FERNANDA APARECIDA PIRES FAZION

ROLE OF PLASMIDS OF *BACILLUS CEREUS* GROUP IN INSECT LARVAE

Londrina
2017

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Thesis presented to the Post-graduation in Genetics and Molecular Biology of Universidade Estadual de Londrina and in Microbiology of Saclay/AgroParisTech.

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“Every time I thought I was being rejected from something good, I was actually being re-directed to something better.”

Dr. Steve Maraboli

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"Ceux qui passent par nous, n'allez pas seul, ne nous laisse pas seuls.
Laisser un peu d'eux-mêmes, prendre un peu de nous."
Antoine de Saint-Exupéry

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LISTS OF ABBREVIATIONS

AI-2	Auto inducer-2
AI	Autoinducers
AIP	Autoinducer peptide
Amp	Ampicillin
AMP	Adenosine monophosphate
AHSL	Acyl homoserine lactone
Bc	*Bacillus cereus*
Bt	*Bacillus thuringiensis*
CADR	Cadherin glycoprotein
Cry	Crystal protein
Cry	*cry* gene
C-terminal	Carboxy terminal of a protein
Cyt	Cytolytic protein
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
h	Hours
HBL	Haemolysin BL
HTH	Helix-turn-helix
Kb	Kilobase
L	Litre
LB	Luria-Bertani medium
Mg	Miligram
Mim	Minute
mL	Milliliter
MLST	Multi-locus sequence typing
NHE	Non-haemolytic enterotoxins
NRPS	Nonribosomal peptide synthesis
N-terminal	Amino-terminal of a protein

OD	Optical density
Opp	Oligopeptide permease
ORF	Open read frames
Pb	Base pair
PCR	Polymerase chain reaction
QS	Quorum sensing
RNPP	QS family composed by **R**ap/**N**prR/**P**lcR/**P**rgX
Rpm	Revolutions per minute
RR	Response regulator
S	Second
SAM	S-adenosylmethionine
TPR	TetratricoPeptide repeat
WT	Wild type
Mg	Microgram
µL	Microliter
µM	Micromolar
°C	Celsius degree
Σ	Sigma factors

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ABSTRACT

FAZION, Fernanda Aparecida Pires. **Role of plasmids of *B. cereus* group in insect larvae**. 2017. Thesis presented to the Post-graduation in Genetics and Molecular Biology of Universidade Estadual de Londrina, Londrina/PR, Brazil and Microbiology of Paris Saclay/AgroParisTech, Paris, France.

ABSTRACT

Bacillus cereus (*Bc*) and *Bacillus thuringiensis* (*Bt*) are two closely related species. *Bc* is a pathogenic species responsible for gastroenteritis by food-borne. *Bt* is an entomopathogenic bacterium, which the lifecycle in insect larvae is controlled by quorum sensing systems, such as Rap/Phr, which regulates processes such as sporulation, biofilm formation and conjugation. The presence of these genes in plasmids has been described, furthermore, plasmids have been involved in bacterial adaptation to their ecological niche. In order to understand the role of the plasmids to these species, two complementary works were carried out. First, insect larvae, a privileged ecological niche of *Bt* strains, were infected with *Bc* and *Bt* strains harboring different plasmid contents. Their fitness were evaluated by vegetative cells and spore counts at four time points. *Bt* and *Bc* strains were classified into five groups according to the bacterial fitness. In these groups, the plasmid affects positively or negatively the bacterial fitness. The results demonstrated that for *B. cereus* group strains, getting a pathogenicity plasmid is not enough to effectively increase bacterial population, colonizing insect hosts. The second study characterized the *rap/phr* system encoded by the cryptic plasmid pHT8_1. The Rap8 protein inhibited the sporulation process in insect larvae. This protein was directly inhibited by the active signaling peptide Phr8. The Rap8/Phr8 system may allow the bacteria to exert a tight control of the sporulation process in the host cadaver for optimizing the multiplication, the survival and the dissemination of the bacteria. Thus, the results of the second study showed that the plasmids can provide advantages for the adaptation and the evolution of *B. thuringiensis* in its ecological niche, while the results of the first study indicate that *B. cereus* group strains must have a suitable genetic background to display a high fitness allowing optimal multiplication and dissemination of the bacterial population within insect larvae.

Key-words: *B. cereus* group. Plasmids. Adaptation. fitness and *rap/phr* system.

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RÉSUMÉ

FAZION, Fernanda Aparecida Pires. **Rôle des plasmides dans le groupe du *B. cereus* chez l'insect larvae**. 2017. Thèse présentée à la Post-graduation en Génétique et Biologie Moléculaire de l'Universidade Estadual de Londrina, Londrina/PR, Brésil et Microbiologie de Paris Saclay/AgroParisTech, Paris, France.

RÉSUMÉ

Bacillus cereus (*Bc*) et *Bacillus thuringiensis* (*Bt*) sont deux espèces génétiquement proches. *Bc* est une bactérie pathogène que peuvent causer des gastro-entérites d'origine aliméntaire. *Bt* est une bactérie entomopathogène, dont le cycle de vie dans la larve d'insecte est contrôlé par des systèmes de quorum sensing, comme le système Rap/Phr, que régule processus tels que la sporulation, la formation de biofilm et la conjugaison. La présence des ces genès a été identifiée dans les plasmides, et ces eleménts ont été associés à l'adaptation des spécies dans sont niche ecologique. Le but de cette étude est de comprendre le rôle des plasmides dans ces bactéries. Pour la première étude l'insecte larvae, le niche privilegie de *Bt*, ont été infectées par souches de *Bc* et *Bt*, avec un contenu plasmidique diffèrent. Le fitness a été evallué par le comptage de cellules végétatives et spores dans quatre temps. Les souches de *Bt* et *Bc* ont été classées dans cinq groups par rapport à sont fitness. Dans ces groups le plasmide a affecté le fitness de la bactérie positive ou négativement. Les résultats ont démontré que les souches du group du *B. cereus* que reçoivent a pathogène plasmid ne est pas suffisant pour une augmentation effectif de la population bactérienne, i.e., coloniser l'hôte. La deuxième étude a permis caractériser le système *rap/phr* porté par le plasmide cryptique pHT8_1. Les résultats démontrent que la protéine Rap8 inhibe la sporulation dans la l'insecte. L'activité de cette protéine est inhibée par le peptide de signalisation Phr8. Le système Rap/Phr8_1 a permis les bactéries exercer un strict contrôle sur la sporulation, un processus important pour assurer la survie et la dissémination des bactéries. L'ensemble des résultats de la deuxième étude montrent que les plasmides peuvent fournir avantages pour l'adaptation et evolution de *B. thuringiensis* dans son niche ecologique, alors que les résultats de la première étude indiqués que les souches de *Bc* group doivent avoir un contenu génétique approprié pour exhiber un fitness élévé en permettant une optimal multiplication and dissemination de populations bactérienne dans l'insect larvae.

Mots-clés: Groupe du *B. Cereus.* Plasmides. Adaptation. fitness et système *rap/phr*.

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RESUMO

FAZION, Fernanda Aparecida Pires. **Role of plasmids of *B. cereus* group em larvas de inseto**. 2017. Tese apresentada ao programa de Genética e Biologia Molecular da Universidade Estadual de Londrina, Londrina/PR, Brasil e Microbiologia da Université Paris Saclay/AgroParisTech, Paris, France. Londrina, 2017.

RESUMO

Bacillus cereus (Bc) e *Bacillus thuringiensis (Bt)* são duas espécies bacterianas estritamente relacionadas. *Bc* é uma espécie patogênica causadora de gastroenterites de origem alimentar. *Bt* é uma bactéria entomopatogênica, cujo ciclo de vida em larvas de insetos é controlado por sistemas de *quorum sensing*, como o sistema Rap-Phr, que regula os processos de esporulação, formação de biofilme e conjugação. A presença desses genes em plasmídeos tem sido identificada e, além disso, plasmídeos tem sido envolvidos na adaptação de bactérias a seus nichos ecológicos. Com objetivo de compreender o papel dos plasmídeos nessas espécies, dois trabalhos complementares foram realizados. Primeiro, larvas de insetos, o nicho ecológico privilegiado de *Bt*, foram infectadas com linhagens de *Bt* e *Bc* possuindo diferente conteúdo plasmidial. O fitness foi avaliado com a contagem de células vegetativas e esporos em quatro tempos. Linhagens de *Bt* e *Bc* foram classificadas em cinco grupos de acordo com o fitness bacteriano. Nesses grupos, o plasmídeo afetou o fitness bacteriano positivamente ou negativamente. Os resultados desse estudo demonstram que linhagens do grupo do *B. cereus* que recebem um plasmídeo patogênico não é o suficiente para um aumento efetivo da população bacteriana., i.e., colonizar o hospedeiro. O segundo artigo caracterizou o sistema *rap/phr* presente no plasmídeo críptico pHT8_1. A proteína Rap8 inibiu o processo de esporulação na larva de inseto. Essa proteína foi diretamente inibida pelo peptídeo sinalizador ativo Phr8. O sistema Rap-Phr8_1 permitiu a bactéria exercer um rigoroso controle da esporulação em cadáveres de insetos permitindo a sobrevivência e disseminação. Assim, os resultados do segundo trabalho demonstram que plasmídeos podem prover igualmente vantagens para a adaptação e evolução de *B. thuringiensis* em seu nicho ecológico, enquanto que os resultados do primeiro trabalho indicam que linhagens do grupo do *B. cereus* devem possuir um conteúdo genético adequado para apresentar um alto fitness permitindo uma ótima multiplicação e disseminação da poulação bacteriana dentro da larva de insetos.

Palavras-Chave: Grupo do *B. cereus.* Plasmídeos. Adaptação. fitness e sistema *rap/phr*

INTRODUCTION

The *B. cereus* group is composed of eight closely related species (Liu *et al*., 2015). Even with a high similarity in nucleotide sequence identity and synteny, these species have a great variability in phenotypic traits. *B. cereus sensu stricto* is an opportunistic pathogen found in many food products, mainly dairy products, which is responsible to a variety of infections (Drobniewski, 1993). *B. thuringiensis* is an insecticidal bacterium used as a biopesticide for many years (Sanchis, 2016). Some studies showed that *B. thuringiensis* is a true pathogen and that the insect larvae is considered as its ecological niche (Raymond *et al*., 2010). Some virulence factors produced by these bacteria are encoded by plasmids, such as the cereulide toxin responsible to emetic syndrome of *B. cereus* (Rasko, Gill and Soberón, 2007) and the Cry toxins responsible to insecticidal activity of *B. thuringiensis* (González, Brown and Carlton, 1982; Lereclus *et al*., 1983).

The infectious lifecycle of *B. thuringiensis* in insect larvae has been extensively studied and is controlled by the sequential activation of quorum sensing (QS) systems responding to cell-population density. The secretion and the detection of specific signaling peptides in the extracellular medium allow bacteria to coordinate gene expression, and consequently change their behaviour in response to conditions and perturbations of the environment (Perego and Hoch, 1996; Lazazzera *et al*., 1997). *B. thuringiensis* lifecycle is controlled by PlcR-PapR, NprR-NprX and Rap-Phr systems which regulate genes related to virulence, survival and sporulation, respectively (Slamti *et al*., 2014). These systems are grouped in the RNPP family named according to their effective regulators Rap, NprR, PlcR and PrgX (Declerck *et al*., 2007). Initially, PlcR-PapR system activates the transcription of many virulence factors causing insect death (Slamti and Lereclus, 2002). Then, NprR-NprX activates genes needed to the necrotrophism lifestyle allowing the bacteria to survive in the insect cadaver (Dubois *et al*., 2012). Moreover, this QS system is also required to connect necrotrophism to the initiation of the sporulation (Perchat *et al*., 2016). The Rap/Phr system is well described in *B. subtilis* for controlling several key processes, including sporulation.

In *Bacilli*, Spo0A-P positively controls sporulation and biofilm formation (Burbulys, Trach and Hock, 1991; Perego and Hoch, 1996; Hamon and Lazazzera, 2001; Fagerlund *et al*., 2014).

Rap proteins regulate the phosphorylation state of Spo0A by dephosphorylating Spo0F-P, thus inhibiting the phosphoryl cascade and consequently the Spo0A-P accumulation. Rap phosphatase is inhibited by its cognate Phr peptide that restores the activity of the phosphorelay allowing to Spo0A-P formation (Ishikawa, Core and Perego, 2002). Phr is produced as a pro-peptide composed by an N-terminal part with a signal peptide necessary to its secretion and a C-terminal part including the mature Phr peptide (Pottathil and Lazazzera, 2003). After being outside processed, the active Phr is reimported *via* oligopeptide permease system (Opp) to the cytoplasm (Perego, 1997). Then, the mature Phr interacts to the Rap protein resulting in the inhibition of Rap activity. Rap proteins have been defined as regulator aspartate phosphatase and are involved in the control of several bacterial processes, as sporulation, competence and transfer of mobile genetic elements. The *rap-phr* genes can be located both on the chromosome and on the plasmid. Recently, several studies reported the regulation of important processes by plasmid-borne Rap-Phr systems in *Bacillus* species (Koetje *et al.*, 2003; Parashar *et al.*, 2013b; Bongiorni *et al.*, 2006; Singh *et al.*, 2013 ; Boguslawski *et al.*, 2015; Yang *et al.*, 2015).

Bacterial species from the *B. cereus* group present an important extrachromosomal set of genes. The plasmid content range in number (from 1 to 17) and size (from 2 to 600 Kb) (Lereclus *et* al., 1982; Reyes-Ramírez *et al*., 2008). These plasmids may represent a significant proportion of the total genetic material of the bacterial genome and have a great importance in the ecology of these bacteria (Patiño-Navarrete and Sanchis, 2016). Therefore is essential to investigate the role of plasmids in this bacterial group.

I- LITERATURE REVIEW

Section I – The *Bacillus cereus* GROUP

1 Generalities

1.1 Biology

The *Bacillus cereus* group comprises eight validated and closely related species, *B. anthracis*, *B. cereus*, *B. thuringiensis*, *B. mycoides*, *B. pseudomycoides*, *B. weihenstephanensis*, *B. cytotoxicus*, *B. toyonensis* (Liu et al., 2015). These Bacillaceae species, belonging to the Firmicutes, are Gram-positive bacteria, rod shaped, spore-forming, with a G+C content around 32% (Figure 1).

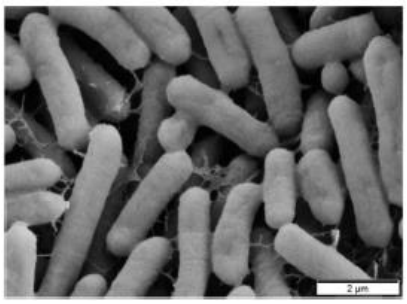

Figure 1. Electron microscopy of vegetative cells of *B. cereus* (Murtey, 2016).

These species are mesophilic, with an optimal growth between 28 °C and 37 °C (Drobniewski, 1993) and are aerobic, facultative anaerobic bacteria. They are ubiquitous species and soil has long been considered their reservoir, although its spores can be isolated from various materials, such as marine water, invertebrates, vegetation, or food (Jensen *et al.*, 2003; Tourasse *et al.*, 2011; Vilas-Bôas *et al.*, 2007). The capacity to survive in several habitats is essentially due their capacity to form spores, which are extremely resistant to environmental conditions such as heat, radiation, freezing and drying (Setlow, 2001, 2006).

Although closely related, these species display different phenotypic traits. Five species of the *B. cereus* group are well-know:

1. *B. cereus* is an opportunistic human pathogen causing food poisoning syndromes, diarrheal and emetic (Drobniewski, 1993).

