra</i> region	<i>ori44</i>	No	(Murawska <i>et al.</i> , 2014)
pBMB76	<i>B. thuringiensis</i> <i>sv. tenebrionis</i> 4AA1	76.9	NZ_CP010580.1	-	<i>ori60</i>	Yes	(Hu <i>et al.</i> , 2020)
pBMB68	<i>B. thuringiensis</i> <i>sv. tenebrionis</i> 4AA1	68.4	NZ_CP010581.1	Putative conjugative region	<i>ori43</i>	No	(Hu <i>et al.</i> , 2020)
pBMB232	<i>B. thuringiensis</i> <i>sv. tenebrionis</i> 4AA1	233	NZ_CP010578.1	Putative conjugative region	<i>orf156/157</i>	No	(Hu <i>et al.</i> , 2020)
pE81-53	<i>B. cytotoxicus</i> E8.1	53.1	NZ_CP066195.1	Putative conjugative region	-	Yes	(Fayad <i>et al.</i> , 2021b)
pE81-84	<i>B. cytotoxicus</i> E8.1	83.6	NZ_CP066196.1	Putative conjugative region, distantly related to pXO2-like plasmids	-	No	(Fayad <i>et al.</i> , 2021b)
pE283-80	<i>B. cytotoxicus</i> E28-3	79.7	NZ_CP066190.1	Putative conjugative region, distantly related to pXO2-like plasmids	-	No	(Fayad <i>et al.</i> , 2021b)

4.1. pAW63 and other pXO2-like plasmids

4.1.1. The broad-host range plasmid pAW63

B. thuringiensis sv. *kurstaki* HD73, a common commercial biopesticide strain, was part of González and colleagues' original study in early 1980's that discovered the transmissible nature of *cry* plasmids (González *et al.*, 1981, 1982). This "*kurstaki*" strain was later shown to contain a second large plasmid, dubbed pAW63, aside from the *cry* plasmid pHT73 (Wilcks *et al.*, 1998) (see Section 4.3). This plasmid exhibited high transfer frequencies, reaching up to 100% efficiency between "*kurstaki*" strains in liquid mating, reminiscent of pXO16 transfer efficiency between strains of *B. thuringiensis* sv. *israelensis* (Wilcks *et al.*, 1998; Van der Auwera *et al.*, 2005) (see next Section). pAW63 was classified as a broad host range plasmid, like plasmid pIP501 initially isolated from *Streptococcus* (Evans and Macrina, 1983). It could indeed transfer to *B. thuringiensis* sv. *israelensis*, *B. cereus*, *Bacillus licheniformis*, *B. subtilis* and *Bacillus (Lysinibacillus) sphaericus* (Wilcks *et al.*, 1998), as well as to more distantly-related species, such as *Listeria innocua* and *E. faecalis* (Van der Auwera *et al.*, 2005). Studies also assessed pAW63 transfer efficiency in food matrices, especially dairy products and plant-based drinks (Van der Auwera and Mahillon, 2008; Modrie *et al.*, 2010) (see Section 6.2).

Studying pAW63 4.1-kb replicon highlighted a strong similarity between pAW63 largest replication protein, Rep63A, and the replication proteins RepR and RepE from pIP501 and pAM β 1, respectively (Wilcks *et al.*, 1999). Both pIP501 and pAM β 1 belong to the pAM β 1-family of theta replicating plasmids, whose replication relies on the host DNA polymerase 1 and is single-stranded DNA-independent. As it shares these features, pAW63 was associated within the same plasmid family (Wilcks *et al.*, 1999). The full sequence of pAW63 was published in 2005 and revealed a modular organization. One 42-kb module forms the *tra* region and encodes 15 CDSs putatively assigned to conjugative functions. Among these, three CDSs share homologies with the ATPases from the Vir secretion system, the CP VirD4, the motor ATPase VirB4 and the ATPase VirB11. Other CDSs share homologies with conjugation proteins from various Gram-positive T4SS (Van der Auwera *et al.*, 2005). Interestingly, two group II-related introns interrupt two genes within the con-

jugative locus (Fig. 2). On the one hand, ORFs 8 and 9 are split by the B.th.I1 intron and are predicted to encode the conjugative cell wall hydrolase. On the other hand, ORFs 14 and 15 are separated by the B.th.I2 intron but form one functional gene encoding the putative VirD4-like CP (Van der Auwera and Mahillon, 2008). Group II introns have also been found on the conjugative plasmid pRS01 from *Lactococcus lactis* (Belhocine *et al.*, 2005) and on other plasmids from *B. cereus* s.l. (Tourasse *et al.*, 2005). It is worth mentioning that most of the current knowledge on pAW63 *tra* region and associated conjugation mechanism was inferred from homologies with other T4SS and does not rely on experimental data.

4.1.2. The family of pXO2-like plasmids

Analyzing pAW63 sequence indicated strong similarities with the "anthracis" plasmid pXO2 from *B. anthracis* and the pBT9727 plasmid from strain 97-27 of *B. thuringiensis* sv. *konkukian* (Van der Auwera *et al.*, 2005). Indeed, among the 76 CDSs predicted on pAW63, 42 are common between the three plasmids, with 50 CDSs shared by pAW63 and pXO2 and 49 by pAW63 and pBT9727. Of specific interest is the high conservation of the region corresponding to the pAW63 transfer module in pXO2 and pBT9727 (Fig. 2) (Van der Auwera *et al.*, 2005). Although pXO2 is mobilizable but non conjugative, pBT9727 was shown to promote its own transfer and to trigger the mobilization of small co-resident mobilizable plasmids (Van der Auwera *et al.*, 2008). Based on comparative sequence analyses, all three plasmids were proposed to derive from a same ancestral conjugative plasmid, from which pAW63 and pBT9727 retained conjugation capacities, while pXO2 genetically drifted and lost the potential to conjugate (Van der Auwera *et al.*, 2008). Most constituents of the *tra* region are intact in pXO2 compared to pAW63 and pBT9727. However, at least two putatively major elements for conjugation display punctual mutations in pXO2, including the *virD4*-like gene, presumably leading to frameshifts and disruption of the conjugative functions (Van der Auwera *et al.*, 2005).

pXO2, pAW63 and pBT9727 compose the core of the pXO2-like plasmid family, but other conjugative – or putatively conjugative – plasmids also share similarities. Three closely related plasmids from *B. cytotoxicus* were

recently described as distantly related to the trio of pXO2-like plasmids (Fayad *et al.*, 2021b). Two of them, pE81-84 and pE283-80, also harbor a region reminiscent of the *tra* region of pAW63 and kin, suggesting a conjugative potential for both plasmids. As illustrated on Fig. 2, these two plasmids are highly similar, and a large portion of their sequences is very similar to that of pXO2. The putative conjugative region encodes three candidates for ATPase functions, including one putative CP, as well as candidates for transglycosylase and relaxase functions. Although they harbor all signature elements of a functional T4SS, experimental data are lacking to confirm the conjugative nature of pE81-84 and pE283-80.

pFR55, a plasmid isolated from *B. thuringiensis* INTA-FR7-4, presents a *tra* region with multiple CDSs sharing similarities to CDSs involved in pXO2-like plasmid conjugation (Fig. 2) (Amadio *et al.*, 2009). pFR55 putative conjugative locus encodes a relaxase, a transglycosylase, a putative CP and an ATPase, all key elements of functional conjugative systems (Goessweiner-Mohr *et al.*, 2013). Given these characteristics, pFR55 has a strong potential for conjugation, but experimental evidence of its self-transferability is lacking. Apart from its *tra* region, pFR55 does not share the common backbone of pXO2-like plasmids (Fig. 2). For instance, its replication region belongs to the family of *ori44*-type replicon, like the well-studied *cry* plasmid pHT73 from *B. thuringiensis* sv. *kurstaki* HD73 (Amadio *et al.*, 2009), and pFR55 forms therefore a separate group from pXO2-like plasmids.

4.1.3. Prevalence of pXO2-like plasmids

As pXO2-like transfer modules exhibit a significant degree of conservation amongst several described conjugative plasmids, the prevalence of such module was evaluated at a larger scale (Hu *et al.*, 2009b, 2009a). pXO2-like plasmids/replicons were widespread in environmental isolates and had therefore a significant potential as conjugative plasmids. However, the proportion of strains demonstrating conjugative elements remained minor compared to the initial population of interest, especially from environmental samples. Indeed, in two independent studies, pXO2-like transfer modules were detected in only 5% of the *B. cereus* s.l. strains selected from both environmental samples and laboratory collections (Hu *et al.*, 2009b, 2009a).

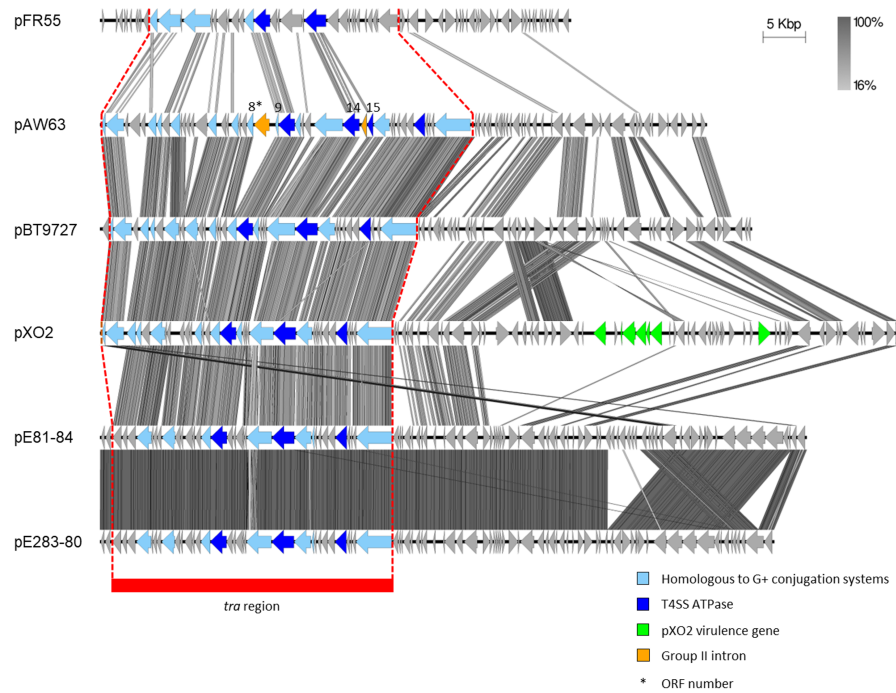


Figure 2: Sequence comparison of the (putative) conjugative plasmids of the pXO2-like family. The *tra* region of the plasmids pFR55, pAW63, pBT9727, pXO2, pE81-84 and pE283-80 share evident similarities. CDSs depicted in light blue share homologies with other known conjugative systems; CDSs depicted in dark blue are the putative ATPases; both CDSs in orange in pAW63 sequence correspond to group II introns interrupting the genes encoded by the CDSs 8-9 and 14-15; CDSs coloured in green encode Cap and Acp virulence factors of pXO2. The region highlighted in red corresponds to the *tra* region. The image was generated by Easyfig 2.0 software. Genbank accession numbers: pXO2: NC_007323.3; others: Table 1.

4.2. pXO16, the unique conjugative master

The conjugative plasmid pXO16, first identified by Reddy *et al.*, in 1987, was thoroughly investigated by different research teams. Much attention was given to its phenotypic description, mainly for the aggregation phenotype induced by pXO16, as well as to the genetic components underlying its conjugation mechanism.

4.2.1. Aggregation-mediated conjugation

Aggregation-mediated conjugation mechanisms have been reported for various conjugative systems. The most studied example is the conjugative system of *E. faecalis*, which involves clumping of donor cells under the influence of a recipient-borne sex pheromone (Dunny *et al.*, 1978). Cell aggregates also mediate plasmid transfer in conjugative systems of some lactic bacteria, such as *L. lactis* and *Lactobacillus plantarum*. In *L. lactis*, the aggregation-inducing factor is actually chromosomally encoded (Walsh and McKay, 1981; Van Der Lelie *et al.*, 1991), while the aggregation-mediated conjugation system of *L. plantarum* is neither plasmid-encoded nor specific and facilitates the formation of mating pairs for various conjugative plasmids (Reniero *et al.*, 1992). Macroscopic cell aggregates were also witnessed in broth matings of *B. thuringiensis* sv. *israelensis* and were attributed to pXO16 conjugation (Andrup *et al.*, 1993; Jensen *et al.*, 1995). pXO16-mediated aggregation was thoroughly studied and brought to light some key features of pXO16 conjugative system, e.g. the transmissibility of the aggregation phenotype alongside pXO16 transfer (Jensen *et al.*, 1996), the mobilization of small co-resident plasmids (Andrup *et al.*, 1996), and the importance of S-layer proteins in pXO16 transfer efficiency (Wiwat *et al.*, 1995).

The interest for pXO16-mediated aggregation got renewed when the aggregation-encoding genes were localized within a 25-kb *agr* region of pXO16 sequence (Makart *et al.*, 2015). After over two decades of hypothesizing that aggregation was a prerequisite for pXO16 transfer, an aggregation-deficient mutant was constructed and exhibited reduced, yet effective, transfer in absence of the aggregation proteins (Makart *et al.*, 2018). Thus, pXO16 aggregation facilitates its transfer but is not required. By studying *B. thuringiensis* sv. *israelensis* aggregation under atomic force microscopy (AFM), surface proteins encoded in the *agr* region were shown to mediate specific interactions between mating partners, resulting in strong adhesion forces ($\sim 2\text{nN}$) (Feuillie *et al.*, 2018).

4.2.2. Kinetics of transfer and DNA capture properties

pXO16 transfer is characterized by high-speed, as transfer is completed in *ca.* 3.5-4 min, by the formation of mating pairs involving one donor and one recipient within large mating aggregates, and by the hypothetical low copy number of the plasmid (Andrup *et al.*, 1998). Primary transfer of pXO16 can be detected within 10 min and peaks at 40-50 min after mating onset (Timmery *et al.*, 2009). A 10-min recovery period is required in between two transfer events from a same donor, and a 40 min maturation time is necessary for newly formed transconjugants to turn into donors themselves (Andrup *et al.*, 1998).

Back in 1987, pXO16 was first reported for triggering the transfer of the mobilizable plasmid pBC16 by a conjugation-like event between *B. thuringiensis* sv. *israelensis* and *B. cereus* s.s. or *B. anthracis* strains (Reddy *et al.*, 1987). Further work dived into pXO16 mobilization capacities and revealed that pXO16 can mobilize mobilizable – *i.e.* usually encoding an *oriT* and a relaxase, but no T4SS – as well as "non-mobilizable" – *i.e.* lacking all elements necessary for conjugation (*oriT*, relaxase and T4SS) – co-resident plasmids (Timmery *et al.*, 2009). It can also trigger the capture of a plasmid from a recipient cell to the pXO16-harboring donor cell via a mechanism known as retro-mobilization (Timmery *et al.*, 2009). More recently, studies reported that pXO16 could trigger the transfer of chromosomal loci between mating partners (Makart *et al.*, 2017). Furthermore, chromosomal fragments of at least 791 kb could be transferred between strains of *B. thuringiensis* sv. *israelensis* and chromosomal markers were successfully transferred by pXO16 between strains of *B. cytotoxicus* (Makart *et al.*, 2018; Koné *et al.*, 2021). pXO16 transfer and capture capacities were also evaluated under different ecological and food-related conditions, such as the midgut of dipteran larvae or milk and rice pudding (Thomas *et al.*, 2001; Van der Auwera *et al.*, 2007) (see Sections 6.1 and 6.2).

4.2.3. Conjugation apparatus, a novel type of T4SS

Despite detailed work on phenotypical features of pXO16 transfer, little was known about the genetics underlying its conjugation mechanism until pXO16 sequence was analyzed in 2015 (Makart *et al.*, 2015). Analysis of the 434 CDSs,

covering more than 85% of the 350-kb sequence, revealed very few relevant homologies with public databases. Among the few CDSs with a predicted functional annotation, none were related to any T4SS components identified so far, suggesting that pXO16 conjugative system is rather unique (Makart *et al.*, 2015). Based on detailed bioinformatics studies and on experimental data, a 25-kb locus involved in conjugation was delimited and referred to as the *transfer of israelensis plasmid (tip)* locus (Makart *et al.*, 2018). Within the 16 CDSs forming the *tip* locus, genes encoding essential conjugative functions were identified, such as two ATPases and one cell wall hydrolase (Makart *et al.*, 2018; Hinnekens *et al.*, 2021). While these functions are widely recognized as essential in canonical T4SS, key elements, particularly the relaxase and the *oriT*, remain unidentified in pXO16 conjugative apparatus.

4.2.4. Transfer of pXO16 across the *B. cereus* group

From first investigations, pXO16 host spectrum was thought to be rather restricted, with only few serovars of *B. thuringiensis* and few strains of *B. cereus* s.s. capable of receiving pXO16 (Jensen *et al.*, 1996). These conclusions were based on the hypothesis that aggregation (Agr) was mandatory for the conjugation-mediated transfer of pXO16. Strains carrying pXO16, designated Agr⁺, could form macroscopic aggregates with some other strains (Agr⁺ strains). Only those Agr⁻ strains were considered as potential recipients for pXO16 (Table 2), while strains which could not form macroscopic aggregates with an Agr⁺ strain were ignored (Jensen *et al.*, 1996). However, as indicated above, an aggregation-deficient variant of pXO16 was capable of conjugative transfer, albeit at dramatically reduced frequencies, indicating that the Agr phenotype is important but not mandatory for pXO16 transfer, as previously stated (Makart *et al.*, 2018). Based on this new finding, previously overlooked non-aggregating *B. cereus* s.l. strains were reported to successfully act as recipients for pXO16 transfer in liquid matings (Makart *et al.*, 2018).

Further work demonstrated that mimicking Agr by using solid mating conditions could compensate Agr deficiency and allow transfer to several *B. cereus* s.l. strains, including *B. cytotoxicus*, *B. mycoides* and strains appertaining to the same clade of *B. anthracis* but lacking the "*anthracis*" virulence genes (Hinneken *et al.*, 2019; Hinneken and Mahillon, 2022) (Table 2). Based on

recent host spectrum data, assuming pXO16 transfer based on the Agr phenotype of the recipient strain is no longer relevant. Similarly, the Agr phenotype should not be confused with the wild-type or aggregation-deficient version of pXO16, as they respectively express strain-specific and plasmid-specific features.

The highly efficient mobilization capacities of pXO16 could be of concern in terms of gene circulation, including antibiotic resistant genes, if these characteristics were to express in natural settings. However, pXO16 transferability within the *B. cereus* group was only detected under tightly controlled laboratory conditions, very distant from environmental conditions. For instance, the most efficient transfer set up tested to date consists in the highly artificial filter mating technique (Hinneken *et al.*, 2019). Also, pXO16 exerts a significant impact on its *B. cereus s.l.* hosts, apart from its natural host, suggesting a co-adaptation between pXO16 and its "*israelensis*" host (Hinneken and Mahillon, 2022). Taken with the natural distribution of pXO16 apparently restricted to *B. thuringiensis* sv. *israelensis* (Gillis *et al.*, 2018), the potential impact of pXO16-mediated conjugation within the *B. cereus s.l.* in natural environments seems rather restricted. Furthermore, to the best of our knowledge, pXO16 has never been transferred by conjugation outside of the *B. cereus* group, even under solid mating conditions, suggesting a limited impact of its incredible transfer features at a wider scale.

Table 2: pXO16 host spectrum. List of all the strains to which pXO16 transfer was successfully demonstrated under liquid or solid mating conditions, either by assessing the aggregation phenotype of the strain or by plating out transconjugants. This table also contains strains in which pXO16 was naturally found and that were used as donors in mating experiments. When appropriate, the aggregation phenotype/clumping status of the strain is mentioned. Original data can be found in the followings: Reddy *et al.*, 1987; Andrup *et al.*, 1993; Jensen *et al.*, 1995, 1996; Makart *et al.*, 2018; Hinneken *et al.*, 2019, 2021; Hinneken and Mahillon, 2022.

Strains	Aggregation phenotype	Clumping group	Mating status	Mating conditions for transfer to R strains	
				Liquid	Solid
<i>B. thuringiensis</i> sv. <i>israelensis</i> 4Q2	Agr ⁺	I	D	-	-
<i>B. thuringiensis</i> sv. <i>israelensis</i> 4Q7	Agr ⁻	II	R	✓	ND
<i>B. thuringiensis</i> sv. <i>israelensis</i> 4Q7(pXO16)	Agr ⁺	I	D	-	-
<i>B. thuringiensis</i> sv. <i>israelensis</i> GBJ001/2*	Agr ⁻	II	R	✓	✓
<i>B. thuringiensis</i> sv. <i>israelensis</i> GBJ001/2(pXO16)	Agr ⁺	I	D	-	-
<i>B. thuringiensis</i> sv. <i>israelensis</i> GSX002	Agr ⁻	II	R	✓	✓
<i>B. thuringiensis</i> sv. <i>israelensis</i> BIS UM1	ND	-	D	-	-
<i>B. thuringiensis</i> sv. <i>israelensis</i> AND508	Agr ⁺	I	D	-	-
<i>B. thuringiensis</i> sv. <i>israelensis</i> NB31	Agr ⁺	I	D	-	-
<i>B. thuringiensis</i> sv. <i>aizawai</i> HD11	0	-	R	< LOD	✓
<i>B. thuringiensis</i> sv. <i>alesti</i> HD4	0	-	R	✓	✓
<i>B. thuringiensis</i> sv. <i>canadensis</i> HD224	Agr ⁻	II	R	✓	ND
<i>B. thuringiensis</i> sv. <i>entomocidus</i> HD9	0	-	R	✓	✓
<i>B. thuringiensis</i> sv. <i>konkukian</i> B9727	ND	-	R	✓	✓
<i>B. thuringiensis</i> sv. <i>kurstaki</i> HD1	Agr ⁻	II	R	✓	ND
<i>B. thuringiensis</i> sv. <i>kurstaki</i> HD73	Agr ⁻	II	R	✓	ND
<i>B. thuringiensis</i> sv. <i>kysusensis</i> HD541	Agr ⁻	II	R	✓	ND

Continued on next page

Strains	Aggregation phenotype	Clumping group	Mating status	Mating conditions for transfer to R strains	
				Liquid	Solid
<i>B. thuringiensis</i> sv. <i>morrisoni</i> HD12	Agr ⁺	II	R	✓	ND
<i>B. thuringiensis</i> sv. <i>pakistani</i> HD395	Agr ⁺	II	R	✓	ND
<i>B. thuringiensis</i> sv. <i>soito</i> HD6	0	-	R	✓	✓
<i>B. thuringiensis</i> sv. <i>tenebrionis</i> BI256-82	Agr ⁺	II	R	✓	ND
<i>B. thuringiensis</i> sv. <i>thompsoni</i> HD542	Agr ⁺	II	R	✓	ND
<i>B. thuringiensis</i> sv. <i>thuringiensis</i> HD2	Agr ⁺	II	R	✓	ND
<i>B. thuringiensis</i> sv. <i>thuringiensis</i> Bt407	ND	-	R	✓	✓
<i>B. thuringiensis</i> sv. <i>tolworthi</i> HD125	0	-	R	✓	✓
<i>B. thuringiensis</i> sv. <i>toumanoffi</i> HD201	Agr ⁺	II	R	✓	ND
<i>B. thuringiensis</i> sv. <i>wilhanensis</i> HD525	Agr ⁺	II	R	✓	ND
<i>B. cereus</i> 569 UM20-1	ND	-	R	✓	ND
<i>B. cereus</i> ATCC10987	Agr ⁺	II	R	✓	ND
<i>B. cereus</i> ATCC10876	Agr ⁺	II	R	✓	ND
<i>B. cereus</i> ATCC14579	Agr ⁺	II	R	✓	ND
<i>B. cereus</i> H3081-97	Agr ⁺ /0	II	R	✓	✓
<i>B. cereus</i> IS075	0	-	R	✓	✓
<i>B. cereus</i> VD021	0	-	R	< LOD	✓
<i>B. cereus</i> CTMA-1571	ND	-	R	✓	✓
<i>B. anthracis</i> Weybridge UM44-1	ND	-	R	✓	ND
<i>B. weihenstephanensis</i> WSBC10204	0	-	R	✓	✓
<i>B. weihenstephanensis</i> KBAB4	0	-	R	✓	✓
<i>B. cytotoxicus</i> E28-3	0	-	R	< LOD	✓
<i>B. pseudomycoides</i> B-617	ND	-	R	-	✓

Continued on next page

Strains	Aggregation phenotype	Clumping group	Mating status	Mating conditions for transfer to R strains	
				Liquid	Solid
<i>B. mycoides</i> JAS391	ND	-	R	-	✓
<i>B. mycoides</i> JAS481	ND	-	R	-	✓
<i>B. mycoides</i> BPN09/1	ND	-	R	-	✓
<i>B. mycoides</i> LMG7128	ND	-	R	-	✓

* *B. thuringiensis* sv. *israelensis* GBJ001 and GBJ002 are isogenic strains, only differing in their respective antibiotic resistance genes Str^R and Nal^R.

Aggregation phenotype: Agr⁺/clumping group I: strains containing pXO16 and acting as donors in mixed mating with Agr⁻/clumping group II strains, as described in Jensen *et al.*, 1996 and Wiwat *et al.*, 1995; Agr⁻/clumping group II: strains lacking pXO16 and forming aggregates in mixed mating with Agr⁺/clumping group I strains, as described in Jensen *et al.*, 1996 and Wiwat *et al.*, 1995; 0: strains lacking pXO16 and forming no aggregate in mixed mating with Agr⁺/clumping group I strains.

Mating status: D = donor strains; R = recipient strain

✓ = transfer successfully demonstrated; < LOD = < limit of detection ($\sim 10^{-7}$ T/D); ND = not determined; - = not relevant

4.3. *Ori44*-type replicon related plasmids

The conjugative potential of the aforementioned pHT73 was first reported in early work on conjugation-like transfer within the *B. cereus* group (González *et al.*, 1982; Wilcks *et al.*, 1998). This *cry* plasmid encodes a *cry1Ac* gene and was successfully transferred to different members of the *B. cereus* group, including *B. mycoides*, under laboratory conditions (Hu *et al.*, 2004). Despite successful transfer, pHT73 stability varied significantly between different recipient strains (Hu *et al.*, 2004). Under environmental selective pressure, such instability could strongly impair the maintenance of the plasmid in those strains. Consequently, the highest stability rate was observed in "*kurstaki*" strains, indicating a biological adaptation of the plasmid to its original serotype (Hu *et al.*, 2004). Such indications confirmed the positive correlation reported previously between the *cry* gene content of a specific strain and the strain serotype (Gaviria Rivera and Priest, 2003). pHT73 host spectrum was described as rather narrow at the time, reminiscent of the host limitations observed in early studies about pXO16 (Jensen *et al.*, 1996). Transfer of pHT73 was later established to strains of *B. anthracis* (Yuan *et al.*, 2010), and the compatibility of this *cry* plasmid with the anthrax plasmids pXO1 and pXO2 and the production of the Cry toxin by the "*anthracis*" recipient were also demonstrated. Such manipulations resulted in a laboratory strain of *B. anthracis* producing Cry toxin, similar to the hybrid *B. cereus* s.s. and *B. anthracis* strains that could produce pXO12-borne Cry toxin upon conjugation-mediated acquisition of pXO12 (Battisti *et al.*, 1985). Although the possibility of formation of such strains with hybrid phenotypes raised concerns about the spreading of insecticidal strains in the environment (Yuan *et al.*, 2010), their actual occurrence in natural settings has so far never been reported, but remains to be further investigated.

Other potentially conjugative plasmids share the *ori44*-type replicon of pHT73, pFR55 (Amadio *et al.*, 2009) (see Section 4.1) and pIS56-63 (Murawska *et al.*, 2014). pIS56-63 sequence exhibits strong similarities with pHT73 as it carries a *cry* gene, multiple MGEs and conjugation-related genes, even though experimental data assessing its conjugative capacities are still missing (Fig. 3). Another plasmid harbors a conjugative locus related to that of pHT73, the *B.*

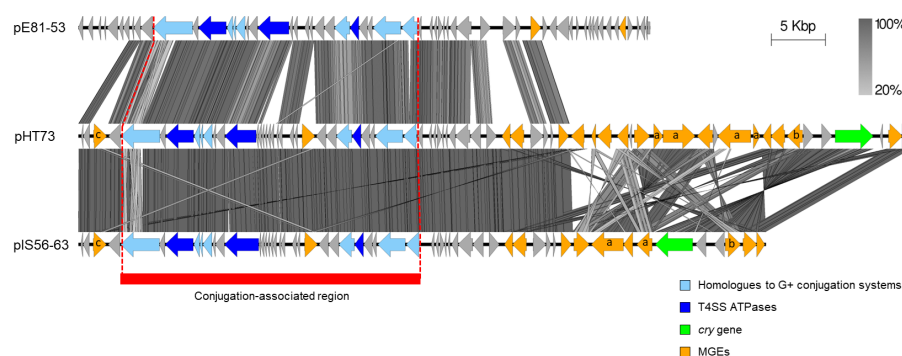


Figure 3: Sequence comparison of the (putative) conjugative plasmids similar to pHT73. The conjugation-associated region of the plasmids pHT73, pE81-53 and pIS56-63 share evident similarities. CDSs depicted in light blue share homologies with other known conjugative systems; CDSs depicted in dark blue are the putative ATPases; CDSs in orange correspond to different types of MGEs (a = Tn; b = group II intron; c = recombinase; no annotation = IS); CDSs in green encode Cry toxins. The region highlighted in red corresponds to the region associated with conjugative functions. The image was generated by Easyfig 2.0 software. Genbank accession numbers: Table 1.

cytotoxicus plasmid pE81-53 (Fig. 3), hosted by the same strain as the pXO2-like plasmid pE81-84 (Fayad *et al.*, 2021b). Contrary to pE81-84, experimental evidence confirmed the conjugation potential of pE81-53 (Table 1). The conjugative loci of pHT73, pE81-84 and pIS56-63 encode three putative ATPases (Fig. 3) and candidates for other conjugative functions, including homologues of TcpD and TcpE, two membrane proteins essential for the conjugative system of the clostridial plasmid pCW3 (Wisniewski *et al.*, 2015).

5. *B. cereus* s.l.: a pool of conjugative candidates

As stated previously, the *B. cereus* group is rich with MGEs, including those with intercellular mobility, such as plasmids, or with intracellular mobility, such as IS, class II Tn and the group-specific *B. cereus* repeats (Bcr). These MGEs are considered as major contributors to the genomic plasticity seen in members of this group. Many studies have attributed the ecological specificity of each member of the group to lineage-specific plasmid acquisition

throughout the species' evolution (Méric *et al.*, 2018). These plasmids differed in number and composition. For the former, a comparison of the average number of distinct plasmid per strain among *B. cereus s.l.* species showed that *B. cereus s.s.* carries an average of 2 plasmids per cell, whereas *B. thuringiensis* average plasmid content pikes at 6-7 plasmids per cell, followed closely by *B. mycoides* at 5-6 plasmids per cell (Fayad *et al.*, 2019). *B. thuringiensis* plasmid numbers in fact ranged from 1 to 14 distinct plasmids per cell. *B. thuringiensis* sv. *israelensis*, for example, usually harbors nine extrachromosomal molecules, among which the toxin-encoding plasmid pBtoxis and the conjugative plasmid pXO16 (Gillis *et al.*, 2018).

While the conjugative nature of some plasmids is classically uncovered through conjugation assays, nowadays, this nature could be highlighted by the *in silico* analysis of sequenced genomes, whose numbers are increasing day-by-day. Therefore, to get a better picture at the distribution of potentially conjugative plasmids within the *B. cereus* group, we compared the sequence of plasmids from sequenced *B. cereus s.l.* genomes with the sequence of known *B. cereus s.l.* conjugative plasmids presented in previous sections. In total, 365 complete *B. cereus s.l.* genomes were recovered from the NCBI genome assembly public database (<https://www.ncbi.nlm.nih.gov/assembly>; last accessed June 15, 2022) mainly from the "*cereus*" (115 genomes), "*thuringiensis*" (74 genomes) and "*anthracis*" (104 genomes) species. This amounted to 1,168 plasmids (Table 3). Among the 20 plasmids reported as conjugative in the *B. cereus* group (Table 1), no sequence nor information relative to the genes involved in conjugation are available for six of them, *i.e.* pXO11 to pXO15 and pGB130. Six other plasmids can be grouped into four classes based on the similarity of their putative transfer apparatus: pAW63 and pBT9727 form class 1, pFR55 forms class 2, pHT73 and pE81-53 form class 3 and pBMB76 forms class 4. A class 5 comprising the case of the conjugative plasmid pXO16 will also be discussed hereafter.

Table 3: Identification of potential conjugative plasmids within the *B. cereus* group. Plasmids retrieved from closed genomes of *B. thuringiensis*, *B. cereus*, *B. anthracis*, *B. cytotoxicus*, *B. toyonensis*, *B. mycoides* and *B. weihenstephanensis*-*B. pseudomycoides* were analyzed for their protein sequence similarities with the model conjugative plasmids of four distinct classes. The potential conjugative plasmids encode at least two ATPase-like and one peptidoglycan hydrolase-like proteins sharing $\geq 60\%$ sequence coverage and $\geq 35\%$ sequence identity with the respective conjugative elements of a model conjugative plasmid.

	Number of genomes	Number of plasmids	Number of potential conjugative plasmids (%)	Number of potential conjugative plasmids per class			
				Class 1 (pAW63/pBT9727)	Class 2 (pFR55)	Class 3 (pHT73/pE81-53)	Class 4 (pBMB76)
<i>B. thuringiensis</i>	74	426	63 (14.8)	14	11	23	15
<i>B. cereus</i>	115	325	27 (8.3)	8	9	10	0
<i>B. anthracis</i>	104	151	1 (0.6)	1	0	0	0
<i>B. cytotoxicus</i>	17	32	13 (40.6)	7*	7*	6	0
<i>B. toyonensis</i>	9	26	7 (26.9)	0	5	2	0
<i>B. mycoides</i>	41	190	30 (15.8)	2	26	2	0
<i>B. weihenstephanensis</i> & <i>B. pseudomycoides</i>	5	18	0 (0)	0	0	0	0

* The same seven plasmids were identified in *B. cytotoxicus* based on the model plasmids from class 1 and 2.

Based on proteins composition of model conjugative plasmids from each of the four classes, a tBLASTn approach was adopted to identify potential conjugative plasmids among the 1,168 analyzed via BLAST+ executables (Camacho *et al.*, 2009; BLAST+ v2.12.0+). Results were then filtered based on the presence of hits with at least 60% sequence coverage and 35% sequence identity with two putative transfer ATPases and one putative conjugative peptidoglycan hydrolase. These three elements are often recognized as the basic components of conjugative T4SS (Goessweiner-Mohr *et al.*, 2013; Schiwon *et al.*, 2013). Plasmids previously described as conjugative (see Table 1) were discarded, from the results, as well as the model plasmids themselves. Overall, 12% (141 plasmids) were labelled as potentially conjugative, *i.e.* with self-transfer potential. Of these, 44.7% originated from *B. thuringiensis*, followed by 21.3% from *B. mycoides* and 19.1% from *B. cereus*. This was somewhat expected given that the highest number of analyzed plasmids (426, *i.e.* 36.47%) originated from *B. thuringiensis*.

A comparison based on the four classes of conjugative plasmids adopted in this mining process showed that 23% of putative conjugative plasmids were identified with the model plasmids from class 1, 41% from class 2, 30% from class 3 and 11% from class 4. The 3D graph shown in Fig. 4 highlights the distribution of the 141 putative conjugative plasmids per model per species. Although the four mined classes were of conjugative plasmids originating from *B. thuringiensis*, this was not always reflected in their distribution per species. For instance, plasmids originating from *B. mycoides* formed 44.83% of the potential conjugative pFR55-like plasmids (class 2), *vs.* 18.97% from *B. thuringiensis*. Class 1, *i.e.* pAW63/pBT9727, both originally *B. thuringiensis* plasmids, were identified mostly in *B. thuringiensis* (43.75%) with *B. cereus* and *B. cytotoxicus* taking on second and third places with 25 and 21.88%, respectively. The distribution of these models across species raises the questions of the early speciation inside the *B. cereus s.l.* group, and whether some of these plasmids, or their likes, date back to the early days of *B. cereus s.l.* evolution. This is one of the reasons some studies suggest that these MGEs should be ignored as to not confound the phylogenetic inference process (Bazinnet, 2017).