2. *B. thuringiensis* is an entomopatogenic bacteria, mainly due the production of crystalline inclusions composed by toxins, which allowing the development of the bacteria in insect larvae (Schnepf *et al.*, 1998).
3. *B. anthracis* is a mammalian pathogen and the etiological agent of Anthrax disease (Mock and Mignot, 2003).
4. *B. weihenstephanensis* consists in psychrotolerant strains (Lechner *et al*., 1998).
5. *B. mycoides* presents a distinctive rhizoidal colony shape in agar plates (Nakamura and Jackson, 1995).

The first three species are the most studied and a summary of their general features is shown in Table 1. A detailed description of each species is present below.

Table 1. Principal phenotypic features of *B. cereus*, *B. anthracis* and *B. thuringiensis*.

Characteristic	***B. cereus***	***B. anthracis***	***B. thuringiensis***
Anaerobic growth	+	+	+
Catalase production	+	+	+
Citrate utilization	+	V	+
Casein hydrolysis	+	+	+
Nitrate reduction	+	+	+
Growth at 60 °C	-	-	-
Acid from glucose	+	+	+
Lysis by γ-phage	-	+	-
Motility	+*	-	+*
Hemolysis on sheep blood agar	+*	-	+*
Resistance to β-lactam antibiotics	+*	-	+*
Glutamyl-polypeptide capsule	-	+	-
Phospholipase C activity	+	-	+
Mutation non-sense in *plcR* regulator	-	+	-
Crystal parasporal inclusion(s)	-	-	+

Note: -, negative; +, positive; V, variable.

*More than 95% of the strains. Data extracted from Jensen *et al*., 2003; Vilas-Bôas, Peruca, Arantes, 2007.

1.2 Ecology

The soil environment is considered to be the reservoir of the species of the *B. cereus* group, meaning soil is where the organisms can normally be isolated and also infect their host. *B. thuringiensis* spores can persist in the soil for several years, however, spore germination and multiplication of bacterial cells are not detected in the soil environment. This is due because spores did not acquire enough nutrients to be able to sustain germination and growth (Ohana, Margalit and Barak, 1987; Thomas *et al.*, 2000, 2001; Vilas-Bôas *et al*., 2000). In contrast, other environments such as rhizosphere and phyllosphere have conditions, such as nutrient availability, which can allow bacterial development (Hendriksen and Hansen, 2002; Saile and Koehler, 2006; Bizzarri and Bishop, 2007).

Although readily isolated from soil, the *Bacillus thuringiensis* is unable to multiply effectively in either bulk soil or water (Ohana, Margalit and Barak, 1987; Yara *et al.*, 1997; Thomas *et al.*, 2000, 2001; Vilas-Bôas *et al.*, 2000; Ferreira *et al.*, 2003; Raymond *et al.*, 2010a). Furthermore, conjugation involving *B. cereus* group species has often been often described in broth culture and in insect larvae using plasmids harboring the *cry* genes (Gonzalez *et al.*, 1982, Battisti *et al.*, 1985, Reddy *et al.*, 1987, Hu *et al.*, 2004, Santos *et al.*, 2010). Also, there is enough evidence that insects are the optimal environment for exchanging genetic material (Jarrett & Stephenson 1990; Vilas-Bôas *et al.*, 1998; Takatsuka & Kunimi 2000; Thomas *et al.*, 2000; 2001; 2002; Suzuki *et al.*, 2004; Santos *et al.*, 2010). Nonetheless, Santos *et al.* (2010) substantiated that there is no positive association between the bacterial multiplication efficiency of the recipient strains and conjugation ability in the infected insects for the used strains.

In addition, analysis of chromosomal genes of *B. cereus* suggests that the natural habitat of the common ancestor of these bacteria was the gut of insects. This is supported by the set of genes that include several factors for invasion, establishment and propagation within this environment (Ivanova *et al*., 2003). So, the *B. cereus* group's ancestor was likely an opportunistic insect pathogen rather than a soil bacterium. Jensen and co-workers (2003) suggest two distinct lifecycles of *B. cereus* in an invertebrate host: (i) a symbiotic lifestyle; or (ii) a pathogenic lifecycle, producing virulence factors and consequently multiplying rapidly, causing infection.

The broad spectrum of phenotypic traits found in *B. cereus* species reflects in the different niches occupied. Then, these bacteria can develop diverse lifecycles, such as symbiotic,

saprophytic and pathogenic. Figure 2 summarizes the *Bacillus anthracis*, *B. thuringiensis* and *B. cereus* possible lifecycles in different hosts (Ceuppens, Boon and Uyttendaele, 2012).

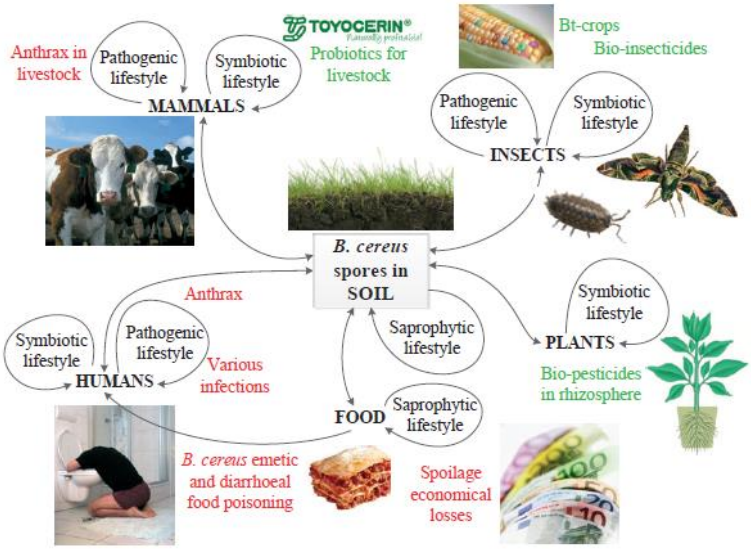

Figure 2. Possible *Bacillus* lifecycles in different hosts. The soil is considered the spore reservoir of *B. cereus* group. In this environment the bacteria can develop a saprophytic lifestyle (Vilain *et al*., 2006). In contact with different hosts, the bacteria are capable to develop a symbiotic, a saprophytic or a pathogenic lifecycle. In humans, *B. cereus* can cause emetic and diarrhoeal food poisoning. *B. anthracis* can also develop a pathogenic lifestyle in humans and others mammals, while *B. thuringiensis* can develop a pathogenic lifestyle in invertebrate hosts. A symbiotic lifestyle is suggest to occur in all these environments, including the phylloplane (Ceuppens, Boon and Uyttendaele, 2012).

1.2.1 *Bacillus cereus sensu stricto*

B. cereus is ubiquitous in nature and an opportunistic pathogen. *B. cereus* strains are known to contaminate food products (particularly dairy products) with spores and/or toxins. After ingestion the host can develop two types of food-borne diseases: emesis and diarrhea (Kotiranta, Lounatmaa and Haapasalo, 2000). The emesis type is associated with foods like rice, noodles, pasta and pastry, and the diarrhoeal type are generally associated with vegetables, meat products, sauces and milk products (Schoeni and Wong, 2005). In most cases the infection symptoms are not aggressive. However, some rare cases of systemic infections can be fatal, mainly in

immunocompromised patients, or patients recovering from surgery (Dierick *et al.*, 2005). Also, *B. cereus* can cause several others diseases, including endocarditis, endophthalmitis, bacteremia, septicaemia and cutaneous infections (Kotiranta, Lounatmaa and Haapasalo, 2000, Callegan, Engelbert and Parke, 2002; Bottone, 2010).

1.2.2 *Bacillus thuringiensis*

B. thuringiensis is characterized by the production of insecticidal proteins during the sporulation phase. These proteins, Cry and Cyt toxins, form crystal inclusions that are effective against several insect and nematode species (Schnepf *et al*., 1998; Wei *et al*., 2003). *B. thuringiensis* is frequently isolated from soil samples due the prevalence of spores in this environment. Raymond and co-workers believe that *B. thuringiensis* is a true pathogen, which has a large arsenal of virulence factors to develop infection without needing the assistance of commensal bacteria (Raymond *et al.*, 2010).

The pathogenic lifecycle is initiated by ingestion of crystals and spores of *B. thuringiensis*. These crystals are solubilized, cleaved by proteases and bind to specific receptors, resulting in pore formation and consequently epithelium cell lysis (Bravo, Gill and Soberón, 2007). All these events generate favorable conditions for spore germination and multiplication of vegetative cells. At this point of infection, several virulence factors are produced for host colonization and to overcome the host defenses (Figure 3). The infection is followed by septicemia, resulting in insect death. Then sporulation is triggered in response to external signals, such as starvation.

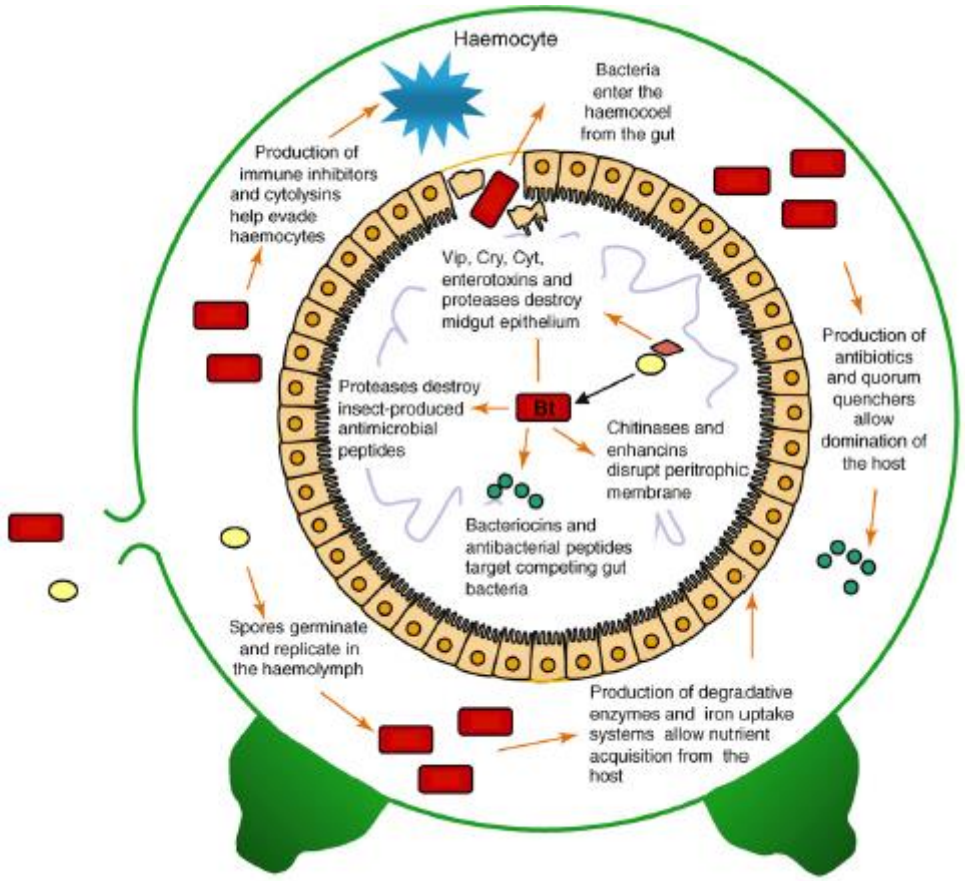

Figure 3. Interactions between *B. thuringiensis* and its host. The schema shows the lumen of midgut to the peritrophic matrix (blue line), midgut epithelial cells, the haemocoel and the cuticle (green line). Red rectangle represent vegetative cells, cream ovals the spores and pink diamonds the crystals. Some compartments, barriers and immune innate response faced to *B. thuringiensis* are represented (Raymond *et al*., 2010).

Transmission, characterized by the transfer of the infection, is an essential phase of bacterial ecology. Two routes of transmission are described, vertical and horizontal. Vertical transmission involves transfer of infection from a parent to its offspring while horizontal infection is the transfer within a single generation (Raymond *et al.*, 2010). *B. thuringiensis* uses horizontal transfer as the main route of transmission, and requires the host's death to proliferate. Horizontal transmission in leaf-eating insects has two possible routes: indirect and direct. In indirect horizontal transmission, the spores and crystals are released from the dead insect larvae and are rapidly deposited in the soil. While crystals are quickly degraded or lost in the soil, spores are disseminated by biotic and abiotic factors (Melnick *et al.*, 2008). Consequently, other insects

can be infected by their ingestion (Raymond *et al.*, 2010). Direct transmission occurs by ingestion of recently released spores and crystals present in leaves. Raymond and co-workers showed that the presence of a cadaver containing spores and crystals in a plant leaf caused the death of a third of uninfected larvae (Raymond *et al.*, 2010). In summary, the transmission routes suggest two important places where toxin production takes place: directly in the host or on plants, where new insect larvae can be infected periodically.

1.2.3 *Bacillus anthracis*

B. anthracis is a pathogenic bacterium, responsible to animal and human disease, and also has a potential role in bioterrorism. Infection is initiated by inhalation, injection, cutaneous contact and/or ingestion of the spores. Respiratory and gastrointestinal infections are severe and frequently associated with high mortality, while cutaneous infection is less severe and is the most common form of infection (Doganay, Metan and Alp, 2010).

Like other bacteria of the *B. cereus* group, the lifecycle of *B. anthracis* comprises two forms: vegetative cells and spores. After infection, the spores reach the lymph nodes, where they can germinate. Subsequently, the vegetative cells multiply, producing large amounts of anthrax toxins, which confer resistance to phagocytosis and the host immune system (Makino *et* al., 2002). Following infection, the toxins are released in the bloodstream, provoking death of the host animal (Hudson *et al*., 2008). In this stage, vegetative cells are expelled by the body orifices of the host and sporulation occurs. This process requires the presence of free oxygen to assure persistence and dissemination of *B. anthracis*. The spores remain dormant until encountering another potential host, achieving a pathogenic lifecycle (Turnbull, 2002). Although the pathogenic lifecycle is extensively known, *B. anthracis* also showed a saprophyte lifestyle in a rhizosphere system (Sale and Koehler, 2006).

1.3 Phylogeny

The taxonomy of the *B. cereus* group been discussed for several years and no consensus has been reached. Initially, these species were classified using morphological and biochemical traits, especially pathogenic patterns. Later, with the advent of molecular biology, several research

groups applied these techniques to investigate these species, but there is still no consensus about their classification.

At the beginning, DNA-DNA hybridization between genomes was used as the standard method. From a prokaryotic standpoint, two specimens are considered members of the same species when they share more than 70% of chromosomal DNA hybridization (Wayne *et al.*, 1987). The genome of the three main species of the *B. cereus* group (*B. anthracis*, *B. thuringiensis* and *B. cereus sensu stricto*) hybridize in approximately 70% limit, so with this method there is no basis to separate them (Carlson, Johansen, Kolstø, 1996). Additionally, analysis of 16S and 23S sequences showed high similarity, suggesting evolution from a common lineage (Ash *et al.*, 1991; Ash and Collins, 1992).

Afterward, several studies using different techniques were conducted to classify these species. Data from multilocus enzyme electrophoresis (MEE) (Helgason *et al.*, 1998; 2000), amplified fragment length polymorphism (AFLP) (Ticknor *et al.*, 2001; Hill *et al.*, 2004; Guinebretière *et al.*, 2008), multilocus sequence typing (MSLT) (Tourasse *et al.*, 2006) and analysis of sigma factor gene sequences (Schmidt, Scott and Dyer, 2011) showed a high nucleotide sequence identity. Consequently, there is no formation of distinct groups comprising each *B. cereus* species. Also, these studies suggest that *B. cereus*, *B. anthracis* and *B. thuringiensis* should be classified as a single species. Tourasse and co-workers (2011) combined MLEE, AFLP and MLST in a single analysis and showed that the *B. cereus* group forms a coherent and dynamic population. A distinction between the various species was not possible and organization in different groups is highly dependent on the strains used in the studies (Tourasse *et al.*, 2006). Another work based in detection of known toxin genes identified seven groups (I-VII). Group I included *B. pseudomycoides* species while groups II, III, IV and V included *B. cereus* and *B. thuringiensis*. *B. anthracis* was found only in group III. Group VI presented *B. weihenstephanensis* and *B. pseudomycoides* (Guinebretière *et al.*, 2010). Finally, group VII included *B. cytotoxicus* species. These analyses showed that a *B. cereus* strain can be closer to a *B. anthracis* strain than to another *B. cereus* one. In addition, Rasko and co-workers (2005) suggested that the main differences between the members of this group are related to gene expression rather than the level of divergent sequences or genetic content.

Some studies using MLST and ribosomal DNA intergenic sequence analysis allow the distinction between *B. anthracis* from *B. cereus* and *B. thuringiensis* and revealed that *B.*

anthracis has recently diverged from the others two (Cherif *et al.*, 2003, Ko *et al.*, 2004, Priest *et al.*, 2004). Also, *B. anthracis* strains have a punctual mutation in *plcR* gene, the master regulator of virulence factors (described below), this reflect in the absence of some phenotypic traits, such as hemolysis on sheep blood agar, motility and γ-phage sensibility (Agaisse *et al*., 1999). However, no specific chromosomal sequence was identified specifically to distinguish *B. anthracis* from the others members from *B. cereus* group (Kolstø; Tourasse; Økstad, 2009).

Cohan (2002) described that bacterial species have genetic, ecological, evolutionary and phylogenetic properties specific to each one. Pursuing this idea, some authors have suggested another view to classify these species by considering their ecological niches. Populations of *B. cereus* or *B. thuringiensis* collected at two separate sites were genetically more similar to each other than to sympatric populations of the other *Bacillus* species (Vilas-Boas *et al.*, 2002). More recently, Zheng and co-workers (2013) phylogenetically evaluated the *csaB* gene, which plays an essential role in the cell maintenance. The 122 strains evaluated were classified into two groups, distinguished according their lifestyle environments: higher animals (group I) and insects (group II). Soil strains were found indiscriminately in the two groups.

All these studies were conducted by investigating the bacterial chromosome. However, the *B. cereus* group has an important extrachromosomal pool. The virulence specificities and adaptation properties of these species are encoded by plasmids, so these elements play a critical role in distinction between these species. In *B. thuringiensis*, Cry and Vip toxins, responsible for entomopathogenic activity, are found in large plasmids (González and Carlton, 1984; Berry *et al*., 2002). In *B. anthracis*, pXO1 and pXO2 plasmids encode the three main toxins and genes involved in capsule synthesis, respectively (Pilo and Frei, 2011). *B. cereus sensu stricto* harbors plasmids that encode the toxin responsible for emetic syndrome (Ehling-Schulz *et al.*, 2006). Two important characteristics of these elements make it difficult to distinguish these species: the plasmids can be lost naturally in the environment (te Giffel *et al.*, 1997) and they have a high potential to disseminate by horizontal transfer, with the exception of pXO1 and pXO2 (Thomas *et al.*, 2001; Yuan *et al.*, 2007; Modrie, Beuls and Mahillon, 2010). Therefore, plasmids contribute to distinction of these species due to the phenotypic traits they encode. In contrast, their high spread among different species can hinder the distinction among the *B. cereus* species.

To conclude, the *B. cereus* group includes species that share high similarities in genome content, but they also have specific genetic elements, such as those encoded by plasmids. Some

strains of the group are possibly adapted to specific hosts and/or environments, exhibiting different phenotypes. So, analysis of *B. cereus sensu lato* is highly dependent on the strains selected for the studies. In addition, these results suggest that the diversification and evolution of the *B. cereus* group was shaped by environmental conditions (Cohan, 2002; Rasko *et al*., 2005).

2 Virulence factors

As described above, members of *B. cereus* group share a high similarity in their genomes, exhibiting a high level of syntheny and protein similarity (Rasko *et al.*, 2005). A set of enterotoxins and degradative enzymes are encoded in their chromosome, however some specific virulence factors are encoded by plasmid-borne genes.

2.1 Plasmid-borne factors

2.1.1 pXO1 and pXO2

The virulent strains of *B. anthracis* carry two not self-transmissible plasmids, pXO1 and pXO2, both encoding factors necessary to virulence: toxin production and capsule formation, respectively. The pXO1 plasmid has 181,7 kb in length and is essential to *B. anthracis* toxicity (Mikesell, Ivins and Ristroph, 1983). This plasmid has 143 open reading frames (ORFs) including 35 with putative functions and nine genes with function experimentally assigned (Okinaka *et al.*, 1999). The most characterized portion of pXO1 is the pathogenicity island (PAI), which comprises 44.8 kb, bordered by inverted elements at each end. PAI of pXO1 has 31 ORFs, including the known toxins genes *cya*, *lef* and *pagA*, the *blsA* gene and toxin regulatory elements (*atxA* and *pagR*). This region also encodes several putative insertion elements and harbors integrases and transposases genes. In addition, pXO1 harbors a cluster composed of three genes *gerXA*, *gerXB* and *gerXC* (Okinaka *et al.*, 1999).

The *pagA*, *cya* and *lef* genes encode the main virulence proteins, called protective antigen (PA), edema factor (EF) and lethal factor (LF), respectively. These proteins are toxic in binary combinations. PA and LF form the lethal toxin, which provokes lethal shock in experimental animals, while PA and EF form the edema toxin, responsible for skin lesions (Collier and Young, 2003). Interestingly, although mutations of the *pagA*, *lef* and *cya* genes abolish the functions as edema and/or lethal toxins, the mutant does not lose its virulence in mouse models (Pezard *et al*.,

1991; Chand *et al.*, 2009). On the other hand, loss of pXO1 substantially decreases the virulence capacity of the *B. anthracis* strain Ames, indicating that other genes present in pXO1 play an important role in virulence (Little and Knudson, 1986). However, it is not clear whether pXO1 confers some advantage to *B. anthracis* in non-host environments (Okinaka *et al.*, 1999).

Other genes found in pXO1 are *blsA* and *gerX* operon. BlsA is an S-layer protein, which is necessary for adherence of vegetative cells of *B. anthracis* to host cells (Kern and Schneewind, 2010). The *gerX* operon is responsible for encoding proteins with high sequence similarities to proteins involved in the germination response in *B. subtilis* (Guidi-Rontani *et al.*, 1999).

The pXO1 plasmid also encodes a master regulator, AtxA (**a**nthrax **t**o**x**in **a**ctivator), which regulates the expression of virulence genes found either on the plasmid or on the chromosome (Bourgogne *et al*., 2003). An *atxA* mutant is unable to produce the three virulence proteins, PA, EF and LF (Uchida *et al*., 1997). In addition, the AtxA regulator is responsible for activating the *cap* operon, evidencing cross-talk in the regulatory process between pXO1 and pXO2 genes (Uchida *et al.*, 1997). PagR, the second regulator found in pXO1, negatively controls *pagA* expression. Therefore, *pagR* expression is positively controlled by *atxA* (Hoffmaster and Koehler, 1999).

The pXO2 plasmid encodes the *capBCADE* operon, involved in capsule synthesis, the *dep* gene associated with capsule degradation and the two regulatory genes, *acpA* and *acpB,* are responsible for activating the transcription of the capsule operon (Vietri *et al*., 1995). The *cap* operon encodes poly-γ-D-glutamic acid (PDGA), which confers resistance to vegetative cells against phagocytosis (Drysdale *et al*., 2005). This set of proteins allows the bacteria to escape from the host immune defenses and provoke septicemia. Strains without pXO2 are noncapsulated and incapable of establishing infection (Keim *et al*., 2006). In addition, a second *pagR* regulator, was recently described in pXO2 plasmid, displaying 70% of homology in amino acid sequence with *pagR* on pXO1. This regulator negatively controls the expression of *pagR* on pXO1. Strains without pXO2 plasmid also showed a reduction in expression of *pagA* and *lef* genes, probably due to the negative control under *pagR* on pXO1, which upregulates toxin genes (Liang *et al.*, 2016).

The interaction between chromosomal and plasmid genes allows *B. anthracis* to develop its complete lifecycle. The regulation of virulence genes and their interactions are represented in Figure 4. The toxin gene expression in the growth phase of *B. anthracis* is associated with *abrB*,

a transition state regulator. AbrB inhibits and activates several genes during transitions of the growth phase: from the lag to the exponential phases and from the exponential to stationary phases (Strauch *et al.*, 1993). Baille, Moir and Manchee (1998) described the expression of anthrax toxins under negative control of *abrB*, observing an eightfold reduction of PA synthesis in an *abrB* mutant. Two *abrB* orthologs were identified in the *B. anthracis* genome, one chromosomal and other plasmid-borne. Chromosomal copying represses the toxin gene expression, but the mechanism is not yet known (Saile and Koehler 2002). AbrB expression is inhibited by Spo0A during the early exponential growth phase (Greene and Spiegelman, 1996). So, Spo0A inhibits *abrB* expression at the start of the exponential phase (O'Reilly and Devine 1997), which allows the syntheses of anthrax toxins.

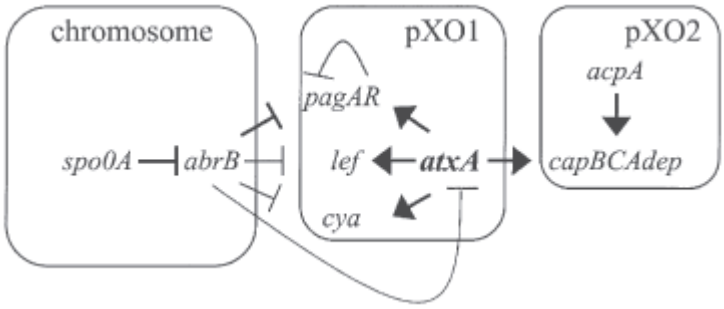

Figure 4. Virulence gene regulation of *B. anthracis* involving chromosomal, pXO1 and pXO2 genes. The pXO1 plasmid gene, *atxA* regulates positively the three anthrax toxins genes, *pagA*, *lef*, and *cya*, while pXO2, *acpA* gene regulates positively genes involved in capsule biosynthesis. Genes involved in toxins and capsule production are expressed in exponential phase. The expression of *atxA*, is repressed by the transitory regulator AbrB inhibiting the toxin expression before exponential phase. When cell density is higher Spo0A inhibit AbrB, allow toxin expression. Black arrows represent a positive effect and black blunt lines represent a negative effect.

2.1.2 Insecticidal toxins

B. thuringiensis strains are characterized by the production of insecticidal proteins, classified into three classes, Cry (Crystal proteins) and Cyt (Cytolytic toxin proteins), which are

produced during sporulation and form crystalline inclusions; and Vip (Vegetative insecticidal proteins), produced during the vegetative phase (Adang, Crickmore and Jurat-Fuentes, 2014).

This species has been used as biopesticide for many years due its entomopathogenic activity against a broad range of insect orders, such as Lepidoptera, Diptera, Coleoptera, Hymenoptera, Hemiptera, and also nematodes (Schnepf *et al.*, 1998; de Maagd *et al.*, 2003). Several structures of Cry proteins have been elucidated using X-ray crystallographic methods, such as Cry1Aa (Grochulski *et al.*, 1995) and Cry5B (Hui *et al.*, 2012). The crystal structures are similar even considering the differences in toxin specificity and amino acid sequence, suggesting a common mode of action. Cry proteins are composed of three domains (Figure 5). The first is located in the N-terminal and consists of seven α-helices in a bundle. This domain has intramolecular activity, and is able to enter a membrane, forming a pore (Li, Carroll and Ellar, 1991). Domain II is composed of antiparallel β-sheets and is linked with Domain I by a short α-helix. This domain is the most variable of the Cry proteins, and is involved in receptor binding, so it determines the toxin specificity (Li, Carroll and Ellar, 1991). Domain III, located in the C-terminal, consists of a lectin-like sandwich with two antiparallel β-sheets and is involved in receptor binding and pore formation, being essential for toxin stability (Li, Carroll and Ellar, 1991; Gómez *et al.*, 2006).

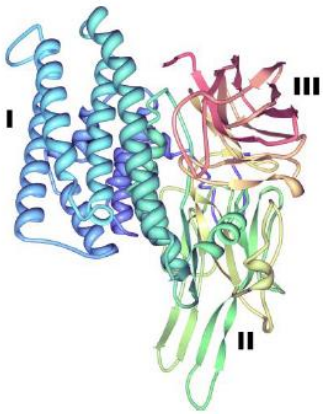

Figure 5. Three-dimensional structure of Cry2Aa protein. Roman numerals indicated the three domains, I- binding membrane domain; II- toxin-receptors interactions domain and III- toxin stability domain (Palma *et al.*, 2014).

Several modes of action of Cry toxins have been described. In Lepidoptera insects (Figure 6), the crystals (inactive form) and spores are ingested by the insect larvae and the Cry toxins are

solubilized by specific proteases, in the midgut fluids to generate their active form. Then, the monomers traverse the peritrophic matrix, reaching the brush border membrane located in the midgut epithelium. In this area, Cry toxins bind to the first receptor located in the membrane of apical cells, a cadherin or a glycol-conjugate receptor. They bind with these receptors, inducing proteolytic cleavage in the α-helix, resulting in an oligomer formation and also the activation of intracellular cell death pathways (G protein, adenylate cyclase and cAMP). Subsequently, the oligomeric toxin binds to another receptor (APN or ALP), inducing a conformation change. Then, the oligomer toxin is inserted into the cellular membrane, leading to pore formation. Cell contents are released and favorable conditions allow the spores' germination and vegetative cell multiplication, resulting in severe septicemia followed by death of the insect (de Maagd, Bravo and Crickmore, 2001; Bravo, Gill and Soberón, 2007; Adang, Crickmore and Jurat-Fuentes, 2014; Zhang, Hua and Adang, 2016).

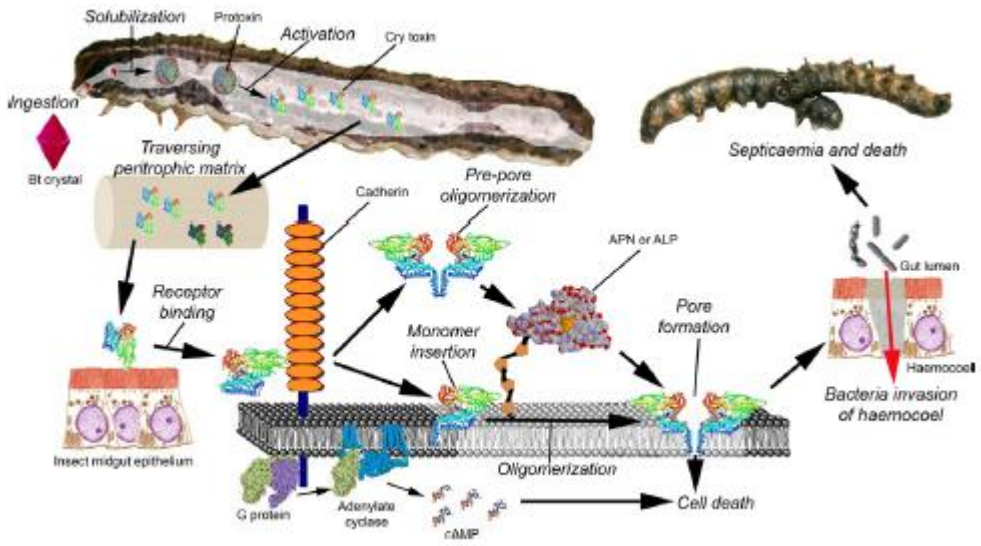

Figure 6. Schema of the current mode of action of Cry toxins. The complete description is found in text above (Adang, Crickmore and Jurat-Fuentes, 2014).