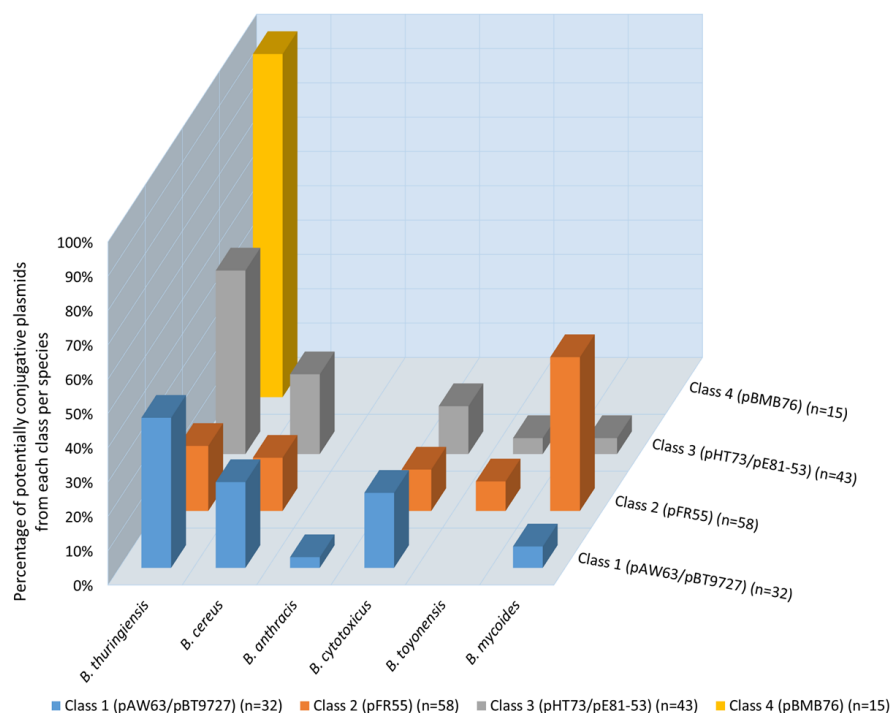


Figure 4: Distribution of potential conjugative plasmids per species based on the four conjugative models (four classes). No hits were obtained with *B. weihenstephanensis* and *B. pseudomycoides*. Each bar shows the percentage of potential conjugative plasmids identified based on the conjugative model of each of the four classes in the corresponding species. The total number of potential conjugative plasmids identified is shown in parenthesis next to the corresponding class.

A fifth class included the conjugative model based on the putative transfer proteins of pXO16. However, when compared to the 1,168 analyzed plasmids, nine hits from *B. thuringiensis* strains matched, five of which are confirmed as "israelensis" strains. The protein sequences from these nine plasmids share at least 99.8% of identity and 99% of coverage with the corresponding protein sequences from pXO16. The reported size of those plasmids is also almost identical to the size of pXO16. The data strongly indicate that the plasmids identified in the nine *B. thuringiensis* strains are actually copies of pXO16 itself. Based on this assumption, the nine plasmids were not counted into the

putative conjugative plasmids and were considered copies of the conjugative model itself.

6. Conjugation into the wild and beyond

6.1. Conjugation under "natural" environmental conditions

Few years after the first description of conjugation-like events in *B. thuringiensis*, interest for plasmid transfer under environmentally relevant conditions started with cadavers of infected lepidopteran larvae as mating matrices (Jarrett and Stephenson, 1990). Transfer was assessed by following either the mobilization of plasmids conferring a specific trait, such as *cry* plasmids (Jarrett and Stephenson, 1990) or the mobilizable Tet^R plasmid pBC16 (Thomas *et al.*, 2000), or by following the transfer of a conjugative plasmid itself, like pHT73 from *B. thuringiensis* sv. *kurstaki* (Vilas-Bôas *et al.*, 1998; Yuan *et al.*, 2007; Santos *et al.*, 2010) or pXO16 from *B. thuringiensis* sv. *israelensis* (Thomas *et al.*, 2001). Over the years, transfer was evaluated in multiple infected lepidopteran larvae, including *Galleria mellonella* and *Bombyx mori* (Jarrett and Stephenson, 1990; Santos *et al.*, 2010), in dipteran larvae (Thomas *et al.*, 2001) and from *B. thuringiensis* donor strains to *B. thuringiensis* or *B. cereus* s.s. recipient strains. As environment-like matrices are much more complex, results were sometimes diverging. For instance, a first study stated that pHT73 could only conjugate in dead larvae among growing vegetative bacteria, after spore germination post infection (Yuan *et al.*, 2007). However, a second study nuanced the previous results by reporting efficient conjugative transfers in environments unsuitable for the multiplication of the recipient strain (Santos *et al.*, 2010).

Preparations of *B. thuringiensis* are widely commercialized as biopesticide to fight against various insect pests. Upon application, numerous spores are released in the environment, especially in soils, where they can survive and multiply. Subsequent studies have focused on conjugal transmission in soils to evaluate the potential dissemination of plasmid-borne genes, *e.g.* *cry* genes. Significant conjugative transfer of the *cry* plasmid pHT73 was reported in non-amended sterile soil, indicating that genetic exchanges between strains

of *B. thuringiensis* could take place in soil (Vilas-Bôas *et al.*, 1998, 2000). However, no transfer could be witnessed when following pBC16 mobilization in non-sterile and non-manipulated soil (Thomas *et al.*, 2000). Instead, rapid sporulation of the strains inoculated for conjugation was observed, which most probably prevented from plasmid exchange (Thomas *et al.*, 2000). These results validated previous observations of transfer of the conjugative plasmid pFT30 between strains of *B. cereus* s.s. and *B. subtilis* in nutrient-amended soil, while no transfer could be detected in non-amended soil (Van Elsas *et al.*, 1987). In this early study, the *B. thuringiensis* donor strain would also quickly germinate after introduction into a soil sample.

From another study run two decades later, only one soil-borne isolate out of 75 exhibited mobilizing activities in triparental matings, *i.e.* carried a conjugative plasmid (Hu *et al.*, 2009a). Such prevalence suggested that conjugation might be rare in soil, although possible, given results described above (Hu *et al.*, 2009a). Moreover, mating conditions used in such studies might not be representative enough of the reality, despite the evident potential for plasmid exchange. Although survival and potential conjugation in soil microcosms is essential for evaluating the impact of *B. thuringiensis*-based pesticide, the question of conjugation impact on the genetic structure of local populations remains. In this context, recent work surveyed the transmissibility and occurrence of conjugative plasmids in confined soil plots (Hu *et al.*, 2020). Within two years post application, the mobility in sprayed plots was 4-5 times higher compared to control plots. Collected data suggested that application of *B. thuringiensis* in specific confined habitats may result in this population supplanting the natural *B. cereus* population and therefore boosting mobility of genetic elements associated with entomocidal properties (Hu *et al.*, 2020). The authors however conceded that the bacterial concentrations used in their study exceeded by far concentrations applied on field for pest management.

Application of *B. thuringiensis* sv. *israelensis*-based products to waterways is essential for the fight against dipteran larvae (*e.g.* mosquitoes and black-flies). Similar to work in soil microcosms, the HGT potential in river water was assessed by using the conjugative plasmid pXO16 (Thomas *et al.*,

2001). Besides significant transfer ratio, an increase in transfer rate at the onset of parental strain sporulation was revealed, suggesting that the optimal cell growth phase for conjugation corresponds to stationary or early sporulation phases (Thomas *et al.*, 2001). Interestingly, another environment explored for the conjugative transfer of pXO16 was the intestine of gnotobiotic rats (Wilcks *et al.*, 2008). In parallel to many studies focusing on conjugative transfer in natural environments between strains of *B. thuringiensis*, HGT between strains of *B. anthracis* was demonstrated within a rhizosphere model set up used to decipher *B. anthracis* survival in the rhizosphere of grass plants (Saile and Koehler, 2006). Although highly important in the case of *B. cereus* *s.l.*, interest for transfer of genetic material in natural environments, and especially in the rhizosphere, goes beyond the *B. cereus* group (Normander *et al.*, 1998; Sørensen and Jensen, 1998; Ronchel *et al.*, 2000; Pearce *et al.*, 2001).

6.2. Conjugation in foodstuffs

For food security and health management, bacterial contamination and gene transfer in food products are key issues. Studies have revealed the potential of genetic material exchange in microenvironments, including home cooking-related situations (Kruse and Sorum, 1994), and in common food products (Coconcelli *et al.*, 2003). Since some members of the group are opportunistic pathogens occasionally associated with food poisoning (Dierick *et al.*, 2005; Naranjo *et al.*, 2011; Delbrassinne *et al.*, 2012), *B. cereus* *s.l.* behavior in food products has been documented, including its DNA exchange potential. Plasmid exchange was mostly illustrated by conjugation of the *B. thuringiensis* plasmids pAW63 and pXO16, as well as by mobilization of the small plasmids pUB110 and pC194. Studies focused on liquid and semi-liquid products, including cow milk, plant-based drinks and rice pudding (Van der Auwera *et al.*, 2007; Timmerly *et al.*, 2009; Modrie *et al.*, 2010). Overall, conjugative transfers usually occurred at least as efficiently in food preparations as in culture broth and mobilization of small plasmids was successfully reported. Kinetics studies revealed an impact of the different food matrices on both the potential for plasmid transfer and the timing of conjugation. Compared to frequencies obtained in typical culture broth, higher transfer frequencies were obtained in food matrices, resulting

from an earlier onset of conjugation, an increased transfer frequency and an extended mating time (Modrie *et al.*, 2010). Similarly, higher transfer frequencies of pHT73 were noticed in milk compared to LB medium (Yuan *et al.*, 2010). Gene transfer can even occur in prepacked ready-to-eat food, and not solely in private or restaurant kitchens. Indeed, pAW63 showed hasty and more efficient conjugation properties in salt conditions mimicking those commonly found in food products like cheese and sausages (Beuls *et al.*, 2012).

In a study looking at *B. cereus* strains used in probiotics, a large proportion of isolated strains were resistant to tetracycline and one of these strains harbored the genetic determinants of tetracycline resistance on an MGE, presenting a risk of antibiotic-resistance gene transfer (Zhu *et al.*, 2016). From other strains of *B. cereus s.l.* isolated from both raw and ultra-heat-treated milk, around 20% of the non-*B. anthracis* strains carried pXO1-like and/or pXO2-like plasmids (Bartoszewicz and Marjańska, 2017). As described previously, the family of pXO2-like plasmids are well known for their conjugative potential (*i.e.* pAW63 and pBT9727, see Section 4.1), emphasizing the potential for conjugation in this case study. Based on work cited hereinabove, food products are proposed to favor exchange of genetic material (Bartoszewicz and Marjańska, 2017). By providing richer media, foodstuffs might indeed sustain bacterial proliferation, resulting in higher bacterial density, extended mating periods and more efficient plasmid transfer (Bartoszewicz and Marjańska, 2017).

6.3. Conjugation in confined environments

Bacteria are well known for their adaptability to challenging environments and for their amazing genetic flexibility due to HGT. Strictly confined environments, such as the Antarctic Concordia Station (ACS) or the International Space Station (ISS), are especially rough due to space confinement, high levels of irradiation and/or microgravity. Microbial genetic exchange in such conditions raised scientists' interest due to potential acquisition of new pathogenic traits. In spacecraft, modifications in microbiome could threaten astronaut health. Significant number of studies evaluated potential effects of real or simulated microgravity on bacterial characters, including

growth, phenotype, virulence, metabolism and gene transfer (Senatore *et al.*, 2018; Prasad *et al.*, 2020; Bijlani *et al.*, 2021; Mahnert *et al.*, 2021). Most of these studies concluded in a strong influence of microgravity on bacterial communities, potentially leading to the evolution of space-specific properties that would not arise on Earth. Research groups also focused on the potential effects of microgravity on HGT. While an increase in genetic exchange between *Acinetobacter* and *Staphylococcus* strains co-cultivated under simulated microgravity was reported, the root cause of this stimulation of genetic transfer was not investigated and transformation was proposed as the main underlying transfer mechanism (Urbaniak *et al.*, 2021). Another research study examined *Staphylococcus* and *Enterococcus* strains isolated from the ISS and the ACS for their conjugative properties. More than 80% of ISS strains and over 50% of ACS strains actually carried plasmids encoding signature transfer genes from well-characterized Gram-positive conjugative plasmids, like pIP501 (Schiwon *et al.*, 2013).

Studying conjugation under space or confined conditions among the members of the *B. cereus* group was not left behind, particularly for strains of *B. thuringiensis*. The first study investigating plasmid-mediated conjugation under space flight conditions involved triparental matings of Gram-positive or Gram-negative bacteria and used *B. thuringiensis* as a Gram-positive model. Not only was it established that genetic exchange could occur by conjugation in space flight, but it was also suggested that conjugation and mobilization capacities were increased in the Gram-positive model (De Boever *et al.*, 2007). However, such conclusions must be nuanced by the poor experimental controls and unexplained negative results in ground-based experiments. Furthermore, work published a couple of years later reported no statistically significant differences in conjugation and mobilization efficiency of *B. thuringiensis* in simulated microgravity – using three different simulation set-ups – compared to standard laboratory conditions (Beuls *et al.*, 2012). Although no stimulation of genetic transfer was revealed, neither was inhibition. Further evidence of HGT in confined space habitats were gathered when one *B. cereus* *s.l.* strain, putatively harboring a conjugative plasmid, was identified among more than 40 *Bacillus* strains isolated from ISS and ACS and tested for conjugative abilities (Timmerly *et al.*, 2011). Strains were also tested for their capacity to re-

ceive foreign DNA by HGT, mimicking their potential recipient role in conjugative matings. Overall, two strains of *B. cereus s.l.* could acquire the conjugative plasmids pAW63 and pXO16, and four additional strains successfully acquired pXO16 (Timmerly *et al.*, 2011). Concerning pXO16 transfer, recent work demonstrated an extended host range of the plasmid via solid mating instead of liquid mating (Hinneken *et al.*, 2019). One should then consider the possibility that more ISS and ACS strains could act as pXO16 recipients when assessing transfer in solid matings. Altogether, collected data indicated that (i) most *B. cereus s.l.* strains analyzed for their plasmid profiles carried at least one plasmid and (ii) plasmid-mediated transfer, *i.e.* conjugation, could occur between *B. cereus s.l.* strains originating from space environments and in space-mimicking conditions (De Boever *et al.*, 2007; Beuls *et al.*, 2009; Timmerly *et al.*, 2011). In light of astronaut health, such considerations are essential as modifications in "strain-plasmid" association could lead to harmless strains developing pathogenic characteristics (Timmerly *et al.*, 2011).

7. Concluding remarks and outlooks

Plasmids are key players in the lifestyle of *B. cereus s.l.*, whether they encode major toxins, provide niche-specific traits, or promote exchange of genetic material. When it comes to conjugation, although a significant proportion of plasmids from *B. cereus s.l.* show potential for self-transferability, more elements come into play. Despite studies analyzing conjugal transfer in specific environments, like insect larvae, soil, foodstuff or confined environments, conjugation was mostly examined in laboratory environments, under highly artificial mating conditions. This applies to studies both in *B. cereus s.l.* and other bacterial groups. However, the natural environment for conjugation seems much more complex, likely occurring in dense bacterial communities in diverse natural niches (Shen *et al.*, 2022). Furthermore, the different members of the *B. cereus* group tend to occupy different environmental niches, limiting their contact and interaction, and thereby limiting conjugation events. This is particularly true for *B. anthracis* who displays a specific and restricted natural lifestyle (Jensen *et al.*, 2003). Moreover, all strains exhibiting a hybrid phenotype, *e.g.* strains of *B. anthracis* or *B. cereus s.s.* with *B. thuringiensis cry* plasmids, were so far obtained in laboratory settings and not isolated from

natural environments (Battisti *et al.*, 1985; Yuan *et al.*, 2010).

In addition to the limited occurrence of "natural" conjugation *per se*, it is important to consider the relative stability of plasmids in different hosts. Broad-host spectrum plasmids have evolved to adapt to multi-host environments. However, in restricted niches, studies showed that in the hosts, plasmids have specifically adapted to alleviate their metabolic cost (Kottara *et al.*, 2016). In other bacteria, maintaining such host-specific plasmids might be more costly, limiting the success of potential transfer overtime. Plasmid stability in a defined host relies on complex interactions between the plasmid itself, the host and the environment, which interact together to determine the balance between plasmid carriage burden and the profit from plasmid-borne genes (Hall *et al.*, 2015; Kottara *et al.*, 2018). More insights into the overall impact and risk of conjugation in the *B. cereus* group, and in other bacterial species, should be brought by further studies focusing on conjugation under environmental-relevant conditions, within complex bacterial communities.

8. Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

9. Author contributions

Conceptualization, P.H., N.F., J.M.; Methodology, P.H., N.F., J.M.; Software, P.H., N.F.; Validation, P.H., N.F., J.M.; Formal analysis, P.H., N.F.; Investigation, P.H., N.F.; Resources, J.M.; Data curation, P.H., N.F.; Writing - original draft preparation, P.H., N.F., J.M.; Writing - review and editing, P.H., N.F., A.G., J.M.; Supervision, J.M.; Project administration, J.M.; Funding, J.M. All authors read and approved the final manuscript.

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Objectives

Since its first description about 35 years ago, the powerful conjugation mechanism of pXO16 has been investigated to understand better its transfer properties and kinetics, its mobilisation capacities and its intriguing aggregation mechanism. Until the thorough analysis of its sequence in 2015, no data were available about the genetics underlying both the aggregation and the conjugation phenotypes. However, with the delimitation of pXO16 transfer locus, *tip*, new perspectives opened up to study the transfer apparatus of this giant plasmid. Also, since reporting aggregation-independent events, interest got renewed about the host spectrum of pXO16.

The primary aim of this work is to further explore the conjugation properties of this giant plasmid, with a particular focus on early transfer steps and some key constituents of the transfer apparatus. To this end, the molecular players potentially governing the initiation of pXO16 conjugative transfer are explored, based on a relaxase-*oriT* interaction hypothesis (Chapter 6.1). In parallel, the function of a predicted peptidoglycan hydrolase is scrutinised to investigate its *bona fide* role in pXO16 conjugation (Chapter 6.2). A second major objective of this study is to evaluate the transfer of pXO16 across different bacterial species. As aggregation is proposed to favour pXO16 transfer, solid-based mating conditions, mimicking this aggregation phenomenon, are thought to help widening the host spectrum of the plasmid (Chapter 7.1). Strains of *B. cereus s.l.* are first examined as potential hosts, before considering other Gram-positive bacterial species. Nonetheless, pXO16 is naturally restricted to *B. thuringiensis sv. israelensis*. Therefore, several tracks are followed to help understand the natural restriction of the plasmid compared to its circulation under laboratory settings (Chapter 7.2).

Part II

Results

Insights into pXO16 transfer apparatus

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In Gram-positive bacteria, conjugation can mediate the transfer of ssDNA (*e.g.* pIP501, pCF10 and pCW3) or dsDNA in the case of *Streptomyces*. For ssDNA transfer, conjugation relies on two main machineries: a relaxosome, composed of a main protein, the relaxase, and an *oriT*, and a transferosome, made of a T4SS. Although it is most likely transferred as ssDNA, the genetic bases underlying pXO16 conjugation remain mostly unknown. This chapter brings new elements to the puzzle of pXO16 transfer apparatus by investigating the relaxosome function (Section 6.1) and by looking at the conjugative PG hydrolase (Section 6.2).

6.1 Inquiries about pXO16 relaxosome

In the course of investigating pXO16 relaxosome, it became evident that the mechanism used by pXO16 in early steps of conjugation must distinguished from mechanisms used by other known conjugative plasmids. In this section are reported the evidence supporting this assumption. First, the search for the *oriT* using mobilisation assays is described. Relaxase and helicase candidates are then investigated by gene knockout experiments and *in vitro* binding assays.

This work is presented as a monograph

**Investing candidate elements for the relaxosome of
pXO16, the conjugative plasmid of *Bacillus*
thuringiensis sv. *israelensis***

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Monograph

Abstract

The mosquitocidal *Bacillus thuringiensis* sv. *israelensis* carries out a large pool of plasmids, including the toxin-carrier pBtoxis and the conjugative master pXO16. Naturally restricted to *B. thuringiensis* sv. *israelensis*, pXO16 can transfer to all members of the *B. cereus* group. pXO16 is characterised by high levels of self-transfer and by successful mobilisation of virtually any co-resident plasmid and chromosomal locus. Over the years, data were gathered about the genetics behind pXO16 transfer mechanism. The *tip* locus was proposed as encoding the transfer genes of pXO16 and several key transfer elements were identified within this specific region. However, information is still lacking about the early steps of pXO16 transfer, mainly the relaxase involved in DNA processing at the *oriT*. In this work, several approaches were explored to delimit the plasmid *oriT*, including *in silico* predictions and experimental delimitation by mobilisation assays of fragmented *tip* sequence. Additionally, the role of two putative relaxosome proteins, TipO and TipP, was investigated with a genetic approach, consisting of building knockout plasmid variants, and a protein approach, based on observing potential nicking activity.

Keywords: pXO16; relaxase; origin of transfer; conjugation; *Bacillus thuringiensis*

1. Introduction

Horizontal gene transfer (HGT) is often defined as "the non-genealogical transmission of genetic material from one organism to another", in opposition to the vertical parental inheritance (Goldenfeld and Woese, 2007). It plays a major role in bacterial evolution by facilitating the acquisition and exchange of genetic material between distantly related organisms (Thomas and Nielsen, 2005; Soucy *et al.*, 2015; von Wintersdorff *et al.*, 2016). One of the major means of HGT is conjugation, a DNA transfer mechanism necessitating a close physical contact between the mating partners (Cabezón *et al.*, 2015). Conjugative plasmids, *i.e.* plasmids capable of promoting their own transfer, encode the two main machineries required for conjugation-mediated transfer: a relaxosome and a transferosome. The latter consists of a type IV secretion system (T4SS) inserted into the donor cell envelope and forming a mating channel for DNA transport to the recipient cell (Goessweiner-Mohr *et al.*, 2013; Waksman, 2019). The relaxosome, on the other hand, is in charge of DNA processing prior to delivery to the T4SS for subsequent transfer. It is composed by a relaxase, a usually large multidomain protein, an origin of transfer (*oriT*) and plasmid or host-encoded accessory proteins (de la Cruz *et al.*, 2010).

The relaxase catalyses a strand- and site-specific transesterification reaction at the *nic* site of the plasmid *oriT*. After nicking, the relaxase remains bound to the nicked *oriT*, forming a nucleoprotein complex recruited by a coupling protein to the transferosome (Virolle *et al.*, 2020). For *oriT* nicking, the relaxase requires a transesterase (or relaxase) activity and additional activities, like DNA helicase or DNA primase, either encoded by distinct proteins or by different domains of the same protein (multidomain relaxase) (Garcillán-Barcia *et al.*, 2009). Relaxases are usually classified into ten MOB families depending on their protein relationship, their catalytic site and the characteristics of their mode of action (Guzmán-Herrador and Llosa, 2019). The *oriT* is a plasmid-borne sequence of 40 to 400-bp long, usually located nearby the relaxase gene, rich in A+T and flanked by one or more inverted repeats (IR) (de la Cruz *et al.*, 2010; O'Brien *et al.*, 2015).

Although bacterial conjugation was mostly studied among Gram-negative bacteria, some Gram-positive conjugative plasmids were also thoroughly described (Goessweiner-Mohr *et al.*, 2013). An intriguing conjugative plasmid is that of the Gram-positive *B. thuringiensis* sv. *israelensis*, pXO16. This plasmid was first described for its ability to induce the formation of macroscopic aggregates between mating partners of its host (Andrup *et al.*, 1993; Jensen *et al.*, 1995). It was also demonstrated to be capable of displacing co-resident non-mobilisable plasmids and host chromosomal loci (Andrup *et al.*, 1996; Makart *et al.*, 2017). Host range, mobilisation capacities and kinetics of transfer of pXO16 have been studied in depth (Andrup *et al.*, 1998; Timmerly *et al.*, 2009; Makart *et al.*, 2018; Hinnekens *et al.*, 2019; Hinnekens and Mahillon, 2022), but the genetic bases underlying its conjugative mechanism remain less understood. Makart *et al.*, (2018) proposed a 25-kb long locus, named *tip*, as the region encoding the genes (*tipA* to *tipP*) responsible for pXO16 conjugation. Within this locus were highlighted two ATPases, some candidate channel components and a peptidoglycan hydrolase (Makart *et al.*, 2018; Hinnekens *et al.*, 2021). However, no information has been gathered on the relaxosome of pXO16 conjugation mechanism yet. In this work, we investigated the localisation of pXO16 *oriT*, as well as the putative function of TipO and TipP as relaxosome proteins.

2. Material and methods

2.1. Bacterial strains and plasmids

Bacterial strains and plasmids used in this work are listed in Table S1 and Table S2, respectively. 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* NEB5 α cells (New England Biolabs™) while these using the pET-30a and pHT304-18Z vectors were transformed into chemically competent *E. coli* 10 β cells (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. Genetic constructs

2.3.1. *oriT* delimitation Fragments of interest were amplified by PCR from pXO16 using the appropriate F/R primers (Table S3 and Fig. 1). The PCR products and pHT304-18Z vector were assembled by restriction/ligation at *Pst*I/*Bam*HI sites. Resulting products were transformed into 10 β *E. coli* cells, then into *dam*⁻/*dcm*⁻ *E. coli* C2925 cells and finally electroporated into GSX002 cells carrying pXO16, as previously described by Mahillon and Lereclus (2000).

2.3.2. *tipO* and *tipP* knockout variants A 1-kb fragment upstream and a 1-kb fragment downstream of the *tipO* or *tipP* gene were amplified by PCR from pXO16 using the *tipO*-up-F/R and *tipO*-down-F/R or *tipP*-up-F/R and *tipP*-down-F/R primers, respectively (Table S3), and the kanamycin resistance cassette (*kan*) was amplified from pDG783 using the *tipO*-Kan-F/R or *tipO*-Kan-F/R primers. For *tipO* knockout, PCR products were cloned into the pMAD thermo-sensitive shuttle vector via the HiFi DNA Assembly method (New England Biolabs™). For *tipP* knockout, the three PCR fragments were digested with the appropriate restriction enzymes (see Table S3) and were ligated together. The resulting product was amplified by PCR using the *tipP*-up-F and *tipP*-down-R primers (PCR on ligation). The amplified fragment and

the pMAD vector were digested by *NcoI*/*Bam*HI and ligated together. The resulting vector was first transformed into NEB5 α *E. coli* cells following HiFi DNA Assembly (*tipO* knockout) or TOP10 *E. coli* cells following PCR on ligation assembly (*tipP* knockout). Then, the construction was transformed into *dam*⁻/*dcm*⁻ *E. coli* C2925 cells and finally electroporated into GSX002(pXO16) cells as previously described by Mahillon and Lereclus (2000). pXO16 Δ *tipO* and pXO16 Δ *tipP* 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, pXO16 Δ *tipO* and pXO16 Δ *tipP* derivatives were also selected based on blue-white screening from growth on LB with X-gal. Replacement of the *tipO* or *tipP* gene by the *kan* gene was verified by PCR.

2.3.3. Cloning of *tipO* and *tipP* for protein expression To further express TipO and TipP, the *tipO* and *tipP* genes were independently cloned into the pET-30a vector. The fragments were amplified by PCR using the appropriate primer pairs (Table S3) and each fragment was ligated into the *NcoI*/*XhoI* sites of pET30a to generate pET-30::TipO and pET-30::TipP. Finally, the constructions were transformed into the expression strain *E. coli* BL21(DE3).

2.4. Mobilisation assays

Mobilisation assays were realised similarly to liquid mating assays described previously (Hinneken *et al.*, 2019). Briefly, separate pre-cultures of donor and recipient cells were grown in 10 mL of LB from fresh plating and incubated overnight, without shaking. Equal amounts of mating partner cells from pre-cultures (maximum 1 mL per partner) were mixed in 10 mL of LB and incubated without shaking for four hours (mating time). After mating, appropriate dilutions were plated on selective LB plates for parental cells and transconjugant cells (*i.e.* recipient cells that have acquired the mobilisable plasmid) and incubated overnight. The mobilisation frequencies were calculated as the ratio of transconjugants per donor cells (T/D) at the end of the conjugation time. Each mating experiment also included a plating control of parental pre-cultures to exclude the presence of potential spontaneous mutants. All matings were repeated at least three times, independently.

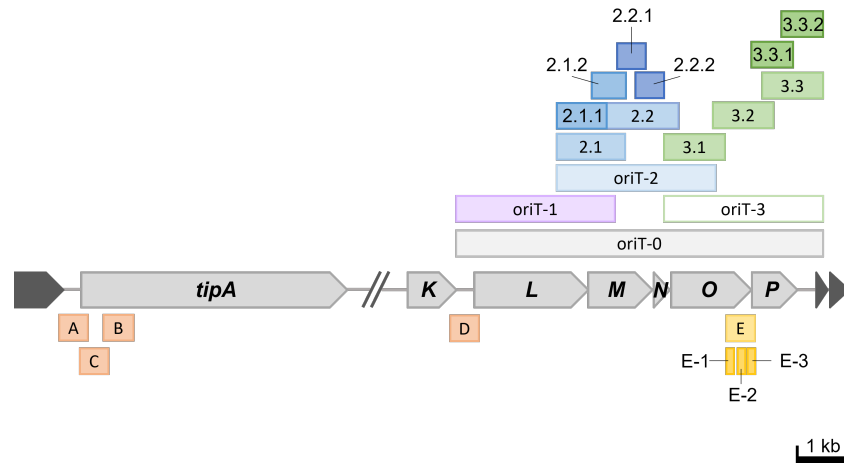


Figure 1: Localisation on pXO16 *tip* locus of the different fragments tested in mobilisation assays. The different fragments were cloned into the pHT304-18Z vector for subsequent mobilisation assays. Arrows in light grey represent the *tip* genes, whereas arrows in dark grey represent genes located right upstream and downstream of the *tip* locus. Rectangles above the *tip* sequence illustrate oriT-0 (7 kb) and its derivative subdivided fragments. Orange boxes (A-D) correspond to fragments upstream the *tip* locus or upstream the *tipL-tipP* operon. The larger yellow rectangle (E) represents the IR-rich region between *tipO* and *tipP*, with the smaller yellow rectangles underneath (E-1, E-2 and E-3) depicting subdivisions of E.

2.5. Protein expression and purification

The genes encoding TipO and TipP were cloned into pET30a and recombinantly expressed in *E. coli* BL21(DE3) cells. The overall process for expression and purification of recombinant proteins is similar to that described by Hinnekens *et al.* (2021). BL21(DE3) containing pET30::TipO or pET30::TipP were cultured overnight in selective LB (Kan). Fresh cultures (LB + Kan) containing 2% (w/v) of glucose were inoculated from overnight cultures (1:10) and incubated until the optical density (OD, 600 nm) reached 0.5-0.8. Protein expression was induced with 0.5 mM of IPTG (Isopropyl β -D-1-thiogalactopyranoside) (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. For subsequent cell lysis, 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 instructions. The final protein buffer consisted in PBS with 300 mM NaCl. Purified proteins were loaded on an SDS-page gel to assess purity. Their concentration was assessed via a Bradford assay (Kruger, 1994).

2.6. Plasmid nicking assays

The potential nicking activity of TipO and TipP was evaluated by plasmid relaxation experiments, inspired by the method described by Wisniewski *et al.* (2016). Plasmid relaxation was evaluated with the constructions containing the different pXO16 "*oriT*" fragments (Fig. 1) cloned into pHT304-18Z and with the empty pHT304-18Z vector as control. Reactions consisted of 1 x binding buffer (20 mM Tris, pH 7.5, 5 mM MgCl₂, 100 mM NaCl) and plasmid DNA incubated either alone or with various concentrations of either *His*₆-TipO, *His*₆-TipP or both TipO and TipP for 30 min at 37°C. Following incubation, the reaction mix was loaded on 0.6 % (w/v) agarose gel (ChemCruz™), stained with ethidium bromide (Sigma-Aldrich™), and submitted to gel electrophoresis for 90 min at 100 V before imaging on a GelDoc™ XR System (Bio-Rad).

2.7. Bioinformatics

The *oriT* finder software (Li *et al.*, 2018) relies on a database of known *oriT* regions for identifying consensus in query sequences. Multiple sequence alignments were then performed using Clustal Omega (Sievers *et al.*, 2011). Inverted repeats (IRs) were localised on pXO16 sequence via Emboss palindrome (Rice *et al.*, 2000). pXO16 sequence from *B. thuringiensis* HD789 can be found using the accession number CP003764.1, TipO and TipP pro-

tein sequences can be found under the accession numbers AFQ30058.1 and AFQ30057.1, respectively.

3. Results and discussion

3.1. Attempts for *oriT* delimitation

3.1.1. *In silico* predictions First approaches for delimiting pXO16 *oriT* relied on bioinformatics predictions, based on major traits of known *oriT*. *oriT* are typically localised nearby other putative plasmid-encoded relaxosome elements, in an A+T rich region and flanked by IRs. As the sequence of pXO16 is naturally rich in A+T, this criterion is not relevant here. *In silico* predictions focused on the *tip* region as the *oriT* is expected to be localised nearby the conjugation locus. The "*oriT*finder" software, which aims at identifying consensus sequences within a query sequence based on known *oriT* sequences, indicated no *oriT* candidates. Multiple sequence alignments were then performed with Clustal Omega, using the consensus *nic* site sequence of plasmids from different MOB families (Table S4). The results obtained were compared to IR localisation (Emboss palindrome) on pXO16 sequence. Those analyses, taken together or separately, provided hundreds of more or less interesting candidate regions within the *tip* locus and on the entire plasmid sequence. Overall, no strong *oriT* candidate could be defined *in silico*, with bioinformatics "*a priori*" approaches.

3.1.2. Experimental approach by mobilisation assays A "*non a priori*" experimental method was developed to identify pXO16 *oriT*. Inspired by Lee and Grossman (2007) work on ICEBs1 *oriT*, we performed mobilisation assays with genetic constructs of a *tip* fragment cloned into the pHT304-18Z vector. When a fragment induced a significant increase in transfer frequencies compared to the empty vector, it was further subdivided and tested in mobilisation assays (Fig. 1). Similarly, when a fragment induced no increase compared to the empty vector, it was dismissed from the candidate pool. We aimed at identifying a minimal fragment necessary for significantly enhancing pHT304-18Z mobilisation. The transfer frequencies associated with the different constructs are represented on Fig. 2.

pXO16-mediated mobilisation was significantly improved when the initial oriT-0 fragment was cloned into pHT304-18Z, compared to the empty vector. oriT-0 was therefore subdivided into three overlapping parts (oriT-1, -2 and -3). As the cloning of oriT-3 could not be achieved, it was divided into three smaller overlapping fragments (oriT-3.1, -3.2 and -3.3). The resulting constructs were tested in mobilisation assays and mobilised at various frequencies. pHT304-18Z::oriT-3.2 exhibited poor mobilisation, while oriT-1 and -3.1 decreased the mobilisation efficiency of the vector compared to oriT-0. oriT-2 was the best candidate as it exhibited transfer frequencies almost identical to oriT-0. Interestingly, oriT-3.3 also showed significant remnant mobilisation efficiency. Both oriT-2 (3,300 bp) and oriT-3.3 (1,200 bp) were further divided into oriT-2.1 and -2.2, and oriT-3.3.1 and -3.3.2, respectively. Fragments descending from oriT-3.3 were mobilised at frequencies significantly different from oriT-0, but not from the empty pHT304-18Z vector. Therefore, they were both discarded from potential *oriT* candidates. The fragments descending from oriT-2, oriT-2.1 and oriT-2.2, both retained a significant transfer frequency, especially for oriT-2.2 which was mobilised at frequencies significantly different from the empty vector but not from the initial oriT-0 fragment. Both oriT-2.1 (1,151 bp) and oriT-2.2 (1,608 bp) were further subdivided into oriT-2.1.1/-2.1.2 and -2.2.1/-2.2.2, respectively. Each subdivision resulted in fragments mobilised at frequencies significantly lower than that of oriT-0, except for oriT-2.2.2, but not significantly higher than that of the empty vector.