Cyt toxins are known to be less toxic against mosquito larvae than Cry toxins (Crickmore, 1995). However, they are an important class of insecticidal toxins. These toxins are composed of a conserved α-β structure (Li, Koni and Ellar, 1996, Cohen *et al.,* 2011). Cyt proteins are also encoded as a protoxin and are cleaved and activated by digestive proteases. In the active form, Cyt toxin displays high affinity for membrane lipids rich in unsaturated acyl chains, characteristic

of the midgut brush border membrane of Diptera insects (Li, Koni and Ellar, 1996). Synergism with Cry toxin has been observed against Diptera insects (Figure 7) (Pérez *et al*., 2005). To act synergistically Cry and Cyt toxins are concomitantly solubilized and activated. So the Cyt toxin serves as a midgut receptor for the Cry toxins (Pérez *et al*., 2005). The interaction with Cyt protein promotes the Cry toxicity, enhancing the bacterial pathogenesis.

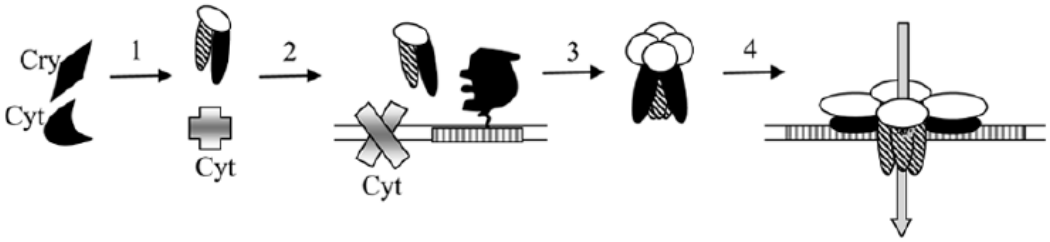

Figure 7. Mode of action of Cyt toxins in synergy with Cry toxins. Both proteins are solubilized and their active form is released (1). Cyt toxin inserts into the membrane and Cry toxin binds to membrane receptors, ALP or Cyt toxin (2). Cry toxins form oligomers (3). Oligomers are inserted into membrane resulting in pore formation (4) (Bravo, Gill and Soberón, 2007).

Vip proteins are produced during the vegetative growth phase and are secreted in the extracellular medium (Struch *et al*., 1996). There are four families of Vip proteins, named Vip1-Vip4. The amino acid sequence of these proteins contains a signal for secretion. In addition, Vip1 and Vip2 contain other conserved signal peptide sequences that indicate cleavage before and after the secretion process (Struch *et al*., 1996; de Maagd *et al*., 2003; Shi *et al*., 2007). Vip1 and Vip2 form a binary protein, in contrast with the single chain of Vip3 toxin. The action mechanism of Vip proteins remains poorly understood, but there is a hypothesis that Vip3 proteins act by pore formation, provoking lysis of the epithelial cells present in the midgut, gut paralysis and consequently the death of the larval insect. Studies suggest that this mode differs from the mode of action of Cry toxin mainly in receptor binding and ion channel properties (Yu *et al*., 1997; Lee *et al*., 2003).

2.1.3 Cereulide

The cereulide toxin is responsible for the emetic syndrome of gastrointestinal disease caused by *B. cereus*, and are produced in food before consumption. The cereulide toxin is produced at the onset of stationary growth phase (Ehling-Schulz *et al.,* 2004). This small toxin

(Figure 8) is extremely stable withstand an enzymatic cleavage, heat and broad pH range, then they are not degraded by gastric acid present in the gastrointestinal tract. In addition, reheating precooked foods is not enough to inactivate the cereulide toxin (Schoeni and Wong, 2005). Cereulide is produced by an enzymatic complex encoded by a 24 kb gene cluster including 7 ORFs named cereulide synthetase (*ces* genes) (Figure 9) (Ehling-Schulz *et al.,* 2005). The *ces* locus is located on a megaplasmid firstly named pBCE4810 and also referred as pCER270 (Rasko, Gill and Soberón, 2007). This plasmid is related to pXO1 of *B. anthracis* and is restricted to emetic strains (Ehling-Schulz *et al.*, 2006). Some strains showed different levels of cereulide production, mainly due to differences in regulation, whereas *ces* genes display a high homogeneity between different strains (Ehling-Schulz *et al.*, 2005). Some studies described that environmental factors such as pH, temperature, oxygen and presence of specific amino acids, influence the production of cereulide (Agata *et al*., 1999; Ehling-Schulz *et al.,* 2004). The mechanisms regulating cereulide synthesis are still largely unknown, however, the differences in cereulide production may result from the cross-talk between the megaplasmids, harboring *ces* genes, and differences in the chromosome background of *B. cereus* strains.

Cereulide acts as a cation ionophore, like valinomycin, inhibiting the mitochondrial fatty acid metabolism resulting in nausea and vomiting, which occur between 30 min and 6 h after ingestion (Mikkola *et al*., 1999; Abee *et al*., 2001). Cereulide can cause cellular damage in animal models and liver failure in humans (Mahler *et al*., 1997).

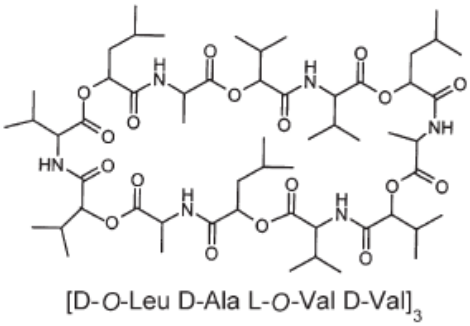

Figure 8. Cereulide structure, a small cyclic dodecadepsipeptide (Agata *et al*., 1994).

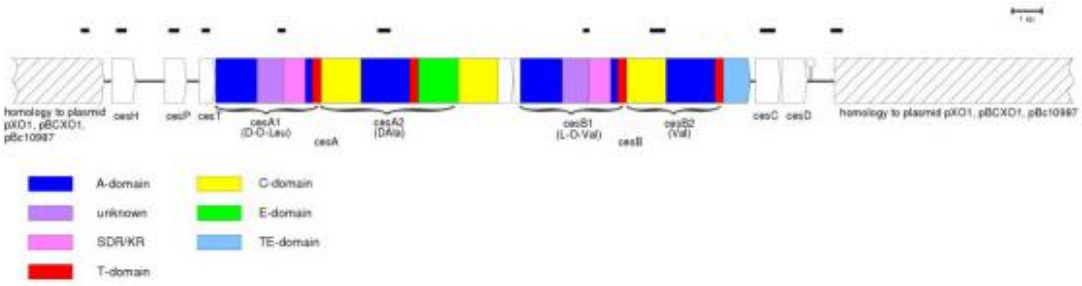

Figure 9. The biosynthetic gene cluster for cereulide synthesis. The flanking regions showing homologies to toxin plasmids from *B. cereus* group members are printed as hatched boxes (Ehling-Schulz *et al*., 2006).

2.2 Chromosomic factors

Several chromosomic factors produced in the early stationary phase are responsible for *B. cereus* virulence (Gilois *et al*., 2007, Guillemet *et al*., 2010). These secreted factors include enterotoxins (haemolytic and non-haemolytic), phospholipases-C, collagenase, zinc metalloproteases, chitinases, collagenases and various haemolysins/cytolysins (Stenfors, Fagerlund, Granum, 2008). These factors are important to complete infection of *B. cereus* and *B. thuringiensis* strains. *B. anthracis* strains also harbor all these chromosomic genes, however the master regulator, encode by *plcR* gene, is truncated (inactive), then all these virulence factors are not produced (Agaisse *et al*., 1999; Mignot *et al*., 2001).

2.2.1 Enterotoxins and hemolysins

Production of several heat-labile enterotoxins is responsible for diarrhoeal syndrome. They are produced by *B. cereus* vegetative cells during stationary phase in the small intestine (Granum, 1997). These classes of toxins include two enterotoxic complexes, hemolysin BL (HBL) and non-haemolitic enterotoxin (NHE), several phospholipases-C, haemolysins/cytolysins (HlyI-HlyIV) and a collagenase.

The enterotoxin Hbl has a broad distribution among species of *B. cereus* group (Prüb *et al*., 1999) and is composed of three subunits Hbl-B (Figure 10A), Hbl-L_1 and Hbl-L_2 encoded by the respectively genes, *hblA*, *hblC* and *hblD*, localized in tandem in the genome (Beecher and Macmillan, 1991). Hemolysin is a membrane-lytic toxin, which is produced and secreted independently during vegetative growth, however, the three components are necessary to

maximum biological activity (Beecher and Macmillan, 1991; Beecher, Schoeni and Wong, 1995). Hbl was first described as causing dermonecrosis (Thompson *et al*., 1983), however, this toxin also causes, hemolysis, vascular permeability and necrosis in rabbit skin (Beecher and Macmillan, 1991, Beecher and Wong, 1994). In addition, Hbl participates in diarrheal syndrome provoking a rapid fluid accumulation in ligated rabbit ileal loops (Beecher, Schoeni and Wong, 1995). The three Hbl proteins bind to erythrocytes independently and attack the membrane forming pores, provoking cellular lysis (Beecher and Wong, 1997).

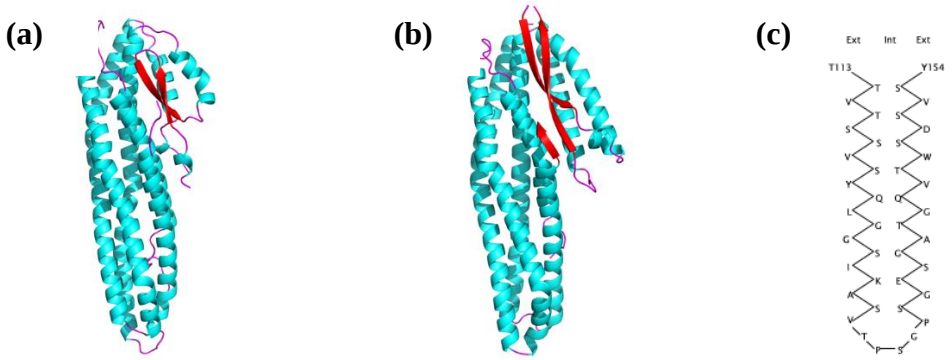

Figure 10. The structure of Hbl-B with α-helices colored cyan, β-strands colored red and loops colored magenta (a), NheA (b) colored as in Hbl-B. Schematic representation of CytK Schematic representation of CytK. The exterior (Ext) and interior (Int) (c) (Lund, De Buyser and Granum, 2000; Ganash *et al*., 2013).

Nhe, is also a tripartite toxin (NheA, NheB and NheC), responsible for the diarrhoeal food-poisoning syndrome caused by *B. cereus* (Figure 10B) (Lund and Granum 1996; Lindbäck *et al*., 2004). These toxins are widely distributed in *B. cereus* strains (Moravek *et al.*, 2006). As Hbl toxins, Nhe toxins are secreted independently, via Sec pathway, however, the maximal toxic activity requires the three toxins (Lindback *et al*., 2004). Nhe toxins have a cytotoxic activity, causing cellular lysis as a result of pore formation in the cell membrane. Previously, Nhe proteins were described as a non-haemolytic enterotoxin, however, studies also show a haemolytic activity towards mammalian erythrocytes (Fagerlund *et al*., 2008).

CytK is another pore-forming toxin with broad distribution and is highly toxic towards human intestinal epithelial cells (Figure 10C) (Hardy, 2001; Ngamwongsatit *et al.*, 2008). CytK has cytotoxic, necrotic, and hemolytic activities, responsible for a huge outbreak of food poisoning in France (Beecher, Schoeni and Wong, 1995). CytK proteins show similarity with

gamma and alfa-heaemolysins, leucocidins of *Staphylococcus aureus* and haemolysin II of *B. cereus*, all these proteins belong to a family of β-barrel channel-forming toxins (Lund, De Buyser and Granum, 2000). CytK possesses also enterotoxic activity against intestinal epithelia cells, which is related to the β-barrel structure (Hardy, 2001).

2.2.2 Phospholipases

Phospholipases C (PLC) are enzymes capable of cleaving phospholipids. This activity is important to various biological processes such as inflammation (Triggiani *et al*., 2006), digestion (Watkins, 1985), infection (Ghelardi *et al*., 2007) and cell membrane homeostasis (Scott, 1984). In *B. cereus* three classes are identified, PC-PLC (active against phosphatidylcholine); PI-PLC (active against phosphatidylinositol) and SM-PLC (active against sphingomyelin).

PC-PLC is a small monomeric enzyme (28 kDa), with three Zn^{2+} atoms in the active site, which are involved in the enzymatic activity and protein stability. Is suggested that the zinc atoms are involved in association with the membrane, then the enzyme is capable of binding to a membrane interface (Figure 11a) (González-Bulnes *et al*., 2010). PC-PLC enhances hemolysis, and is toxic to retinal tissue *in vitro*, retinal necrosis *in vivo* and septic endophthalmitis in rabbits (Beecher and Wong, 2000). PC-PLC is encoded by *plc* gene, which is associated in tandem with *sph* gene, encoding sphingomyelinase (SM-PLC), together they form a complex known as cereolysin AB. Previous studies demonstrated a cooperativity between these two enzymes, provoking erythrocyte haemolysis (Gilmore *et al*., 1989).

PI-PLC is an enzyme produced and secreted by several organisms, including bacterial pathogens (Griffith and Ryan, 1999). This enzyme has cytotoxic activity toward different eukaryotic cell types by cleaving glycosylphosphatidylinositol-anchored proteins (Figure 11b). *B. thuringiensis* is a strong producer of the small secreted protein, PI-PLC, which contains only a monomeric catalytic domain, responsible for membrane binding (Cheng *et al*., 2013).

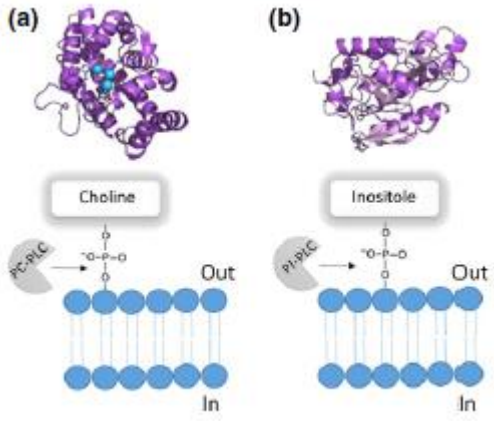

Figure 11. X-ray structure and mechanism of action of PC-PLC (a) and PI-PLC (b) (Celandroni *et al*., 2014).

2.2.3 Proteases

Proteases are enzymes capable of degrading proteins to short peptides and/or free amino acids, they are mostly extracellular, thermostable, broad range pH activity and are produced in considerable amounts. These proteins are found in a wide variety of organisms, including bacteria of *B. cereus* group, 48 CDS were found in *B. anthracis* and 51 in *B. cereus* genome (Ivanova *et al*., 2003). Among the proteases identified in *B. cereus* group are NprA (neutral protease), InhA (immune inhibitor A), Sfp (serine protease) and ColB (Gohar *et al*., 2002).

NprA, also designated NprB and Npr599, is a zinc metalloprotease of the thermolysin family (peptidase M4) (Donovan, Tan and Slaney, 1997). NprA acts as extracellular serine protease and is a potential virulence factor, due to its degradative properties against human tissue proteins (Chung *et al*., 2006). Any experiment with NprA was essayed using an animal model. In *B. thuringiensis*, *nprA* deletion does not affect the insecticidal activity (Donovan, Tan and Slaney, 1997). NprA is produced and exported during the first stage of the sporulation process and is controlled by the cell-cell communication system NprR/NprX (Perchat *et al*., 2011).

InhA is another zinc metalloprotease, belonging to M6 family. It digests several substrates, such as extracellular matrix proteins and cleaves tissue components, including fibronectin, laminin and type I and IV collagens (Chung *et* al., 2006). Lethal effect of InhA, was already described after injection into the insect hemocoel, this is probably due to cleavage of antibacterial peptides produced by the insect hosts, as cecropin and attacin (Dalhammar and Steiner, 1984).