Others demonstrated that the *oriT* of known Gram-positive plasmids could be located at the upstream extremity of the conjugation locus, nearby the relaxase and other relaxosome elements, in intergenic regions or at the extremity of a coding sequence (Kurenbach *et al.*, 2002, Lee and Grossman, 2007, Wisniewski *et al.*, 2016). Therefore, small fragments (A-E) located at strategic positions within the *tip* locus were also tested in mobilisation assays. Fragments A-C correspond to the very upstream section of the locus, within the intergenic region upstream *tipA* and at the 5' extremity of *tipA*, whereas fragment D covers the intergenic region between *tipK* and the putative coupling protein-coding gene *tipL*. The last fragment (E) and its subdivided sections (E.1, E.2 and E.3) correspond to an IR-rich zone overlapping *tipO* and *tipP*, which are both putative relaxosome elements. Upon insertion into

pHT304-18Z, none of the fragments oriT-A to -E was mobilised at frequencies significantly higher than the empty vector.

As illustrated with the horizontal dotted lines on Fig. 2, a majority of the fragments originating from the initial oriT-0 fragment resulted in mobilisation with a range of efficiency comprised within the transfer efficiencies of pHT304-18Z and pHT304-18Z::oriT-0. Even if some fragments initially exhibited high mobilisation frequencies, they remained extremely large compared to known *oriT* regions and were therefore further subdivided. Those subdivisions systematically and drastically reduced the transfer frequencies, usually no longer different than the transfer frequencies of the empty vector. Some matings were additionally repeated under filter mating conditions, which did not provide any further information (data not shown).

Given the number of fragments tested and the large sequence covered by the multiple mobilisation assays, we doubt that pXO16 encodes a typical *oriT* within the *tip* locus. Several alternatives could be hypothesised. The *oriT* could first be much larger than known *oriT*, and could therefore be located within the fragments oriT-2 or oriT-2.2. It could also be encoded by another region of the plasmid. Alternatively, pXO16 could rely on "*oriT*-like" regions or its relaxase could recognise sequences with low specificity to initiate its transfer. Its *oriT* could at last either belong to a novel type of *oriT*, or pXO16 could use an alternative system to prime its transfer, different from the typical relaxase-*oriT* recognition system hypothesised so far.

3.2. Putative relaxosome proteins

While describing the *tip* locus as the conjugation locus of pXO16, Makart *et al.* (2018) indicated the absence of homologue to any described conjugative relaxase within the *tip* locus. However, the one before last *tip* gene, *tipO*, is predicted to encode a helicase. Helicase domains are often carried out by the relaxase protein itself or by an accessory protein (Garcillán-Barcia *et al.*, 2009). Due to its proximity to the predicted helicase TipO, TipP was therefore indicated as a potential candidate for the relaxase function. In this work, we investigated the potential role of both TipO and TipP in pXO16 conjugation.

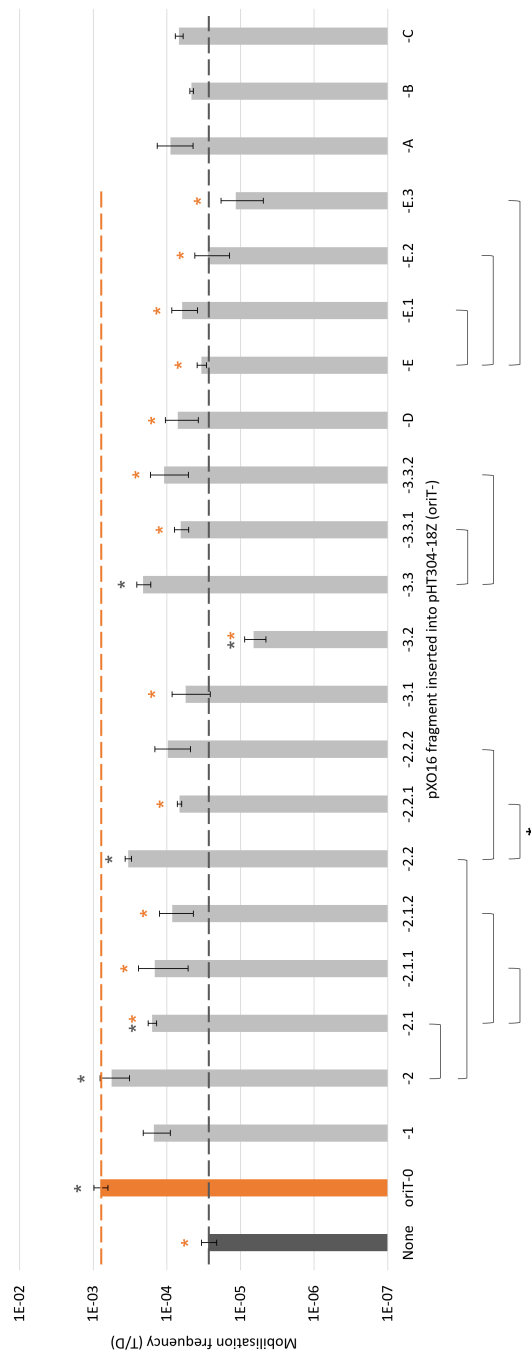


Figure 2: Transfer efficiency of the different genetic constructs with pHT304-18Z and fragments from pXO16, in mobilisation assays. Mobilisation frequencies are expressed as transconjugants per donor cells (T/D). Each frequency corresponds to the mean $\pm$ SEM out of three replicates. An orange star indicates that the differences in frequencies with the oriT-0 fragment (orange bar) were significant, following an unpaired Student's *t*-test ($p < .05$). Similarly, a gray star indicates that the differences in frequencies with the empty pHT304-18Z vector (gray bar) were significant. A black star indicates that the differences between frequencies of the fragments over the same bracket were significant, following an unpaired Student's *t*-test ($p < .05$). Two reference lines are drawn: the dotted orange line reports the mobilisation frequency of pHT304-18Z containing the initial oriT-0 fragment and the dotted gray line represents the transfer frequency of the empty pHT304-18Z vector by pXO16. All fragments under the dotted orange line cover a section of the original oriT-0 fragment. Other fragments (oriT-A, B and C) cover another section of the *tip* locus (see text and Fig. 1).

3.2.1. Knockout variants of pXO16 for *tipO* and *tipP* To first evaluate the importance of *tipO* and *tipP* for pXO16 transfer, we replaced each gene (one at a time) by a kanamycin-resistance (*kanR*) cassette in pXO16 sequence via double recombination (see Material and methods). To confirm gene replacement and assess the integrity of the knockout variant, multiple PCR were performed. When primers hybridising in the region upstream and in the region downstream of the gene of interest (Fig. 3 (a)) were used, two bands were systematically observed on the resulting gel electrophoresis. Fig. 3 (b) features one gel obtained for the screening of *tipP* knockout variants. Given that *tipP* and *kanR* sizes differ by around 400 bp, we could determine that the upper band (framed in green in Fig. 3 (b)) corresponded to the amplification of a fragment with *kanR* and the lower band (framed in pink in Fig. 3 (b)) to the amplification of a fragment with *tipP*. Similar results were obtained when trying to replace *tipO* by *kanR* (data not shown).

In both knockout attempts, the resulting variants somehow still harboured both versions of pXO16, one with the target gene and one with the *kanR* cassette. These data indicate that both *tipO* and *tipP* display an essential role in pXO16 maintenance and support the hypothesis of their implication in a mechanism related to DNA processing and replication. Therefore, pXO16 could encode a replicative relaxase, like the conjugative transposon Tn916. In the case of Tn916, the *oriT* also serves as an origin of replication and the element replicates by a rolling-circle replication (RCR) mechanism (Wright and Grossman, 2016). However, with its size of 350 kb, pXO16 is almost twenty times larger than Tn916 (18 kb) and, to the best of our knowledge, RCR has never been reported for large plasmid molecules.

3.2.2. *In vitro* activity of TipO and TipP on plasmid DNA TipO and TipP were incubated in varying concentrations with plasmid DNA to assess their potential DNA binding and nicking activity. Plasmid DNA corresponds to the different constructions used in mobilisation assays (Fig. 1). When incubating DNA with increasing concentrations of TipO, a shift in DNA mobility was observed (Fig. 4 (A)). Indeed, in absence of protein, both the supercoiled (CCC) and the open circular (OC) conformations of the vector were visible, while the linearised (L) conformation became visible in presence of TipO (Fig.

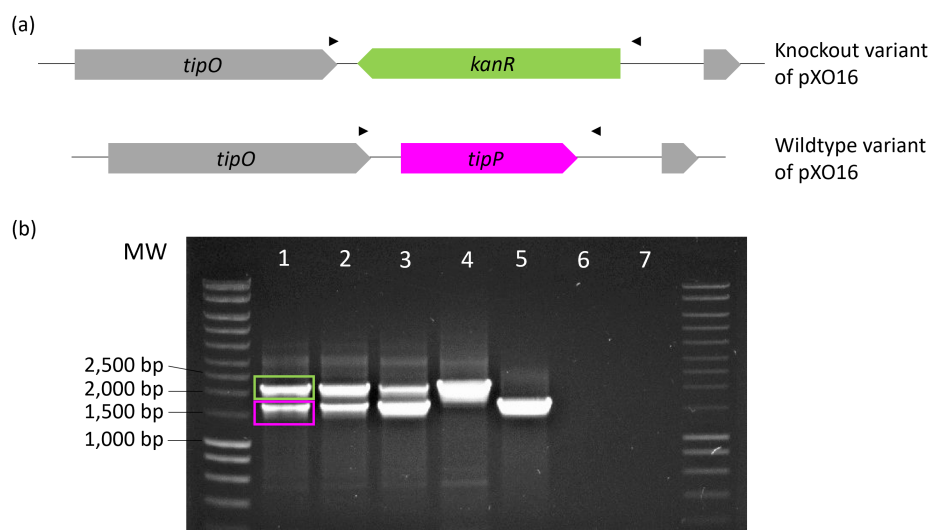


Figure 3: Simultaneous presence of the *tipP* knockout variant and the wildtype variant of pXO16. (a) Schematic representation of both variants of pXO16 and the hybridisation site of the primers (black arrow heads) used for generating (b). (b) Gel electrophoresis illustrating the simultaneous presence of two bands in knockout candidates (lanes 1 and 2). The band framed in green has the expected size of the knockout variant (*kanR* cassette replacing *tipP*, upper (a)), whereas the band framed in pink has the expected size of the wildtype variant (lower (a)). 1, 2: knockout candidates; 3: GSX002(pXO16, pMAD::KOTipPKan); 4: C2925(pMAD::KOTipPKan); 5: GSX002(pXO16); 6: GSX002; 7: null control; MW: molecular weight.

4 (A), line 1 *vs* lines 2-4).

On the contrary, no shift was observed when incubating DNA with TipP. No matter the protein concentration, the pattern remained unchanged on gel (Fig. 4 (B)). In a third experiment, TipO and TipP were added at increasing concentrations to DNA, either alone or simultaneously (Fig. 4 (C)). In presence of TipO, the OC and L conformations were visible (lines corresponding to L and CCC conformations tend to confound in a single bottom line). In presence of TipP only, no conformational change could be observed. When DNA was incubated with the same concentration of both TipO and TipP, the conformation pattern remained identical to that observed

for TipO alone. Taken together, these results indicate that TipO is responsible for the conformational change observed in those mobility assays and carries a DNA nicking activity.

Fig. 4 (D) illustrates the pattern obtained after incubating plasmid DNA (here pHT304-18Z::oriT-C) alone (lanes 1 and 4) or with 3 pmol of TipO (lanes 2 and 5). The plasmid DNA used in lanes 1 to 3 was methylated, whereas the plasmid DNA used in lanes 4 to 6 was non-methylated. Interestingly, when DNA was non-methylated, a complex appeared at the top of the gel (white arrow on Fig. 4 (D)), but not when DNA was methylated. This observation was made with all other genetic constructs tested and might indicate that TipO binds to DNA, forming a nucleoprotein complex stuck at the top of the agarose gel. However, preliminary treatments with SDS and proteinase K did not help understanding the nature of this complex so far. Beside the OC and L forms of the plasmid visible in lanes 2 and 5 (red frames in Fig. 4), a smear formed at the size of the OC conformation when DNA was methylated (lane 2), while a band became visible within the smear when the DNA was non-methylated (lane 5).

Although these observations tend to confirm the nicking activity of TipO, the same patterns were obtained for all plasmids tested, even for the empty pHT304-18Z vector and for other vectors (data not shown). No specificity could be noted for any fragment of pXO16 inserted into pHT304-18Z. Nonetheless, TcpM, the relaxase encoded by the conjugative clostridial plasmid pCW3, was also shown to nick plasmid DNA in absence of any fragment of pCW3 sequence, but no further information was provided about this unspecific activity (Wisniewski *et al.*, 2016). We might therefore hypothesise a relaxase-helicase role for TipO in pXO16 conjugation, with specificity coming from the action of one (or more) unknown accessory protein(s), although more data are required to back up this hypothesis.

4. Conclusions

Despite thorough study of the *tip* locus, and especially the C-terminal section from *tipL* to *tipP*, no candidate region for the *oriT* stood out from mobilisation

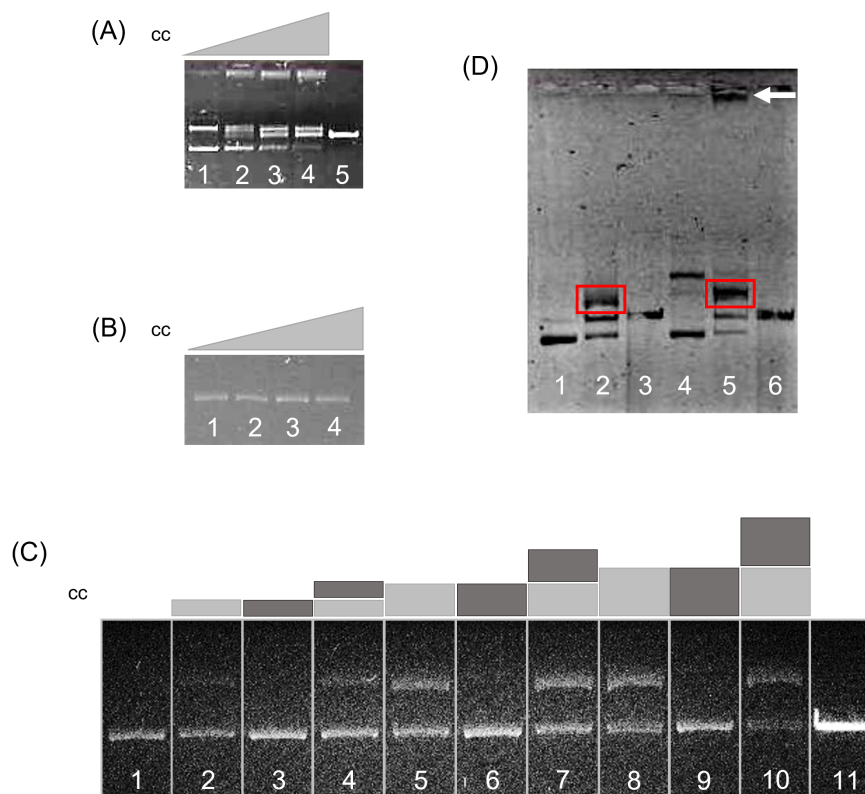


Figure 4: DNA nicking and binding activity of TipO and TipP. Incubation of plasmid DNA with varying concentrations of TipO and/or TipP for 30 min at 37°C and subsequent gel electrophoresis for 90 min at 100V. (A) Incubation of 300 ng of plasmid DNA (here: pHT304-18Z::oriT-E-1) with increasing concentrations of TipO. 1: 0 pmol TipO; 2: 1.5 pmol TipO; 3: 3 pmol TipO; 4: 4.5 pmol TipO; 5: plasmid DNA linearised by enzymatic restriction. (B) Incubation of 200 ng of plasmid DNA (here: pHT304-18Z::oriT-2.1.1) with increasing concentration of TipP. 1: 0 pmol TipP; 2: 1 pmol TipP; 3: 2 pmol TipP; 4: 3 pmol TipP. (C) Incubation of 200 ng of plasmid DNA (here: pHT304-18Z::oriT-2.2.2) with increasing concentrations of TipO and/or TipP. 1: plasmid DNA alone; 2, 5, 8: 1, 2, 3 pmol TipO; 3, 6, 9: 1, 2, 3 pmol TipP; 4, 7, 10: 1, 2, 3 pmol TipO + 1, 2, 3 pmol TipP; 11: plasmid DNA linearised by enzymatic restriction. (D) Incubation of 0.05 pmol of plasmid DNA (here: pHT304-18Z::oriT-C) alone or with 3 pmol of TipO. 1-3: methylated plasmid DNA (extracted from *E. coli* 10β); 4-6: non-methylated DNA (extracted from *dam*⁻/*dcm*⁻ *E. coli* C2925); 1, 4: plasmid DNA alone; 2, 5: plasmid DNA + TipO; 3, 6: plasmid DNA linearised by enzymatic restriction. The white arrow indicates a band at the top of lane 5. Red frames highlight two bands of interest in lanes 2 and 5 (for details, see text).

assays. The more the sections of pXO16 sequence were fragmented, the less they impacted the mobilisation of pHT304-18Z by pXO16 itself. Similarly, no *oriT* candidate could be defined by bioinformatics analyses based on typical *oriT* characteristics. These data suggest that pXO16 might have developed a low-specificity system or an alternative mechanism for DNA processing prior to transfer. On the other hand, investigating the potential roles of TipO and TipP in pXO16 relaxosome revealed the importance of both protein-encoding genes in pXO16 maintenance. TipO also clearly interacts with DNA by unspecific nicking and by binding under specific conditions. Based on data presented hereinabove, we propose that TipO could act as pXO16 relaxase, but more information about potential accessory proteins is required to evaluate the specificity of its nicking activity. At last, we suggest that *tipO* and *tipP* are somehow involved in pXO16 replication and that pXO16 *oriT* might serve as the origin of replication, suggesting that pXO16 replicates via an RCR-like mechanism. Further experimental evidences should bring light onto these hypotheses by focusing on pXO16 replication and the putative function of TipO as pXO16 relaxase.

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Supplementary data

Table S1: Bacterial strains used in this work.

Strains	Main features	Sources
<i>E. coli</i> strains		
<i>E. coli</i> NEB5 α	Chemically competent	New England Biolabs™
<i>E. coli</i> 10 β	Chemically competent	New England Biolabs™
<i>E. coli</i> TOP10	MC1061-derivative	Invitrogen™
<i>dam</i> ⁻ / <i>dcn</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::TipO)	<i>E. coli</i> BL21(DE3)-derivative containing pET-30::TipO (Kan ^R)	This study
<i>E. coli</i> BL21(DE3)(pET-30::TipP)	<i>E. coli</i> BL21(DE3)-derivative containing pET-30::TipP (Kan ^R)	This study
<i>B. thuringiensis</i> sv. <i>israelensis</i> strains		
GSX002	Plasmid-less derivative of strain GBJ002 (Nd ^R)	(Gillis <i>et al.</i> , 2017)
GSX002(pXO16)	GSX002-derivative containing wild-type pXO16	(Hinnekens <i>et al.</i> , 2021)
GBJ001	Sm ^R spontaneous mutant of reference strain 4Q7 cured of its plasmids (except pBtic235)	(Jensen <i>et al.</i> , 1995)
GSX002(pXO16, pHT304-18Z)	GSX002-derivative containing pXO16 and pHT304-18Z (Ery ^R)	This study
GSX002(pXO16, pHT304-18Z::oriT-0)	GSX002-derivative containing pXO16 and pHT304-18Z::oriT-0 (Ery ^R)	This study
GSX002(pXO16, pHT304-18Z::oriT-1)	GSX002-derivative containing pXO16 and pHT304-18Z::oriT-1 (Ery ^R)	This study
GSX002(pXO16, pHT304-18Z::oriT-2)	GSX002-derivative containing pXO16 and pHT304-18Z::oriT-2 (Ery ^R)	This study
GSX002(pXO16, pHT304-18Z::oriT-2.1)	GSX002-derivative containing pXO16 and pHT304-18Z::oriT-2.1 (Ery ^R)	This study
GSX002(pXO16, pHT304-18Z::oriT-2.1.1)	GSX002-derivative containing pXO16 and pHT304-18Z::oriT-2.1.1 (Ery ^R)	This study
GSX002(pXO16, pHT304-18Z::oriT-2.1.2)	GSX002-derivative containing pXO16 and pHT304-18Z::oriT-2.1.2 (Ery ^R)	This study

Continued on next page

Strains	Main features	Sources
GSX002(pXO16, pHT304-18Z::oriT-2.2)	GSX002-derivative containing pXO16 and pHT304-18Z::oriT-2.2 (Ery ^R)	This study
GSX002(pXO16, pHT304-18Z::oriT-2.2.1)	GSX002-derivative containing pXO16 and pHT304-18Z::oriT-2.2.1 (Ery ^R)	This study
GSX002(pXO16, pHT304-18Z::oriT-2.2.2)	GSX002-derivative containing pXO16 and pHT304-18Z::oriT-2.2.2 (Ery ^R)	This study
GSX002(pXO16, pHT304-18Z::oriT-3.1)	GSX002-derivative containing pXO16 and pHT304-18Z::oriT-3.1 (Ery ^R)	This study
GSX002(pXO16, pHT304-18Z::oriT-3.2)	GSX002-derivative containing pXO16 and pHT304-18Z::oriT-3.2 (Ery ^R)	This study
GSX002(pXO16, pHT304-18Z::oriT-3.3)	GSX002-derivative containing pXO16 and pHT304-18Z::oriT-3.3 (Ery ^R)	This study
GSX002(pXO16, pHT304-18Z::oriT-3.3.1)	GSX002-derivative containing pXO16 and pHT304-18Z::oriT-3.3.1 (Ery ^R)	This study
GSX002(pXO16, pHT304-18Z::oriT-3.3.2)	GSX002-derivative containing pXO16 and pHT304-18Z::oriT-3.3.2 (Ery ^R)	This study
GSX002(pXO16, pHT304-18Z::oriT-A)	GSX002-derivative containing pXO16 and pHT304-18Z::oriT-A (Ery ^R)	This study
GSX002(pXO16, pHT304-18Z::oriT-B)	GSX002-derivative containing pXO16 and pHT304-18Z::oriT-B (Ery ^R)	This study
GSX002(pXO16, pHT304-18Z::oriT-C)	GSX002-derivative containing pXO16 and pHT304-18Z::oriT-C (Ery ^R)	This study
GSX002(pXO16, pHT304-18Z::oriT-D)	GSX002-derivative containing pXO16 and pHT304-18Z::oriT-D (Ery ^R)	This study
GSX002(pXO16, pHT304-18Z::oriT-E)	GSX002-derivative containing pXO16 and pHT304-18Z::oriT-E (Ery ^R)	This study
GSX002(pXO16, pHT304-18Z::oriT-E.1)	GSX002-derivative containing pXO16 and pHT304-18Z::oriT-E.1 (Ery ^R)	This study
GSX002(pXO16, pHT304-18Z::oriT-E.2)	GSX002-derivative containing pXO16 and pHT304-18Z::oriT-E.2 (Ery ^R)	This study
GSX002(pXO16, pHT304-18Z::oriT-E.3)	GSX002-derivative containing pXO16 and pHT304-18Z::oriT-E.3 (Ery ^R)	This study
GSX002(pXO16, pMAD::KO <i>tip</i> OKan)	GSX002-derivative containing wild-type pXO16 and pMAD::KO <i>tip</i> OKan (Kan ^R , Ery ^R)	This study
GSX002(pXO16, pMAD::KO <i>tip</i> PKan)	GSX002-derivative containing wild-type pXO16 and pMAD::KO <i>tip</i> PKan (Kan ^R , Ery ^R)	This study

Table S2: Plasmids used in this work.

Plasmids	Main features	Sources
pXO16	Conjugative plasmid naturally occurring in strain 4Q2 of <i>B. thuringiensis</i> sv. <i>israelensis</i>	(Jensen <i>et al.</i> , 1995)
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::TipO	Derivative of pET-30a, containing TipO coding sequence	This study
pET-30::TipP	Derivative of pET-30a, containing TipP coding sequence	This study
pDG783	Plasmid carrying a kanamycin resistance cassette	(Guérout-Fleury <i>et al.</i> , 1995)
pMAD	Gram ⁺ /Gram ⁻ shuttle vector containing a thermo-sensitive Gram ⁺ replicon; Ery ^R , Amp ^R	(Arnaud <i>et al.</i> , 2004)
pMAD::KO <i>tipO</i> Kan	Derivative of pMAD containing antibiotic resistance cassette with upstream and downstream regions of <i>tipO</i> , Kan ^R	This study
pMAD::KO <i>tipP</i> Kan	Derivative of pMAD containing antibiotic resistance cassette with upstream and downstream regions of <i>tipP</i> , Kan ^R	This study
pXO16Δ <i>tipO</i>	Derivative of pXO16, carrying the replacement of <i>tipO</i> gene by Kan ^R cassette	This study
pXO16Δ <i>tipP</i>	Derivative of pXO16, carrying the replacement of <i>tipP</i> gene by Kan ^R cassette	This study
pHT304-18Z	Shuttle vector; Ery ^R	(Agaisse and Lereclus, 1994)
pHT304-18Z::oriT-0	Derivative of pHT304-18Z containing the oriT-0 fragment	This study
pHT304-18Z::oriT-1	Derivative of pHT304-18Z containing the oriT-1 fragment	This study
pHT304-18Z::oriT-2	Derivative of pHT304-18Z containing the oriT-2 fragment	This study
pHT304-18Z::oriT-2.1	Derivative of pHT304-18Z containing the oriT-2.1 fragment	This study
pHT304-18Z::oriT-2.1.1	Derivative of pHT304-18Z containing the oriT-2.1.1 fragment	This study
pHT304-18Z::oriT-2.1.2	Derivative of pHT304-18Z containing the oriT-2.1.2 fragment	This study
pHT304-18Z::oriT-2.2	Derivative of pHT304-18Z containing the oriT-2.2 fragment	This study
pHT304-18Z::oriT-2.2.1	Derivative of pHT304-18Z containing the oriT-2.2.1 fragment	This study

Continued on next page

Plasmids	Main features	Sources
pHT304-18Z::oriT-2.2.2	Derivative of pHT304-18Z containing the oriT-2.2.2 fragment	This study
pHT304-18Z::oriT-3.1	Derivative of pHT304-18Z containing the oriT-3.1 fragment	This study
pHT304-18Z::oriT-3.2	Derivative of pHT304-18Z containing the oriT-3.2 fragment	This study
pHT304-18Z::oriT-3.3	Derivative of pHT304-18Z containing the oriT-3.3 fragment	This study
pHT304-18Z::oriT-3.3.1	Derivative of pHT304-18Z containing the oriT-3.3.1 fragment	This study
pHT304-18Z::oriT-3.3.2	Derivative of pHT304-18Z containing the oriT-3.3.2 fragment	This study
pHT304-18Z::oriT-A	Derivative of pHT304-18Z containing the oriT-A fragment	This study
pHT304-18Z::oriT-B	Derivative of pHT304-18Z containing the oriT-B fragment	This study
pHT304-18Z::oriT-C	Derivative of pHT304-18Z containing the oriT-C fragment	This study
pHT304-18Z::oriT-D	Derivative of pHT304-18Z containing the oriT-D fragment	This study
pHT304-18Z::oriT-E	Derivative of pHT304-18Z containing the oriT-E fragment	This study
pHT304-18Z::oriT-E.1	Derivative of pHT304-18Z containing the oriT-E.1 fragment	This study
pHT304-18Z::oriT-E.2	Derivative of pHT304-18Z containing the oriT-E.2 fragment	This study
pHT304-18Z::oriT-E.3	Derivative of pHT304-18Z containing the oriT-E.3 fragment	This study

Table S3: Primers used in this work. Underlined: restriction site.

Target	Primer	Sequence (5' → 3')
oriT-0	oriT-0-F	aaaa
	oriT-0-R	aaaa
oriT-1	oriT-1-F	cac <u>ctg</u> cagaggaggggtagacaatgaat
	oriT-1-R	ctgga <u>tcct</u> catcatcgctctaacaattcttc
oriT-2	oriT-2-F	cac <u>ctg</u> cagataatcaagttccgcatgatg
	oriT-2-R	cacgga <u>tcct</u> cataggtgtattcttcatttctgc
oriT-2.1	oriT-2-F	See hereinabove
	oriT-1-R	See hereinabove
oriT-2.1.1	oriT-2-F	See hereinabove
	oriT-2.1.1-R	tatgga <u>tcct</u> atgctctattgactcgaa
oriT-2.1.2	oriT-2.1.2-F	tatctgcaggtcaagtaaaagcgaagag
	oriT-2.1.2-R	tatgga <u>tcct</u> gttcaggctcaggttca
oriT-2.2	oriT-2.2-F	cac <u>ctg</u> caggactctattctaccacatgaga
	oriT-2.2-R	ctgga <u>tcct</u> gtttcaatgagtaacttccg
oriT-2.2.1	oriT-2.2.1-F	tat <u>ctg</u> caggaagaccttgaagaagacttt
	oriT-2.2.1-R	tatgga <u>tcct</u> cgatgaagtcttaggta
oriT-2.2.2	oriT-2.2.2-F	tat <u>ctg</u> cagcacctcaaaacctgag
	oriT-2.2.2-R	tatgga <u>tcct</u> ccgacatcgctctctatctg
oriT-3.1	oriT-3.1-F	cac <u>ctg</u> caggagacgatgtcggaatag
	oriT-2-R	See hereinabove
oriT-3.2	oriT-3.2-F	gaactgcaggtattagagtatggacatggtca
	oriT-3.2-R	gaaggat <u>ccca</u> actgctctacctaataatgag
oriT-3.3	oriT-3.3-F	gaactgcagcgtcagaaagtatatatgtaatggta
	oriT-3.3-R	caaggat <u>ccgt</u> tgcaagcatgaaccc
oriT-3.3.1	oriT-3.3.1-F	tatctgcaggtggatgaattaagagtgaatgag
	oriT-3.3.1-R	actgga <u>tcct</u> catttccgattatcatccttct
oriT-3.3.2	oriT-3.3.2-F	tat <u>ctg</u> cagcggttcgattttcggtagtg

Continued on next page

Target	Primer	Sequence (5' → 3')
	oriT-3.3-R	See hereinabove
oriT-A	oriT-A-F	gagctgcagaatggcttagttggttaac
	oriT-A-R	atcggatccatgacagtgacaatacgc
oriT-B	oriT-B-F	atcctgcagcaaggtgggtgaagtaattg
	oriT-B-R	atcggatcctgtgcaaaagtataaagggtg
oriT-C	oriT-C-F	actctgcagggtatcgtgaaaagatac
	oriT-C-R	actggatccgataggaactgcacgtc
oriT-D	oriT-D-F	atcctgcagcgggtgaaatcgtagtaaac
	oriT-D-R	actggatccagagacaaagtttcgtgag
oriT-E	oriT-E-F	taactgcaggatatccattcgatgaaga
	oriT-E-R	actggatccattcactcttaattcatccac
oriT-E.1	oriT-E-F	See hereinabove
	oriT-E.1-R	attggatccccggtttgtaataatcgcc
oriT-E.2	oriT-E.2-F	agcctgcagacttcagttcagaaagg
	oriT-E.2-R	atcggatcctgactgtaaggcttcttaac
oriT-E.3	oriT-E.3-F	ctactgcagtctagtgacgatttaagtgc
	oriT-E-R	See hereinabove
tipO-up	tipO-up-F	agatctatcgatgatgccacatgaacctgagcctg
	tipO-up-R	gtacatccgcttaaccttctcctaattaaaatag
tipO-down	tipO-down-F	ctatcacctcgtctatagggtttggtaatg
	tipO-down-R	cggatccatatgacgtcgaccatccttctataatgaggttg
tipP-up	tipP-up-F	aaacatgggtatgcagaactattaagaaggatgc
	tipP-up-R	accggaattcttttagccctacctttaattttatacc
tipP-down	tipP-down-F	aaagtcgacgcagtacagaagcaataatttcagc
	tipP-down-R	cgaggatccttactgtttcgacatttcatcaa
KanR cassette for <i>tipO</i> knockout	Kan-F-tipO	ggaagggttaagcggatgtacttcagaaaag
	Kan-R-tipO	ccctatagacgaggtgatagtaagattatac
KanR cassette for <i>tipP</i> knockout	Kan-F-tipP	aaaagaattcaggtgatagtaagattataccgagg
	Kan-R-tipP	aaagtcgacgcggatgtacttcagaaaagattagatg

Continued on next page

Target	Primer	Sequence (5' → 3')
TipO	tipO-F	tctccatggaagcgaaacgaacggtaaatact
	TipO-R	tatactcgagttaatccataaggtttagtaacatttcaactact
TipP	TipP-F	tctccatggatgatgaattaagagtgaatgagaaatca
	TipP -R	tatactcgagtcattccgattatcatccttct

Table S4: Consensus sequence of the *nic* site of plasmids from six main MOB families. A consensus sequence was determined for each MOB subgroup by alignment of available *oriT* of plasmids from different MOB groups.

MOB family	MOB subgroup	Consensus <i>nic</i> site sequence
<i>MOB_F</i>	<i>MOB_{F1}</i>	CGCTGACACC
	<i>MOB_{F11}</i>	CnATAnACA
	<i>MOB_{F12}</i>	nnnCCACACn
	<i>MOB_{F13}</i>	TTTTCGCACA
<i>MOB_Q</i>	<i>MOB_{Q1}</i>	AnnGCnCA
	<i>MOB_{Q2}</i>	AGGGCGCAA
	<i>MOB_{Q3}</i>	ATnnnTnCcCnT
	<i>MOB_{Qu}</i>	AnGGCGC
<i>MOB_P</i>	<i>MOB_{P11}</i>	CGGGCAGGA
	<i>MOB_{P12}</i>	CGGGACAGGA
	<i>MOB_{P13}</i>	GCGGCGGnnG
	<i>MOB_{P14}</i>	nnnACAGGA
	<i>MOB_{P2}</i>	TTTGnCAGGA
	<i>MOB_{P3}</i>	CGAnnCAGGA
	<i>MOB_{P4}</i>	TAnnGCAGGA
	<i>MOB_{P5}</i>	TTAAGCCAGT
	<i>MOB_{P6}</i>	AnAGGCAnGA
	<i>MOB_{P7}</i>	AAnCnnAAGGGG
<i>MOB_V</i>	<i>MOB_{V1}</i>	TAACnnAC
	<i>MOB_{V2}</i>	GTATACTCAC
	<i>MOB_{V3}</i>	TAGnnTTC
	<i>MOB_{V4}</i>	TATAGCnCAC
	<i>MOB_{Vu}</i>	TAACnnnC
<i>MOB_H</i>	<i>MOB_{H11}</i>	CTAnTTAnTnnnG
	<i>MOB_{H12}</i>	AGAGnGAAATnTGA
<i>MOB_C</i>	<i>MOB_{C1}</i>	GGGTnnnCnG

6.2 Crossing the cell wall during conjugation

The main components of T4S-like systems in Gram-positive bacteria are a CP, ATPases, channel components and a PG hydrolase. In this section, TipB is proposed as the PG hydrolase of pXO16 transfer apparatus. Bioinformatics analyses led to identifying TipB (accession number: AFQ30071.1) as the putative PG hydrolase, which was further reinforced by experimental data focusing on TipB lytic activity.

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**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 a 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 a 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.

Keywords: *Bacillus thuringiensis*; conjugation; large plasmid; pXO16; conjugative cell wall hydrolase

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 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 *B. 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 T4SS encoded by the 25-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 CwlT 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).

Table 1: Bacterial strains and plasmids used in this work.

Strains/Plasmids	Main features	Sources
<i>E. coli</i> strains		
<i>E. coli</i> NEB5 α	Chemically competent	New England Biolabs™
<i>E. coli</i> 10 β	Chemically competent	New England Biolabs™
<i>dam⁻/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)	GSX002-derivative containing wild-type pXO16 and pMAD::KO <i>tipB</i> Kan (Kan ^R , Ery ^R)	This study
pMAD::KO <i>tipB</i> Kan	GSX002-derivative containing pXO16 Δ <i>tipB</i> (Kan ^R)	This study
GSX002(pXO16 Δ <i>tipB</i>)	GSX002-derivative containing pXO16 Δ <i>tipB</i> and pHT304-18Z:: <i>tipB</i> _comp (Kan ^R , Ery ^R)	This study
GSX002(pXO16 Δ <i>tipB</i> , 18Z:: <i>tipB</i> _compl)	GSX002-derivative containing pXO16 Δ <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-30a, containing TipB coding sequence	This study
pET-30::TipB-2TM	Derivative of pET-30a, containing TipB-2TM coding sequence	This study
pET-30::CTR _{TipB}	Derivative of pET-30a, containing CTR _{TipB} coding sequence	This study
pET-30::CHAP _{TipB}	Derivative of pET-30a, 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::KO <i>tipB</i> Kan	Derivative of pMAD containing antibiotic resistance cassette with upstream and downstream regions of <i>tipB</i> ; Kan ^R	This study
pXO16 Δ <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

2.2. General molecular techniques

PCR amplifications were performed using the Q5 High-Fidelity DNA polymerase (New England *Biolabs*TM, Leiden, The Netherlands). Restriction enzymes were obtained from New England *Biolabs*TM and the T4 DNA Ligase from Promega (Leiden, The Netherlands). PCR and restriction products were purified and recovered by GeneEluteTM PCR Clean-Up Kit (Sigma-AldrichTM, Overijse, Belgium). Constructions with pMAD vector were transformed into chemically competent *E. coli* NEB5 α cells (New England *Biolabs*TM) while these using the pET-30a vector were transformed into chemically competent *E. coli* 10 β (New England *Biolabs*TM). Transformants were checked by PCR and plasmids DNA were extracted using the GeneEluteTM Plasmid MiniPrep Kit (Sigma -AldrichTM) 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*TM). The resulting vector 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]. pXO16 Δ *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, pXO16 Δ *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].