InhA synthesis occurs mainly during stationary phase and requires specific sigma factors (Dalhammar and Steiner, 1984). Granvalet and co-workers (2001), demonstrated that *inhA* transcription is negatively regulated by AbrB. Three genes encoding InhA metalloproteases are found *in silico* analyses on the genome of *B. cereus* group and named, *inhA1*, *inhA2* and *inhA3*. InhA1 of *B. anthracis* shares 91% of similarity with InhA1 of *B. cereus* (Chitlaru *et al*., 2006). The *inhA1* deletion does not affect the bacterial virulence in various insect models (Fedhila, Nel and Lereclus, 2002). InhA1 is also produced and secreted during the growth cycle of *B. cereus* and associated with the spore exosporium (Charlton *et al*., 1999). When spores are internalized by macrophages, the metalloprotease InhA1 promotes the survival and escape from this hostile environment (Ramarao and Lereclus, 2005). The second metalloprotease identified is InhA2. Contradictory to InhA1, InhA2 has a role in virulence against *Galleria mellonella* after oral infection (Fedhila, Nel and Lereclus, 2002). InhA2 alone is not sufficient to virulence and requires other PlcR-regulated genes (Fedhila *et al*., 2003). The third metalloprotease, named InhA3, has 72% identity to InhA1 and is secreted in low amounts by some species of *B. cereus* group (Gilois *et al*., 2007). Its function is poorly understood, however, is knows that *inhA3* is controlled by NprR (Dubois *et al*., 2012). In addition, neither InhA2 nor InhA3 are involved in circumventing the immune system (Guillemet *et al*., 2010).

Recently, the metalloproteinase ColB, belonging to M9 superfamily, was described as a virulence factor in *B. thuringiensis*, acting against nematodes and insects. ColB has collagenase activity, with high specificity to collagen, destroying intestine cells of *Caenorhabditis elegans* and *Helicoverpa armigera* (Deng *et al*., 2016).

These virulence factors are mainly, under control of the transcriptional regulator PlcR, the master regulator of virulence in *B. cereus* group (Gohar *et al*., 2008). The activation mode of this quorum sensing system is described below. All these virulence factors promote the destruction of epithelial cell layers and the access to others sources of nutrients allowing vegetative cells multiplication and consequently the development of infection. Although these virulence factors are found in all species of *B. cereus* group, *B. anthracis* has a nonsense mutation in the *plcR* gene, which disables this species to encode these proteins (Agaisse *et al*., 1999; Slamti *et al*., 2004). This can be explained by the incompatibility of *plcR* and *atxA* (a transcriptional regulator present on pXO1 plasmid of *B. anthracis*). Strains harboring both *plcR* and *atxA* genes are

incapable to sporulate, suggesting that the PlcR regulon was counter selected to promote sporulation in *B. anthracis* strains (Mignot *et al*., 2001; Fouet and Mock, 2006).

3 Persist and survive

3.1 Sporulation

Sporulation is an important process which consists of the formation of endospores in response to external signals, including nutrient limitation. The endospore is stress-resistant and metabolically inert, able to persist in the environment thousands and even millions of years (Higgins and Piggot, 2012). This process has been extensively studied in *B. subtilis* and starts with asymmetric cell division, which forms a mother cell and a forespore (prespore). Different gene expressions in these two compartments result in distinct outcomes.

3.1.1 Sporulation stages

The sporulation process is divided in eight stages (0 from VII), represented in Figure 12.

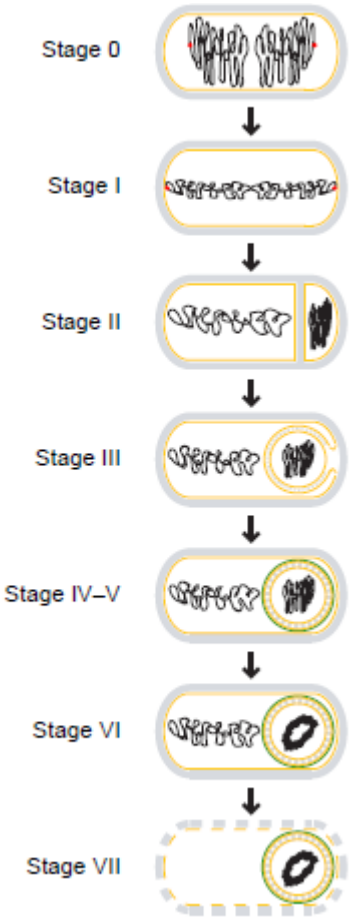

Figure 12. Schematic representation of morphological changes during sporulation process of *B. subtilis*. All stages are mentioned and described in the text (Tan and Ramamurthi, 2014).

Sporulation is a costly process which is irreversible at a certain stage, so this is an important response by bacterial cell (Veening *et al*., 2008). The first stage (Stage 0) is marked by the decision to sporulate. Nutrient limitation is a well-known signal for entry into sporulation, however other factors remain elusive. Stage I is characterized by chromosome duplication and axial filamentation. The two chromosomes form the axial filament, which stretches in the cell length. The RacA protein helps the proper chromosome segregation by anchoring the two chromosomes to the cell poles (Ben-Yehuda, Rudner and Losick, 2003). Stage II is characterized by the asymmetric septation, after this stage the sporulation process becomes irreversible. Two proteins are involved in the polar septum formation, FtsZ and SpoIIE. They form filaments, homologs to tubulin, which redistribute the FtsZ protein to subpolar positions, determining the fates of the prespore and the mother cell (Figure 13) (Carniol *et al.*, 2005).

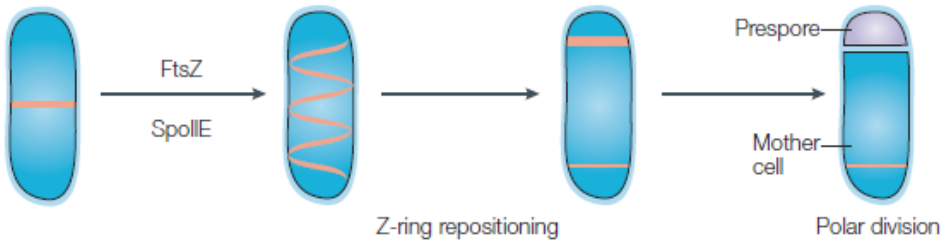

Figure 13. Cell division during sporulation in *B. subtilis*. The FtsW protein is accumulated and orientated into two separated rings, one in each cell pole, providing the septum formation and determining the fates of the prespore and the mother cell (Errington, 2003).

Stage III is characterized by engulfment. After asymmetric division, the polar septum curves around the forespore, resulting in a double-membrane encompassing the forespore in the mother cell cytosol. Stages IV and V corresponds to cortex and coat assembly. These two shells are separated by the outer forespore membrane and protect the spore from fluctuations in environmental conditions (Setlow, 2006). Four distinct layers are identified in the coat: the basement layer, inner coat, outer coat and crust. Each layer is composed of one major morphogenetic protein which defines each one (McKenney, Driks and Eichenberger, 2013). The cortex protects the spore from heat and desiccation and is formed by specialized peptidoglycans and lipids, synthetized from precursors named Mur proteins, which are upregulated by σ^K (Vasudevan, *et al*., 2007). Stage VI refers to spore maturation, corresponding to the formation of a multilayered proteinaceous coat outside the cortex. Finally, Stage VII is characterized by lysis of the mother cell, allowing the release of the mature spore and its dissemination in the environment.

3.1.2 Regulation of sporulation

The different stages of sporulation described above are controlled by sequential activation of sigma factors. These factors allow RNA polymerase to transcribe the specific genes of each subsequent stage of sporulation in the two compartments of the bacterial cell (Figure 14). In *B. subtilis*, Spo0A is the master regulator of sporulation, directly controlling the transcription of 121 genes (Molle *et al*., 2003). Phosphorylation of Spo0A is directed by phosphorelay, consisting of

five histidine kinases (KinA to KinE). In response to starvation signals, kinases are autophosphorylated. The phosphoryl group is transferred from kinases to Spo0F (Spo0F-P) and then to Spo0B (Spo0B-P). Then, Spo0A is phosphorylated to generate Spo0A-P (Burbulys, , Trach and Hock, 1991). At the end of the exponential phase, the phosphorylated form of Spo0A (Spo0A-P) accumulates progressively in the cytoplasm (Fujita and Losick, 2005). Spo0A-P at high levels regulates a set of genes involved in sporulation. Spo0A-P positively controls the transcription of *spoIIE* and the two operons *spoIIGA-sigE* and *spoIIAA-spoIIAB-spoIIAC* (Molle *et al*., 2003; Phillips and Strauch, 2002). The *spoIIAC* gene encodes the transcriptional factor Sigma-F and *sigE* encodes Sigma-E. These genes are transcribed in both cellular compartments, however in an inactive form. The activation of Sigma-F depends on the SpoIIAA protein, which forms a complex with SpoIIAB, which in turn releases the active form of Sigma-F (Duncan and Losick, 1993). Sigma-F actives the transcription of *spoIIIG* and *spoIIR*, which encode the precursor of the factor Sigma-G and the SpoIIR protein, respectively. SpoIIR activates the protease SpoIIGA, which is located in the septum and is responsible for cleaving the precursor of Sigma-E, activating this transcriptional factor (Hofmeister *et al*., 1995). Sigma-E activates the transcription of *spoIID* and also of the *spoIVFA-spoIVFB* and *spoIIIA* operons (Eichenberger *et al*., 2004; Steil, 2005). SpoIID prevents a second polar division of the sporulating cell (Eichenberger, Fawcett and Losick, 2002). In addition, SpoIID actives the transcription of *spoIVCA*, which encodes the recombinase SpoIVCA. This recombinase excises a fragment of 48 kb, named *skin*, which interrupts the *sigK* gene, so the *spoIIIC* and *spoIVCB* genes are correct organized, resulting in a functional copy of *sigK* (Takemaru *et al*., 1995). Sigma-K is encoded in the mother cell, in an inactive form. The activation of Sigma-K depends on SpoIVFB protease, encoded by the gene *spoIVFB*. This protease is located in the membrane of the mother cell and is kept inactive by the proteins SpoIVFA and BofA (Cutting, Roels and Losick, 1991; Ricca, Cutting and Losick, 1992). To activate Sigma-K, the SpoIVFB and CtpB proteases cleave SpoIVFA and/or BofA, activating SpoIVFB (Campo and Rudner, 2006). SpoIVFB in its active form cleaves the pro-Sigma-K factor in the mother cell. The *spoIIIA* operon is also activated by Sigma-E factor and encodes eight proteins, named SpoIIIAA-SpoIIIAH. This protein complex is involved in pore formation in the forespore membrane, connecting the mother cell and the forespore (Meisner *et al*., 2008). Then the mother cell transfers small molecules that enable the forespore to continue expressing the genes necessary for sporulation (Camp and Losick, 2009).

The SpoIIIJ protein, located in the prespore, interacts with SpoIIIAE protein, activating the Sigma-G factor (Camp and Losick, 2008; Serrano *et al*., 2008). Once active in their specific compartments, Sigma-K and Sigma-G factors regulate the transcription of genes involved in the late stages of sporulation, such as assembly, maturation and release of the spore (Errington, 2003; Hilbert and Piggot, 2004).

After asymmetric septation, the sequential activation of sporulation specific sigma factors: σ^F in the forespore, σ^E in the mother cell, then σ^G in the forespore, and σ^K in the mother cell.

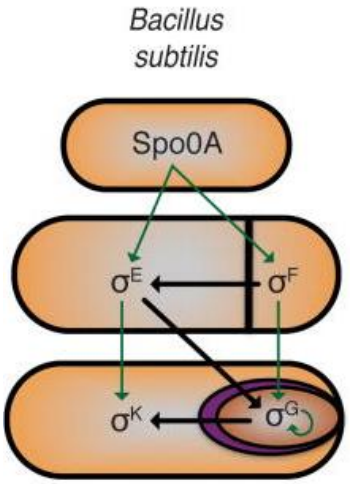

Figure 14. Sequential activation of sigma factors in the forespore (σ^F and σ^G) and mother cell (σ^E and σ^K) (Fimlaid and Shen, 2015).

Spores are resistant to several adverse environmental conditions, such as heat, dehydration, radiation and chemicals (Setlow, 2006). In addition, *B. cereus* spores have adhesive traits that facilitate their attachment (Klavenes *et al*., 2002). These dormant structures are capable of surviving for millions of years (Vreeland, Rosenzweig and Powers, 2000). All these characteristics ensure survival and dissemination. Once in favorable conditions, the spores can germinate and a pathogenic bacterium can develop its lifecycle. Consequently, these structures cause food spoilage and food-borne disease (Setlow, 2006).

3.2 Biofilm

3.2.1 Components, formation and molecular regulation

Biofilm is defined as "aggregates of microorganisms in which cells are frequently embedded in a self-produced matrix of extracellular polymeric substances (EPS) that are adherent to each other and/or a surface" (Vert *et al*., 2012). The biofilm lifestyle is completely different from that of free-living cells and has an essential role in bacterial communities, such as social cooperation, protection, resource capture and enhanced survival from exposure to antimicrobials (Flemming *et al.*, 2016). This bacterial lifestyle is very common and is found on almost all natural and artificial surfaces (Hall-Stoodley, Costerton and Stoodley, 2004). Biofilms have been widely studied because they represent an intriguing example of bacterial adaptation as well as because of the problems the cause in industrial processes (Hall-Stoodley and Stoodley, 2009). Figure 15 illustrates the different stages of *B. subtilis* biofilm formation. First, some motile cells adhere to each other and to a surface and became non-motile. These cells produce the extracellular matrix, allowing the biofilm development by cell multiplication (Branda *et al*., 2006). In a mature biofilm, some matrix-producing cells sporulate. In advanced stages, some cells secrete enzymes that degrade the matrix compounds, allowing cell dispersion in the environment. Thus, biofilms are composed of heterogeneous populations, including the three main cell types: motile cells, matrix-producing cells and sporulated cells, which coexist as subpopulations (Vlamakis *et al*., 2008). Matrix production creates microenvironments where nutrients are severely limited, stimulating Spo0A activation and a high level of spore formation (El-Khoury *et al*., 2016).

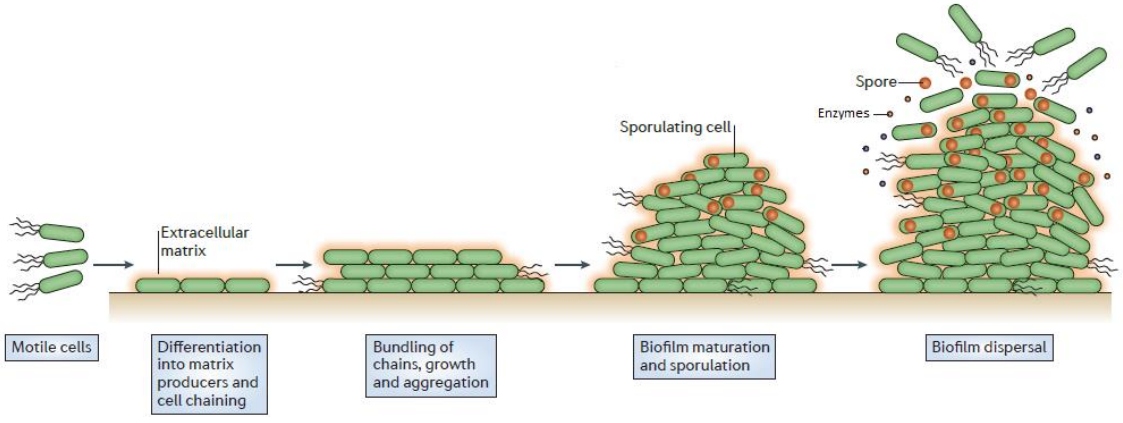

Figure 15. Stages of biofilm formation. The figure is described in the text. (Vlamakis *et al*., 2013).