Table 2: Primer pairs used in this work.

Target	Primer	Sequence 5'→3'	Reference
tipB-up	tipB-up-F	agatctatcgatgcatgccaacgggtattccaactatgaag	This work
	tipB-up-R	gtacatccgctccttttagctgctagattg	
tipB-down	tipB-down-F	ctatcacctctgataaaacgtaacgaagtataaaac	This work
	tipB-down-R	cggatccatatgacgtcgacttttcttcttaacgagttg	
Kan ^R cassette	tipB-Kan-F	actaaaaggagcggatgtacttcagaaaag	This work
	tipB-Kan-R	gttttatcaggaggtataggttaagattatac	
tipB_compl	tipB-comp-F	gagctgcagaaggagggtaaaccatttgattgc	This work
	tipB-comp-R	ctcggatcccataagatgacctccttctacttctaa	
tipB	tipB-F	tatccatggctattgctctcggtggtgttta	This work
	tipB-R	tatggatccttatttgattacattaccatagaatt	
tipB-2TM	tipB-2TM-F	tatccatggctgcttctgtacaaacactagac	This work
	tipB-R	tatggatccttatttgattacattaccatagaatt	
CTR domain	tipB-2TM-F	tatccatggctgcttctgtacaaacactagac	This work
	CTR-tipB-R	ttaggatccctatctagccctcatatagtc	
CHAP domain	CHAP-tipB-F	tatccatggctttaaccgctttactaacagg	This work
	CHAP-tipB-R	tatggatccatatccgaacatacttccc	

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 μ m filter, which was then collected and placed on non-selective LB plate. 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 *NcoI*/*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 μ m (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 DeWitt and Grossman [6], with some modifications. Briefly, a 4 h culture (OD₆₀₀ 0.8-0.9) 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 OD₅₉₅ between 0.8-1. 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 OD₅₉₅ 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 performed using InterPro [22], HHpred [23], Phyre2 [24] and I-Tasser [25] softwares 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) do-

main, 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 suggest that *tipB* encodes an atypical SLT domain that has not been identified yet or that no SLT domain is encoded, reinforcing the uniqueness of TipB compared to other described conjugative PG hydrolases.

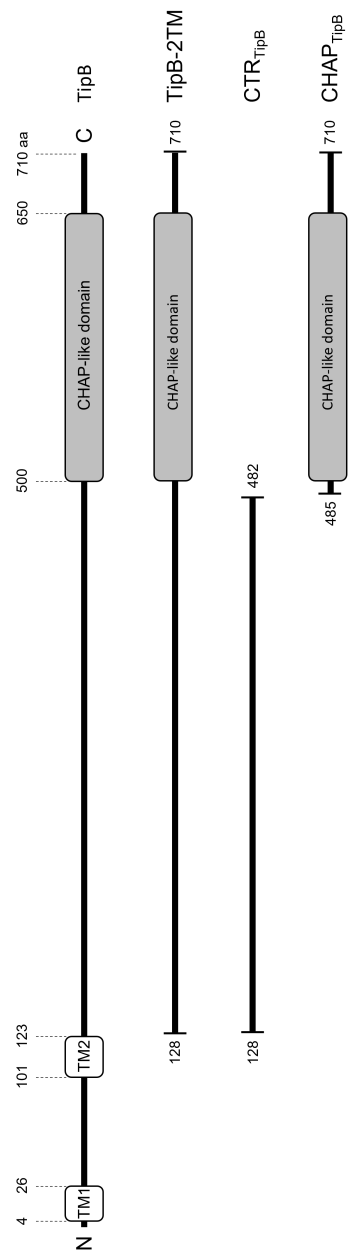


Figure 1: Structure of TipB and truncated variants. The predicted domains were depicted based on bioinformatics analyses. The CHAP-like domain was delimited based on structural homologies retrieved from HHpred analyses. Each truncated variant is represented with its corresponding amino acid (aa) location.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

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 (pXO16 Δ *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(pXO16 Δ *tipB*) as donor. As shown in Fig. 2, the derivative mutant showed a reduction of pXO16 Δ *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 pXO16 Δ *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(pXO16 Δ *tipB*, pHT304-18Z::*tipB*_compl) reached transfer frequencies of 1.5×10^{-1} T/D, demonstrating a significant rise compared to pXO16 Δ *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

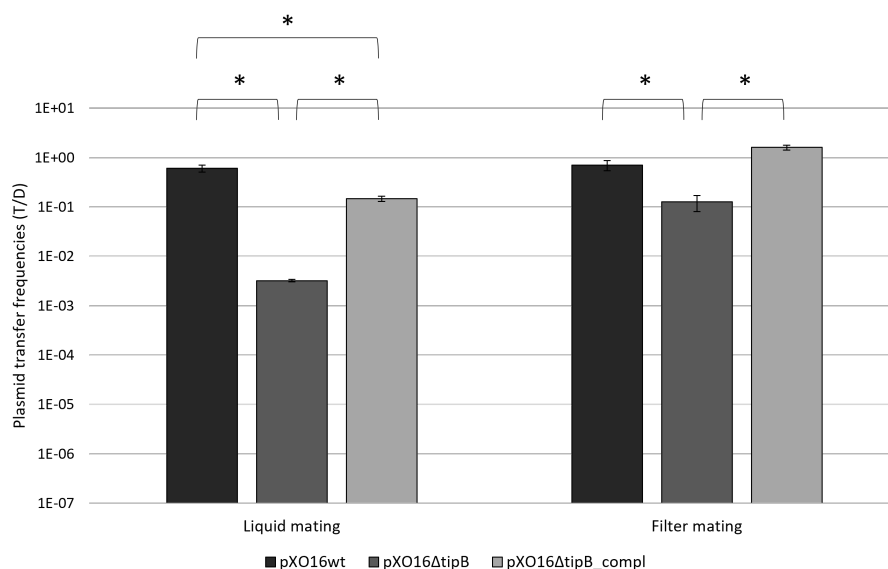


Figure 2: Conjugative transfer frequencies of pXO16wt, pXO16ΔtipB and pXO16ΔtipB_compl (the complemented pXO16ΔtipB) in liquid and filter matings. Transfer frequencies are expressed as transconjugants per donor cells (T/D). Each frequency corresponds to the mean $\pm$ SEM out of three replicates. A star 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.

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 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 incu-

bation (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 μ M concentration (*i.e.* 100 μ 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.

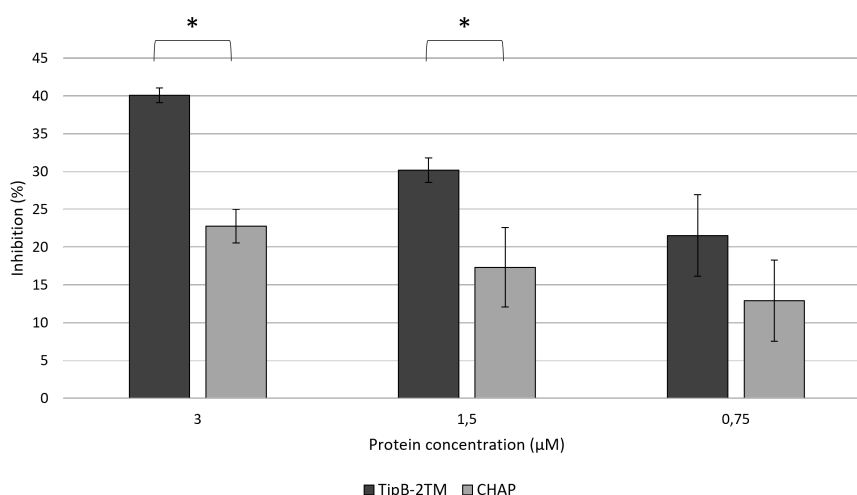


Figure 3: Bacteriolytic activity of TipB-2TM and CHAP_{TipB} on *B. thuringiensis* GBJ001. The reduction in OD induced by TipB-2TM and CHAP_{TipB} on suspensions of GBJ001 was monitored during 1 h at 30°C. Equimolar concentrations of each protein were tested. The experiment was done in triplicate. A 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.

4. Discussion

Since its first description in 1987 [29], pXO16 conjugation has been widely studied phenotypically. However, little information on 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 bioinformatics 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 (pXO16 Δ *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 1,000-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 cod-

ing 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 CDSs encodes 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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Transfer and behaviour of pXO16 in *B. cereus s.l.* hosts

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Conjugative plasmids can promote their own transfer from a donor to a recipient strain. In *Bti*, the large plasmid pXO16 is capable of mediating its own transfer at high frequencies, alongside displacing co-resident plasmids and host chromosomal loci. Although the natural occurrence of pXO16 is restricted to *Bti*, its extremely efficient self-transfer mechanism could help its dissemination within the closely-related species of the *B. cereus* group. In this chapter, two manuscripts report the study of pXO16 host spectrum (Section 7.1), as well as the transferability of pXO16 among strains of *B. cereus s.l.* and its impact on host cells (Section 7.2).

7.1 The extended host spectrum of pXO16

Since the discovery of the dispensability of aggregation for pXO16 conjugation-mediated transfer, previously overlooked strains were described as potential hosts for the plasmid (Makart *et al.*, 2018). In this section, the transfer of pXO16 is investigated based on a solid mating approach, to artificially mimic the effect of aggregation. Solid-based conjugation assays are also compared to the liquid-based method in terms of transfer efficiency.

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pXO16, the large conjugative plasmid from *Bacillus thuringiensis* serovar *israelensis* displays an extended host spectrum

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Abstract

pXO16, the large conjugative plasmid from *Bacillus thuringiensis* serovar *israelensis* is able to efficient self-transfer, to mobilize and retro-mobilize non-conjugative plasmids, including "non-mobilizable" plasmids, and to transfer chromosomal loci. It also displays a remarkable aggregation phenotype associated with conjugation under liquid conditions. However, it was recently shown that aggregation boosts pXO16 transfer but is not mandatory. In this paper, we have further explored pXO16 transfers under various mating conditions and with different members of the *Bacillus cereus* group. The results indicated that colony or filter mating largely compensate the transfer deficit observed when using a pXO16 aggregation-minus mutant. Using filter mating, pXO16 transfer efficiency and host range were both improved. For instance, pXO16 was shown to transfer itself, and to mobilize the small pUB110 plasmid, from *B. thuringiensis* serovar *israelensis* to the thermotolerant *Bacillus cytotoxicus* at frequencies of 3.3×10^{-3} and 5.2×10^{-4} transconjugants per donor (T/D), respectively. All together, these results indicate that pXO16 can potentially "circulate" among members of the *Bacillus cereus* group. Yet, this is contrasting with pXO16's known natural distribution, which is apparently limited to the *israelensis* serovar of *B. thuringiensis*.

Keywords: *Bacillus cytotoxicus*; *Bacillus thuringiensis*; *Bacillus weihenstephanensis*; conjugation; large plasmid; pUB110; pXO16

1. Introduction

pXO16 is a large conjugative plasmid associated with the entomopathogen *Bacillus thuringiensis* serovar *israelensis*. It is used as biopesticide to control insect larvae of mosquitoes and blackflies, the vectors of animal and human diseases. This entomopathogenic activity results from the production, during sporulation, of delta-endotoxins (Cry) and cytolytins (Cyt), which form crystals in the bacterial sporanges (Lacey, 2007; Palma *et al.*, 2014). *B. thuringiensis* belongs to *Bacillus cereus sensu lato* (s.l.), a group of closely related bacteria whose members display differential virulence (Jensen *et al.*, 2003; Okinaka and Keim, 2016). *Bacillus anthracis* is a mammal and human pathogen, while some strains of *B. cereus sensu stricto* are known as foodborne pathogens, provoking emesis or diarrhoea with a potentially lethal outcome. Interestingly, most of these bacteria harbour a plethora of plasmids, including conjugative and mobilizable ones. Moreover, some of the extrachromosomal molecules contain the genetic determinants for the virulent genes, as is the case for the virulent plasmids pXO1 and pXO2 of *B. anthracis*, the causative agent of anthrax (Hu *et al.*, 2009).

One intriguing element of this extrachromosomal pool is pXO16. This 350-kb conjugative plasmid transfers itself in a fast and efficient way (100% within < 5 min) (Andrup *et al.*, 1998). Another peculiar feature of pXO16 is its macroscopic aggregation phenotype displayed during conjugation (Andrup *et al.*, 1993; Jensen *et al.*, 1995). Typically, within minutes of contact, donor and recipient cells aggregate and form visible clumps that last for several minutes until pXO16 transfer is achieved. Recently, a 25-kb "aggregation" locus was identified in pXO16 sequence. Deletion of this region hampered the appearance of aggregation. However, although the transfer efficiency was reduced in the aggregation-minus mutant, pXO16 could be transferred at frequencies of *ca.* 10^{-4} transconjugants per donor cell (T/D). Similar transfer reductions were observed for mobilization of small plasmids (Makart *et al.*, 2018).

In this paper, we report the transfer of pXO16 aggregation-minus mutant in colony and filter matings. We have also further investigated pXO16 con-

jugation and mobilization properties among other members of the *B. cereus* group using filter mating. In these conditions, and contrary to what was previously thought, the host range of this *B. thuringiensis* serovar *israelensis* plasmid appears to cover various members of the group, including its most distantly related kin, the thermotolerant *Bacillus cytotoxicus*.

2. Materials and methods

2.1. Bacterial strains and plasmids

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. When appropriate, antibiotics (Sigma) were added at the following concentrations (μg mL⁻¹): Sm, streptomycin (100); Nd, nalidixic acid (15); Tc, tetracycline (10); Cm, chloramphenicol (10); Rf, rifampicin (50); Km, kanamycin (50).

2.2. Spontaneous antibiotic-resistant mutants

Spontaneous antibiotic-resistant mutants were obtained by plating the equivalent of at least 15 mL of an overnight culture (*ca.* 10⁸ CFU mL⁻¹) on LB plates with the appropriate antibiotics from which growing colonies were retrieved and confirmed on new antibiotic-containing LB plates.

2.3. pXO16 mating assays

2.3.1. Matings in liquid media. Plasmid transfers in liquid media were performed as previously described in Timmerly *et al.* (2009) with some modifications. Briefly, the mating partners (donor and recipient cells) were streaked separately on LB plates containing the appropriate antibiotic(s) for strain and plasmid selection and incubated at 30°C overnight. Separate pre-cultures from initial plates were grown in 10 mL of LB medium without antibiotics and incubated overnight at 30°C without shaking. Equal amounts of mating partner cells from pre-cultures (maximum 1 mL per pre-culture) were mixed in 10 mL of LB medium. The mixture was incubated at 30°C, without shaking, during

the mating time (4 h). After mating, appropriate dilutions were plated on selective media for parental and transconjugant strains and incubated overnight at 30°C. Each mating experiment included a plating control of donor and recipient cell pre-cultures on selective media to exclude 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. All mating experiments were repeated at least three times, independently.

2.3.2. Filter matings. Filter mating experiments were performed three times during 4 h, as described hereinabove. The distinction with liquid matings is the establishment of contact between conjugative cells. Equal amounts of donor and recipient cells were successively passed through a 0.20 μm filter, which was then collected, placed on a LB plate without antibiotic and incubated at 30°C during the defined mating time. After mating, cells were collected from the filter by resuspension in LB medium, before plating out the appropriate dilutions on selective media.

2.3.3. Drop-on-drop or colony mating. Drop-on-drop mating experiments were performed three times, as described above, during 4 h. Five μL of overnight pre-cultures of donor and recipient cells were placed on the same spot of an LB plate without antibiotic and incubated at 30°C. After mating, the resulting colony was collected from the plate with a sterile loop and resuspended in LB medium, before plating out the appropriate dilutions on selective media.

2.3.4. Mobilization experiments. Mobilization of the small Km^{R} plasmid pUB110 (Table 1) by pXO16 was performed as previously described (Timmery *et al.*, 2009), except that the matings were done on filter, as detailed above for pXO16.

Table 1: Bacterial strains and plasmids used in this work.

Strains/Plasmids	Mean features	Sources
Bacterial strains		
<i>B. thuringiensis</i> sv. <i>israelensis</i> GBJ001	Sm ^R spontaneous mutant of reference strain 4Q7 cured of its plasmids	Jensen <i>et al.</i> , 1995
<i>B. thuringiensis</i> sv. <i>israelensis</i> GBJ002	Nd ^R spontaneous mutant of reference strain 4Q7 cured of its plasmids, except pBhc235	Jensen <i>et al.</i> , 1996; Gillis <i>et al.</i> , 2017
<i>B. thuringiensis</i> sv. <i>israelensis</i> GBJ002(pXO16)	GBJ002-derivative containing pXO16::Tn5401 (Tc ^R)	Timmery <i>et al.</i> , 2009
<i>B. thuringiensis</i> sv. <i>israelensis</i> GBJ002(pXO16Δ <i>agr</i>)	GBJ002-derivative containing pXO16Δ <i>agr</i> (Cm ^R)	Makart <i>et al.</i> , 2018
<i>B. thuringiensis</i> sv. <i>israelensis</i> GBJ002(pXO16, pUB110)	GBJ002-derivative containing pXO16::Tn5401 (Tc ^R) and pUB110 (Km ^R)	Timmery <i>et al.</i> , 2009
<i>B. thuringiensis</i> sv. <i>entomocidus</i> HD9	Wild-type isolate, BGSC-ID: 414	BGSC ^a
<i>B. thuringiensis</i> sv. <i>entomocidus</i> HD9.1	Sm ^R spontaneous mutant of strain HD9	Makart <i>et al.</i> , 2018
<i>B. thuringiensis</i> sv. <i>aizawai</i> HD11	Wild-type isolate, BGSC-ID: 414	BGSC ^a
<i>B. thuringiensis</i> sv. <i>aizawai</i> HD11.1	Sm ^R spontaneous mutant of strain HD11	Makart <i>et al.</i> , 2018
<i>B. weihenstephanensis</i> WSBC10204	Wild-type isolate	Lechner <i>et al.</i> , 1998
<i>B. weihenstephanensis</i> WSBC10204.1	Sm ^R spontaneous mutant of strain WSBC10204	Makart <i>et al.</i> , 2018
<i>B. cereus sensu stricto</i> (s.s.) H3081.97	Wild-type emetic isolate	Hoffmaster <i>et al.</i> , 2008
<i>B. cereus</i> s.s. H3081.97.1	Sm ^R spontaneous mutant of strain H308197	Makart <i>et al.</i> , 2018
<i>B. cereus</i> s.s. IS075	Wild-type emetic isolate	Hoton <i>et al.</i> , 2009
<i>B. cereus</i> s.s. IS075.1	Sm ^R spontaneous mutant of strain IS075	Makart <i>et al.</i> , 2018
<i>B. cereus</i> s.s. VD021	Wild-type isolate	Van der Auwera <i>et al.</i> , 2013
<i>B. cereus</i> s.s. VD021.1	Sm ^R spontaneous mutant of strain VD021	This work
<i>B. cytotoxicus</i> E28.3	Wild-type isolate from potato flakes	Koné <i>et al.</i> , submitted
<i>B. cytotoxicus</i> E28.3.1	Sm ^R spontaneous mutant of strain E28.3	This work
<i>B. cytotoxicus</i> E28.3.1(pXO16)	E28.3.1-derivative containing pXO16::Tn5401 (Tc ^R)	This work
<i>B. cytotoxicus</i> E28.3.2	Rf ^R spontaneous mutant of strain E28.3	This work
Plasmids		
pXO16::Tn5401	Derivative of pXO16, conjugative plasmid naturally occurring in strain 4Q2 of <i>B. thuringiensis</i> sv. <i>israelensis</i> , Tc ^R	Jensen <i>et al.</i> , 1996
pXO16Δ <i>agr</i>	Aggregation-deficient pXO16-derivative due to the deletion of its <i>agr</i> region, Cm ^R	Makart <i>et al.</i> , 2018
pUB110	Mobilizable plasmid, Km ^R	Keggins <i>et al.</i> , 1978

^a BGSC: *Bacillus* Genetic Stock Center, Ohio State University, USA.

3. Results

3.1. pXO16 aggregation is dispensable in filter and colony matings

One of the predictions of the study on pXO16 aggregation-minus mutant (Makart *et al.*, 2018) was that the transfer deficit, in liquid medium, might be compensated by providing alternative physical contact between donor and recipient cells. In order to test this hypothesis, two complementary experiments were performed: the mating partners were either mixed together as drops on the surface of LB plates (drop-on-drop or colony mating) or were first deposited on a single filter and then incubated on LB plates (filter mating).

As shown in Fig. 1, transfer of wild type pXO16 between *B. thuringiensis* sv. *israelensis* strains GBJ002 and GBJ001 was equally efficient, irrespective of the conditions, with average transfer frequencies of 6.1×10^{-1} , 3.2×10^{-1} and 7.1×10^{-1} T/D for liquid, drop-on-drop and filter matings, respectively. As shown before, the pXO16 Δ *agr* mutant displayed a reduction of *ca.* 10^3 log in transfer efficiency in liquid conditions (Makart *et al.*, 2018). However, when the transfer of pXO16 Δ *agr* was performed with either colony or filter mating, the transfer efficiency observed of the wild-type pXO16 was restored, although with a slight decrease in the case of filter mating (1.7×10^{-1} versus 7.1×10^{-1} T/D) (Fig. 1).

3.2. Filter mating allows pXO16 to move to various members of the *B. cereus* group

Based on the observation that filter mating provided appropriate conditions for pXO16 transfer among *B. thuringiensis* sv. *israelensis*, we further explored this mating set-up for various members of the *B. cereus* group and compared the transfer efficiencies between liquid and filter matings (Fig. 2). Three distinct situations could be observed. In the case of pXO16 transfer between *B. thuringiensis* sv. *israelensis* strain GBJ002 and the emetic strain IS075.1 of *B. cereus*, no significant differences were noticed between liquid and filter matings with frequencies of *ca.* 1.6×10^{-2} T/D. In two other cases, where the recipient strains were either *B. thuringiensis* sv. *entomocidus* HD9.1 or the psychrotolerant *B. weihenstephanensis* strain WSBC10204.1, nine and ninety-five fold increase, respectively, were observed when the matings took

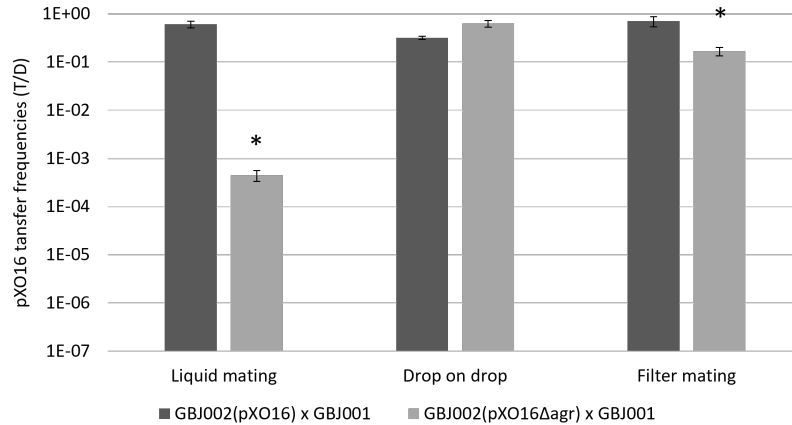


Figure 1: Conjugative transfer frequencies of pXO16 and pXO16Δagr under different mating conditions. Matings are always noted as "Donor x Recipient". Transfer frequencies are expressed as transconjugants per donor cells (T/D). Each frequency corresponds to the mean $\pm$ SEM out of three replicates. A star indicates that the differences between frequencies were significant, following a non-parametric Wilcoxon-Mann-Whitney test ($p < .05$). Otherwise, the differences were not significant.

place in filter rather than liquid conditions. Yet, the more striking observation was made for the matings with strains *B. thuringiensis* sv. *aizawai* HD11.1, *B. cereus* VD021.1 and the thermotolerant *B. cytotoxicus* E28.3.1. In each case, while no transfer could be detected under liquid condition, efficiencies of 2.5×10^{-2} , 3.7×10^{-3} and 3.2×10^{-3} T/D, respectively, were recorded when filter mating was used (Fig. 2).

These results demonstrate that, provided that the donor and recipient cells are in close contact with each other, pXO16 can "circulate" among a much broader spectrum of bacteria inside the *B. cereus* group, with frequencies of transfer reaching 8.6×10^{-2} T/D. However, in order to further investigate the influence of aggregation in plasmid transfer to these strains, a wild type and an aggregation-minus mutant of pXO16 were tested under condition of liquid matings. As shown in Fig. 3, while in the case of the emetic strain IS075.1 of *B. cereus* no noticeable difference in transfer was observed in the absence of aggregation, less efficient pXO16 transfers were noted between

B. thuringiensis sv. *israelensis* strain GBJ002 and either *B. weihenstephanensis* WSBC10204.1 (9-fold drop) or H3081.97.1 (19-fold drop), another emetic strain of *B. cereus*. These observations confirmed the importance of the aggregation phenomenon, but also indicated the influence of the partner cells in the extent of its contribution.

3.3. Plasmid mobilization by pXO16 is also efficient in filter mating

Under liquid conditions, pXO16 was shown to mobilize non-conjugative plasmids among strains of *B. thuringiensis* sv. *israelensis* (Timmery *et al.*, 2009). Using filter-mating experiments, the Km^R plasmid pUB110 was transferred from *B. thuringiensis* sv. *israelensis* GBJ002(pXO16, pUB110) to *B. cytotoxicus* E28.3.1. The transfer of pUB110 was observed with an average frequency of 5.2×10^{-4} T/D, tenfold less than for pXO16 itself.

3.4. pXO16 can circulate among members of the *B. cereus* group

As indicated above, in the conditions of filter mating (Fig. 2), pXO16 can transfer itself to the thermotolerant *B. cytotoxicus*, certainly the most "divergent" member within the *B. cereus* group (Guinebretière *et al.*, 2013). It was therefore interesting to see whether this pXO16 move could be followed by further migration across members the *B. cereus* group. First, a pXO16 "back-transfer" from *B. cytotoxicus* E28.3.1 to *B. thuringiensis* sv. *israelensis* GBJ002 was tested using a combination of appropriate antibiotic-resistant mutants. As shown in Fig. 4, this transfer was at least 100-fold more efficient than the original transfer (4.9×10^{-1} versus 3.3×10^{-3} T/D). Second, the transfer between two antibiotic-resistant mutants of *B. cytotoxicus* (E28.3.1 and E28.3.2) was also rather efficient, with an average frequency of 1.7×10^{-1} T/D (Fig. 4). Again, these results indicate that, under filter mating conditions, pXO16 can travel at relatively high frequencies across the different species of *B. cereus* s.l.

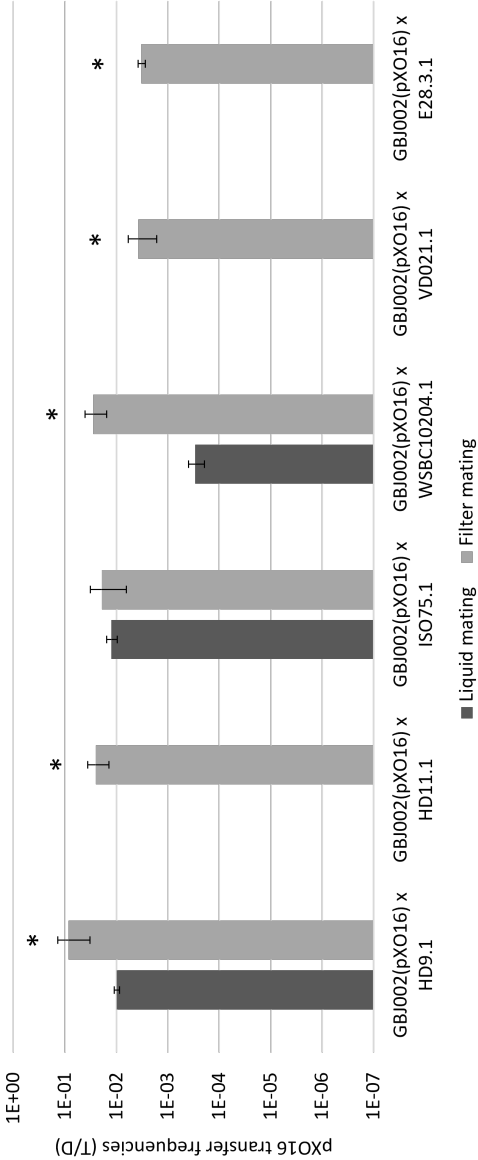


Figure 2: Comparison of conjugative transfer frequencies of pXO16 to *B. cereus* s.l. strains under conditions of liquid and filter matings. The recipient strains are *B. thuringiensis* sv. *entomocidus* HD9.1, *B. thuringiensis* sv. *aizawai* HD11.1, emetic *B. cereus* ISO75.1, *B. weihenstephanensis* WSB10204.1, *B. cereus* VD021.1 and *B. cytotoxicus* E28.3.1. A star indicates that the differences between frequencies were significant, following a non-parametric Wilcoxon-Mann-Whitney test ($p < .05$). Otherwise, the differences were not significant.

4. Discussion

Discovered in 1979, in a mosquito pond in Israel, *B. thuringiensis* sv. *israelensis* rapidly became an important biopesticide widely used to control mosquitoes and blackflies, in particular in countries where these insects contribute to the spread of human and animal diseases (Ben-Dov, 2014). Also named serotype H14, these bacteria contain a large pool of extrachromosomal molecules, including small, large and very large circular plasmids, as well as a linear prophage (Bolotin *et al.*, 2017; Gillis *et al.*, 2017; Gillis *et al.*, 2018). pXO16 is one of the two largest plasmids and was found to display an efficient aggregation-associated conjugation (Andrup *et al.*, 1998) that allows the transfer of "non-mobilizable" plasmids (Andrup *et al.*, 1998) and even chromosomal loci (Makart *et al.*, 2017). Until recently, the transfer of pXO16 was thought to be dependent of its aggregation, and only mating pairs forming clumps were considered and studied. However this is not the case. pXO16 aggregation is not mandatory for its DNA transfer. Rather, it helps donor and recipient cells to get into close contact and increases the transfer efficiency by 1,000 to 10,000 folds (Makart *et al.*, 2018). In the present paper, the extent of the aggregation contribution was further investigated, together with alternative transfer conditions (*i.e.* filter and colony matings).

The first and foremost observation is the fact that pXO16 aggregative phenotype is designed for DNA exchanges in "liquid" conditions, with "select" partners. In these conditions, transfers of almost 100% can be easily reached. However, the specific features displayed by the select recipient cells recognised in pXO16 aggregation remain so far unknown. The recent development of an exquisite assay using AFM (Feuillie *et al.*, 2018) should bring more insights into this relevant question of cognitive interactions between the mating partners.

The second output of this work is the discovery of alternative routes for pXO16 DNA transfer, either inside colonies of mixed mating pairs or on artificially compacted cell partners (filter mating). These set-ups not only elude the necessity for aggregation, but also often increase pXO16 transfer frequencies and broaden its host spectrum. Using filter mating, the plasmid was indeed

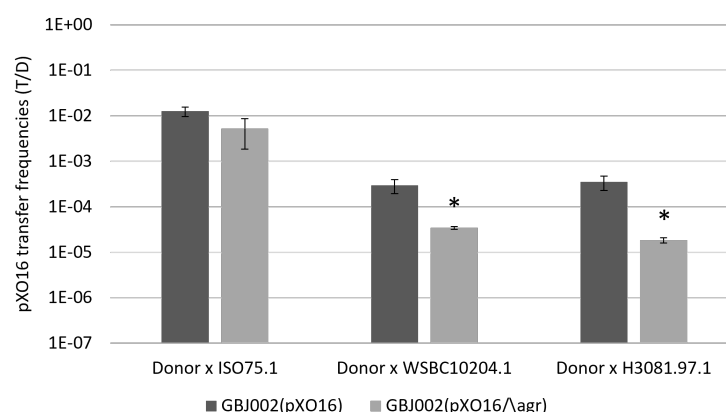


Figure 3: Comparison of conjugative transfer frequencies of pXO16 and pXO16Δagr to *B. cereus* s.l. strains under liquid mating conditions. Detailed legend as for Fig. 1.

shown to move from *B. thuringiensis* sv. *israelensis* to various members of the *B. cereus* group, including strains of the psychrotolerant *B. weihenstephanensis* and the thermotolerant *B. cytotoxicus*, at average frequencies from ca. 3.3×10^{-3} to 8.6×10^{-2} (T/D) (Fig. 2). This extends pXO16 host range, which was so far limited to a few strains of *B. thuringiensis*, *B. cereus* (Jensen *et al.*, 1996) or *B. anthracis* (Reddy *et al.*, 1987). It should be noted that attempts to transfer the plasmid outside the *B. cereus* group (e.g. *Bacillus pumilus*) by filter mating have so far failed (data not shown) as reported before in liquid conditions (Jensen *et al.*, 1996).

This paper also indicates that pXO16 is able to move among different members of the *B. cereus* group, as illustrated in the case of *B. cytotoxicus* (Fig. 4). One should however mention that in these transfers, the presence of large and potentially conjugative plasmids in *B. cytotoxicus* might have contributed to the transfer of pXO16. Nevertheless, the fact that pXO16 is able to migrate, at rather high frequencies, and to mobilize other non-conjugative plasmids (e.g. pUB110, the Km^R plasmid) along its migration, is potentially relevant in the scope of gene exchanges between members of *B. cereus* s.l.

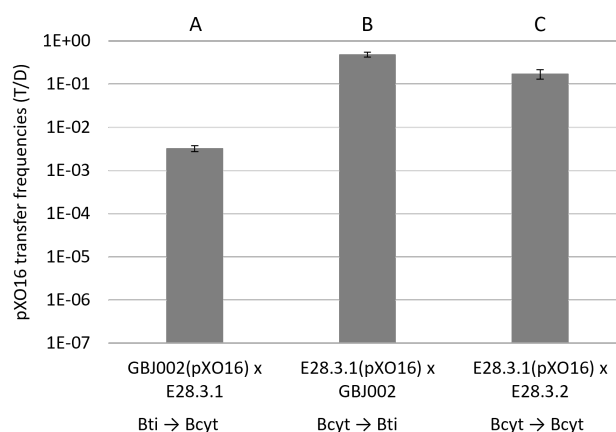


Figure 4: Conjugative transfer frequencies of pXO16 between *B. thuringiensis* serovar *israelensis* and *B. cytotoxicus*. Different letters on frequencies indicate that the differences between the differently labelled frequencies were significant, following a non-parametric Wilcoxon-Mann-Whitney test ($p < .05$).

This brings us to the following paradox: why then is pXO16 only observed in strains of the *israelensis* serovar (Gillis *et al.*, 2018)? One possible explanation is its potential instability in the new host. This was indeed shown in the case of *B. cytotoxicus* where pXO16 is highly unstable, albeit at high temperature (50°C, data not shown). Another possible reason for pXO16's restricted natural host range is to be found in its *raison d'être*: indeed, *B. thuringiensis* sv. *israelensis* peculiar habitat, mosquito larvae, may act as positive selection for the presence of pXO16 in the bacterium. This hypothesis remains however to be tested.

Finally, since biofilms are a natural and common lifestyle of bacteria, and members of the *B. cereus* are no exception (Majed *et al.*, 2016; Candela *et al.*, 2018), it would be particularly interesting to further explore the transfer behavior of pXO16 in biofilms, including under flow cell conditions which may be closer to the actual lifestyle of its bacterial host.

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7.2 pXO16 transfer and impact among the *B. cereus* group

The paradox between the natural restriction of pXO16 to *Bti* and the potential transfer of pXO16 to multiple *B. cereus* group species could be solved by a co-evolutionary hypothesis. First insights into resolving this paradox are proposed in this section, where are considered the impact of pXO16 on its host phenotype and the stability of the plasmid in different conditions.

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Conjugation-mediated transfer of pXO16, a large plasmid from *Bacillus thuringiensis* sv. *israelensis*, across the *Bacillus cereus* group and its impact on host phenotype

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Abstract

pXO16, the 350 kb-conjugative plasmid from *Bacillus thuringiensis* sv. *israelensis* promotes its own transfer at high efficiency, triggers the transfer of mobilisable and non-mobilisable plasmids, as well as the transfer of host chromosomal loci. Naturally found in *B. thuringiensis* sv. *israelensis*, pXO16 transfers to various strains of *Bacillus cereus sensu lato* (s.l.) at a wide range of frequencies. Despite this host diversity, a paradox remains between the relatively large host spectrum and the natural occurrence of pXO16, so far restricted to *B. thuringiensis* sv. *israelensis*. Proposing first insights exploring this paradox, we investigated the behaviour of pXO16 among different members of the *B. cereus* group. We first looked at the transfer of pXO16 to two new host clusters of *B. cereus* s.l., *Bacillus mycoides* and *Bacillus anthracis* clusters. This examination brought to light the impairment of the characteristic rhizoidal phenotype of *B. mycoides* in presence of pXO16. We also explored the stability of pXO16 at different temperatures as some members of the *B. cereus* group are well-known for their psychro- or thermo-tolerance. This shed light on the thermo-sensitivity of the plasmid. The influence of pXO16 on its host cell growth and on swimming capacity also revealed no or limited impact on its natural host *B. thuringiensis* sv. *israelensis*. On the contrary, pXO16 affected more strongly both the growth and swimming capacity of other *B. cereus* s.l. hosts. This reinforced the running hypothesis of a co-evolution between pXO16 and *B. thuringiensis* sv. *israelensis*, enabling the maintenance of the plasmid without impairing the host strain development.

Keywords: *Bacillus cereus*; conjugation; large plasmid; pXO16; fitness

1. Introduction

Bacillus cereus sensu lato (s.l.) is a group of ubiquitous Gram-positive bacteria displaying differential virulence (Jensen *et al.*, 2003; Ehling-Schulz *et al.*, 2019). While *Bacillus anthracis*, the etiological agent of anthrax disease, and *Bacillus cereus sensu stricto* (s.s.), an opportunistic pathogen occasionally associated with foodborne infections, can infect humans, *Bacillus thuringiensis* is an entomopathogen widely used as biopesticide to control insect pests (Kolstø *et al.*, 2009; Ceuppens *et al.*, 2013; Jouzani *et al.*, 2017). Apart from these, other species are usually mentioned as *B. cereus* group members. Two members depict opposite temperature profiles, as *Bacillus weihenstephanensis* is characterised by a resistance to temperatures as low as 4°C, while *Bacillus cytotoxicus* is thermophilic and able to grow at temperatures higher than 50°C (Lechner *et al.*, 1998; Guinebretière *et al.*, 2013). *Bacillus (pseudo-)mycoides* exhibits a peculiar growth phenotype, forming rhizoid-like colonies on solid medium (Flügge, 1886; Nakamura, 1998). While these main members of the *B. cereus* group display specific characteristics, they are at the heart of a taxonomy controversy due to their strong genetic relatedness (Okinaka and Keim, 2016; Vilas-Bôas *et al.*, 2007). Indeed, major characteristics differentiating the members of the *B. cereus* group are often encoded on plasmids, highly subjected to horizontal gene transfer. For example, *B. anthracis* main characteristics, *i.e.* causing the anthrax disease, resides within the presence of two plasmids, pXO1 and pXO2, responsible for the production of the anthrax toxins and capsule, respectively (Baillie and Read, 2001). However, multiple studies have reported cases of other *B. cereus* strains responsible for anthrax-like infections and harbouring plasmids related to pXO1 and/or pXO2 (Antonation *et al.*, 2016; Hoffmaster *et al.*, 2004; Ireng *et al.*, 2020; Klee *et al.*, 2010). Similarly, typical delta-endotoxins and cytotoxins of *B. thuringiensis* are encoded by *cry* and *cyt* genes, respectively, located on extrachromosomal genetic elements (Bravo *et al.*, 2007; Palma *et al.*, 2014). The *israelensis* serovar of *B. thuringiensis* is well known for the specific toxicity of its toxins against dipteran larvae, especially mosquitoes and blackflies. Preparations of *B. thuringiensis* sv. *israelensis* toxins have been used worldwide to fight against mosquito-borne diseases, such as malaria and dengue fever (Ben-Dov, 2014; WHO, 1999). The genetic determinants of *B. thuringiensis* sv. *israelensis* Cry

toxins are encoded on a 128-kb plasmid, called pBtoxis (Berry *et al.*, 2002). In 2005, Hu and colleagues demonstrated that pBtoxis could be transferred to other *B. cereus* s.l. strains by the mean of natural conjugation (Hu *et al.*, 2005). The potential transfer of toxin-encoding plasmids participates in blurring the lines between the different members of the *B. cereus* group and further fuels the ongoing debate concerning their classification.

Besides the toxin-encoding pBtoxis, *B. thuringiensis* sv. *israelensis* also carries a large conjugative plasmid, named pXO16. Highly efficient and fast transfer characterises this 350-kb element, with transfer frequencies reaching 100% between strains of its favourite host (Jensen *et al.*, 1996; Andrup *et al.*, 1998). pXO16 is also able to move about co-resident mobilisable and non-mobilisable plasmids, as well as fragments of its host chromosome (Makart *et al.*, 2017). pXO16 was originally studied for its ability to induce the formation of macroscopic aggregates of donor and recipient cells in liquid mating (Jensen *et al.*, 1995). First host spectrum studies assumed that aggregation was mandatory prior pXO16 transfer between mating partners (Jensen *et al.*, 1996). However, recent work identified a 25-kb "aggregation" locus within pXO16 sequence. Deleting this locus hampered aggregate formation but did not prevent pXO16 from transfer, both in liquid and solid matings. pXO16 transfer was then also observed between non-aggregating mating partners and the host spectrum of the plasmid was extended to different serovars of *B. thuringiensis* and different strains of *B. cereus* s.s., *B. weihenstephanensis* and *B. cytotoxicus* (Makart *et al.*, 2018; Hinnekens *et al.*, 2019). At time of its discovery, pXO16 was also shown to successfully transfer itself from *B. thuringiensis* sv. *israelensis* to *B. anthracis*, even though no transfer frequency was determined at the time (Reddy *et al.*, 1987). The same study also revealed the ability of pXO16 to induce the mobilisation of the capsule-encoding plasmid pXO2. Besides *B. anthracis*, most strains of *B. cereus* s.l. carry a large pool of extrachromosomal genetic elements, offering numerous candidates for mobilisation by co-resident conjugative plasmids. In this context, the case of pXO16 is of prime interest and the implications of its efficient transfer capacities require clarifications.

Despite previous work on the plasmid host spectrum, the potential impact of pXO16 on its new hosts remained to be addressed. To this end, we investigated pXO16 transfer to the rhizoid-like forming *B. mycoides* and reported impaired formation of rhizoid-like structures after pXO16 transfer. We also analysed the transmissibility of pXO16 amongst different *B. cereus s.l.* strains, highlighted the thermo-sensitivity of pXO16 and looked at its effect on its bacterial host growth and motility. Overall, this paper aims at bringing new insights into the influence of a highly powerful conjugative plasmid – both for its own transfer and for triggering the transfer of co-resident plasmids – on *B. cereus s.l.* hosts.

2. Materials and methods

2.1. Bacterial strains and plasmids

Bacterial strains and plasmids used in this study and their specific characteristics (incl. antibiotic resistance) are reported in Table 1. Bacteria were grown in Lysogeny Broth (LB) medium containing NaCl (5 g/L), yeast extract (5 g/L) and Tryptone (10 g/L). LB was solidified with 1.4% (w/v) agar for agar plates. Strains were grown at 30°C, or 37°C for *B. cytotoxicus* strains, unless mentioned otherwise. When appropriate, antibiotics (Sigma) were added to LB medium at the following concentrations ($\mu\text{g/mL}$): Sm, streptomycin (100); Nd, nalidixic acid (15); Tc, tetracycline (10); Cm, chloramphenicol (10); Rf, rifampicin (50); Sp, spectinomycin (250).

2.2. Spontaneous antibiotic-resistant mutants

Spontaneous *Bacillus* antibiotic-resistant mutants were obtained as described previously (Makart *et al.*, 2018; Hinnekens *et al.*, 2019). Briefly, the equivalent of at least 15 mL of an overnight culture (*ca.* 10^8 CFU/mL) were pelleted and plated out on LB plates with the appropriate antibiotic. Growing colonies were retrieved and confirmed on new antibiotic-containing LB plates.

Table 1: Bacterial strains and plasmids used in this work.

Strains/Plasmids	Main features	Sources or references
Bacterial strains		
<i>B. thuringiensis</i> sv. <i>israelensis</i> GBJ001	Sm ^R spontaneous mutant of reference strain 4Q7 cured of its plasmids, except pBtic235	(Jensen <i>et al.</i> , 1995)
<i>B. thuringiensis</i> sv. <i>israelensis</i> GBJ001(pXO16)	GBJ001-derivative containing pXO16::Tn5401	(Timmery <i>et al.</i> , 2009)
<i>B. thuringiensis</i> sv. <i>israelensis</i> GBJ002	Nd ^R spontaneous mutant of reference strain 4Q7 cured of its plasmids, except pBtic235	(Jensen <i>et al.</i> , 1996; Gillis <i>et al.</i> , 2017)
<i>B. thuringiensis</i> sv. <i>israelensis</i> GBJ002(pXO16)	GBJ002-derivative containing pXO16::Tn5401	(Timmery <i>et al.</i> , 2009)
<i>B. thuringiensis</i> sv. <i>israelensis</i> GBJ002(pXO16Δagr)	GBJ002-derivative containing pXO16Δagr	(Makart <i>et al.</i> , 2018)
<i>B. thuringiensis</i> sv. <i>aizawai</i> HD11	Wild-type isolate, BGSC-ID: 4J4	BGSC ^a
<i>B. thuringiensis</i> sv. <i>aizawai</i> HD11.1	Sm ^R spontaneous mutant of strain HD11	(Makart <i>et al.</i> , 2018)
<i>B. thuringiensis</i> sv. <i>aizawai</i> HD11.1(pXO16)	HD11.1-derivative containing pXO16::Tn5401	This study
<i>B. thuringiensis</i> sv. <i>aizawai</i> HD11.2	Rf ^R spontaneous mutant of strain HD11	This study
<i>B. thuringiensis</i> sv. <i>thuringiensis</i> Bt407	Wild-type isolate, active against Lepidoptera	(Lereclus <i>et al.</i> , 1989)
<i>B. thuringiensis</i> sv. <i>thuringiensis</i> Bt407.1	Rf ^R spontaneous mutant of strain Bt407	This study
<i>B. thuringiensis</i> sv. <i>thuringiensis</i> Bt407.1(pXO16)	Bt407.1-derivative containing pXO16::Tn5401	This study
<i>B. weihenstephanensis</i> WSBC10204	Wild-type isolate	(Lechner <i>et al.</i> , 1998)
<i>B. weihenstephanensis</i> WSBC10204.1	Sm ^R spontaneous mutant of strain WSBC10204	(Makart <i>et al.</i> , 2018)
<i>B. weihenstephanensis</i> WSBC10204.1(pXO16)	WSBC10204.1-derivative containing pXO16::Tn5401	This study
<i>B. weihenstephanensis</i> WSBC10204.2	Rf ^R spontaneous mutant of strain WSBC10204	This study
<i>B. cereus</i> s.s. H3081.97	Wild-type isolate	(Hoffmaster <i>et al.</i> , 2008)

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Strains/Plasmids	Main features	Sources or references
<i>B. cereus</i> s.s. H3081.97.1	Sm ^R spontaneous mutant of strain H3081.97	(Makart <i>et al.</i> , 2018)
<i>B. cereus</i> s.s. H3081.97.1(pXO16)	H3081.97.1-derivative containing pXO16::Tn5401	This study
<i>B. cereus</i> s.s. H3081.97.2	Rf ^R spontaneous mutant of strain H3081.97	This study
<i>B. cereus</i> s.s. IS075	Wild-type isolate	(Hoton <i>et al.</i> , 2009)
<i>B. cereus</i> s.s. IS075.1	Sm ^R spontaneous mutant of strain IS075	(Makart <i>et al.</i> , 2018)
<i>B. cereus</i> s.s. IS075.1(pXO16)	IS075.1-derivative containing pXO16::Tn5401	This study
<i>B. cereus</i> s.s. IS075.2	Rf ^R spontaneous mutant of strain IS075	This study
<i>B. cytotoxicus</i> E28.3	Wild-type isolate from potato flakes	(Koné <i>et al.</i> , 2019)
<i>B. cytotoxicus</i> E28.3.1	Sm ^R spontaneous mutant of strain E28.3	(Hinnekens <i>et al.</i> , 2019)
<i>B. cytotoxicus</i> E28.3.1(pXO16)	E28.3.1-derivative containing pXO16::Tn5401	(Hinnekens <i>et al.</i> , 2019)
<i>B. cytotoxicus</i> E28.3.2	Rf ^R spontaneous mutant of strain E28.3	(Hinnekens <i>et al.</i> , 2019)
<i>B. pseudomycoides</i> B-617	Wild-type isolate; rhizoidal growth	(Nakamura and Jackson, 1995)
<i>B. pseudomycoides</i> B-617.1	Sm ^R spontaneous mutant of strain B-617	This study
<i>B. mycoides</i> JAS391	Wild-type isolate; rhizoidal growth	(Fiedoruk <i>et al.</i> , 2021)
<i>B. mycoides</i> JAS391.1	Sm ^R spontaneous mutant of strain JAS391	This study
<i>B. mycoides</i> JAS481	Wild-type isolate; rhizoidal growth	(Fiedoruk <i>et al.</i> , 2021)
<i>B. mycoides</i> JAS481.1	Sm ^R spontaneous mutant of strain JAS481	This study
<i>B. mycoides</i> BPN09/1	Wild-type isolate; rhizoidal growth	(Fiedoruk <i>et al.</i> , 2021)
<i>B. mycoides</i> BPN09/1.1	Sm ^R spontaneous mutant of strain BPN09/1	This study
<i>B. mycoides</i> LMG7128	Wild-type isolate; rhizoidal growth	BCCM/LMG ^b
<i>B. mycoides</i> LMG7128.1	Sm ^R spontaneous mutant of strain LMG7128	This study
<i>B. thuringiensis</i> sv. <i>konkukian</i> B9727	Isolated from a soldier war wound	(Hernandez <i>et al.</i> , 1998)
<i>B. thuringiensis</i> sv. <i>konkukian</i> B9727.1	Sm ^R spontaneous mutant of strain B9727	This study
<i>B. cereus</i> CTMA-1571	Soil sample, Namibia	(Irege <i>et al.</i> , 2020)

Continued on next page

Strains/Plasmids	Main features	Sources or references
<i>B. cereus</i> CTMA-1571.1	R ^R spontaneous mutant of strain CTMA-1571	This study
<i>B. cereus</i> CTMA-1571.1(pXO16)	CTMA-1571.1-derivative containing pXO16::Tn5401	This study
<i>B. cereus</i> CTMA-1571.2	Sp ^R spontaneous mutant of strain CTMA-1571	This study
Plasmids		
pXO16::Tn5401	Derivative of pXO16, conjugative plasmid naturally occurring in strain 4Q2 of <i>B. thuringiensis</i> sv. <i>israelensis</i> , Tc ^R	(Jensen <i>et al.</i> , 1996)
pXO16Δ <i>agr</i>	Aggregation-deficient pXO16-derivative due to the deletion of its <i>agr</i> region, Cm ^R	(Makart <i>et al.</i> , 2018)

^a BGSC: *Bacillus* Genetic Stock Center, Ohio State University, USA.
^b BCCM/LMG: Belgian Coordinated Collections of Microorganisms, UGent, Ghent, Belgium.

2.3. Mating assays

Liquid and filter mating assays were performed as described previously (Hinnekens *et al.*, 2019), with some specific modifications for *B. mycoides* cultures. Briefly, independent pre-cultures of donor and recipient cells from fresh separate plating were grown in 10 mL of LB medium without antibiotics and were incubated overnight without shaking. For *B. mycoides* strains, cultures were grown from fresh plating in 15 mL LB medium without antibiotic, in 100 mL baffled Erlenmeyer and shaken overnight at 30°C. The overnight cultures were diluted 100-fold in LB medium and grown until reaching a similar OD₆₀₀ than that of the *B. thuringiensis* sv. *israelensis* mating partner pre-culture (OD₆₀₀ 0.5-0.6). For liquid mating, mating partners were mixed in equal amount in 10 mL of LB medium and incubated for 4 h. Mating partners for filter mating were successively passed through a 0.2 µm filter and the filter was retrieved and incubated on LB plate without antibiotic for a 4-h mating period. Cells were then resuspended from the filter in LB medium. In both liquid and filter matings, dilutions were plated out on selective media for parental and transconjugant strains. Each mating experiment also included a plating control of donor and recipient cell pre-cultures on selective media to exclude the presence of potential spontaneous mutants. Transfer frequencies were calculated as the ratio of transconjugants per donor cells (T/D) at the end of the mating time. All mating experiments were repeated at least three times, independently.

2.4. Stability assay

Selective liquid cultures were initiated from fresh solid plating and grown overnight with shaking. Overnight cultures were then diluted 100-fold in LB medium without antibiotics and grown in parallel at 50°C and 30°C, with shaking, for 5 h. Samples were taken every hour from the cultures and dilutions were plated out in parallel on LB agar only selecting the strain (LB + Sm) and on LB agar selecting both the strain and pXO16 (LB + Sm + Tc) (Table 1). Results were expressed in CFU/mL and compared the number of CFUs recovered on plasmid selecting medium with the number of CFUs obtained on strain selecting medium. All stability assays were repeated at least three times, independently.

2.5. Measurement of bacterial growth in liquid medium

Bacterial growth in liquid medium was followed up as described by Gillis and Mahillon in 2014, with some modifications. Overnight selective liquid cultures were diluted 100-fold in liquid medium and 200 μ L/well were inoculated into a 96-well plate. Bacterial growth was monitored by measuring the OD₅₉₅ in a Multiskan™ FC Microplate spectrophotometer (Thermo Fisher Scientific, Waltham, USA) every half hour over 16 h of incubation at 30°C (or 37°C for *B. cytotoxicus* strains). Control wells consisting of non-inoculated LB medium were added on each lane. For data analyses, LB medium was considered as the reference with an adjusted OD₅₉₅ of 0. All bacterial growth studies were repeated at least three times, independently. To further verify the stability of pXO16, cultures were screened by PCR after the 16-h incubation period in the spectrophotometer (primer pair: pXO16-F: 5'-TGAAAGAGTTGTACC-CCCTGC-3'/pXO16-R: 5'-TACGAACTTCGGTTACGGGC-3').

2.6. Mobility assays

The swimming ability of different strains was evaluated on LB soft 0.3% (w/v) agar by measuring the bacterial growth area, as described elsewhere (Houry *et al.*, 2010; Attieh *et al.*, 2020). A volume of 10 μ L of fresh static overnight bacterial culture (OD₆₀₀ $\sim$ 0.4-0.5) was spotted in the agar plate centre, and plates were incubated overnight. Colony diameters were measured 20 h after inoculation. After incubation, colonies were also screened by PCR to check the stability of pXO16 (pXO16-F/pXO16-R, as defined hereinabove). The colony diameter of strains carrying pXO16 were compared to the corresponding strains lacking pXO16 to evaluate the impact of the plasmid on the strain motility. All mobility assays contained three replicates each and were repeated three times, independently.

3. Results

3.1. pXO16 interferes with *B. mycoides* colony morphology

The large conjugative plasmid pXO16 from *B. thuringiensis* sv. *israelensis* raised multiple questions about its transferability to other bacteria since early host spectrum studies (Jensen *et al.*, 1996). Previous work on transfer in liquid

and solid mating conditions revealed that pXO16 could promote its own transfer from its original host - *B. thuringiensis* sv. *israelensis* - to different members of the *B. cereus* group (Makart *et al.*, 2018; Hinnekens *et al.*, 2019). However, conjugative transfer to *B. mycoides*, distinct from other *B. cereus* s.l. by its rhizoidal growth, remained overlooked so far. To address this, different strains of *B. mycoides* and *B. pseudomycoides* forming rhizoid-like colonies were chosen as potential hosts for pXO16 conjugation (Table 1). Plasmid transfer was demonstrated in filter mating at a wide spectrum of frequencies, ranging from 7.9×10^{-6} T/D up to 9.3×10^{-1} T/D (Fig. 1). The aggregation-minus version of pXO16 (pXO16 Δ agr) was also transferred to the same *B. mycoides* recipients. pXO16 Δ agr transferred to the five strains tested at similar frequencies than pXO16, except for the JAS481.1 strain to which transfer was 600-times more efficient when using pXO16 Δ agr (Fig. 1). Although not significant, pXO16 Δ agr also transferred more efficiently to LMG7128.1 than pXO16. Increased transfer frequencies when using pXO16 Δ agr instead of pXO16 in filter mating were also noted with other strains than *B. mycoides*, such as *B. cytotoxicus* E28.3 and *B. thuringiensis* sv. *thuringiensis* Bt407 (P. Hinnekens, J. Mahillon, unpublished).

Noteworthy was the change in colony morphology when pXO16 was transferred to any of the tested rhizoid-like forming strains. Transconjugant colonies displayed a star-shaped or round morphology, completely different from the recipient morphology prior to transfer (Fig. 2a). When spotted on non-selective solid medium and incubated for 2 days, rhizoid-like structures developed on the periphery of the transconjugant colonies (Fig. 2b). When these pseudo-rhizoids started developing, pXO16 was no longer detected in the cells, as demonstrated by PCR (data not shown). The same transconjugants were spotted in parallel on selective medium, but they formed no rhizoid-like colonies after 2 days of incubation and pXO16 was still detected in the cells. Interestingly, when *B. mycoides* transconjugants were grown in successive non-selective liquid cultures, pXO16 was maintained, even over several days (data not shown). These data first suggest that the presence of pXO16 in *B. mycoides* cells is interfering with the formation of rhizoid-like colonies. They then indicate that the loss of pXO16 from *B. mycoides* could

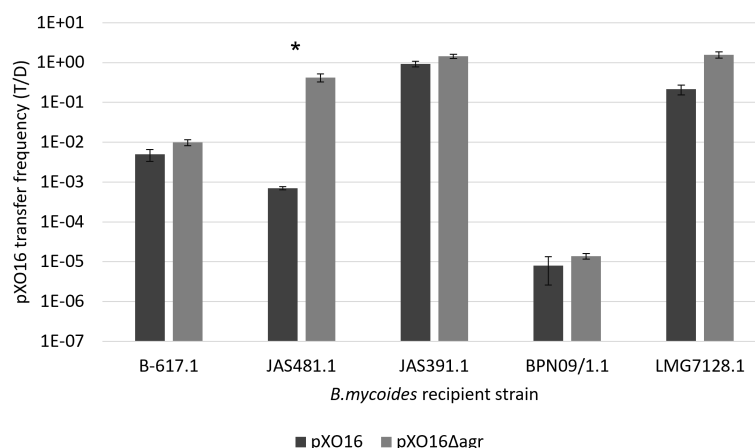


Figure 1: Comparison of conjugative transfer frequencies of pXO16 and pXO16Δagr to different strains of *B. mycoides*, under filter mating conditions. The donor strain is GBJ002(pXO16) or GBJ002(pXO16Δagr). Each frequency corresponds to the mean $\pm$ SEM out of three replicates. Frequencies of a same pair labelled with a "*" indicate that the differences between these frequencies were significant, following an unpaired Student's *t*-test ($p < 0.05$). Otherwise, differences between frequencies of a same pair were not significant.

somehow be triggered by the interference between the plasmid and the formation of rhizoid-like structures on solid medium.

3.2. pXO16 transfers to *B. anthracis* clade and amongst strains of *B. cereus* s.l.

To deepen the study of pXO16 conjugative transfer to *B. cereus* s.l., the plasmid transfer efficiency was evaluated to strains related to *B. anthracis*. In 1987, Reddy and colleagues first described pXO16 and suggested its transfer to *B. anthracis* (Reddy *et al.*, 1987). However, transfer efficiency was not evaluated at the time, leaving room for further investigation. Fig. 3 illustrates the transfer frequencies of pXO16 to *B. thuringiensis* sv. *konkukian* Bt9727 and to *B. cereus* CTMA-1571 (Table 1), both under liquid and filter mating conditions. Ireng *et al.*, previously classified both strains in the *B. anthracis* cluster, using single nucleotide polymorphism (SNP)-based phylogenomic analyses (see

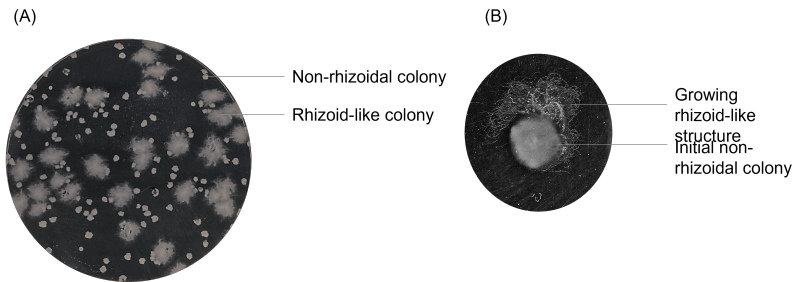


Figure 2: Impact of pXO16 on the rhizoid-like phenotype of *B. mycoides*. (A) Phenotype alteration after pXO16 transfer to *B. mycoides* JAS391.1. Following filter mating, when bacteria were plated on selective medium for recipients, two phenotypes could be observed. Rhizoid-like colonies do not carry pXO16 and are actual recipients, while non-rhizoidal colonies carry the plasmid and correspond to transconjugants, *i.e.* recipients that have acquired the plasmids. (B) Rhizoid-like structures growing from a colony of B-617.1(pXO16) after spotting on non-selective medium. From plates obtained at the end of mating assays, non-rhizoidal colonies were spotted on non-selective medium and incubated for 2 days at 30°C. pXO16 was no longer detected in the growing rhizoid-like section.

Fig. 1 in Irengue *et al.*, 2020). pXO16 transfer to CTMA-1571 was significantly enhanced in filter mating compared to liquid mating, while no difference was observed for Bt9727. Transfer frequencies to each of the two strains are also very different from each other. Similar observations were previously collected when transferring pXO16 to other *B. cereus s.l.* strains (Makart *et al.*, 2018; Hinnekens *et al.*, 2019). Taken together, these data strongly indicate that the efficiency of pXO16 transmission from *B. thuringiensis* sv. *israelensis* is strain dependent, rather than species or clade/cluster dependent.

Looking at pXO16 potential impact on *B. cereus s.l.* horizontal exchanges, it is crucial to investigate the transfer of pXO16 amongst its *B. cereus* hosts, independently from *B. thuringiensis*. So far, most of pXO16 host spectrum studies indeed focused on the transmission of the plasmid from its favourite host *B. thuringiensis* sv. *israelensis* to other strains of *B. cereus s.l.* Here, we looked at the dissemination of pXO16 within six strains of *B. cereus s.l.*, one *B. thuringiensis*, two *B. cereus s.s.*, one *B. weihenstephanensis*, one *B. cytotoxicus* and one from *B. anthracis* clade (Fig. 4). We also compared the transfer frequen-

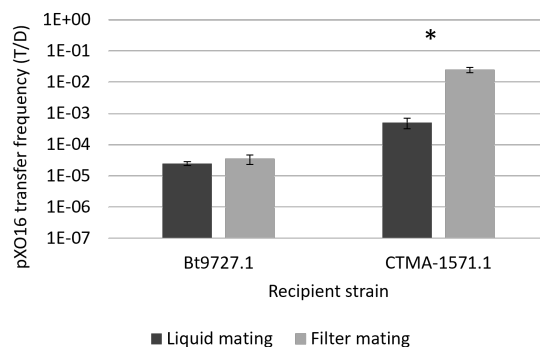


Figure 3: Transfer frequencies of pXO16 to strains belonging to the *B. anthracis* clade. Comparison of conjugative transfer frequencies of pXO16 to *B. anthracis*-closely related strains under liquid and filter mating conditions. The donor strain is *B. thuringiensis* sv. *israelensis* GBJ002(pXO16) and the recipient strains are *B. thuringiensis* sv. *konkukian* Bt9727.1 and *B. cereus* CTMA-1571.1. Each frequency corresponds to the mean $\pm$ SEM out of three replicates. Frequencies of a same pair labelled with a "*" indicate that the differences between these frequencies were significant, following an unpaired Student's *t*-test ($p < 0.05$). Otherwise, differences between frequencies of a same pair were not significant.

cies of the "homologous" matings (*i.e.* using isogenic strains, only differing in their antibiotic-resistance genes, in the mating pair) to those obtained for the corresponding "heterologous" matings in which *B. thuringiensis* sv. *israelensis* was the donor strain. The transfer efficiency of the plasmid was significantly enhanced in two homologous matings (*B. thuringiensis* sv. *aizawai* HD11 and *B. cytotoxicus* E28.3), while it was strongly decreased in the homologous mating involving *B. cereus* IS075, compared to the corresponding heterologous matings. For the other three matings, difference in plasmid transfer efficiency was not significant between homologous versus heterologous matings. Interestingly, homologous matings exhibited a much wider diversity in pXO16 transfer frequencies than the corresponding heterologous matings, and transfer efficiency was always lower than what was observed between cells of *B. thuringiensis* sv. *israelensis*. Experimental data also showed that pXO16 could transfer between strains of *B. mycoides* strains (data not shown). These data collectively demonstrate the ability of *B. cereus* group strains to exchange pXO16, even independently from its favourite *israelensis* host.

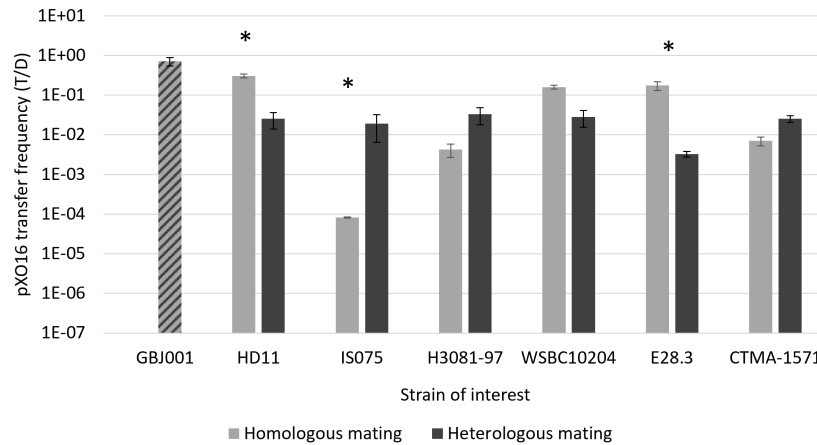


Figure 4: Transfer of pXO16 amongst *B. cereus* s.l. Heterologous and homologous matings were performed under filter mating conditions. The different strains illustrated are *B. thuringiensis* sv. *israelensis* GBJ001, *B. thuringiensis* sv. *aizawai* HD11, *B. cereus* IS075, *B. cereus* H3081-97, *B. weihenstephanensis* WSBC10204, *B. cytotoxicus* E28.3 and *B. cereus* CTAM-1571. In heterologous matings, the donor strain is GBJ002(pXO16) and the recipient strain is the version 1 of the strain of interest (e.g. HD11.1). In homologous matings, the donor strain is the version 1 of the strain of interest carrying pXO16 (e.g. HD11.1(pXO16)) and the recipient strain is the version 2 of the strain of interest (e.g. HD11.2). Both versions of each strain of interest only differ by antibiotic resistance from spontaneous mutation (Table 1). The hatched bar indicates the reference transfer frequency of pXO16 in *B. thuringiensis* sv. *israelensis* ("GBJ002(pXO16) x GBJ001"). Each frequency corresponds to the mean $\pm$ SEM out of three replicates. Frequencies of a same pair labelled with a "*" indicate that the differences between these frequencies were significant, following an unpaired Student's *t*-test ($p < 0.05$). Otherwise, differences between frequencies of a same pair were not significant. The transfer frequencies of the following matings were first described in Hinnekens *et al.* (2019): GBJ002(pXO16) x GBJ001, x HD11.1, x IS075.1, x WSBC10204.1, x E28.3.1 and E28.3.1(pXO16) x E28.3.2.

3.3. pXO16 stability under specific thermal conditions

Despite its relatively high efficiency in transferring among *B. cereus* s.l., pXO16 seems confined into the *israelensis* serovar of *B. thuringiensis* (Gillis *et al.*, 2018). A first hypothesis to resolve this paradox might be the relative (in-)stability of the plasmid throughout the wide range of temperatures encountered by *B. cereus* s.l. Thus, pXO16 stability was first assessed in *B. cytotoxicus* at 50°C and

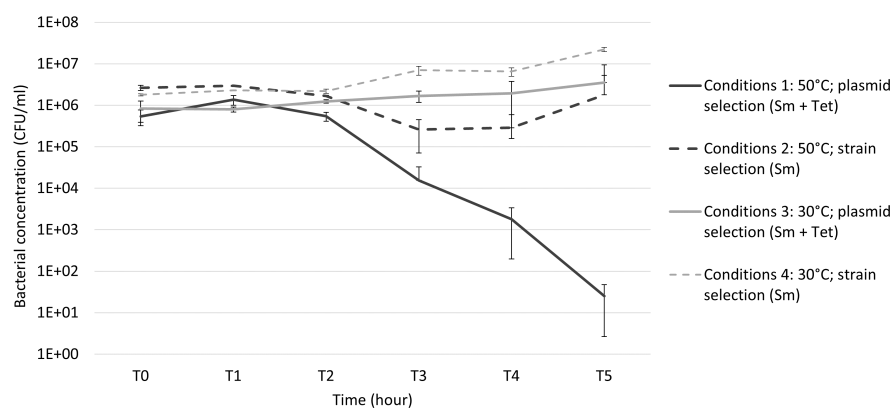


Figure 5: Comparison of pXO16 stability in *B. cytotoxicus* at 50°C and 30°C. Each concentration corresponds to the mean $\pm$ SEM out of three replicates.

at 30°C. As shown in Fig. 5, a strong instability of the plasmid was observed in the absence of selection at 50°C, with a dramatic drop in the concentration of plasmid-carrying bacteria within only a few hours. On the other hand, no difference was noticed between bacterial concentrations with or without plasmid selection at 30°C, indicating that the temperature is responsible for pXO16 loss at 50°C. On the contrary, comparison of pXO16 maintenance in the presence or absence of selection in *B. weihenstephanensis* at 8°C revealed that the plasmid is maintained over a period of at least 3 days (data not shown). These results suggest that pXO16 maintenance depends on the temperature and that the plasmid replicon might be thermo-sensitive.

3.4. pXO16 impacts the cell growth and motility of its new host cells

To further evaluate the impact of pXO16 on its hosts, we investigated the effect of the plasmid on the host cell growth. Optical density was monitored for cultures of different strains and was compared to cultures of the same strains harbouring pXO16 (Fig. 6). Cell growth was monitored over 16 h in the absence of any selective pressure. On Fig. 6 is illustrated the impact of pXO16 on the growth of its original host, of *B. thuringiensis* sv. *thuringiensis* Bt407 and of *B. weihenstephanensis* WSBC10204. Concerning *B. thuringiensis* sv. *israelensis*, curves illustrating the growth in presence and in absence of pXO16

blend into one another. This suggests that pXO16 visibly affects neither the growth speed nor the growth level of its *israelensis* host. On the contrary, growth monitoring revealed a slower growth and a significantly lower final optical density in presence of pXO16 for both illustrated *B. cereus s.l.* strains compared to the pXO16-less strains. Cell growth was also monitored for other *B. cereus s.l.* strains with and without pXO16. The growth of these strains followed the same pattern as that reported on Fig. 6 (B) and (C) for *B. weihenstephanensis* and *B. thuringiensis* sv. *thuringiensis* (data not shown). Overall, for all *B. cereus s.l.* strains different from *B. thuringiensis* sv. *israelensis*, the growth was affected by the presence of pXO16.