Biofilms are formed by microbial cells and an extracellular matrix, composed of polysaccharides, proteins, lipids and extracellular DNA (eDNA), mostly produced by the organisms present in the biofilm. This matrix provides mechanical stability and shapes cell distribution (Flemming and Wingender, 2010; Neu and Lawrence, 2015). The matrix has several functions in biofilms, such as adhesion, aggregation of bacterial cells, protective barrier formation, nutrient source, cohesion of biofilms and exchange of genetic information, among other functions (Flemming and Wingender, 2010). The major components of biofilm matrixes are the exopolysaccharides. A mutant that cannot synthetize these molecules is unable to form a mature biofilm (Danese, 2000; MA, 2009). In *B. subtilis*, the exopolysaccharides required for biofilm formation are mainly encoded by the *epsA-O* operon (Branda *et al*., 2001; Kearns *et al*., 2005). Deletion of *epsA-O* results in a non-structured and fragile biofilm pellicle (Lemon, *et al*., 2008). *B. cereus* species have two *eps* operons, named *eps1* and *eps2*, responsible for pellicle and ring formation, respectively (unpublished data, Majed, 2017). In *B. cereus*, *eps1* locus deletion does not affect biofilm formation (Gao, *et al*., 2015). The deletion of both *eps* operons of *B. thuringiensis* completely inhibits biofilm formation (unpublished data, Majed, 2017). In *B. subtilis*, three other proteins are important components of the biofilm matrix, TasA, TapA and BslA (Vlamakis *et al*., 2013). BlsA forms a hydrophobic envelope surrounding the biofilm (Hobley et al., 2013). TasA and TapA are responsible for forming a strengthened fiber network (Romero *et al*., 2011). These two proteins are encoded by the *tapA-sipW-tasA* operon, while *sipW* encodes for a signal peptidase, responsible for releasing TasA and TapA in the extracellular medium. There is no paralog of *tapA* and *blsA* genes in *B. cereus*, but *tasA* has two paralogs. These two paralogs are *tasA*, present in the *sipW-tasA* operon, and CalY, located next to the *sipW-tasA* operon (Caro-Astorga *et al*., 2015). CalY and TasA are involved in fiber production, and deletion of these genes or *sipW* leads defective biofilm formation (Caro-Astorga *et al*., 2015). As in *B. subtilis*, biofilm formation in *B. cereus sensu lato* is repressed by the master regulator SinR. In *B. subitlis*, SinR negatively controls the transcription of the *epsA-O* and *tapA-sipW*-tasA operons. In *B. cereus sensu lato*, SinR negatively controls the *sipW*, *tasA* and *calY* genes, but not the *eps* operon (Pflughoeft, Sumby and Koehler, 2011). SinR is antagonized by SinI, and in both species, deletion of *sinI* completely inhibits biofilm formation. Additionally, the phenotype is reversed upon deletion of SinR (Kearns *et al*., 2005; Fagerlund *et al*., 2014). In both species, the master regulator of sporulation, Spo0A, is required for biofilm formation, activating *sinI*

transcription (Shafikhani *et al*., 2002) and repressing *abrB* (Strauch *et al*., 1990). Also, AbrB represses biofilm formation in both species, inhibiting the two polycistronic operons *tapA-siW-tasA* (Hamon and Lazazzera, 2001; Fagerlund *et al*., 2014). Figure – summarizes the network biofilm regulation in *B. cereus sensu lato.* There are some differences in biofilm formation between *B. cereus* group species and *B. subtilis*: (i) *B. subtilis epsA-O* is included in the SinR regulon, contrary to *eps* of *B. thuringiensis*; (ii) AI2 autoinducer represses biofilm formation in *B. cereus* (Auger *et al*., 2006), while it induces formation in *B. subtilis* (Assaf, Steinberg and Shemesh, 2015); and (iii) biofilm formation is activated by the DegU regulator in *B. subtilis* (Kobayashi, 2007), while *B. cereus sensu lato* has none of these homologs.

In *B. thuringiensis* there is interaction between biofilm formation and other processes of the lifecycle in insects, such as virulence and necrotrophism (Majed et al., 2016). PlcR, the master regulator of virulence factors, also promotes transcription of NprR, the necrotrophic lifestyle regulator (Dubois *et al*., 2013). NprR positively regulates the transcription of the *krs* locus, needed to produce kurstakin (Dubois *et al*., 2012), which promotes biofilm formation (Gélis-Jeanvoine *et al*., 2016). All these network connections suggest involvement of biofilm in the pathogenic, commensal or necrotrophic lifestyles of *B. cereus sensu lato* (Majed, *et al*., 2016).

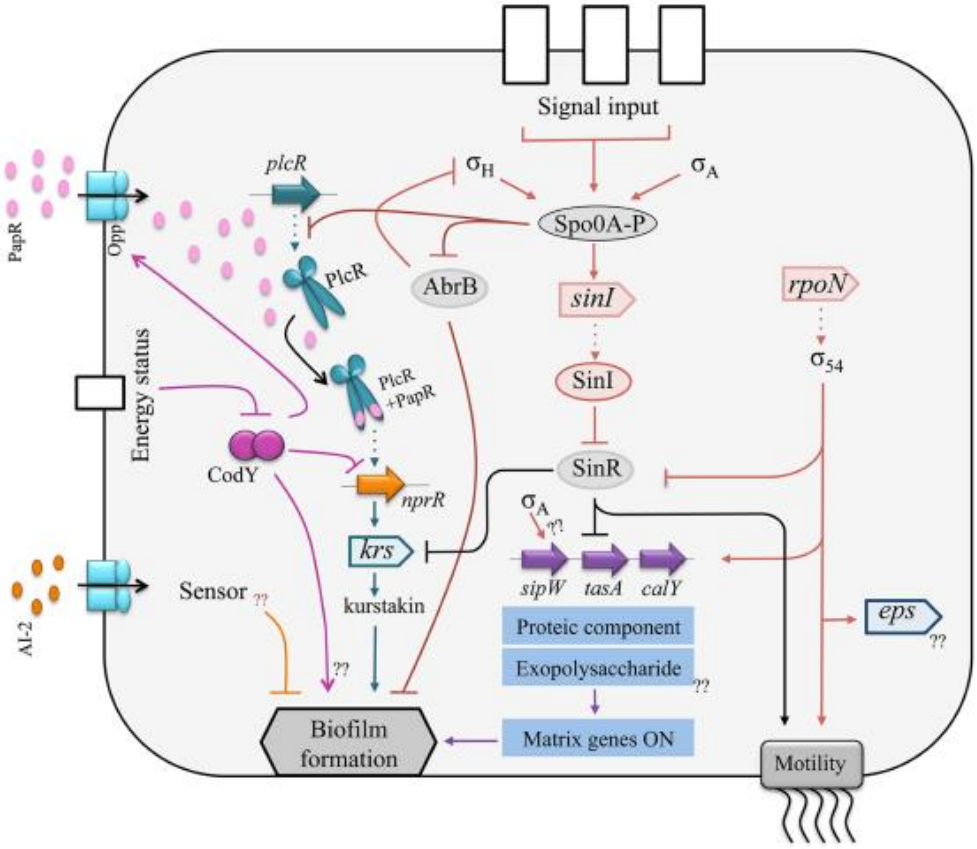

Figure 16. The regulatory network controlling biofilm formation in *B. cereus*. Circles symbolize proteins, triangles symbolizes open read frames (ORFs). Arrows indicate activation and blunt lines indicate repression. Dotted arrows represent transcription. All regulation and interactions in the pathways are described in the text (Majed, *et al*., 2016).

Biofilms provide a suitable environment for sporulation, an important process for persistence and dissemination of bacteria. In addition, this mode of life confers to bacteria protection against stress, such as antibiotics, toxic compounds and desiccation (Lee *et* al., 2007; Weaver *et al*., 2015). The conjugation process is up to 700-fold more efficient in biofilms compared with free-living cells (Król *et al*., 2013). Due to these characteristics, biofilms are also a concern to the food industry as a potential source of contamination of food and water (Van Houdt and Michiels, 2009; Wingender and Flemming, 2011), resulting in spoilage or foodborne illness (Rajkovic *et al*., 2008).

4 Evolution plasmids

Bacterial populations are in constant evolution to cope with environmental changes. The major contributor to this adaptation is horizontal gene transfer (HGT), which occurs at high rates. This phenomenon is so important that some theoretical models hypothesize that HGT is essential for microbial survival (Koonin, 2016). Three mechanisms of horizontal transfer are described: transformation (transfer of extracellular DNA to a recipient cell); transduction (phage-assisted transfer); and conjugation (direct transfer of plasmids between two cells) (Figure 17). All these processes contribute to extensive genome differences among bacterial species. Studies of Popa and co-workers (2011) estimated that roughly 20% of genes have recently been acquired by these methods.

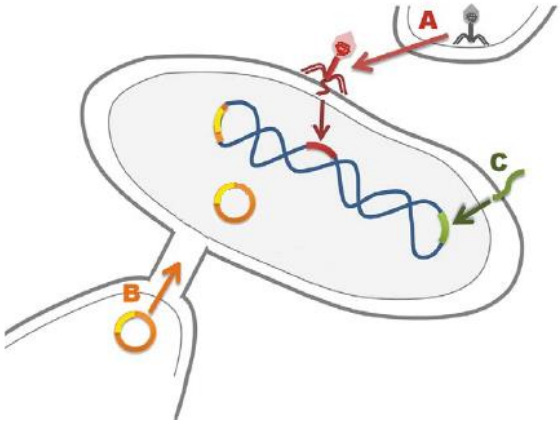

Figure 17. Mechanisms of DNA transfer between and within bacteria. (A) Transduction, (B) conjugation and (C) transformation (Gyles and Boerlin, 2014).

Transformation or natural competence is the ability of some microorganisms to integrate exogenous DNA into their genome. This process has so far not been evidenced in *B. cereus*, even orthologs of most proteins involved in this process have been identified in these bacteria. In addition, it has been demonstrated that *B. cereus* ATCC14579 can become naturally competent, evidencing that the *B. cereus* genome has a sufficient structural system for DNA uptake (Mirończuk *et al*., 2008). However, some genes are lacking or can be non-functional, inhibiting natural competence in *B. cereus* species.

Transduction is also an important mechanism for HGT. Temperate phages can infect cells where they develop lytic or lysogenic cycles. The lytic cycle leads to lysis of the bacterial cells, while in the lysogenic cycle the phage genome integrates the bacterial genome. Recently, a report was published indicating that phages that infect *Bacillus* species rely on small molecules to

communicate. If the concentration of the signaling peptide is sufficiently high, the lysogenic cycle is activated (Erez *et al*., 2017). Besides this, communication is phage-specific, due to different structures of the signaling peptide.

Conjugation is the third HGT method and involves the circular extra-chromosomal genetic elements, the plasmids. Plasmids generally carry genes that encode for several traits, allowing bacteria to adapt to different stresses and niches. These elements are able to spread in bacterial communities by conjugation, playing an essential role in bacterial genome evolution. However, the main difficulty is understanding the routes, causes and consequences of horizontal gene flow.

4.1 Conjugation in Gram-positive bacteria

The transfer of mobile genetic elements (MGE) enables the fast spread of genes between genomes. MGE have been described as "agents of open source evolution" (Frost *et al*., 2005), among them there is the plasmids. Conjugative plasmids encode all the protein apparatus for their own transfer between two cells. They are generally large with low copy numbers: about 15% of the fully sequenced plasmids were predicted to be conjugative (Smillie *et al*., 2010). In addition, some plasmids are classified as mobilizable. They carry *oriT* and *mob* region, encoding specific relaxome components. However, they do not encode for other components of the mating pair formation. These plasmids can be transmitted using additional conjugative functions encoded by conjugative plasmids. These plasmids tend to be small and present in high copy numbers (Garcillán-Barcia, Francia and de la Cruz, 2009). In addition, some plasmids are classified as nonmobilizable. These plasmids lack all components described above, *oriT*, *mob* region and genes for mating pair formation. Despite the lack of all elements, these plasmids can be transferred by recombination with a conjugative plasmid (Andrup *et al*., 1996).

Plasmids are involved in adaptation and evolution of bacteria, harboring these elements affords their hosts access to a huge genetic resource, benefiting bacteria in some environmental conditions. In the *B. cereus* group, several virulence factors are encoded by plasmids. The location of *cry* genes in plasmids has been reported in several studies, including the classic experiment by González and Carlton (1984), which demonstrated conjugation between *B. thuringiensis* strains with a plasmid carrying a *cry* gene. Other studies have also evidenced the location of *cry* genes in conjugative plasmids, showing the dissemination of this important virulence factor (González, Brown and Carlton, 1982; Lereclus *et al*., 1983; Battisti, Green and Thorne, 1985; Reddy, Battist and Throne, 1987; Hu *et al.*, 2004; Van der Auwera *et al*., 2007;

Santos *et al*., 2010). The cytolytic toxin Cyt1Ca is also encoded by a gene on pBtoxis plasmid of *B. thuringiensis* subps. *israelensis* (Berry *et al*., 2002). Finally, *vip* genes are generally found in association with *cry*1A and *cry*2 genes, and are also carry by plasmids (Mesrati, Tounsi and Jaoua, 2005; Hernández-Rodríguez *et al*., 2009). The three anthrax toxins and capsule necessary for *B. anthracis* toxicity are encoded by pXO1 and pXO2 plasmids (Pilo and Frei, 2011). The cereulide synthetase gene cluster was also detected on a mega virulence plasmid related to pXO1 plasmid (Ehling-Schulz *et al*., 2006).The *ces* cluster was identified in *B. weihenstephanensis* strains, which suggests the lateral transfer between species of the *B. cereus* group (Thorsen *et al*., 2006). In addition, in that group, some genes involved in quorum sensing are carry by plasmid, such as Rap/Phr systems involved in the control of sporulation (Bongiorni, *et al*., 2006). The presence of all these plasmid-borne genes reveals the importance of these elements to the *B. cereus* group.

Conjugation of *B. thuringiensis* in insect larvae, its ecological niche, was firstly investigated by Jarret and Stephenson (1990) that described dead larvae as hot spots for genetic transfer between different strains of this species. Then, plasmid transfer can occour in nature and has an important impact in the *B. thuringiensis* ecology, producing new genetic combinations in bacterial population. Several studies regarded conjugation in labolatory conditions, in soil, and in insects between different *B. thuringiensis* strains. The classic study of Gonzalez and Carlton (1982) between different *B. thuringiensis* strains (Cry+, donor and Cry-, recipient) confirmed the location of *cry* genes in plasmids. Gonzalez and co-workers (1982) also showed the conjugation between *B. cereus* and *B. thuringiensis*. The conjugation was also detected between the releated species belonging to the *B. cereus* group (Hu *et al*., 2004). The conjugation was more efficient between the same strain of *B. thuringiensis* than *B. cereus*, *B. mycoides* or *B. weihenstephanensis* strains, suggesting that the donor strain might prefer to establish conjugal mating with another *B. thuringiensis*. However, Yuan and co-workers (2007) used the same group of strains used by Hu and co-workers (2004). The conjugation results in culture broth and lepidopteran larvae were conflicting. Some strains were capable to conjugate in culture broth and unable to conjugate in lepidopteran larvae. This differences can be explained by the differences in ecological niche between the species used in these studies. Spores of *B. cereus* and *B. mycoides* strains were enable to germinate in insect larvae inhibiting the conjugation process. Santos and co-workers (2010) evaluated the conjugation and multiplication frequency of *B. cereus* and *B. thuringiensis*

strains. These authors suggested that the conjugal transfer is dependent of spore germination and not vegetative cell multiplication, concluding thar the limitant factor is the presence of viable vegetative cells. In addition, Suzuki, *et al.* (2004) discussed the **t**oxin **s**pecificity (TS), ability of a *B. thuringiensis* strains kill the insect and **h**ost **s**pecificity (HS), ability of a *B. thuringiensis* strain to colonize the insect larvae. The conclusions showed that even a strain not be able to kill the insect (without TS ability) it is capable to multiply in the insect larvae. The conjugation frequency is influenced by the germination and multiplication rates, which can vary in accordance with the ecological niche of each species. The insect larvae is the environment in which the *B. thuringiensis* strains can germinate the spores and multiply the vegetive cells more efficiently. Then, this environment can be considered the ecological niche of this bacteria, where propably the genome evolution occurs through different mechanisms such as genetic exchange.