Motile bacteria developed the ability to migrate over substrates through different motility processes, *e.g.* swimming and swarming (Kearns, 2010). As defined by Henrichsen in 1972, swimming is the movement of individual bacterial cells, powered by flagella, and taking place in liquid environments or on semi-solid media. Swarming, on the other hand, describes a multicellular surface translocation of bacteria, also dependent on the action of flagella (Kearns, 2010; Henrichsen, 1972). Numerous *B. cereus s.l.* are flagellated and motile bacteria, characterised by swimming and/or swarming abilities on medium surfaces. Looking at the swimming ability of different strains of *B. cereus s.l.* revealed that the presence of pXO16 within the strain significantly decreases the swimming zone of the strain, *i.e.* the surface of the plate covered by the swimming bacteria (Fig. 7). To quantitatively evaluate the effect of pXO16 on swimming capacities, we performed swimming assays comparing the swimming zone of a strain carrying pXO16 to that of the same strain without pXO16 (reference strain) (Fig. 7a). The swimming area of the strain carrying pXO16 was expressed as a percentage of the swimming zone of the reference strain (Fig. 7b). Results indicate a reduced impact of pXO16 on the swimming capacity of *B. thuringiensis* sv. *israelensis* GBJ001 and *B. cereus s.s.* IS075, while a much stronger impact was revealed for both *B. cytotoxicus* E28.3 and *B. weihenstephanensis* WSBC10204. Amongst the seven *B. cereus s.l.* strains tested, the swimming zone of the strain carrying pXO16 ranged from 15% to 75% of the zones displayed by the related reference strains. Swarming assays, however, revealed no significant impact of pXO16 on the multicellular motility of the same strains (data not shown). Reminiscent of transfer frequency variations,

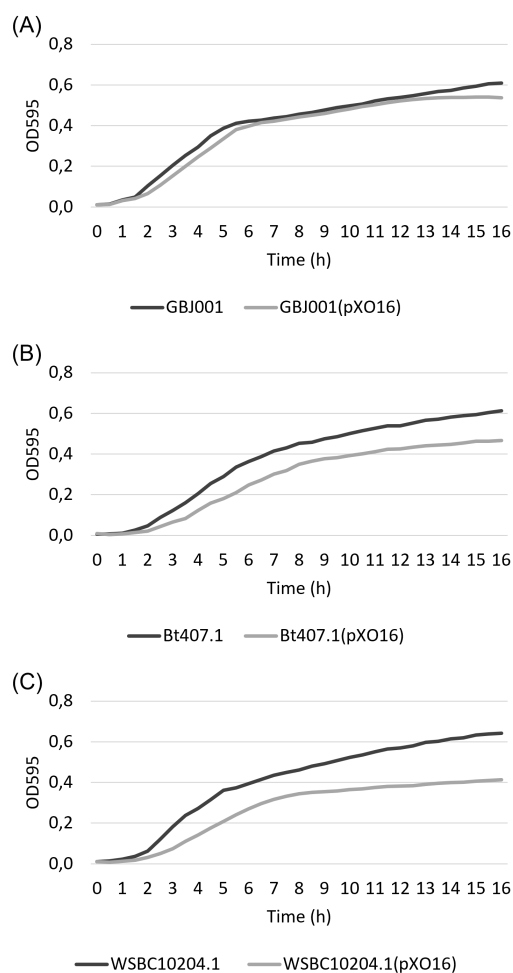


Figure 6: Impact of pXO16 on host cell growth. The impact of pXO16 was assessed for the growth of different strains, at 30°C. (A) *B. thuringiensis* sv. *israelensis* GBJ001; (B) *B. thuringiensis* sv. *thuringiensis* Bt407.1; (C) *B. weihenstephanensis* WSBC10204.1. Each curve corresponds to the mean out of three replicates. Error bars were not indicated on the graphs for clarity purpose.

the extent of pXO16 impact on individual cell motility in *B. cereus s.l.* strongly varies from strain to strain.

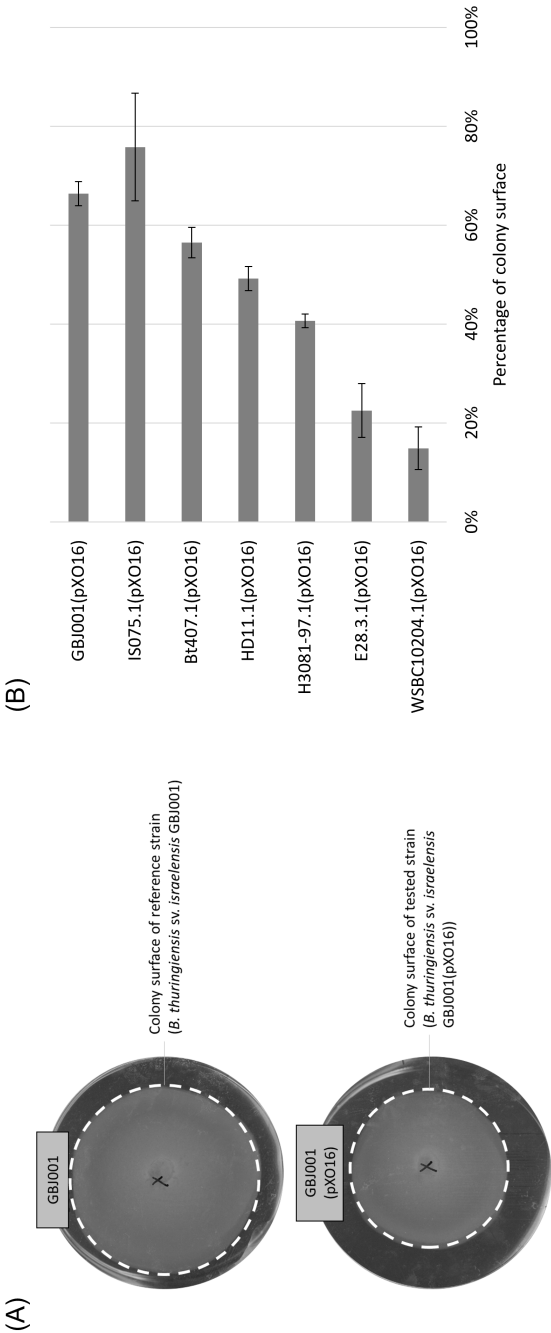


Figure 7: Effect of pXO16 on host relative swimming capacity. (A) Illustration of the comparison between the colony surface of the reference strain and the colony surface of the strain carrying pXO16 (tested strain). (B) For each strain, the colony surface is given as a percentage of the colony surface of the strain without pXO16. The colony diameter of each strain without pXO16 is taken as reference (100%). Each value corresponds to the mean $\pm$ SEM out of nine replicates (three biological replicates $\times$ three experimental replicates).

4. Discussion

First considered narrow (Jensen *et al.*, 1996), pXO16 host spectrum was extended in recent years (Makart *et al.*, 2018; Hinnekens *et al.*, 2019). Since demonstrating the dispensable action of aggregation in pXO16 transfer, the range of potential hosts increased until including a large diversity of *B. cereus s.l.* strains. In the present study, we first report the transfer of pXO16 to so far-overlooked potential hosts amongst *B. cereus s.l.* including *B. mycoides*.

Transferring pXO16 to several strains of *B. mycoides* revealed strong phenotypic alterations of the recipient strains post-conjugation. The selected *B. mycoides* were all characterised by a rhizoidal-like growth on solid LB medium prior to transfer, whereas none of the observed transconjugants showed any rhizoid-like phenotype and rather formed small round colonies. In addition, cultures on solid medium showed an instability of the plasmid in *B. mycoides*, while it was maintained stably in liquid cultures over several days. To further grasp the extent of pXO16 impact on *B. mycoides*, the aggregation-defective variant of pXO16, pXO16 Δ *agr*, was transferred in place of pXO16. On the one hand, two out of the five *B. mycoides* strains (JAS481 and BPN09/1) retained their rhizoidal morphology after acquiring pXO16 Δ *agr*. Differential stability and phenotypic interferences indicate that pXO16 hampers the rhizoid-like morphology of *B. mycoides*, limiting its stability in this *B. cereus s.l.* member. Although the plasmid-encoded aggregation proteins apparently cause morphology interference, additional factors most probably come into play as removing the aggregation proteins did not permit all tested strains to maintain their rhizoidal-like morphology post-transfer.

On the other hand, the transfer efficiency to JAS481 was increased by 600 times when the aggregation-defective plasmid was used instead of the wild-type plasmid. To the best of our knowledge, this is the first time that an increase in transfer efficiency is reported with the *agr*-deficient variant of pXO16 compared to the wildtype plasmid. Indeed, aggregation is thought to favour conjugation by bringing together donor and recipient cells (Makart *et al.*, 2018). Previous experimental data tend to confirm this hypothesis as the transfer of pXO16 Δ *agr* was less, or rarely equally, efficient than the transfer of

pXO16 (Makart *et al.*, 2018; Hinnekens *et al.*, 2019). However, these previous data were obtained in liquid mating. In filter mating, the situation is more variable. The transfer efficiency is usually very similar in presence or absence of the aggregation proteins, but for some strains, the transfer frequencies are increased in absence of the aggregation proteins (P. Hinnekens, J. Mahillon, unpublished). These experimental data indicate that the aggregation proteins, although facilitating mating pair formation in *B. thuringiensis* sv. *israelensis*, might disrupt or impair plasmid transfer to other strains. The presence of pXO16-encoded aggregation proteins at the cell surface might interfere with components of the cell surface of some recipients. Further investigations would however be required to understand the molecular bases underlying these interferences.

Horizontal gene transfer is genuinely recognised as a fundamental driving force in bacterial evolution and circulation of genetic material by conjugative plasmids makes no exception in *B. cereus* s.l. (Boto, 2010; Zheng *et al.*, 2013). As pXO16 has proven to transfer effortlessly to all *B. cereus* members and to move about mobilisable and non-mobilisable co-resident plasmids or chromosomal loci, we decided to investigate the capacity of pXO16 to transfer amongst members of the *B. cereus* group, independently of *B. thuringiensis* sv. *israelensis*. After assessing the plasmid transmissibility to *B. mycoides* and strains associated with the *anthracis* cluster, we assessed pXO16 transfer in "homologous matings". Compared to the related "heterologous matings" (*i.e.* mating between *B. thuringiensis* sv. *israelensis* and the *B. cereus* s.l. strain of interest), "homologous matings" exhibited a wider range of transfer frequencies. Nevertheless, pXO16 could transfer amongst *B. cereus* s.l., even out of its natural host.

Amongst all strains tested here and elsewhere (Makart *et al.*, 2018; Hinnekens *et al.*, 2019), variations in transfer frequencies seem to rely on one, or several, characteristic(s) of the recipient strain, rather than on the strain phylogeny. Different hypotheses can be drawn to explain the variations of pXO16 transfer efficiencies across *B. cereus* s.l., amongst which the plasmid content of the recipient strain, potentially limiting pXO16 transmission by incompatibility or exclusion mechanisms (Novick, 1987; Getino and de la

Cruz, 2018). Rather than the transfer mechanism *per se*, differences in the stability of pXO16 in the different hosts could also explain the discrepancies in terms of transfer efficacy. As plasmid stability is thought to rely on cross-talks between the plasmid and its host chromosome and not solely on the plasmid (Kottara *et al.*, 2018), different host genetic backgrounds can lead to different stability rates. As some strains of *B. cereus s.l.* are characterised by extreme thermal resistance, the thermal stability of pXO16 within these strains was explored. Although it was stably maintained in *B. weihenstephanensis* at low temperatures, pXO16 was rapidly lost from *B. cytotoxicus* at 50°C. In its favourite host, *B. thuringiensis* sv. *israelensis*, pXO16 also tends to be lost overtime at temperatures above 42°C (data not shown). These data suggest that pXO16 replicon is thermosensitive, providing first hints on pXO16 replication since the delimitation of its putative replication region by Makart *et al.* (2015).

Given that pXO16 was recently demonstrated to mobilise a co-resident plasmid and displace chromosomal loci from one strain of *B. cytotoxicus* to another (Hinneken *et al.*, 2019; Koné *et al.*, 2021), a similar scenario could occur amongst other *B. cereus* species. Besides, as many key features of *B. cereus s.l.* are located on plasmids, pXO16 might blur the lines by moving about critical plasmids, like the capsule-encoding plasmid pXO2 from *B. anthracis* (Reddy *et al.*, 1987). At a wider scale, pXO16 transfer could eventually occur outside of the *B. cereus* group. Conjugative matings were therefore attempted with other species such as *Listeria spp.*, *Bacillus subtilis* or *Staphylococcus spp.*, but remained so far unsuccessful. This could be explained by recurrent observations that plasmid host range is often restricted to the recipient strains in which they are able to replicate (Guiney, 1982; Virolle *et al.*, 2020). However, pXO16-mediated mobilisation of a co-resident mobilisable plasmid, which is able to replicate in *B. subtilis* and *Streptococcus spp.*, was observed at rare occasions from *B. thuringiensis* sv. *israelensis* to a specific strain of *B. amyloliquefaciens* in presence of pXO16 (P. Hinneken, R. Jaumain, J. Mahillon, unpublished). This enhances the interrogations about the potential impact of pXO16 extraordinary conjugative and mobilisable abilities on the extrachromosomal pool of species beyond *B. cereus s.l.*

All plasmids are thought to exert physiological costs on their carrier bacterial cells, which might be compensated by accelerating the acquisition of new traits (Harrison and Brockhurst, 2012). Given its size (~350 kb), pXO16 maintenance most probably involves significant metabolic costs. In addition, experimental data indicate that the acquisition of plasmids is often correlated with reduced growth of host cells, compared to plasmid-free cells (San Millan and MacLean, 2017). Following the growth of pXO16 host cells indicated that the presence of the plasmid slowed down and reduced the growth of its *B. cereus s.l.* hosts in liquid cultures. Nonetheless, no effect of its presence was noted on the growth of *B. thuringiensis* sv. *israelensis*. Besides affecting host cell growth, conjugative plasmids can also manipulate their host phenotype, for instance by reducing their mobility to promote sustained cell contact and therefore favour horizontal transmission (Billane *et al.*, 2022). The mobility of *B. cereus s.l.* strains, including *B. thuringiensis* sv. *israelensis*, is indeed impacted by the presence of pXO16, as demonstrated by mobility assays. The extent of mobility impairment varied from one strain to another, reminiscent but not correlated to the variations in pXO16 transfer efficiency. Similarly to pXO16 differential impact on its hosts, other studies also reported that a same plasmid can weigh differently on different hosts and that the plasmid-host relationship highly depends on the environmental context (Kottara *et al.*, 2018; De Gelder *et al.*, 2007; Humphrey *et al.*, 2012).

Why is pXO16 so efficient in transferring itself across *B. cereus s.l.* and yet seems naturally restricted to *B. thuringiensis* sv. *israelensis* (Gillis *et al.*, 2018)? The data collected in the present paper brought some pieces of information laying the first steps towards answering this major question about pXO16 ecology. pXO16's impact on its host fitness on *B. cereus s.l.* strains compared to *B. thuringiensis* sv. *israelensis* might lead to poorer survival of the plasmid in *B. cereus s.l.* Also, segregational loss of the plasmid was shown in some conditions. As the plasmid usually transfers less efficiently to *B. cereus s.l.* recipients than to *B. thuringiensis* sv. *israelensis*, conjugation-mediated transmission might not compensate the loss of the plasmid overtime. If the rate of segregational loss is higher than the efficiency of conjugative transfer, pXO16 would be lost from its host, especially because of its low copy-number (Andrup *et al.*, 1998; Carroll and Wong, 2018). Furthermore, the transmissibility of pXO16 to

B. cereus s.l. under tightly controlled laboratory conditions must be nuanced by its impact on cell growth and growth yield. In natural environments, such impact could lead to a less competitive bacterial strain, reducing the chance of survival of *B. cereus s.l.* strains harbouring pXO16. So far, no specific phenotype attributed to pXO16 could explain its strong stability and its low impact on its host, despite its impressive size and costly conjugative apparatus. As proposed to apprehend the plasmid paradox concept (Harrison and Brockhurst, 2012; Carroll and Wong, 2018), the impact of pXO16 on strains of *B. cereus s.l.* compared to *B. thuringiensis* sv. *israelensis* reinforces the hypothesis that pXO16 conjugation and coexistence with *B. thuringiensis* sv. *israelensis* can only be understood with a strong co-evolution between the natural host and the plasmid itself. Further work should consequently focus on the interactions between pXO16 and its *B. thuringiensis* sv. *israelensis* host. Such analyses should bring more information to understand the intimate host-plasmid relationship between pXO16 and *B. thuringiensis* sv. *israelensis* and help resolve the ongoing paradox around pXO16 conjugation and host spectrum.

Declaration of competing interest

The authors declare no conflict of interest.

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Part III

Discussion, conclusions and perspectives

General discussion

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Horizontal dissemination of genetic content is a core phenomenon in bacterial evolution, and relies on mechanisms like conjugation (Soucy *et al.*, 2015; von Wintersdorff *et al.*, 2016). Plasmids are key drivers of conjugation and conjugation systems have been extensively described for plasmids of both Gram-negative and Gram-positive origins (Goessweiner-Mohr *et al.*, 2013a; Grohmann *et al.*, 2017; Waksman, 2019; Virolle *et al.*, 2020). Among Gram-positive bacteria, the group of *B. cereus* is a cluster of genetically closely related bacteria with a high content of plasmids and other MGEs (Ehling-Schulz *et al.*, 2019; Fayad *et al.*, 2019). Plasmids play a crucial role in *B. cereus s.l.* as most characteristic toxins are plasmid-borne and therefore susceptible to HGT, especially through conjugation. Among the pool of conjugative plasmids from *B. cereus s.l.* is pXO16, the conjugative master of *Bti*. Despite intense work performed previously, the conjugative apparatus of pXO16 remains poorly understood and apparently diverge from known models. The present work aimed at proposing new insights into both the molecular aspects of the unique

conjugative apparatus of this plasmid and its transfer and behaviour among *B. cereus* s.l.

8.1 The mysterious transfer apparatus of a giant

Conjugation requires the transfer of plasmidial DNA between partner cells, from the donor to the recipient. As detailed in the introductory section, conjugative transfer relies on a T4SS, or transferosome, and a relaxosome (Chapter 2.2). Over the years, the conjugation apparatus of several plasmids, including plasmids of Gram-positive origin, was investigated and thoroughly described. Although models can be inferred from these described systems, the mechanism of conjugation used by pXO16 seems rather unique. While the transfer locus was mapped on pXO16 sequence by Makart *et al.* (2018), this work focused on specific components of pXO16 transfer apparatus, including relaxosome candidates (Chapter 6.1), the conjugative PG hydrolase of pXO16 (Chapter 6.2) and other *tip* elements.

8.1.1 Essential building blocks of pXO16 conjugation

As more and more data are collected, the T4S-like structure used by pXO16 for its self-transfer is progressively deciphered. When first describing the *tip* locus, putative functions were attributed to most Tip proteins based on bioinformatics predictions and on analogies with other Gram-positive conjugative plasmids, mostly pCW3 from *C. perfringens* and ICEBs1 from *B. subtilis* (Chapter 2.3) (Makart *et al.*, 2018). Based on these predictions, further work focused on specific elements of the Tip system, including the putative ATPases TipH and TipL, the putative PG hydrolase TipB, the predicted helicase TipO and the membrane proteins TipA and TipE, and added up to previous knowledge (Table 8.1).

Energising the conjugative transfer: ATPases

At the origin of the *tip* locus delimitation stands the FtsK/SpoIIIE ATPase TipL and a second putative AAA ATPase, TipH. Both *tipH* and *tipL* were demonstrated as essential genes for pXO16 conjugative transfer. TipL was proposed as pXO16 CP, whereas TipH was suggested as the VirB4-like motor ATPase of

pXO16 transfer apparatus (Makart *et al.*, 2018). Both protein types are essential components of canonical conjugative systems and VirB4-like ATPases are the signature of all T4SS identified so far.

Table 8.1: Overview of the predicted functions, features and importance of the Tip proteins in pXO16 conjugation. The putative function of each Tip protein was first established based on bioinformatics predictions, and further assessed experimentally for seven of them (see fifth column). When determined, the importance - or value - of the Tip proteins is reported with a colour code: green: essential; yellow: important but not essential; orange: important in maintenance-related functions; gray: not determined. TMD: transmembrane domain; SP: signal peptide; KO: gene knockout; -: no experiment performed.

Tip protein	Predicted function	CDS features	Value	Method
TipA	Channel protein, VirB6-like	5 TMD, SP	Green	KO
TipB	PG hydrolase	2 TMD, NlpC/P60 domain	Yellow	KO; protein function analysis (Chapter 6.2)
TipC	Unknown	SP	Gray	
TipD	Unknown	SP	Gray	
TipE	Channel protein, VirB8-like	2 TMD	Green	KO
TipF	Unknown	None	Gray	-
TipG	Unknown	None	Gray	-
TipH	ATPase	AAA ATPase domain	Green	KO
TipI	Unknown	3 TMD, TcpE-like (pCW3)/ConC-like (ICEBs1)	Gray	-
TipJ	Channel protein, VirB3-like	2 TMD, TcpD-like (pCW3)/ConD-like (ICEBs1)	Gray	-
TipK	Unknown	SP	Gray	-
TipL	CP	FtsK/SpoIIIE ATPase domain, 2 TMD	Green	KO
TipM	Unknown	None	Gray	-
TipN	Unknown	Stage V sporulation protein G domain	Gray	-
TipO	Helicase	Helicase domain	Orange	KO; protein function analysis (Chapter 6.1)
TipP	Unknown	None	Orange	KO; protein function analysis (Chapter 6.1)

Spanning the bacterial membrane: channel components

The main elements composing the channel of Gram-positive T4SS are VirB6-like and VirB8-like proteins (Goessweiner-Mohr *et al.*, 2013a). In the case of pXO16, two genes were proposed to encode VirB6-like and VirB8-like functions, *tipA* and *tipE*, respectively (Makart *et al.*, 2018). TipA presents five TMD and shares some distant similarities with other VirB6-like proteins, despite its unusual signal peptide and large size (1,834 aa). VirB6-like proteins are typically 250-350-aa long, but larger variants have been described previously, such as TraG from *E. coli* R1 plasmid (938 aa) and TcpH from *C. perfringens* pCW3 plasmid (832 aa) (Goessweiner-Mohr *et al.*, 2013a).

VirB8-like protein classification relies solely on structural similarities, with a typical N-terminal TMD, often followed by a predicted coiled-coil (CC) and a C-terminal periplasmic domain (Goessweiner-Mohr *et al.*, 2013a). In the absence of other putative function, the membrane-inserted TipE was proposed as pXO16 VirB8-like protein (Makart *et al.*, 2018), although its topology differs from the canonical VirB8-like structure (Fig. 8.1). Based on bioinformatics modelling using Interpro and Phyre2 (Hunter *et al.*, 2009; Kelley *et al.*, 2015), most of TipE presents a cytoplasmic orientation due to two, instead of one, N-terminal TMD. It also bears one CC in its last C-terminal third and contains a large putative C-terminal cytoplasmic domain.

To better grasp the importance of TipA and TipE for pXO16 conjugative transfer, KO variants of pXO16 were built (pXO16 Δ *tipA* and pXO16 Δ *tipE*), in which *tipA* or *tipE* was replaced by a kanamycin resistance cassette by homologous recombination. Deleting *tipA* or *tipE* totally impaired pXO16 ability for self-transfer as no transconjugant could be retrieved from liquid- or solid-based conjugative matings (P. Hinnekens, unpublished data). For both channel component candidates, data on their function, localisation and interactions with other Tip proteins should be collected by structure prediction - especially for TipA -, specific domain expression or mutation, fluorescence tagging and double-hybrid assays, for example. These supplementary experimental data are required to confirm the role of TipA and TipE in pXO16 conjugative channel.

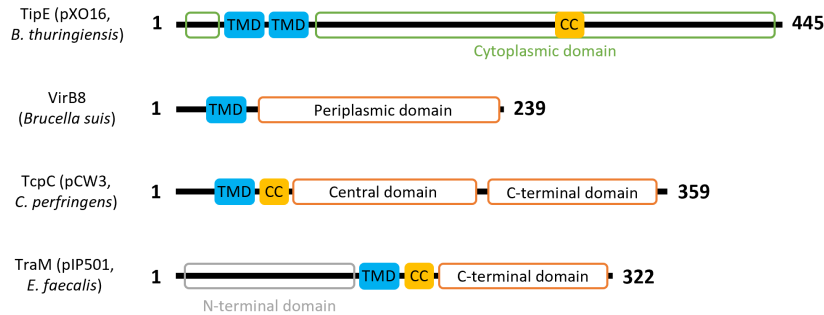
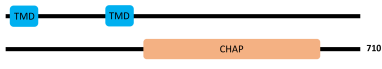
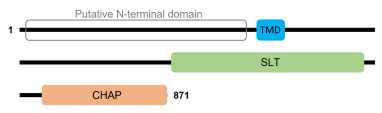
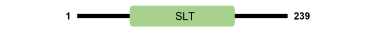


Figure 8.1: Comparison between TipE from pXO16 and other VirB8-like proteins. TipE is compared to three prototypes of VirB8-like proteins. VirB8 from *Brucella suis* contains one N-terminal TMD and a periplasmic C-terminal domain. TcpC from pCW3 carries an N-terminal TMD, a coiled-coil (CC) and a double domain (central and C-terminal). TraM from pIP501 possesses a N-terminal domain (putative) upstream the typical TMD and CC, and a C-terminal domain. The domains of these proteins are periplasmic (orange), while TipE is mostly predicted as cytoplasmic (green). TipE is also slightly longer than the different prototypes and carry two N-terminal TMD, while a CC is predicted in the C-terminal half of the protein. Adapted from Goessweiner-Mohr *et al.* (2013a).

A pair of small membrane proteins, TipI and TipJ, were also proposed to play a role in pXO16 conjugative channel, based on their resemblance with the pairs ConC/ConD and TcpD/TcpE from ICEBs1 and pCW3, respectively. Experimental data demonstrated that these two pairs of proteins are essential for their respective conjugation systems (Leonetti *et al.*, 2015; Wisniewski *et al.*, 2015). ConC (83 aa) and TcpE (122 aa) both exhibit three TMD, similarly to TipI (121 aa), and ConC was proposed as a Gram-positive specific T4SS protein in the absence of any suitable analogue (Auchtung *et al.*, 2016). ConD (175 aa) and TcpD (115 aa) both contain two TMD, like TipJ (73 aa), and were mentioned as putative VirB3 analogues (Wisniewski *et al.*, 2015; Auchtung *et al.*, 2016). Given the conserved topology and size between the three protein pairs, TipI/TipJ could have a similar role in pXO16 conjugation than ConC/ConD and TcpD/TcpE in their related T4SS. Experimental data, starting by the construction of knockout variants for each gene, are however required to evaluate the importance of *tipI* and *tipJ* for pXO16 transfer.

Table 8.2: Comparison of TipB and other conjugative PG hydrolases. TMD (blue): transmembrane domain; SLT (green): soluble lytic transglycosylase domain; CHAP (orange): cysteine, histidine-dependent amidohydrolases/peptidases domain; LOD: limit of detection. Adapted from Goessweiner-Mohr *et al.* (2014) and Hinnekens *et al.* (2021).

PG hydrolase (Plasmid)	Size (aa)	Impact of deletion	Topology
TipB (pXO16)	710	200-times reduction	
TcpG (pCW3)	334	1,000-times reduction	
CwIT (ICEBs1)	328	< LOD	
TraG (pIP501)	369	< LOD	
PrgK (pCF10)	871	< LOD	
VirB1 (pTi)	239	< 100-times reduction	

Crossing the cell wall barrier: the PG hydrolase

PG hydrolases play a key role in conjugation by locally degrading the PG to insert the T4SS into the bacterial cell envelope (Grohmann *et al.*, 2018). PG hydrolases from Gram-positive T4SS, as PrgK from pCF10, TraG from pIP501 and CwIT from ICEBs1 (Arends *et al.*, 2013; DeWitt and Grossman, 2014; Laverde Gomez *et al.*, 2014), are essential for conjugation and present a typical topology with one N-terminal TMD followed by two enzymatic domains, one central soluble lytic transglycosylase (SLT) domain and one C-terminal cysteine, histidine-dependent amidohydrolases/peptidases (CHAP) domain (Table 8.2). Conjugative PG hydrolases of Gram-negative origin are usually not essential for conjugation and only carry one SLT domain (Table 8.2).

In the *tip* locus of pXO16, early bioinformatics analyses revealed a putative conjugative PG hydrolase encoded by *tipB* (Makart *et al.*, 2018). However, TipB differs from other Gram-positive conjugative hydrolases by

its size (710 aa *versus* 200-400 aa typically), its topology and its importance in conjugative transfer. Indeed, similarly to TcpG from pCW3 (Bantwal *et al.*, 2012), TipB is not essential for self-transfer of pXO16. Also, TipB harbours two N-terminal TMD and one C-terminal CHAP-like domain, separated by a large uncharacterised region. Although the hydrolytic activity of TipB on bacterial cell wall was demonstrated, only part of the PG hydrolysis could be attributed to the C-terminal CHAP-like domain of the protein. The large uncharacterised central region of the protein remained so far insoluble and its activity could therefore not be tested. In the aim of purifying the large central domain, we could purify it along with an inactive variant of the CHAP domain (= TipB-2TM*, where the star indicates that the CHAP domain is inactivated). Such inactivation could come from a punctual mutation of the active cysteine residue (Fig. 8.2). A cysteine residue and a histidine residue are indeed recognised as invariant residues of CHAP domains (Bateman and Rawlings, 2003). With the CHAP domain being inactivated, any remaining PG hydrolysis activity would be attributable to the large unknown domain. However, this domain could also play a different role than enzymatic, by stabilising the CHAP domain or by displaying an interacting or anchoring function.

Interestingly, *tipB* was shown as an important but not essential gene for pXO16 conjugation (Chapter 6.2) (Hinneken *et al.*, 2021). Deletion of *tipB* only slightly affected the transfer efficiency of the plasmid. This is likely due to redundant PG hydrolases encoded on the plasmid or on the host chromosome that might substitute and compensate the loss of TipB (Koraimann, 2003; Zahrl *et al.*, 2005; Hinneken *et al.*, 2021). As mentioned in Hinneken *et al.* (2021), pXO16 encodes at least three putative hydrolases, including one peptidase M15 and two putative N-acetylmuramoyl-L-alanine amidases. Besides, chromosomal cell wall hydrolases remain poorly described for strains of *B. cereus s.l.* and they were only reported in few studies (Moriyama *et al.*, 1996; Chen *et al.*, 2000; Tran *et al.*, 2010). To date, chromosomal PG hydrolases of *B. thuringiensis* were mainly studied for their importance in the context of sporulation, mother cell lysis and the release of mature spore and toxin crystal (Yang *et al.*, 2013; Chen *et al.*, 2018b).

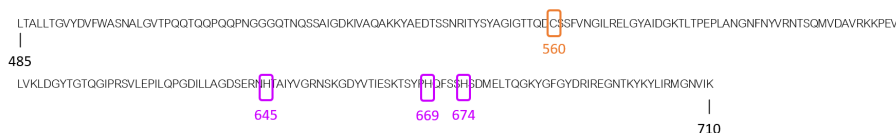


Figure 8.2: Amino acid sequence of the CHAP domain of TipB. Numbers refer to the aa position. The putative active cystein residue (Cys₅₆₀) is framed in orange and the three histidine residues identified within the CHAP sequence (His₆₄₅, His₆₆₉ and His₆₇₄) are framed in purple.

Further information could also be collected on TipB activity by determining its target in the PG meshwork. PG is made of glycan strands of alternating N-acetylmuramic acid and N-acetylglucosamine, connected by glycosidic bonds and cross-linked by short peptides (Vollmer *et al.*, 2008). PG is therefore susceptible to cleavage at different sites, by different types of PG hydrolases (*e.g.* peptidase, muramidase, glucosaminidase). Defining the nature of TipB hydrolysis could be achieved either by analysing the degradation products collected upon PG hydrolysis, or by performing differential hydrolysis assays, such as endopeptidase or glycosidase activity assays (Pritchard *et al.*, 2004).

8.1.2 Quid of the early transfer steps?

Increasing information becomes available about pXO16 conjugation and the picture of its transfer apparatus is getting clearer. Yet, the early steps of pXO16 conjugative transfer remain poorly known, especially the components taking part in plasmid processing prior to the recruitment to the conjugative channel, *i.e.* the relaxase and the *oriT*. Conjugation-mediated transfer is indeed initiated by nicking of the conjugative element by the relaxase and its accessory proteins at the *oriT* (de la Cruz *et al.*, 2010; Virolle *et al.*, 2020).

In this work, *a priori* and non *a priori* approaches were attempted *in silico* and *in vivo* to delimit a potential *oriT* region within the *tip* locus of pXO16 (Chapter 6.1). Although no typical *oriT* stood out from these analyses, new information was provided for supporting further hypotheses. On the one hand, the *oriT* could be located outside of the *tip* locus, or belong to a novel type of *oriT*, differing from those of known *MOB* families. Indeed, among

the ten recognised *MOB* families, two families, *MOB_L* and *MOB_K*, were recently proposed (Ramachandran *et al.*, 2017; Nowak *et al.*, 2021). More *MOB* groups could therefore be identified in coming years, among which one could host pXO16 *oriT*. Alternatively, initiation of pXO16 transfer could rely on *oriT*-mimicry, for which the relaxase could recognise "*oriT*-like" sequences with less specificity. Such scenario would be further supported by the unique ability of pXO16 for transferring any type of co-resident plasmid and chromosomal locus. Those genetic elements could indeed carry "*oriT*-like" sequences recognised with differential efficiency by pXO16 relaxase, inducing *in trans* mobilisation at various frequencies. On the other hand, the mechanism used by pXO16 to initiate its own transfer could also rely on a completely different and so far unknown process.

Conjugative relaxases catalyse a transesterification reaction at the *oriT* to initiate plasmid transfer by conjugation. They are often accompanied by a second enzymatic activity, like DNA helicase, either encoded by a second domain of the relaxase itself or by an accessory protein (Garcillán-Barcia *et al.*, 2009). Their intrinsic function requires relaxases to both bind and nick DNA. Additionally, accessory proteins can be involved for part of these activities. No relaxase candidate could be identified within pXO16 sequence from *in silico* analyses. However, data indicate that TipO, the one before last Tip protein, might be a candidate for an atypical relaxase. InterPro software analyses (Hunter *et al.*, 2009) mainly highlighted a DNA-binding domain, an ATP-binding domain and a C-terminal helicase-like domain within TipO sequence. Homology-based structure predictions using HHPred (Söding *et al.*, 2005) and Phyre2 (Kelley *et al.*, 2015) softwares further associated TipO with DNA helicases with high score values. Although *in silico* predictions for a helicase-like domain cover 79-83% of TipO sequence (528 aa), experimental data gathered in this work suggest both DNA binding and DNA nicking activities for TipO, indicative of a putative relaxase function. Nevertheless, DNA nicking assays presented in this work still necessitate some fine-tuning. It would for example be relevant to add ATP, Mg²⁺ or Mn²⁺ to the reaction mixture to evaluate the effect on TipO activity, or SDS and proteinase K to eventually released nicked DNA from the protein, and to increase the

resolution of the gel electrophoresis.

As reported in Table 8.3, the size of conjugative relaxases typically ranges between 200 and 700 aa, although larger relaxases have also been reported. For example, the relaxase of the F plasmid from *E. coli* is over 1,700 aa-long, and harbours one relaxase domain, one helicase domain and one C-terminal domain interacting with other conjugative elements (Guogas *et al.*, 2009; Dostál and Schildbach, 2010; Cheng *et al.*, 2011). However, relatively small proteins have been demonstrated as *bona fide* relaxases. TcpM, from the conjugative plasmid pCW3, is smaller than TipO, with only 267 aa, and was successfully demonstrated to nick pCW3 DNA (Wisniewski *et al.*, 2016). Nevertheless, the nicking activity of TcpM seemed rather unspecific, and the authors suggested that another protein might intervene for specific recognition of the plasmid *oriT* (Wisniewski *et al.*, 2016). As unspecific DNA nicking was also observed for TipO (Chapter 6.1), we could hypothesise that one, or several, accessory protein(s) is essential for specific activity. Based on data gathered throughout this work, we would like to propose TipO as a novel putative relaxase involved in pXO16 conjugation. Furthermore, relaxosome components, especially the relaxase, are expected to interact with the CP upon recruitment to the T4SS for DNA transfer (Smillie *et al.*, 2010). Preliminary data of double hybrid assays indicated that TipO indeed interacts with TipL, the putative CP of pXO16 (P. Hinnekens, unpublished data). Focusing on new double hybrid assays involving more Tip proteins should bring a greater picture of the interactions between pXO16 relaxosome and CP, as well as with other T4SS components.