4.2 Plasmid and adaptation

In 1979, Godwin and Slater discussed plasmids and their costs to bacterial cells. Since them, research is focused in their persistence, replication and spread. This is mainly due to their clinical importance, carrying many virulence and antibiotic resistance genes.

It is known that plasmids have an energy cost that can arise from several mechanisms, such as metabolic burden, plasmid copy number, expression of plasmid-borne genes and interference between the regulatory systems of plasmid and host cell (Harrison and Brockhurst, 2012). Theory predicts that if plasmids encode beneficial traits to their hosts, they are probably maintained by selection and the integration of the benefits genes into the bacterial genome. This could avoid the costs of plasmid carriage (Bergstrom et al., 2000). Contrary, if the plasmids have no beneficial traits they can be lost (Dahlberg and Chao, 2003). All these issues difficult the understanding how plasmids can persist in bacterial populations, and this evolutionary dilemma can be referred as the ‘plasmid paradox’ (Harrison and Brockhurst, 2012). Plasmids have a high metabolic cost to bacterial cells, to an intriguing question is: How are plasmids able to remain in a bacterial population? Four key factors are mentioned: (i) advantages of genes carried by the plasmid, (ii) horizontal transfer, (iii) compensatory adaptation, and (iv) persistence mechanisms. The main advantage brought by the plasmids is their function as a simple maintenance mechanism that acts in the short term, while the environmental conditions require them. The horizontal transfer

mainly occurs by conjugation and allows the spread of plasmids, so they are maintained in bacterial populations as parasites. The segregational loss is offset by the transfer of plasmids to a greater number of individuals. Compensatory adaptation involves mutations in chromosome and plasmid which alleviate the cost of plasmid carriage. Finally, several persistence mechanisms have been described that ensure plasmid presence in bacterial populations (Bahl, Hansen and Sørensen, 2009; San Millan *et al*., 2014).

Nowadays, evolutionary biology and ecology of plasmids are little investigated. Distinct views about the relationship between plasmids and their bacterial hosts are investigated, some researchers considerate the plasmids as (i) genetic parasitic, that exploits the host machinery and offset their costs by conjugating and infecting new hosts, then they persist in a bacterial population (Modi and Adams, 1991; Lili et al., 2007; Smith, 2010); (ii) elements that carry intermittent selected traits (Lilley and Bailey, 1997); (iii) 'domesticated' accessory genomes, as pXO2 that loses its conjugational ability (Hu et al., 2009) or (iv) altruist elements, encoding extracellular traits that promote the fitness of non-producer cells (Raymond and Bonsall, 2013). Although these controversy plasmids are essential tools for adaption and evolution in prokaryotes (Gogarten and Townsend, 2005).

Accessory genes encoded by plasmids can be classified into three functional groups: (i) the genes encoding for resistance and toxin genes such as antibiotics and heavy metals, (ii) the genes involved in virulence, which allow the host to inhabit and exploit other organisms, and (iii) the genes related to metabolic functions, such as nitrogen fixation (Harrison and Brockhurst, 2012). The well-known example of plasmid adaptation described involves antibiotic resistance genes, which was first recognized in Japan when susceptible and multiresistant strains were isolated from the same patient. This fact suggests that multiple resistance was acquired from genetic determinants in a single step and not by several mutations (Ramirez *et al*., 2014). Tamminen and coworkers (2012) identified 2.678 sequences putatively associated with antibiotic resistance, belonging to 501 different plasmids. Another study with hospitalized patients identified several antibiotic-resistant genes in gut microbiome plasmids. In addition, the same genes were also found in a healthy control subject, although they were not linked with antibiotic usage or plasmid type, indicating that the mechanism involving the spread of these genes is complex (Jitwasinkul, *et al*., 2016). Nowadays, the spread of these genes is a threat to human health due to the emergence of innumerous multiresistant pathogenic bacterial strains (Alekshun and Levy, 2007).

Virulence capacity may be also controlled by plasmids, as evidenced in a study with *Vibrio nigripulchritudo*, which requires both pB1067 and pA1066 plasmids, finding a high level of shrimp mortality. In addition, isolates without these plasmids are non-pathogenic. This allows concluding that the plasmids are driving forces for the emergence of pathogenic strains (Le Roux, 2011). In the *Bacillus cereus* group, plasmids encode important phenotypic traits, such as Cry and Vip toxins in *B. thuringiensis*, cereulide in *B. cereus* and tripartite toxin and capsule in *B. anthracis*. The plasmid profile of this group is complex. In *B. thuringiensis*, for example, it varies in size (2-80 MDa) and number (1-17) (Lereclus, *et al*., 1982). The horizontal transfer mechanism has an important role in bacterial diversity and evolution, leading to the acquisition of novel functions that allow colonization of new environmental niches. A recent study demonstrated that *B. thuringiensis* strains with higher insecticidal activity also possessed a larger number of different plasmid types, explaining their ability to adapt to different hosts (Zhu, *et al.* 2015). In addition, Patiño-Navarrete and Sanchis (2016) compared the proportion between total plasmid size and whole genome size. In *B. thuringiensis*, the extrachromosomal elements have a higher proportion of total genome than *B. cereus* and *B. anthracis*, suggesting that this high plasmid content is linked with the ability to adapt to invertebrate hosts (Zhu, *et al.* 2015). Even with this information, these plasmids encoded several proteins not already characterized (unknown functions). Thus, additional studies are important to describe genes potentially involved in *B. thuringiensis* adaptation to specific niches.

Besides adaptation, plasmids are also tools for bacterial genomic evolution. Some studies have demonstrated that evolved plasmids confer a fitness advantage to bacteria, such as the R1 and RP4 plasmids in *Escherichia coli* and *Salmonella enterica* cells (Dahlberg and Chao, 2003; Dionisio *et al*., 2005). Another important question is how the plasmids themselves adapt to their hosts. Recent studies have observed the role of bacterial host species heterogeneity in plasmid evolution. Analyzing, successive plasmid passages through single-host and multi-host environments, the results showed that the plasmid stability was enhanced after several passages in a single host. In addition, they observed a fitness trade-off in plasmids adapted to a single host, and this trade-off is circumvented when the plasmid is exposed to multiple hosts. In other words, the plasmids exposed to single host were selected for host-specialist plasmids becoming less costly, while the adaptation of plasmids exposed to multiple hosts was not constrained, leading to the evolution of a host-generalist plasmids (Kottara, 2016).

Thus, plasmids are strong drivers of the evolution of specialism and have strong influence in bacterial communities, carrying genes with ecologically important traits for bacterial adaptation and also evolution of their genomes (Slater *et al*., 2008). Besides carry important genes, plasmids allow the rapid dissemination of these traits in a bacterial population (Smillie *et al*., 2010).

Section II-CELLULAR COMMUNICATION AND LIFECYCLE

1 Quorum Sensing

1.1 Definition

Quorum sensing (QS) is a bacterial cell-cell communication process responding to fluctuation in cell-population density. The release in the extracellular medium and the detection of specific chemical signals called autoinducers (AIs) allow bacteria to coordinate gene expression, and consequently to behave as a cohesive group. In this view, QS systems could be considered an early step in the development of multicellularity (Miller and Bassler, 2001). Important cellular processes are regulated and coordinated by QS systems, allowing the bacterial community to cope with the environmental changes (Rutherford and Bassler, 2012; Bassler and Vogel, 2013). Several systems are described in Gram-positive and Gram-negative bacteria and three sorts of QS are classified according to the chemical molecules used for communication: acyl-homoserine lactones (AI-1) used by Gram-negative bacteria; small auto-inducing oligopeptides (AIP) used by Gram-positive bacteria and cyclic furanone-based compounds (AI-2) used by both Gram-negative and Gram-positive bacteria (Rutherford and Bassler, 2012). In all systems, after synthesis, the autoinducers (signaling molecules) are exported and their concentration increases according to population density. When the autoinducer concentration reaches a certain threshold, the signal is detected by a specific sensor located in the cytoplasm or in the membrane of the related bacteria. This event triggers the expression of a set of genes which determines a group behavior (Slamti *et* al., 2014; Hawver, Jung and Ng, 2016).

1.2 Gram-negative systems

Gram-negative bacteria use small molecules as AIs to communicate: acyl-homoserine lactones (AHSLs), alkyl quinolones, α-hydroxyketones and fatty acid-like compounds (Hawver, Jung and

Ng, 2016). These signaling molecules are synthetized from a common substrate, S-adenosylmethionine (SAM) and AHL production seem to be restricted to Proteobacteria species (Cha, 1998; Wei, 2011). There are two mechanisms of action depending on the detection mode, described in Figure 18. The first type is described as one-component QS system. In this mode of action, the signaling molecules (AIs) are produced by the AI synthase and exported to the extracellular medium. Then, they are imported back into the cytoplasm and bind to quorum sensors acting as transcriptional regulators (Figure 18a).

In the second type, named two-component QS system, the signaling molecules (AIs) are also produced and released into the extracellular environment. Then, they bind to a transmembrane receptor (Figure 18b). This interaction triggers a phosphorelay controlling the activity of a transcription factor which modulates gene expression (Rutherford and Bassler, 2012; Hawver, Jung and Ng, 2016). In Gram-negative bacteria, these systems involve two mainly proteins, an R protein acting as a transcriptional regulator and an I protein acting as autoinducer synthase, which synthetizes the signaling molecule (AHL) (Lazdunski, Ventre and Sturgis, 2004).

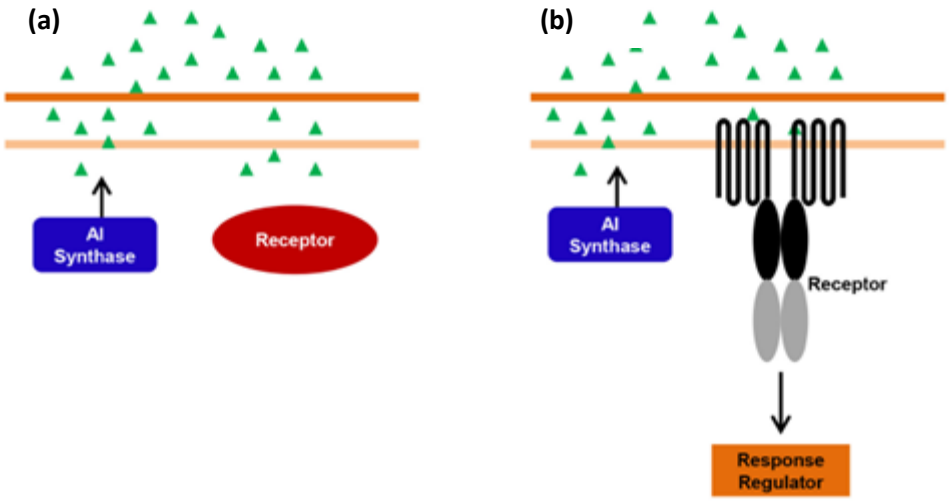

Figure 18. Quorum sensing systems in Gram-negative bacteria. One-component system (a), the autoinducers are produced and released into the extracellular medium, then they are imported back into the cytoplasm, where they are detected by QS specific sensors acting as transcriptional regulator. Two-component system (b) involves a transmembrane receptor that controls the activity of the response regulator (Hawver, Jung and Ng, 2016).

In Gram-negative bacteria several processes are coordinated by QS systems (Table 2). The first and the most known QS system (LuxI-LuxR) was described more than forty years ago in the Gram-negative bacterium *Vibrio fischeri* and controls the bioluminescence induction. LuxI-LuxR system controls the expression of the luciferase operon and regulates the light production (Nealson, 1970). In this system, the AI synthase, LuxI, catalyzes the production of the signaling molecules (AIs), used as cognate signals by LuxR (Figure 19) (Stevens and Greenberg, 1997; Ng and Bassler, 2009). LuxR serves as both: the cytoplasmic AI receptor and the transcriptional activator of the *lux* operon. LuxR is composed of two distinct domains, a ligand binding domain located in N-terminal and a DNA-binding domain located in the C-terminal (Shadel, Young, and Baldwin, 1990; Choi, 1991, 1992). LuxR proteins are very unstable and rapidly degraded when they are not bound to the AI. At high intracellular concentrations, AI binds to LuxR, inducing conformational changes, which allows the bind to DNA and then the transcriptional activation of target genes, including the *lux* operon (Zhu and Winans, 2001).

Table 2. Examples of QS systems in Gram-negative bacteria.

Organism	***I/R* genes**	**QS-regulated phenotypes**	**References**
Vibrio fischeri	*luxR,luxI*	Bioluminescence Colonization factors	Stevens and Greenberg, 1997
Pantoea stewartii	*esaR,esaI*	Exopolysaccharide production	von Bodman, Majerczak and Coplin, 1998
Agrobacterium tumefaciens	*traR,traI*	Virulence plasmid copy number and conjugal transfer	Fuqua and Winans, 1994
Pseudomonas aeruginosa	*lasR,lasI* *rhlR,rhlI*	Virulence, biofilm formation other cellular functions	Schuster *et al*., 2003
Rhizobium leguminosarum biovar *viciae*	*rhiR,rhiI* *raiR,raiI* *bisR,traR, traI* *cinR, cinI*	Nodulation efficiency Function unknown Plasmid transfer Growth inhibition	González and Marketon, 2003; Gilson, Kuo and Dunlap, 1995; Lithgow *et al*., 2000.

A LuxI/LuxR system has been described in more than 100 other bacteria species (Case, Labbate and Kjelleberg, 2008). The QS AIs share a common homoserine lactone group and chemical diversity between the intraspecies is due to differences in length and decorations of the side chains which confer a high specificity (Ng and Bassler, 2009).

Another well-known QS system is the TraI-TraR system described in *Agrobacterium tumefaciens*. This system regulates the transfer of the Ti plasmid from the bacterium to the plant cell, causing tumor formation in the host. The TraR regulator activates *tra* and *tbr* genes which stimulate the transfer of Ti plasmid (Piper, von Bodman and Farrand, 1993; Zhu and Winans, 2001).