In parallel, experimental data highlighted the importance of both TipO and TipP in pXO16 maintenance and probably replication. Based on the putative relaxase function of TipO, we could extend its function to a replicative relaxase, similar to Orf20 from Tn916 (Wright and Grossman, 2016). pXO16 could therefore utilise its so far unidentified *oriT* as an origin of replication and replicate according to an RCR mechanism, although RCR has never been reported for large plasmids to date. Work on pXO16 replication would be crucial to further evaluate such hypothesis and to elucidate the potential role of TipO and TipP in pXO16 replication-related maintenance. Two other CDS lo-

Table 8.3: Comparison of TipO and relaxases of different MOB families.

Relaxase	MGE	Size (aa)	MOB family (if relevant)
TipO	pXO16	528	
TraI	F plasmid	1756	MOB_F
TraA	pIP501	660	MOB_Q
MobA	pC221	324	MOB_P
PcfG	pCF10	395	
VirD2	pTi	424	
MobM	pMV158	494	MOB_V
TraX	pAD1	216	MOB_C
Orf20	Tn916	203	MOB_T
NicK	ICEBs1	350	
TcpM	pCW3	267	MOB_K
RelLS20	pLS20	410	MOB_L

cated in the putative replication region of pXO16 are annotated with potential relaxase accessory functions ("Replicative helicase" (AFQ30028, 481 aa) and "DNA primase" (AFQ30016, 190 aa) in Fig. 8.6) (Makart *et al.*, 2015). Given their spatial distance with the *tip* locus, their function was not investigated in this work, but further comparison with TipO activity on DNA could provide supplementary information about the potential relaxase-related function of TipO. Circling back to the original objective of investigating the importance of TipO and TipP in pXO16 conjugation, *tipO* and *tipP* could be placed under the control of an inducible promoter to complement the knockout variants. Upon induction, TipO (or TipP) would be produced *in trans*, facilitating its deletion from pXO16 sequence. After verification of the knockout variant, the expression of *tipO* could be turned off to finally assess the impact of its absence on pXO16 behaviour.

8.2 From one species to another: transfer of pXO16 across *B. cereus* s.l.

Early studies about pXO16 described a narrow host spectrum, limited to some serovars of *B. thuringiensis*, a few strains of *B. cereus* s.s. and one strain of *B. anthracis* (Reddy *et al.*, 1987; Jensen *et al.*, 1996). At the time, the transmission of pXO16 was followed by the transmission of pXO16-mediated aggregation phenotype, and potential recipients were assumed to be limited to strains capable of forming aggregates with the pXO16-carrying donor strain. More recent work demonstrated that pXO16 transfer was not restricted to aggregating mating pairs and that its host spectrum was wider than first established (Makart *et al.*, 2018).

8.2.1 Invading *B. cereus* s.l. by all means

While pXO16 host spectrum was first considered narrow, studying the aggregation phenotype led to expanding the range of potential hosts. This work participated in further investigating susceptible hosts for pXO16 conjugative transfer and its transfer efficiency under different mating settings.

To aggregate or not to aggregate

For clumping cells together and bringing susceptible mating partners in close vicinity, conjugation can rely on mechanisms like aggregation. Aggregation was for instance reported for the conjugative transfer of pheromone-sensitive streptococcal plasmids (Dunny *et al.*, 1978) and for conjugation between lactic bacteria (Walsh and McKay, 1981; Reniero *et al.*, 1992). The formation of macroscopic aggregates was also observed between *Bti* strains exchanging pXO16 in liquid mating set-ups (Andrup *et al.*, 1993; Jensen *et al.*, 1995). As aggregation could only be witnessed under circumstances of conjugative transfer, it was assumed that only aggregating partners could transfer pXO16 (Jensen *et al.*, 1995). Two decades later, an aggregation-deficient variant of pXO16 (pXO16 Δ *agr*) was constructed by removing the *agr* region (Fig. 8.3), responsible for the aggregation phenotype (Makart *et al.*, 2018). The absence of *agr* proteins strongly impaired, but did not annihilate, pXO16 transfer. Subsequent investigations revealed that pXO16 could actually transfer between

8.2. From one species to another: transfer of pXO16 across *B. cereus* s.l. 231

non-aggregating mating partners in liquid mating (Makart *et al.*, 2018), and opened up perspectives of a wider host spectrum for pXO16.

In absence of aggregation proteins, the transfer efficiency of pXO16 was reduced by over 1,000 times in liquid mating. By mimicking cell aggregation with solid mating conditions, transfer frequencies could return to wild-type levels, even in absence of aggregation proteins (Chapter 7.1). Experimental data from this work further demonstrated that solid mating conditions allowed the transfer of pXO16 to new recipient strains, including strains of *B. cytotoxicus* and *B. mycoides*. Also, in most cases, the use of solid mating increased pXO16 transfer frequencies compared to liquid mating conditions (Fig. 8.4). Similarly, *agr*-deficient pXO16 often transferred less efficiently than pXO16. These data suggest that aggregation tends to enhance the conjugation of pXO16, most probably by bringing and maintaining the cell partners in close contact. Once pXO16 is transferred, intimate cell contact is no longer required and cells can separate. Disaggregation is indeed observed in liquid mating after the transfer of the plasmid (Makart *et al.*, 2018).

However, pXO16 Δ *agr* showed higher transfer frequencies than pXO16 when transferring to about one quarter of the tested recipients (*B. thuringiensis* Bt407 and Bt9727, *B. weihenstephanensis* KBAB4, *B. cytotoxicus* E28-3, *B. cereus* CTMA15-71 and *B. mycoides* JAS481) in solid mating and to one strain (*B. weihenstephanensis* KBAB4) in liquid mating (Fig. 8.4). For these strains, aggregation proteins negatively impacted pXO16 transfer. As most of the proteins encoded in the *agr* region are predicted as proteins impacting the cell surface (Fig. 8.3), they might interfere with proteins at the surface of the recipient cell, limiting or destabilising the mating pair. On the contrary, aggregation-mimicry by solid mating conditions was only reported as improving - or at least not affecting - pXO16 transfer efficiency (Fig. 8.4). In this situation, solid mating conditions can favour conjugation by forcing the contact between the cell partners. While surface proteins are involved in pXO16-encoded aggregation, no such proteins come into play in solid mating settings. Therefore, solid mating reduces the potential interferences between donor and recipient cell surface proteins compared to natural aggregation.

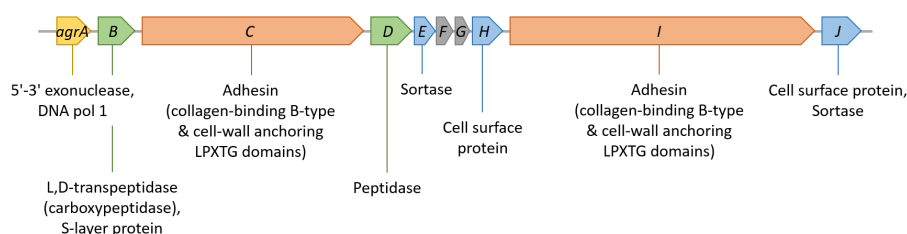


Figure 8.3: Genetic map of the *agr* region of pXO16. The ten genes composing the *agr* region are represented by arrows and alphabetically named *agrA* to *agrJ*. The predicted functions of the Agr proteins are indicated under the corresponding genes. Genes coloured identically are predicted to encode a similar function. Genes in grey encode hypothetical proteins.

Overall, transfer frequencies greatly varied from one recipient strain to another. For some strains, the presence or absence of *agr* proteins or the mating technique influenced the transfer efficiency, while not for others. For example, transfer frequencies of pXO16 to *B. thuringiensis* HD11 and HD125 and *B. cereus* IS075 were not affected by any of the tested parameters (Fig. 8.4). Data obtained in this work highlighted that pXO16 transfer efficiency is strain-dependent and relies on strain-specific features, including surface-exposed proteins that might interfere with pXO16-encoded *agr* proteins. Although some work was initiated on the *agr* region, it only compared pXO16 to pXO16 Δ *agr* (Feuillie *et al.*, 2018; Makart *et al.*, 2018). Further work should concentrate on specifics of the 29-kb *agr* region, especially by focusing on the two large predicted adhesins, AgrC (2,559 aa) and AgrI (3,527 aa) (Fig. 8.3). To pursue the investigation initiated by Feuillie *et al.* (2018), adhesion forces relying solely on AgrC or AgrI could be determined by AFM. Adhesion forces could also be assessed between mating partners different than *Bti*, to better understand the implication of aggregation for pXO16 conjugation across the *B. cereus* group.

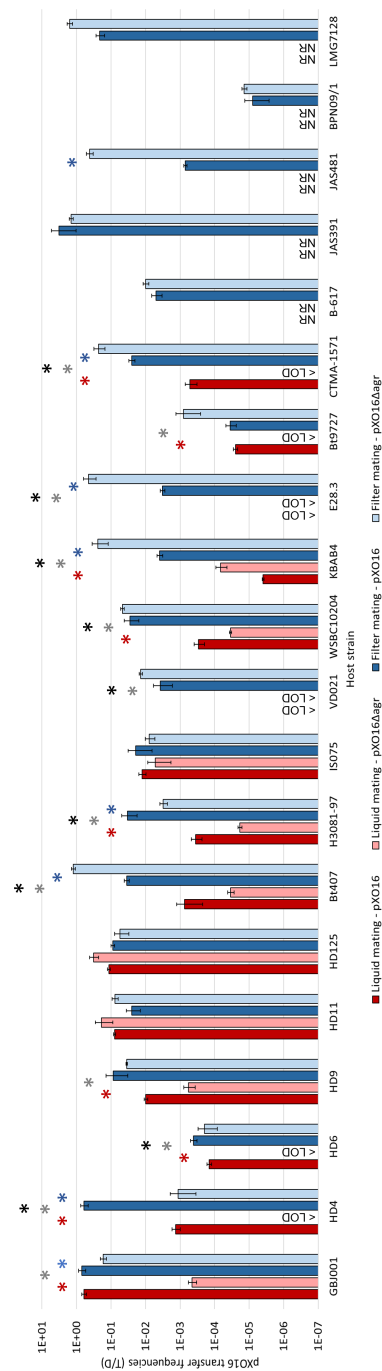


Figure 8.4: Transfer of pXO16 to members of the *B. cereus* group. Transfer of pXO16 (dark colour) and pXO16Δagr (light colour) to 20 strains of *B. cereus* s.l. under liquid (red) or solid (blue) mating conditions. The donor strain is *Bti* carrying pXO16 (GSX002(pXO16) or GSX002(pXO16Δagr)). Transfer frequencies are expressed as transconjugants per donor cells (T/D). Each frequency corresponds to the mean ± SEM out of three replicates. A red star indicates that the differences between frequencies in liquid mating (red bars) were significant, following an unpaired Student's *t*-test ($p < .05$). Similarly, a blue star indicates that the differences between frequencies in filter mating (blue bars) were significant, a black star indicates that the differences between frequencies of pXO16 (dark bars) were significant and a grey star indicates that the differences between frequencies of pXO16Δagr (light bars) were not significant. Otherwise, the differences were not significant.

Conquest of the entire group

As already mentioned, first studies concerning the host spectrum of pXO16 restricted the transfer of the plasmid to seven serovars of *B. thuringiensis* (*canadensis*, *israelensis*, *kurstaki*, *morrisoni*, *pakistani*, *thuringiensis* and *wuhanensis*) and two strains of *B. cereus* s.s., all able to form aggregates with the donor *Bti* strain (Jensen *et al.*, 1996). Earlier work also indirectly witnessed pXO16 transfer to one strain of *B. anthracis* (Weybridge UM44) by following the mobilisation of a small plasmid and detecting pXO16 in transcient cells (Reddy *et al.*, 1987). The host spectrum of the plasmid was first widened when pXO16 transfer was reported between non-aggregating mating pairs (Makart *et al.*, 2018). With this work, we further expanded pXO16 host range by using solid-based matings, which allowed the detection of transconjugants of *B. weihenstephanensis*, *B. cytotoxicus*, *B. anthracis*-related strains and even *B. mycoides* from mating with the *Bti* donor (Fig. 8.5).

Although pXO16 was successfully transferred from *Bti* to various strains of *B. cereus* s.l. and amongst strains of *B. cereus* s.l. (Chapter 7.2), its transfer is so far restricted to the *B. cereus* group. Indeed, in interspecies matings between a donor *Bti* strain and other Gram-positive bacteria, like *B. subtilis*, *Streptococcus* spp. and *Listeria* spp., no transconjugant carrying pXO16 could be isolated (Fig. 8.5). Interestingly, some occurrences of pXO16-mediated mobilisation of the small mobilisable plasmid pUB110 to *B. amyloloquefaciens* were reported at extremely low frequencies (P. Hinnekens, unpublished data). Such mobilisation event could not be reproduced with any other mobilisable plasmid or bacterial strain tested. Most likely, the mobilisation event is very rare and occurs at frequencies around the limit of detection (10^{-7} T/D). Even more plasmids and recipient strains could be tested and adjustments, such as the initial ratio of donor and recipient cells and the mating time, could lower the limit of detection and increase the chance of reproducing this mobilisation event.

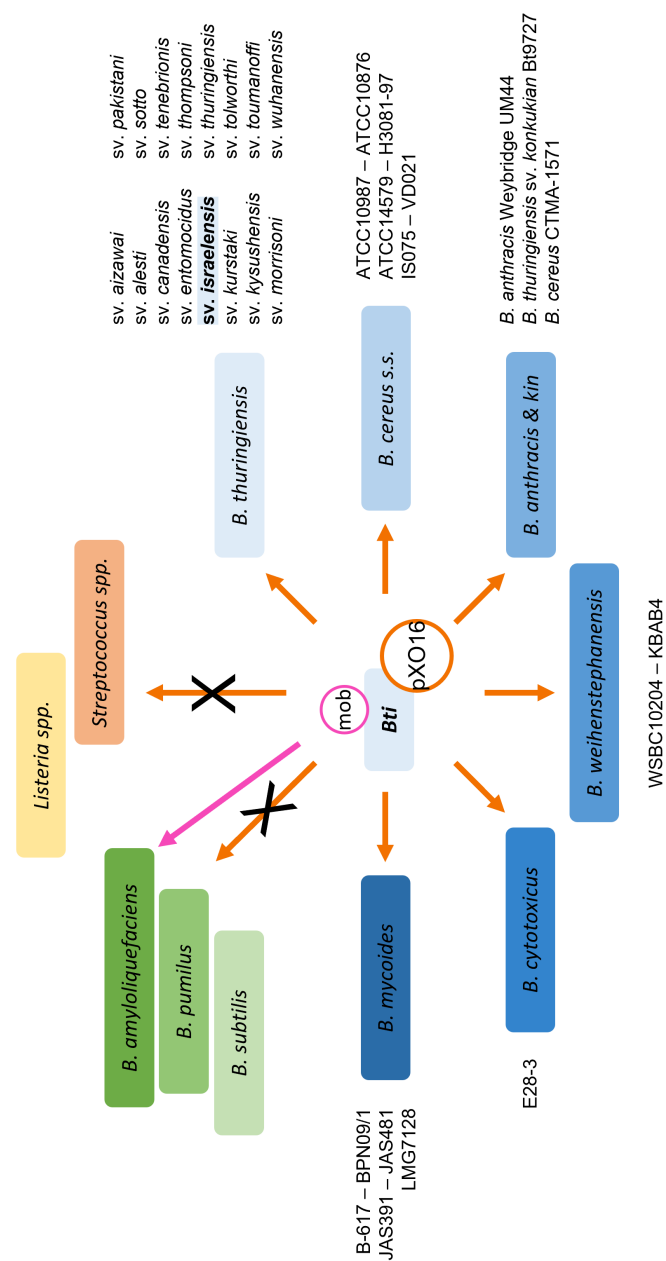


Figure 8.5: Illustration of the host spectrum of pXO16 as defined to date. pXO16 (orange circle), originally found in *Bti*, is capable of transferring itself to the different main species of the *B. cereus* group (orange arrows). For each species, all tested strains or serovars are indicated. No transfer of pXO16 was observed to strains of the *B. subtilis* group, nor to *Streptococcus* spp. and *Listeria* spp. (crossed orange arrows). However, a small plasmid (mob, pink circle) was sometimes mobilised by pXO16 to one specific strain of *B. amyloliquefaciens* (pink arrow).

Compared to broad-host range (BHR) plasmids like pIP501 from *S. agalactiae* or pAW63 from *B. thuringiensis* sv. *kurstaki* (Van der Auwera *et al.*, 2005; Kohler *et al.*, 2018b), the host spectrum of pXO16 remains rather narrow and restricted to the *B. cereus* group. As described in the introductory section of this manuscript, different parameters can limit plasmid transfer, such as incompatibility between the conjugative plasmid and other plasmids of the recipient cell, the inability for the bacterial partners to form stable mating pairs and the capacity of the plasmid to replicate within the recipient cell. Concerning the host spectrum of pXO16 transfer, plasmid incompatibility can hardly explain the limitation of pXO16 transfer to different strains of Gram-positive bacteria. The Gram-positive bacterial strains tested as recipients for pXO16 were diverse and most likely harboured various plasmid content. Then, mixed mating pairs with *B. cereus* s.l. and other Gram-positive bacteria should be stable enough for pXO16 transfer, because previous work focusing on the host spectrum of pAW63 relied on similar mating partners (Van der Auwera *et al.*, 2005). Therefore, the transfer of pXO16 to different Gram-positive bacteria is probably limited by its ability to replicate in some strains (Guiney, 1982; Virolle *et al.*, 2020), rather than by its ability to penetrate those strains. Contrary to BHR plasmids, pXO16 replicon could be specific to *B. cereus* s.l., shaping its host range.

8.2.2 Living with no trace ?

The existence of plasmids is rarely traceless in bacterial cells or at the population level (Billane *et al.*, 2022; Brockhurst and Harrison, 2022). At the population scale, pXO16 is capable of shuffling gene content by triggering the mobilisation and retromobilisation of plasmids and the displacement of chromosomal loci. As illustrated by recent experiments with *B. cytotoxicus* (Appendix A), pXO16 mobilisation power can be detoured to transfer a mutation from one strain to another. Specifically, a variant defective in the *cytK1* gene was built within a first strain by the classical double recombination method. Then, a similar variant of a second strain was obtained from pXO16-mediated transfer of the mutated chromosomal locus of interest (Appendix A). Given how hardly transformable *B. cereus* s.l. strains are, pXO16-mediated transfer of engineered mutations or constructions represents an alternative to

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standard transformation methods.

At the cellular level, pXO16 is thought to exert a physical cost on its bacterial host cells, as for all plasmids (San Millan and Maclean, 2017). The large size of pXO16 further suggests significant metabolic costs involved in plasmid maintenance over generations. Plasmid maintenance costs can be alleviated by facilitating the acquisition of new traits (Harrison and Brockhurst, 2012). However, none of pXO16 CDS has so far been predicted as homologous to any known toxin, antibiotic resistance gene, or other traits displaying obvious interest. As expected from previous studies (San Millan and Maclean, 2017; Billane *et al.*, 2022), pXO16 indeed reduces the growth of its host cells in liquid cultures and their mobility on semi-solid medium (Chapter 7.2). Furthermore, pXO16 and its *agr* proteins strongly interfere with the rhizoidal morphology of *B. mycoides*. Experimental data collected in this work are the first to document phenotypical differences between host cells in the presence and absence of pXO16. Interestingly, however, the impact of pXO16 was significantly reduced, or even absent, on the cell development of its original host, *Bti*.

The natural distribution of pXO16, restricted to *Bti* and absent from other *B. cereus s.l.* strains, could arise from the significant impact of pXO16 on *B. cereus s.l.* host cells compared to *Bti* cells. The deleterious effect of pXO16 on *B. cereus s.l.* host cells might reduce plasmid maintenance in those strains (Prensky *et al.*, 2021). Segregational loss of pXO16 was indeed noticed under specific conditions, *i.e.* solid cultures of *B. mycoides* and high temperature conditions, and natural environments could exacerbate plasmid instability in absence of selective plasmid-borne traits. Besides, knowledge about pXO16 host spectrum was collected under tightly controlled laboratory conditions. In natural environments, strains of *B. cereus s.l.* occupy various environmental niches, reducing the probability for plasmid exchange. Also, given the negative impact of pXO16 on the fitness of *B. cereus s.l.* host cells, cells carrying pXO16 would be less competitive, reducing their life expectancy, especially in the absence of any known favourable pXO16-borne phenotype.

8.3 pXO16 and the *israelensis* serovar, an intimate relationship

Despite its ability to transfer itself across the *B. cereus* group, pXO16 is naturally restricted to the *israelensis* serovar of *B. thuringiensis* (Gillis *et al.*, 2018). A major paradox then stands between the transferability of the plasmid and its natural distribution. With this work, we reported a significant impact of pXO16 on its *B. cereus s.l.* hosts (cell growth, growth yield, motility and colony morphology) and of host thermal niche on pXO16 stability, as pXO16 replicon was shown as thermosensitive. Notably, none of the tests performed so far revealed any influence of the plasmid on *Bti*. Therefore, the natural and impact-free coexistence of pXO16 with *Bti* suggests a strong co-evolution between the plasmid and its host. However, no particular phenotype has ever been attributed to the presence of pXO16, except for the formation of macroscopic aggregates in liquid matings (Andrup *et al.*, 1993; Makart *et al.*, 2015). So far, the only known function of aggregation is promoting pXO16 conjugative transfer.

As the role of pXO16 for *Bti* ecology remains unknown, the role of the extremely efficient conjugal transmission of pXO16 is still to be determined. At first, we hypothesised that conjugation could compensate some segregational loss of the plasmid over successive bacterial generations. By comparing the stability of pXO16, the variant pXO16 Δ *agr* and a variant of pXO16 deficient for transfer (pXO16 Δ *tipL* elaborated by Makart *et al.* (2018)), no evidence indicated any relative instability of pXO16 Δ *tipL* or pXO16 Δ *agr* overtime (up to six days) (P. Hinnekens, unpublished results). These preliminary data suggest that the conjugal transfer of pXO16 does not seem essential for the maintenance of the plasmid in *Bti* population, at least under laboratory settings. However, more parameters should be tested to further challenge this hypothesis, for example by comparing the growth of the different strains at different phases of their growth curve or by evaluating the stability of pXO16 in the presence and in the absence of selection in solid cultures instead of liquid cultures.

A second hypothesis for the role of pXO16 conjugation could reside in the mobilisation capacities of pXO16 and not in its own transfer. *Bti* is typically characterised by eight extrachromosomal molecules in addition to pXO16, including the major toxin plasmid pBtoxis (Gillis *et al.*, 2018). pXO16 could therefore serve as a mean to spread one (or several) of these elements and its related functions or characteristics, such as toxins, into the bacterial population. In this way, pXO16-encoded aggregation would help increasing the transfer efficiency of these elements. Given that the mosquitocidal toxins of *Bti* are encoded on pBtoxis, intense pXO16-mediated dissemination of pBtoxis would ensure the toxic action of any *Bti* cell. However, such hypothesis does not provide any explanation for the capacity of pXO16 to mobilise other plasmids than those co-residing in *Bti*, nor its ability to mobilise chromosomal loci, and requires experimental investigation.

From the 350 kb of pXO16 sequence, only ~30 kb encode the aggregation proteins and other ~25 kb form the *tip* locus. From the remaining ~295 kb of the sequence, a certain proportion should be dedicated to the replication and maintenance of the plasmid - putatively ~30-50 kb flanking the *tip* locus. It is reasonable to assume that the ~245-265 kb left should encode proteins involved in multiple functions, including interactions between the plasmid and its host. Strong interactions between pXO16 and *Bti* could explain their natural co-occurrence and help understanding their intimate relationship. From the analysis of the 434 CDS of pXO16 sequence, only 30% have a predicted functional annotation. These putative proteins can be grouped into seven categories: cell wall-associated proteins, DNA/RNA-interacting proteins, transcriptional regulators, membrane proteins, ATPases, phage-like proteins and proteins associated with regulatory pathways (Fig. 8.6). Most CDS with a predicted function can either be classified as DNA/RNA-interacting proteins (26.5% of CDS with predicted functions - 8% of total CDS) or as phage-like proteins (25% of CDS with predicted functions - 7.6% of total CDS).

The first group, gathering the putative DNA/RNA-interacting proteins, is of peculiar interest when investigating pXO16-*Bti* relationship. The corresponding CDS are indeed scattered throughout pXO16 sequence (Fig. 8.6 (A)), and not restricted to the putative replication region. Apparently, pXO16

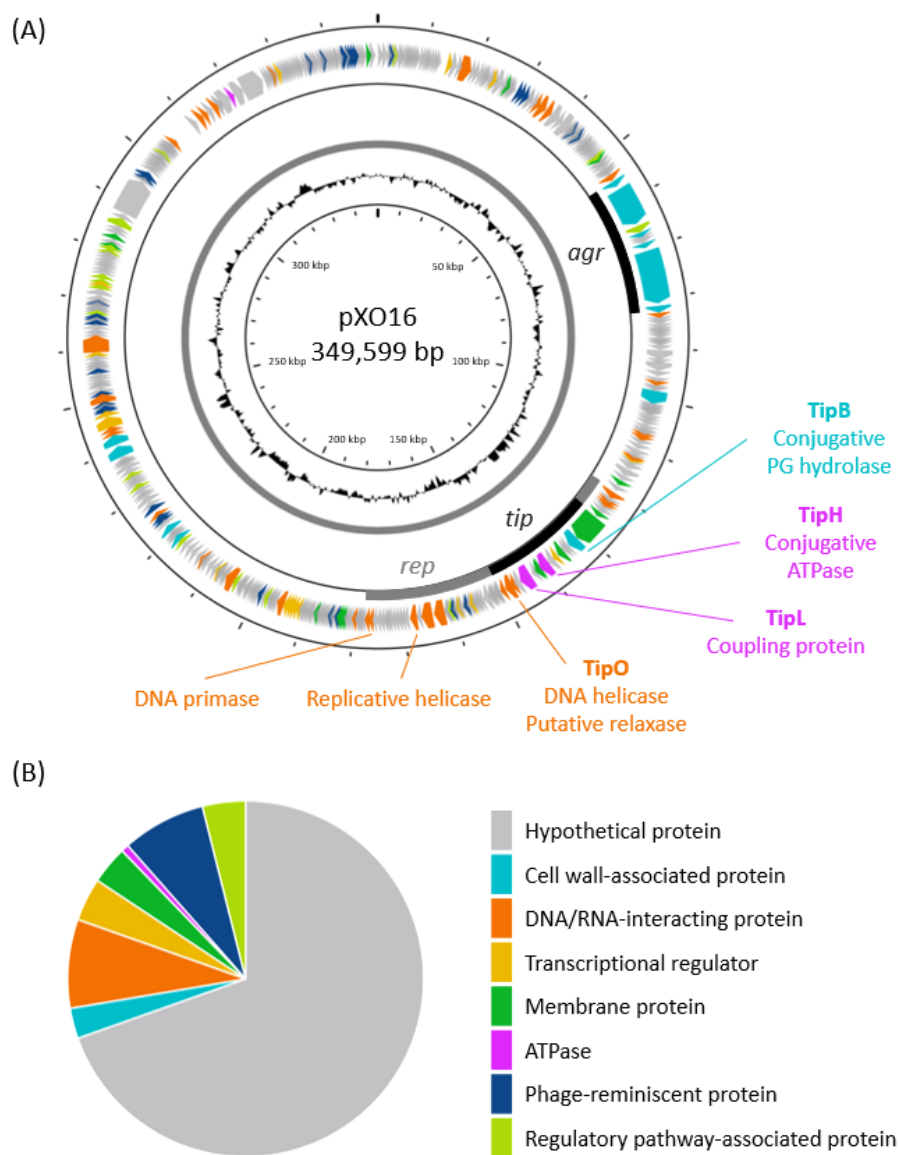


Figure 8.6: Annotation of pXO16 sequence based on latest CDS function prediction. Continued on next page.

Figure 8.6: (A) Circular map of pXO16 sequence. Block arrows in the outer circle represent the predicted CDS in their direction of transcription, with or without functional annotation. CDS are coloured based on their predicted functions. The black inner circle illustrates the GC content. The black bars represent the *agr* region (top right) and the *tip* locus (bottom right), and the grey bar delimits the putative replication (*rep*) region. Illustration generated by submitting pXO16 sequence from *B. thuringiensis* HD789 (CP003764.1) to Proksee (<https://proksee.ca/>). (B) Proportion of CDS annotated from each highlighted function type. 70% of total CDS are hypothetical proteins with no predicted function, while 30% of total CDS have a function associated with one of the seven highlighted function groups. The colour code is identical for both diagrams.

makes a great deal of DNA interactions. Additionally, 13% of the CDS with a predicted function are potential transcriptional regulators, enhancing the importance of DNA, DNA regulation and gene expression in pXO16 function. Whether these proteins are restricted to interactions and regulation of pXO16 itself or are involved in regulating processes of *Bti* chromosome or co-residing extrachromosomal elements remains to be elucidated.

The second main category of predicted proteins groups CDS reminiscent of phage CDS. As reported previously, the sequence of pXO16 exhibits peculiar features usually associated with the sequence of phage genomes, such as the unidirectional orientation of the 434 CDS, the high coding capacity and the large proportion of potential paralogues throughout the plasmid sequence (Makart *et al.*, 2015). However, no experimental proof of phage features, such as lysis plaques, could be observed to date for pXO16, which is often the case for jumbo phages. Jumbo phages are phages featured by large genome sizes (> 200 kb) and remarkable functionalities in terms of replication, metabolic adaptation and traits acquisition (Iyer *et al.*, 2021). Based on its common characteristics with jumbo phages, pXO16 might have evolved from a phage-like molecule.

At last, a significant proportion of pXO16 CDS (6.2% of total CDS) are predicted as hypothetical membrane proteins (11.3% of the CDS with a predicted function) or cell wall-associated proteins (9% of the CDS with a predicted function). Several of the putative cell wall-associated proteins are obviously

located in the aggregation region, but others are found elsewhere (Fig. 8.6 (A)). It implies that pXO16 most likely impacts the surface of its bacterial host cells by modelling or shaping their envelope. We could then expect to notice differences between pXO16-carrying and pXO16-free cells. Experiments performed so far failed at highlighting such differences. Thus alternative approaches are required to identify a differential phenotype which would help better grasping the role of pXO16 in its natural host, *Bti*. An interesting approach could consist in comparing the expression of host genes and proteins in the presence or in the absence of pXO16 in order to identify a potential pattern or influence of the plasmid on *Bti*. Comparing gene and protein expression should also be performed at the onset of conjugation, in mixed cultures of donor and recipient cells, in aggregating *versus* non-aggregating mating pairs, as well as in newly-formed transconjugants. These situations represent key steps where pXO16 is inclined to exert the greatest influence on its host cell, based on current knowledge about its conjugation and aggregation properties.

Conclusions and perspectives

Plasmids and other MGEs play a major role in the ecology, toxicity and classification of the members of the *B. cereus* group (Fayad *et al.*, 2019). They are at the very center of genome rearrangement and gene transfer, both intra- and inter-chromosomally. Inter-chromosomal movements, or horizontal transfer of genetic material, among *B. cereus* *s.l.* mainly involve conjugation of plasmids. Plasmids represent a large proportion of the genome of *B. cereus* group species, especially for *B. thuringiensis*. For instance, the serovar *israelensis* of *B. thuringiensis* typically carries nine extra-chromosomal molecules, including the mosquitocidal plasmid pBtoxis and the conjugative plasmid pXO16 (Gillis *et al.*, 2018). pXO16 was first described over 30 years ago (Reddy *et al.*, 1987) and its phenotypic features, like aggregation and transfer kinetics, have been thoroughly described over the years (Jensen *et al.*, 1995, 1996; Andrup *et al.*, 1998; Timmerly *et al.*, 2009). The genetic bases underlying its powerful conjugative system remain however poorly known. This thesis first focused on the study of defined elements of pXO16 conjugal apparatus, including early transfer steps and crossing of the bacterial cell wall. A second part of this thesis investigated the extend of pXO16 host spectrum and the impact of the plasmid on its host cells.

Conjugation-mediated transfer of conjugative plasmids or transposons relies on a physical contact between a donor and a recipient cell. It involves DNA processing at the *oriT* by a relaxase, followed by the recruitment of the processed plasmid to a T4S structure by a CP. The overall conjugation mechanism has been studied in depth in Gram-negative bacteria, whereas less is known about Gram-positive conjugation systems. Gram-negative T4SS are usually constituted by a core complex embedded into the OM and an IMC, as well as a pilus-associated complex involved in bringing the mating

partners together. In Gram-positive bacteria, cell-to-cell contact does not require any pilus and is often established by the mean of adhesins. The T4S-like systems described in Gram-positive bacteria are less complex and less conserved than their Gram-negative counterparts. They usually involve a channel reminiscent of the Gram-negative IMC and inserted into the bacterial thick PG layer thanks to the local PG degradation by a conjugative PG hydrolase. The best described conjugative plasmids of Gram-positive origin are those of the broad-host range plasmid pIP501, the pheromone-induced plasmid pCF10, the clostridial plasmid pCW3 and the conjugative transposon ICEBs1, whose T4SS serve as references.

The genes encoding the T4S-like system of pXO16 are gathered within the 25-kb *tip* locus within the putative replication region on the plasmid sequence. Except for two major conjugative ATPases, TipH and the CP TipL, information about pXO16 conjugative components was strictly based on bioinformatics predictions. With this thesis, much attention was dedicated to the early steps of pXO16 transfer, especially its relaxase and *oriT*. From both experimental data and detailed bioinformatics analyses, no typical *oriT* could be localised within the *tip* locus. Different scenarios can be drawn, including a low-specificity recognition of *oriT*-like sequences by pXO16 relaxosome to prime plasmid transfer or the use of several *oriT* with different degrees of specificity. On the other hand, TipO was presented as a candidate for the relaxase function based on its DNA binding and nicking activities. Although it is predicted as a DNA helicase, TipO could act as a relaxase protein and form the main constituent of pXO16 relaxosome, alongside accessory proteins which remain to be identified. Investigating the activity of mutated variants of TipO, especially for specific amino acids, its action on DNA in the presence of other relaxosome constituents and its interactions with other Tip proteins should help further elucidating its putative relaxase function. Interestingly, together with TipP, TipO is most probably involved in pXO16 maintenance, and perhaps replication, suggesting a potential RCR-like mechanism for pXO16 replication. In such context, pXO16 would use the same sequence as both an *oriT* and an origin of replication. Whether pXO16 replicates via RCR or not, understanding the replication mechanism of pXO16 would most

certainly provide new insights into this giant and unique conjugative plasmid.

Besides considerations about pXO16 relaxosome, this thesis brought new elements concerning pXO16 T4SS. The PG hydrolase involved in pXO16 conjugation, TipB, has been characterised and presents unique features. It is indeed very large compared to most described conjugative PG hydrolases and lacks the typical central enzymatic domain (SLT domain) in addition to the C-terminal CHAP-like domain. However, the large central uncharacterised region of the protein could encode a putatively novel enzymatic domain or exert a structural function. Two *tip* genes, *tipA* and *tipE*, predicted as channel components of pXO16 transfer apparatus, were also demonstrated as essential for pXO16 self-transfer. To further confirm their implication in pXO16 T4SS, the exact role of TipA and TipE should be investigated by first looking at their spatial localisation and their interactions with other Tip elements. Data should also be collected about the pair of small proteins TipI and TipJ to determine their importance for pXO16 conjugation and their potential similarities with the TcpD/TcpE and ConC/ConD pairs from the conjugative systems of pCW3 and ICEBs1, respectively. In addition, cloning the entire *tip* locus of pXO16 into a BAC vector, a shuttle vector designed for large fragment cloning (Liu *et al.*, 2009), would be an interesting alternative to study the conjugation system encoded by the *tip* locus. If the BAC vector containing *tip* transfers as efficiently as pXO16, it would constitute a great tool to work on the components of the conjugation system.

Formation of macroscopic aggregates for transfer is the major trait associated with pXO16 conjugation. Although previous work demonstrated the dispensable role of aggregation proteins in pXO16 self-transfer, this study highlighted the importance of aggregation or aggregation-mimicking conditions to favour pXO16 transmission. Under solid mating conditions, *i.e.* filter and colony mating, pXO16 could transfer from *Bti* to all *B. cereus s.l.* species and among *B. cereus s.l.* So far, no evidence showed an effective transfer of pXO16 itself to other Gram-positive bacteria, such as *B. subtilis*, *Streptococcus* spp. and *Listeria* spp. However, preliminary data indicate that pXO16 could trigger the mobilisation of small plasmids from *Bti* to *B. amyloliquefaciens* under tightly controlled conditions. By further optimising

mating conditions, pXO16-mediated mobilisation could serve as a tool to transfer genetic constructs from rather easily-transformable *Bti* strains to Gram-positive species, like *Listeria* spp., that can hardly undergo typical transformation. pXO16-mediated mobilisation was successfully used to transfer a chromosomal mutation from one strain of *B. cytotoxicus* to another, providing a first glimpse at the potential of a pXO16-based mobilisation tool.

Despite its rather extended host spectrum under strictly regulated mating conditions, the natural host spectrum of pXO16 seems restricted to *Bti*. Monitoring *B. cereus s.l.* host cells and following the plasmid stability under various conditions revealed that pXO16 weighs on the development of its host bacterial cells, except for *Bti*, and that it carries a thermosensitive replicon. Development impairment and plasmid thermal restriction, in addition to distinct natural niches of *B. cereus s.l.* may explain why pXO16 is not naturally present in those strains. However, the *raison d'être* of pXO16 and its powerful conjugation system in *Bti* remains to be elucidated. Of peculiar interest are its apparent inability to interfere with any of *Bti* development parameters and the lack of apparent pXO16-associated phenotype, besides aggregation and conjugation. Given the large proportion of uncharacterised CDS constituting pXO16 sequence, we can expect the plasmid to provide some sort of beneficial traits to *Bti*, compensating the metabolic cost involved in its maintenance. Cross-talks and interactions between pXO16 and its natural host, both under laboratory conditions and in natural niches like *Bti* target insects, surely represent a goldmine of new details concerning both the plasmid itself and its co-evolutionary relationship with *Bti*.

To conclude, results gathered during this thesis expanded the knowledge on the key elements playing essential roles in the conjugative machinery of the giant plasmid pXO16, as well as on the comprehension of the relationship between the plasmid and its *B. cereus s.l.* potential hosts. Beyond its contributions to the characterisation and understanding of these fundamental elements of pXO16 biology, this work highlighted the potential of a pXO16-based mobilisation tool for cloning in strains of *B. cereus s.l.* and brought to light new perspectives to further grasp the ins and outs of pXO16 conjugation and relationship with its *israelensis* host.

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Appendices

APPENDIX A

Cytotoxin CytK1 from *B. cytotoxicus*

B. cytotoxicus, the thermotolerant member of the *B. cereus* group, has been found associated with foodborne infections. *B. cytotoxicus* is systematically characterised by CytK-1, a highly cytotoxic and necrotic variant of CytK. To gain knowledge about the implication of CytK-1 into the toxicity of *B. cytotoxicus*, cytotoxicity assays were set up in this work to compare the cytotoxicity of wildtype strains and knockout variants.

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New insights into the potential cytotoxic role of *Bacillus cytotoxicus* cytotoxin K-1

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Abstract

The thermotolerant representative of the *Bacillus cereus* group, *Bacillus cytotoxicus*, reliably harbors the coding gene of cytotoxin K-1 (CytK-1). This protein is a highly cytotoxic variant of CytK toxin, initially recovered from a diarrhoeal foodborne outbreak that caused the death of three people. In recent years, the cytotoxicity of *B. cytotoxicus* has become controversial, with some strains displaying a high cytotoxicity while others show no cytotoxicity towards cell lines. In order to better circumscribe the potential pathogenic role of CytK-1, knockout (KO) mutants were constructed in two *B. cytotoxicus* strains, E8.1 and E28.3. The complementation of the *cytK-1* KO mutation was implemented in a mutant strain lacking in the *cytK-1* gene. Using the tetrazolium salt (MTT) method, cytotoxicity tests of the *cytK-1* KO and complemented mutants, as well as those of their wild-type strains, were carried out on Caco-2 cells. The results showed that *cytK-1* KO mutants were significantly less cytotoxic than the parental wild-type strains. However, the complemented mutant was as cytotoxic as the wild-type, suggesting that CytK-1 is the major cytotoxicity factor in *B. cytotoxicus*.

Keywords: *Bacillus cereus*; *Bacillus cytotoxicus*; CytK-1; cytotoxicity; Caco-2

Key Contribution: The current work created viable *Bacillus cytotoxicus* mutant strains lacking the *cytK-1* gene. Compared to their wild-type counterparts, the mutant strains showed very low cytotoxicity effects toward Caco-2 cells. Complemented mutant was as cytotoxic as the wild-type strain, suggesting that cytotoxin K-1 is the major cytotoxicity factor in *B. cytotoxicus*.

1. Introduction

Cytotoxin K-1 (CytK-1) is a highly cytotoxic and necrotic variant of cytotoxin K (CytK). It was initially recovered from a specific *Bacillus cereus* strain isolated from a food poisoning outbreak fatal to three elderly people in France in 1998. This *B. cereus* strain and its kin were later described as a new species, *Bacillus cytotoxicus*, the thermotolerant representative of the *B. cereus* group [1,2]. This group, also known as *B. cereus sensu lato* (s.l.), contains Gram-positive, spore-forming, and facultative anaerobic bacteria. Although numerous new species have been described as *B. cereus* s.l. members, the primary members of the group are *B. cereus sensu stricto* (s.s.), *Bacillus thuringiensis*, *Bacillus anthracis*, *Bacillus mycoides*, *Bacillus pseudomycoides*, and *Bacillus weihenstephanensis*. The *B. cereus* group comprises both beneficial and pathogenic members. While *B. mycoides*, *B. pseudomycoides*, and *B. weihenstephanensis* have not reportedly been implicated in any human infections or foodborne diseases yet [3], many *B. thuringiensis* have been used for several decades as bio-pesticides in agriculture and control of disease vectors, due to their ability to produce insecticidal molecules [4].

Because of its plasmid-encoded anthrax toxins, *B. anthracis* is highly pathogenic to mammals, including humans [5], and some *B. cereus* s.s. strains have been implicated in human extra-gastrointestinal infections, as well as in foodborne illnesses [6]. Indeed, an ingestion of food containing particular *B. cereus* strains can lead to two types of foodborne diseases, the emetic and diarrhoeal syndromes. The former is caused by a plasmid-encoded, in-food produced, heat-stable, acid-resistant and ring shape 1.2 kDa peptide, the cereulide [7,8]. The diarrhoeal syndrome is presumably caused by one or a combination of several enterotoxins produced in the small intestine after ingestion of *B. cereus* contaminated food. These enterotoxins are chromosomally encoded and prominently include the hemolysin BL (Hbl), the non-hemolytic enterotoxin (Nhe) and CytK [9]. The expression of most of these potential enterotoxins is regulated by the Pap/PlcR regulatory system [10]. Hbl and Nhe toxins are both three-component toxins. While almost all *B. cereus* s.l. members harbor the Nhe genes (*nheABC*), about 40 to 60% contain the Hbl genes (*hblABCD*) [11,12]. It has also been shown that the

Nhe toxin initiate cell apoptosis in Vero cells [13], and more recently, it was reported that Hbl and Nhe can act synergistically to trigger inflammation [14].

Encoded by the *cytK* gene, CytK is a 34-kDa single-peptide toxin for which two variants have been reported, CytK-1 and CytK-2. The latter forms smaller pores in phospholipidic bilayer membrane, is less cytotoxic, and is mainly found in some mesophilic *B. cereus* strains. It shares 87% amino acid identities with CytK-1 toxin which is more cytotoxic, and whose coding gene is restricted to *B. cytotoxicus* strains [15]. However, NVH 883-00, a *B. cytotoxicus* strain originating from spices, was shown to under-express CytK-1 toxin and be less cytotoxic than the reference strain, NVH 391-98, isolated from the fatal outbreak in France [16]. Moreover, based on whole genome sequencing, four genomic clades (A to D) have been described and clade A reportedly gathered the most potentially cytotoxic strains, including strains NVH 391-98 and CH_213 [17,18]. It is also noteworthy that the presence of toxin genes does not by itself lead to their expression and to the bacterium cytotoxicity. Indeed, the expression of *B. cereus* enterotoxin genes is thoroughly regulated and is dependent on the strain and its microenvironment [19–21]. It has been suggested that the cytotoxicity of *B. cytotoxicus* could have been overestimated in the past. As for other potentially enterotoxigenic *B. cereus*, the cytotoxicity of *B. cytotoxicus* should not be deduced solely based on the presence/absence of the *cytK-1* gene and genetic profile should thus be complemented with (cyto)toxicity assays [20,22].

Therefore, there was a need for an in-depth exploration of the implication of CytK-1 toxin in *B. cytotoxicus* toxicity. To the best of our knowledge, no study has yet focused on the deletion of *cytK-1* gene in order to assess the role of its toxin. Hence, the current study aimed at constructing *B. cytotoxicus* mutants lacking their *cytK-1* gene and comparing their cytotoxicity with that of the parental wild-type strains.

2. Results and Discussion

2.1. Construction of E28.3^{cytK-1-KO-A} and E8.1^{cytK-1-KO-B} *B. cytotoxicus* mutants lacking *cytK-1*

Since the initial isolation of *B. cytotoxicus* from a fatal food poisoning incident [1], its toxicity has become somewhat controversial. Indeed, it has even been suggested that the cytotoxicity of this species was overestimated in the past [20]. To help circumscribe the role of CytK-1 toxin, we knocked out the *cytK-1* gene in two *B. cytotoxicus* strains, E28.3 and E8.1. Both strains were isolated from potato flakes. In contrast to the reference strain NVH 391-98 showing no visible plasmid, E28.3 and E8.1 display large and small plasmids. These two *B. cytotoxicus* strains share the same RAPD profile, which is different from that of NVH 391-98 [23]. They also display distinct plasmid profiles. While strain E28.3 is one of the rare *B. cytotoxicus* of the collection to be susceptible to electroporation, E8.1 is the only strain in which the *cytK-1* KO mutation could be successfully transferred via conjugation (see below).

Using a double-recombination approach based on a thermo-sensitive shuttle plasmid, a KO-mutant of the *cytK-1* gene was built in strain E28.3 by replacing this locus by a kanamycin-resistance (*kanR*) gene. Five recombinant strains (A–E) were screened for *cytK-1* and kanamycin resistance genes. As shown in Figure 1, all the potential recombinant clones carried the resistance gene (Lanes a2 to a6), while they all lost the *cytK-1* gene (Lanes b2 to b6). Additional PCR experiments confirmed the validity of the gene swap (data not shown). Based on these results, E28.3^{cytK-1-KO-A} recombinant clone was retained for further experiments.

It was recently shown that the mega-plasmid pXO16 could mobilize chromosomal loci between members of the *B. cereus* group during its conjugation [24]. Using this approach, the KO-mutation of E28.3^{cytK-1-KO-A} was transferred to strain E8.1 via filter mating conjugation. The kanamycin-resistant (Kan^R) candidate transconjugants were then verified by RAPD profiling [23] to confirm that they displayed the same pattern as their parental strain E8.1. As shown in Figure 2, the clone present in Lane 2 displayed the RAPD pattern identical to that of its parental strains E8.1. Additional PCR confirmed that

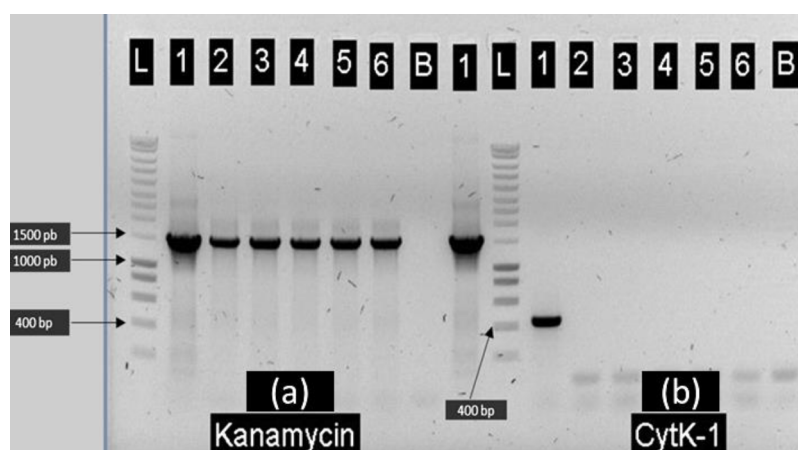


Figure 1: PCR-based confirmation of the five *B. cytotoxicus* KO-mutants selected after homologous recombination. L: DNA molecular weight markers (200 bp to 10 kb); B: negative control (buffer only). Panel (a): PCR-screening of the Kanamycin resistance gene: lanes 1 to 6: positive control (pDG173), E28.3^{*cytK-1*-KO-A}, E28.3^{*cytK-1*-KO-B}, E28.3^{*cytK-1*-KO-C}, E28.3^{*cytK-1*-KO-D} and E28.3^{*cytK-1*-KO-E}. Panel (b): Detection of the *cytK-1* gene: lanes 1 to 6: positive control (E28.3 wild-type), E28.3^{*cytK-1*-KO-A}, E28.3^{*cytK-1*-KO-B}, E28.3^{*cytK-1*-KO-C}, E28.3^{*cytK-1*-KO-D} and E28.3^{*cytK-1*-KO-E}.

this clone (named E8.1^{*cytK-1*-KO-B}) had acquired the locus where *cytK-1* was replaced by the *Kan^R* gene (data not shown). This clone was retained for the cytotoxicity experiments. Given that viable mutants of *B. cytotoxicus* strains lacking the *cytK-1* gene were obtained, it can be suggested that this gene is not essential to *B. cytotoxicus*. However, it is worth mentioning that attempts to create a similar mutant in the reference type-strain NVH 391-98 through electroporation, as well as via pXO16 conjugation, have failed (data not shown). The reasons for these unsuccessful attempts remain so far unknown. Future trials to construct mutants of other highly cytotoxic *B. cytotoxicus* strains, such as CH_213 [18], should certainly be considered.

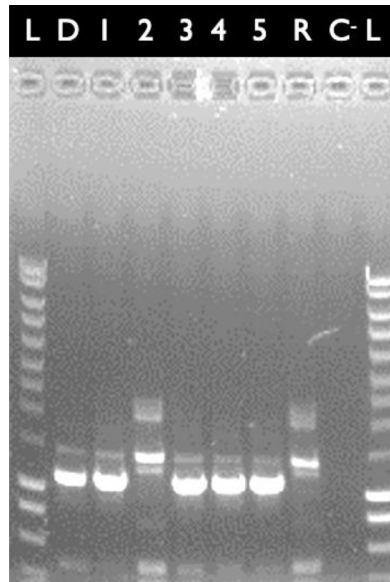


Figure 2: RAPD pattern of *B. cytotoxicus* strains using OPA9 primer. L: DNA molecular weight markers (200 bp to 10 kb); D: donor strain E28.3^{cytK-1-KO-A}; R: recipient strain E8.1; C: negative control (buffer only); Lanes 1 to 5: candidate transconjugants. Lane #2 contains a *bona fide* E8.1 transconjugant containing the desired genetic loci (*i.e.* *kan*^R gene replacing the *cytK-1* gene). It was named E8.1^{cytK-1-KO-B}.

2.2. Cytotoxicity of *B. cytotoxicus* wild-type strains E28.3 and E8.1, their derived mutants lacking the *cytK-1* gene and the complemented mutant

Caco-2 cells were exposed to the *B. cytotoxicus* supernatant of the reference strain NVH 391-98, as well as that of the wild-type strains E8.1 and E28.3 and that of the derived *cytK-1* KO-mutants. The cytotoxicity was assayed using the tetrazolium salt method (MTT) which assesses cell viability. After two hours, the highly cytotoxic strain, NVH 391-98, was able to impair Caco-2 viability while little cytotoxic effect was observed for the wild-type strains E8.1 and E28.3. However, after twelve hours of exposure, these two strains were almost as cytotoxic as NVH 391-98 (Figure 3).

Interestingly, deleting the *cytK-1* gene had a striking effect: in both genetic backgrounds, removing the gene reduced by more than 90% the deleterious

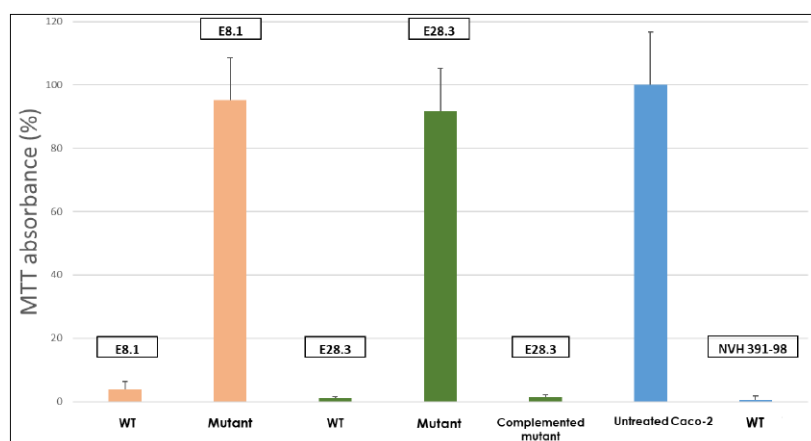


Figure 3: Tetrazolium salt method (MTT) used to assess viability of Caco-2 cells after 12 h of exposure to *B. cytotoxicus* supernatants. Cells treated with supernatant of wild-type (WT) NVH 391-98 and untreated Caco-2 cells were used as positive and negative control, respectively. Mutants (E8.1^{cytK-1-KO-B}) and E28.3^{cytK-1-KO-A}) lacking the *cytK-1* gene are less cytotoxic than the WT strains of E8.1 and E28.3 ($p < 0.05$). The complemented mutant of E28.3^{cytK-1-KO-A} is as cytotoxic as the WT E28.3

effect of the E28.3^{cytK-1-KO-A} and E8.1^{cytK-1-KO-B} supernatants on Caco-2 cell viability, indicating a drastic reduction of cytotoxicity and suggesting that CytK-1 toxin plays a major role in *B. cytotoxicus* cytotoxicity. In order to confirm that the observed effects were directly related to the *cytK-1* gene deletion, a complementation experiment was conducted by cloning *cytK-1* into the pHT304-18Z shuttle vector and introducing it in the E28.3^{cytK-1-KO-A} mutant by electroporation. As shown in Figure 3, the resulting complemented mutant displayed almost the same activity as its wild-type counterpart, reinforcing the idea that CytK-1 is responsible for most of the *B. cytotoxicus* cytotoxicity, at least on Caco-2 cells.

The toxicity of NVH 391-98, initially isolated from the fatal case in 1998, is in line with previous reports [1,20]. Recently, Stevens *et al.* [17] explored the relationship between genomic diversity and cytotoxicity among strains pertaining to four *B. cytotoxicus* genomic clades (A–D). It was then suggested that potentially highly cytotoxic strains are gathered in clades A

and B, while those of clades C and D are presumably less cytotoxic. More recently, the draft genome of another highly cytotoxic *B. cytotoxicus* strain, CH_213, pertaining to genomic clade A has been released [18]. In addition, whole genome sequencing of wild-type strains E8.1 and E28.3 indicated that they relate to clade C [25]. With clade C strains displaying relatively high cytotoxicity toward cell lines, the current findings indicate that highly cytotoxic *B. cytotoxicus* strains are not necessarily restricted to clades A and B. Nevertheless, a better understanding of the link between genomic grouping and virulence would require cytotoxic studies on other *B. cytotoxicus* strains, from different food matrices and genomic clades.

The inactivation of *cytK-1* correlates with a drastic drop in the cytotoxicity of our *B. cytotoxicus* strains. This is in contrast to the findings of Ramarao and Lereclus, in which there was no significant cytotoxicity reduction (both on Caco-2 and HeLa cell lines) in a *B. thuringiensis* mutant lacking its *cytK-2* gene [26]. It is noteworthy that the *cytK-2* gene harbored by *B. thuringiensis* encodes for the less cytotoxic variant of the CytK toxin [15]. Assessing the various wild-type *B. cytotoxicus* and *B. thuringiensis* strains and their *cytK*-KO mutants in the same assay could be valuable to resolve this seeming discrepancy.

Despite displaying an impaired cytotoxicity, our KO-mutants potentially possessed remaining toxicity (Figure 3) that could be imputable to other to-be-investigated enterotoxins or virulence factors in *B. cytotoxicus*. In fact, previous reports have shown that *B. cytotoxicus* lacks the Hbl genes but harbors a novel variant of Nhe genes [1,16]. Nhe is a three-component toxin that was shown to be necrotic at high concentrations, while inducing apoptosis in sub-necrotic concentrations. It has also been recently reported to trigger inflammation synergistically with the Hbl toxin [13,14]. As for several *B. cereus* enterotoxins, Nhe and CytK-1 are under the control of the PlcR regulator [10], and it has been reported that NVH 391-98 overproduces the CytK-1 toxin [16]. We can therefore speculate that the high cytotoxicity of some *B. cytotoxicus* strains could be imputable to a synergistic action of large amount of produced CytK-1 and/or Nhe toxins. It remains to be seen whether the remaining toxicity displayed by the *cytK-1* KO-mutants can be attributed to these other putative enterotoxins.

3. Conclusions

In conclusion, viable *B. cytotoxicus* mutants lacking the *cytK-1* gene were successfully created. Cytotoxicity tests showed that these mutants were less cytotoxic than the parental wild-type strains. To give further credence to these observations, a complementation of the knockout mutants with the wild-type *cytK-1* gene was constructed. The complemented mutant displayed cytotoxic activity comparable to that of the wild-type strains. Together, these results suggest that CytK-1 toxin is indubitably implicated in *B. cytotoxicus* cytotoxicity. The exact contribution of this activity to the diarrhoeal syndrome caused by the *B. cytotoxicus* strains remains, however, to be clarified. Similarly, it is plausible that high cytotoxicity observed in certain *B. cytotoxicus* strains could be imputable to the association of CytK-1 with one or more additional enterotoxin(s) and/or enzyme(s).

4. Materials and Methods

4.1. Bacterial strains, growth media and plasmids

The bacterial strains and plasmids used in this study are summarized in Table 1. *B. cytotoxicus* strain E28.3 was used to create the *cytK-1* KO mutant through the homologous recombination method (see below). NEBuilder HiFi DNA Assembly Cloning Kit (New England Biolabs™, Ipswich, MA, USA) was used to insert the PCR amplicons into the thermo-sensitive plasmid pMAD [27]. Chemically competent *Escherichia coli* NEB 5-alpha and *dam*⁻/*dcm*⁻ C2925 strains (New England Biolabs™, Ipswich, MA, USA) were used for the cloning steps. Plasmid pXO16 conjugation was used to transfer, via mobilization, the *cytK-1*-KO mutation into strain E8.1. Bacterial strains were grown at 37°C (*E. coli*) or 30°C (*B. cytotoxicus*) on Luria-Bertani (LB) medium (broth or supplemented with 1.5% agar) containing the appropriate antibiotics: ampicillin (Amp, 100 µg/mL), erythromycin (Ery, 10 µg/mL), kanamycin (Kan, 50 µg/mL) or tetracycline (Tet, 50 µg/mL).

4.2. Construction of the E28.3 mutant strain lacking the *cytK-1* gene

A fresh overnight colony of *B. cytotoxicus* strain E28.3 from LB agar was mixed in 50 µL of deionized water for the DNA extraction. Using Q5 High-Fidelity

Table 1: Bacterial strains and plasmids used.

Strains	Main features	References
<i>B. cytotoxicus</i> strains		
E8.1	Wild-type strain isolated from potato flakes; RAPD pattern A, plasmid profile PP10	[23]
E8.1 ^{cytK-1-KO-B}	<i>cytK-1</i> KO mutant of E8.1	This work
E28.3	Wild-type strain isolated from potato flakes; RAPD pattern A and plasmid profile PP8	[23]
E28.3 ^{cytK-1-KO-A}	<i>cytK-1</i> KO mutant of E28.3	This work
E28.3.1	Derivative of E28.3 containing pXO16::Tn5401, Tet ^R	[29]
E28.3.1 ^{cytK-1-KO-A}	Derivative of E28.3 ^{cytK-1-KO-A} containing pXO16::Tn5401, Tet ^R	This work
E28.3 ^{cytK-1-KO-A} (pHT304-18Z::cytK-1)	Complementation of the mutant lacking <i>cytK-1</i> gene	This work
NVH 391-98	Reference strain; highly cytotoxic	[1]
<i>E. coli</i> strains		
<i>dam</i> ⁻ / <i>dcm</i> ⁻ <i>E. coli</i> C2925	K12-derivative deficient in adenine and cytosine methyl-transferases, chemically competent	New England Biolabs™
<i>E. coli</i> NEB 5-alpha	DH5-alpha derivative, T1 phage resistant and <i>endA1</i> deficient, chemically competent	New England Biolabs™
Plasmids		
pHT304-18Z	Shuttle vector used in the complementation; Ery ^R	[30]
pHT304-18Z::cytK-1	<i>cytK-1</i> gene of strain E28.3 cloned into the pHT304-18Z shuttle vector	This work
pMAD	Gram ⁺ /Gram ⁻ shuttle vector containing a thermo-sensitive Gram ⁺ replicon; Ery ^R , Amp ^R	[27]
pMAD::UD	pMAD derivative containing the Kan ^R cassette with up- and down-stream regions of <i>cytK-1</i> gene; Ery ^R , Amp ^R , Kan ^R	This work
pDG783	Plasmid carrying a Kan ^R cassette; Kan ^R	[28]
pXO16::Tn5401	Large conjugative plasmid from <i>B. thuringiensis</i> sv. <i>israelensis</i> , tagged with a Tetracycline-resistance gene; Tet ^R	[31]

polymerase (Promega, Leiden, The Netherlands), PCR amplification of upstream (987 bp) and downstream (1,001 bp) regions of *cytK-1* gene, as well the kanamycin resistance gene from plasmid pDG783 [28], were performed. The primers used in this study are listed in Table 2. Electrophoresis gel migration of the PCR products was performed as described elsewhere [23].

The PCR amplicons were purified using GenElute™ PCR Clean-Up Kit (Sigma-Aldrich™, Overijse, Belgium) and the DNA quality was checked with the Nanodrop spectrophotometer (Isogen, De Meern, The Netherlands).

As previously described by Gibson *et al.* [32], the purified PCR amplicons were mixed with the PCR-opened pMAD shuttle vector [27], deionized water, and NEBuilder HiFi DNA Assembly MasterMix. The HiFi DNA assembly reaction product was chemically transformed into *E. coli* NEB 5-alpha and incubated at 37°C (1 h, 180 rpm). The bacterial culture was then spread on a LB agar supplemented with ampicillin and incubated overnight at 37°C. Next, *E. coli* transformants were PCR-checked for the presence of the expected construct. The recombinant plasmid, pMAD::UD (containing the Kan^R cassette flanked by the upstream and downstream sequences of *cytK-1*) was extracted using GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich™, Overijse, Belgium) and checked through DNA sequencing (MacroGen Europe, Amsterdam, The Netherlands). Thereafter, the construct was demethylated in *E. coli* C2925 before subsequent electroporation into *B. cytotoxicus*.

The *B. cereus* electroporation protocols previously described [34,35] were adapted for *B. cytotoxicus*. Briefly, a single fresh colony of E28.3 was sub-cultured in 25 mL of brain-heart infusion (BHI) (Bio-Rad, Richmond, CA, USA) and incubated O/N (30°C, 120 rpm). Next, the bacterial culture was centrifuged (6000 rpm, 4°C, 10 min), the cell pellet washed three times with chilled deionized water (4°C) and resuspended in 400 µL PEG6000 (40% w/v). Using the Gene Pulser and in a 2 mm cuvette (Bio-Rad, Richmond, CA, USA), the electro-competent *B. cytotoxicus* cells were electroporated with 1 to 2 µg of demethylated pMAD::UD plasmid. Electroporated bacterial cells were immediately suspended in 1 mL of LB broth and incubated at 30°C, (1 h, 120 rpm). Next, 100 µL of the bacterial suspension were spread on LB agar supplemented with erythromycin and incubated O/N at 30°C. The transformants were PCR-checked for the construct presence.

Swapping of the *cytK-1* gene by the *kan^R* gene in *B. cytotoxicus* strain E28.3 was performed following the plasmid homologous recombination approach described by Makart *et al.* [24,36]. *B. cytotoxicus* transformants containing the

shuttle pMAD::UD underwent successive non-permissive incubation cycles as follow: two at 43°C, one at 45°C and a last one at 50°C. Up to 10^{-7} serial dilutions were made from the last cycle culture (50°C) and spread on LB agar supplemented with kanamycin and 20 $\mu\text{g}/\mu\text{L}$ of X-Gal (Sigma-Aldrich™, Overijse, Belgium). Since pMAD contains a β -galactosidase cassette, five white colonies (indicating pMAD absence) on X-gal LB agar were re-streaked on LB agar supplemented with kanamycin and incubated overnight at 30°C.

Table 2: Primer pairs used in the cloning steps.

Designation	Primers	Sequences (5' to 3')	T _m (°C)	Amplicon (bp)	Reference
<i>kan^R</i> gene	Kana-R	GTTTTACTATCGAT- ACAAATTCCTCGTAG	60	1.490	This study
	Kana-F	ACATATATCGTGATA- AACCCAGCGAACC			
<i>cytK-1</i> upstream region	cytK-1-Up-R	GGGTTTATCACGATA- TATGTCGTATTTCAC- ATATATC	58	987	This study
	cytK-1-Up-F	AGATCTATCGATGCA- TGCCAGAAGTTTtag- GTTcATACATTG			
<i>cytK-1</i> downstream region	cytK-1-Down-R	CGGATCCATATGACG- TCGACAATGCGAGAG- ACGTTGCG	62	1.001	This study
	cytK-1-Down-F	TTGTATCGATAGTAA- AAAACAACACTGACA- AACTC			
<i>cytK-1</i> gene	cytK-1-R	CCTCGTGCATCTGTT- TCATGAG	61	436	This study
	cytK-1-F	CAATTCCAGGGGCAA- GTGTC			

4.3. Transfer of the *cytK-1* KO locus from strain E28.3 to strain E8.1

Using filter mating as described by Hinnekens *et al.* [29], the *cytK-1* KO mutation was mobilized from *B. cytotoxicus* strain E28.3.1^{*cytK-1*-KO-A} (derivative of E28.3^{*cytK-1*-KO-A} containing pXO16::Tn5401) into E8.1 via conjugation [24]. The

candidate transconjugants strains were PCR-screened to check the effective replacement of *cytK-1* gene by *kan^R* gene. RAPD patterns of the final transconjugant were also checked as previously described by Koné *et al.* [23].

4.4. Complementation of *cytK-1* KO mutant

Primers flanked with overlapping restriction sites sequences of *Bam*HI and *Pst*I enzymes (New England Biolabs™, Ipswich, MA, USA) (Table 2) were used to amplify the *cytK-1* gene from *B. cytotoxicus* wild-type strain E28.3. As described by Makart *et al.* [24], the PCR product was cloned in the shuttle pHT304-18Z and transformed into chemically competent *E. coli* 10-beta cells. The resulting construct pHT304-18Z::*cytK-1* was retrieved using the GeneE-lute Plasmid Miniprep Kit (Sigma-Aldrich™, Overijse, Belgium) and checked through sequencing (Macrogen Europe, Amsterdam, The Netherlands). The pHT304-18Z::*cytK-1* construct was then demethylated by passing it through *E. coli* C2925 and subsequently electroporated into the *B. cytotoxicus* strain *cytK-1*-KO mutant to obtain E28.3^{*cytK-1*-KO-A}(pHT304-18Z::*cytK-1*).

4.5. Cytotoxicity of E28.3, E8.1, their mutants lacking *cytK-1* and the complemented strain

4.5.1. Cell culture Caco-2 cell culture described by Rajkovic *et al.* [37] was adapted as follows. The human colorectal carcinoma cell line Caco-2 (HTB-37™) was obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells were maintained in high glucose, Glutamax Dulbecco's Modified Eagle Medium (DMEM) (Gibco-Thermo Fisher, Geel, Belgium), supplemented with (i) 10% heat-inactivated foetal bovine serum (Greiner Bio One, Wommel, Belgium), (ii) 1% non-essential amino acids (Gibco-Thermo Fisher, Geel, Belgium), (iii) 1% penicillin/streptomycin (Gibco-Thermo Fisher, Geel, Belgium) in the humidified 10% CO₂ atmosphere at 37°C (Mettmert GmbH & Co., Nurnberg, Germany). Cell morphology was regularly checked with phase-contrast microscopy (VWR, Leuven, Belgium) and the growth medium was changed every other day. After reaching 80–90% of confluency, the cells were sub-cultured by trypsinization using 0.5% trypsin-EDTA (Gibco-Thermo Fisher, Geel, Belgium) until cells were realized from the bottom of 75 cm² neck tissue culture flasks (SPL Life Sciences, Gyeonggi-do, Korea). The cells were

counted with a Bürker counting chamber according to the Trypan blue staining method. Next, they were seeded in a 96-well plate at a concentration of 20,000 cells per well to achieve optimal distribution of cells. In all experiments, the cells were passaged less than 25 times.

4.5.2. Preparation of bacterial cell-free supernatants To assess the effects of the wild-type *B. cytotoxicus* strains E28.3, E8.1, their mutant *cytK-1-KO* derivatives and the complemented strain E28.3^{*cytK-1-KO-A*}(pHT304-18Z::*cytK-1*), cell-free supernatants were prepared. *B. cytotoxicus* reference strain NVH 391-98 cell-free supernatant and the untreated Caco-2 cells were used as positive and negative controls, respectively. Single separated colonies of selected strains were inoculated in serum and antibiotic-free DMEM and incubated for overnight at 37°C. The cultures were centrifuged at 5,000 g for 10 min, and supernatants were filter-sterilized through a 0.22 µm filter (Millipore Inc., Billerica, MA, USA) and used freshly. Two-fold diluted supernatants were used for Caco-2 cell exposure, to better discriminate cytotoxicity of the strains. Exposure was performed for 2, 4, and 12 h. The effects were the most notable after 12 h of exposure.

4.5.3. MTT assays Cytotoxic effects were characterized via the tetrazolium salt or MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. This method assesses the cell's mitochondrial activity as an indicator of cell viability and cytotoxicity, initially described by Mosmann [38]. After exposure, 20 µL of MTT (5 mg/mL in PBS) was added to each well, and the plates were incubated at 37°C for 2 h. Upon incubation, liquid was discarded, and the purple formazan crystals were solubilized in dimethyl-sulfoxide (DMSO). SpectraMax plate reader (Molecular Devices, Sunnyvale, CA, USA) was used to record the absorbance at 570 nm.

4.5.4. Statistical analysis The normality of the data was investigated using the Kolmogorov–Smirnov test. Microsoft Excel 2016 was used to compute mean values and standard deviations ($N = 2$, $n = 6$) for each test condition. To determine whether the data were significantly different ($p < 0.05$), a *t*-test (two-tailed with unequal variance) was performed with SPSS Statistics 26 (Chicago, IL, USA).

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Scientific communications

B.1 Oral presentations

- **Hinnekins P.** and Mahillon J. (2021). *tip*, the intriguing transfer locus of pXO16, a conjugative plasmid from *Bacillus thuringiensis* sv. *israelensis*. **9th International Conference on *Bacillus anthracis*, *B. cereus* and *B. thuringiensis*- Bacillus ACT 2021** - Online
- **Hinnekins P.** and Mahillon J. (2022). Insights into the intriguing conjugation system of pXO16 from *Bacillus thuringiensis* sv. *israelensis*. **International Plasmid Biology Meeting 2022** - Toulouse (France)

B.2 Posters

- **Hinnekins P.**, Koné M.K., Fayad N., Leprince A. and Mahillon J. (2019) An extended host range displayed by pXO16 from *Bacillus thuringiensis* sv. *israelensis*. **20th International Conference on Bacilli and Gram-Positive Bacteria** - Washington (USA); **Belgian Society for Microbiology (BSM) - Microbes without Frontiers** - Brussels (Belgium)
- Nuytten M., Verhaegen M., **Hinnekins P.**, Leprince A., Morandini L., Komagbé G., Gillis A. and Mahillon J. (2022) *B. cereus*: friends or foes? **ELIday** - Louvain-la-Neuve (Belgium)