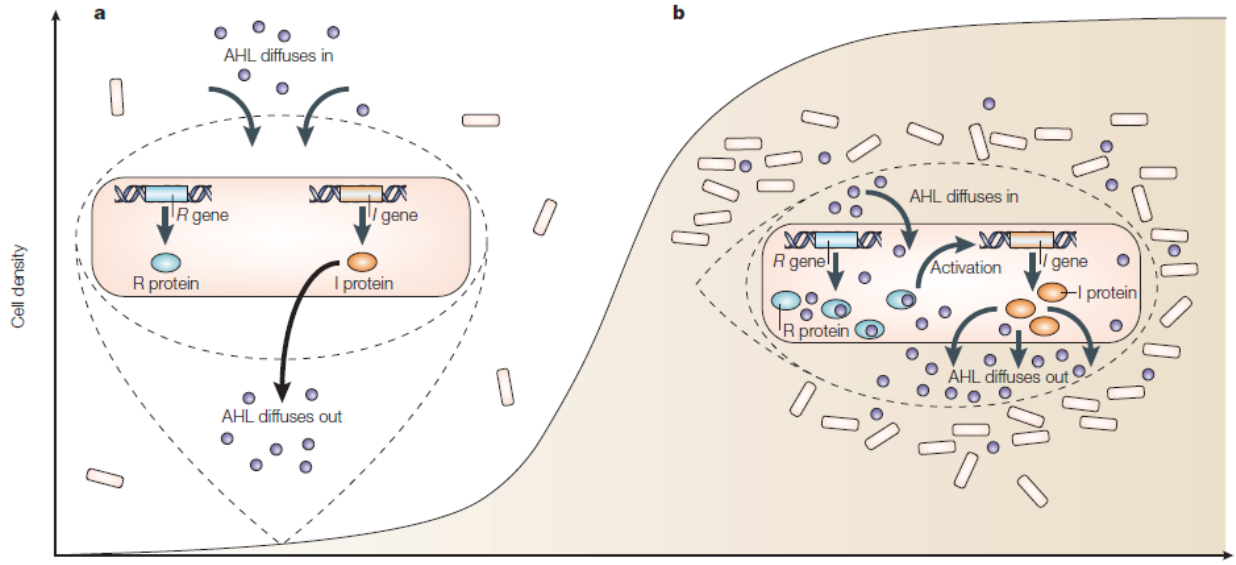

Figure 19. Mode of action of R/I QS systems. The *R* genes encode a transcriptional regulator and the *I* genes encode an AHL synthase. The AHL are synthetized, exported and accumulate in the extracellular medium. When density is high and a threshold of AHL molecules is reached, The R proteins bind to their cognate AHL forming a complex that controls the transcription of target genes. The amplification of the AHL signal is obtained by a positive feedback, resulting from the activation of *I* gene transcription by the complex R protein/AHL (Lazdunski, Ventre and Sturgis, 2004).

1.3 Gram-positive systems

Communication in Gram-positive bacteria relies on the principles common to all QS systems. However, the signaling molecules are mainly oligopeptides (AIs) (Table 3). Gram-positive communication involves several steps: propeptide production, export, extracellular processing and reimport. The oligopeptides are encoded as precursors (inactive form) and differ from each other in sequence and structure (Stephenson *et al*., 2003; Okada *et al*., 2005; Thoendel *et al*., 2011). They are exported via the secretory pathway and are processed in the extracellular medium, generating their active form. The signaling molecules can act through two different pathways (one and two-component systems): (i) they are imported into the bacteria by an oligopeptide permease (Opp) system and binds to a cytoplasmic receptor (Figure 20a), or (ii) they bind to a transmembrane sensor kinase, which is autophosphorylated in conserved histidines (Figure 20b). Then the phosphoryl group is transferred to a cognate cytoplasmic response regulator protein (RR), which controls the expression of the target genes.

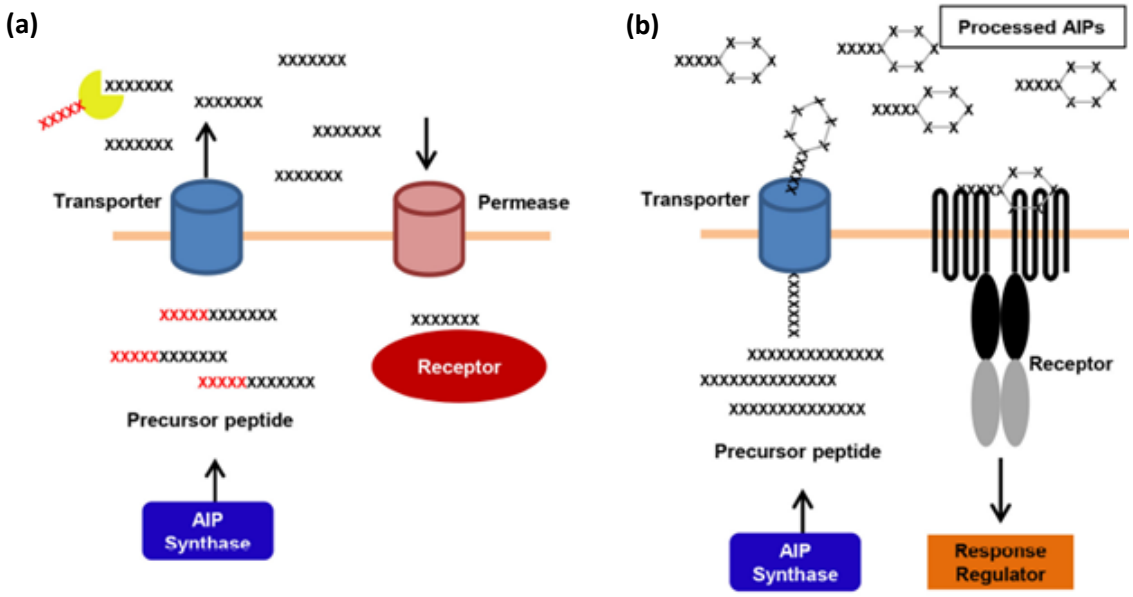

Figure 20. Gram-positive QS systems. The one-component system (a). The oligopeptides are synthetized and released in the extracellular medium in a pro-active form through a transporter of the secretory machinery. Then, they are processed and the active peptides are reimported through a permease (Opp). In the cytoplasm, the signaling molecules bind to a QS receptor acting as a transcriptional regulator or by modulating protein function. The two-component system (b). The peptides bind a transmembrane receptor which triggers a phosphorelay cascade controlling the activity of QS response regulators (Hawver, Jung and Ng, 2016).

Table 3. Examples of QS systems in Gram-positive bacteria (Gobbetti *et al*., 2007).

Organism	Signaling molecules	QS-regulated phenotypes	References
B. thuringiensis	PapR	Virulence	Agaisse, *et al*., 1999; Gohar *et al*., 2002, 2008
B. thuringiensis	NprX	Necrothrophism	Chitlaru *et al.*, 2006; Perchat *et al.*, 2011
B. anthracis	Phr	Sporulation, conjugation, biofilm, protease production	Koetje *et al.*, 2003; Bongiorni *et al*., 2006; Singh *et al*., 2013; Boguslawski, Hill and Griffith, 2015
B. subtilis	ComX, CSF subtilin	Competence, sporulation and lantibiotic synthesis	Auchtung, Lee and Grossman, 2006
Carnobacterium maltaromaticum	AMP-like peptide pheromone (CS)	Class II bacteriocin synthesis	Rohde and Quadri, 2006
Enterococcus faecalis	GBAP, FsrB, CyIL AMP-like peptide pheromone (EntF)	Virulence Class II bacteriocin synthesis	Haas, Shepard and Gilmore, 2002
Lactococcus lactis	Nisin	Lantibiotic synthesis	Kuipers *et al*., 1998
Staphylococccus aureus	AIP, AgrD (agr system)	Virulence	Ji, Beaves and Nocivk, 1995; Novik *et al*., 1995
Streptococcus pneumoniae	CSP, BlpC	Virulence, competence	Cvitkovitch, Li and Ellen, 2003

A well-studied QS system in Gram-positive bacteria is the Agr system of *Staphylococcus aureus* (Figure 21). *S. aureus* is a benign human commensal *Bacillus* that can become a lethal

pathogen in the host's tissues (Massey *et al*., 2006). The Agr system controls gene expression in function of cell density: in low concentration, bacteria develop a colonization lifestyle and when the density is high they shift to a dissemination lifestyle, secreting of toxins and proteases (Lyon and Novick, 2004). The Agr system consists of a propeptide, encoded by *agrD*, a kinase encoded by *agrC*, a transmembrane transporter encoded by *agrB* and a response regulator encoded by *agrA* (Ji, Beaves and Nocivk, 1995; Novick *et al*., 1995). First, the propetide (AgrD) is synthetized and exported by the AgrB protein, which also contributes to maturation of the propeptide (Saenz *et al*., 2000). In the extracellular medium, the active form of AgrD binds to AgrC, inducing phosphorylation of AgrA (AgrA-P). AgrA-P induces: (i) expression of a regulatory RNA (RNAIII) (P3 promoter) that suppresses expression of cell adhesion proteins and activates the expression of secreted factors; and (ii) expression of the *agrBDCA* operon (P2 promoter), which leads positive feedback, increasing the peptide concentration (Novick *et al*., 1993).

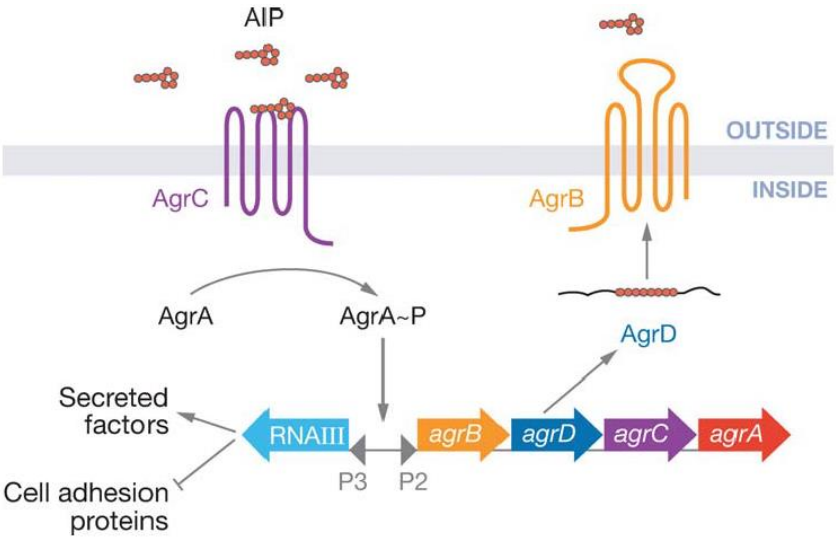

Figure 21. QS using a two-component system in *Staphylococcus aureus*. The mechanism of action is described in the text (Waters and Bassler, 2005).

2 The RNPP Family

2.1 General description

The study of QS in Gram-positive bacteria led to the characterization of a new family of QS regulators, the RNPP family, named according to their regulatory proteins: **R**ap phosphatases from *B. subtilis* and *B. cereus group*, **N**prR from *B. cereus* group, **P**lcR from *B. cereus* group, and

PrgX from *Enterococcus faecalis* (Declerck *et al*. 2007). These systems involve signaling molecules that are sensed intracellularly by the cytosolic regulatory proteins (one-component model). These proteins share three similarities: (i) they are found only within the Firmicute phylum; (ii) the oligopeptides necessary for QS regulation are produced in pro-active form, after they are exported, processed and reimported into the bacteria, where they interact directly with their regulatory protein; and (iii) the signaling peptides are encoded by a small gene located downstream from the regulatory protein coding sequence (Declerck *et al*., 2007). Furthermore, the RNPP members have six to nine tetratricopeptide repeats (TPR) in the C-terminal, know to allow interactions with other proteins or peptides (Perego, 2001; Declerck *et al*., 2007).

All RNPP members, except Rap proteins, have an additional helix-turn-helix (HTH) domain, in the N-terminal, which interacts with DNA (Figure 22). All those shared characteristics suggest a common ancestor for the proteins grouped in the RNPP family, where Rap would be the ancestor, followed by the other members that incorporated the HTH domain (Perchat *et al*., 2011; Perchat, *et al*., 2016).

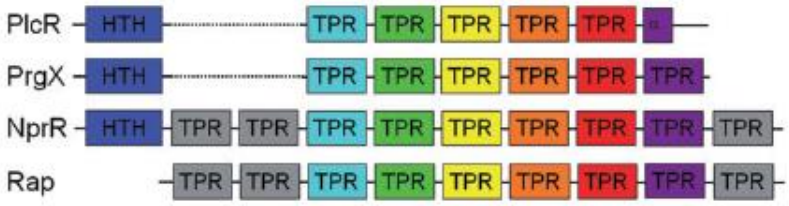

Figure 22. Schematic representation of the structure of RNPP proteins. HTH DNA binding domain, located in the N-terminal are represented in blue. The others colored boxes represent the TRP domains (six to nine) (Perchat *et al*., 2011).

All these systems share the similar mechanism of signaling described above (Gram-positive one-component system). The binding of oligopeptides to regulatory protein in the cytoplasm induces allosteric changes, which activates or inhibits the regulatory protein activity (Perego, 2013; Grenha, *et al*., 2013).

2.1.1 PrgX/cCF10

The QS system of *Enterococcus faecalis*, PrgX/cCF10, is encoded by genes located on the plasmid pCF10. PrgX positively regulates the expression of conjugative transfer genes present in the plasmid (Kozlowicz, Dworkin and Dunny, 2006). The mode of action of this system is represented in Figure 23.

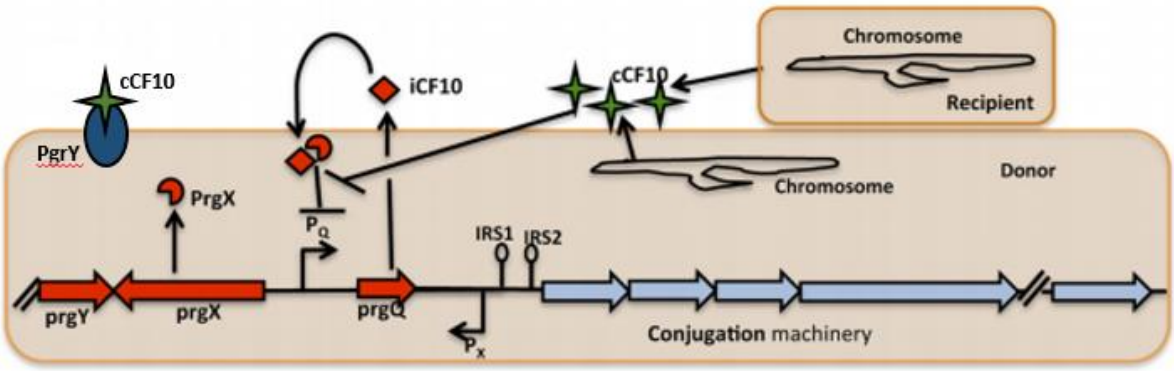

Figure 23. Schematic representation of the QS system of *Enterococcus faecalis*, PrgX/cCF10. Mechanism of action is described in the text (Adapted from Singh and Meijer, 2014).

Contrary to the previous examples, the PrgX/cCF10 system mediates communication between two different cell types: the donor cells harboring the plasmid pCF10 and the plasmid free recipient cells. This system regulates the expression of the conjugation genes present in the donor cells in response to recipient cell density. The pheromone (cCF10) is produced by both cell types; however, evolution selected a mechanism to avoid self-induction, and thus conjugation is stimulated only by the pheromone produced by recipient cells (Kozlowicz, Dworkin and Dunny, 2006). This mechanism involves two proteins, PrgY, a transmembrane protein that sequester, modify or degrade the pheromone produced by the donor cell, without affecting the interaction with the pheromones produced by the recipient cell. The second is iCF10, an inhibitor peptide that neutralizes the donor pheromone (Chandler *et al*., 2005; Kozlowicz, Dworkin and Dunny, 2006). In donor cell, the regulation of genes under control of the transcriptional regulator, PrgX, depends on the ratio cCF10/iCF10. In low cell density, the iCF10 is complexed with PrgX (tetramer form), inhibiting its activity, and repressing the main conjugation promoter, P_Q. In high cell density, the concentration of the heptapeptide cCF10 produced by recipient cells increases

and competes with iCF10, forming the complex cCF10/PrgX (dimer form). Both peptides, cCF10 and iCF10, interact with PrgX, however, to be active these peptides must be processed by proteases. The active molecules correspond to the C-terminal end of the peptides. They are composed of seven amino acids: LVTLVFV and AITLIFI for cCF10 and iCF10, respectively.

The complex PrgX/cCF10 induces a conformation change in PrgX which releases the repression of the P_Q promoter (Shi *et al*., 2005). The activation of the P_Q promoter allows the expression of conjugative transfer genes, also the expression of Asc10, a protein that promotes aggregation and iCF10 to ensure the system to return to the default OFF state (Dunny, 2007).

2.1.2 PlcR/PapR

PlcR is the major regulator of virulence in *B. cereus* group and regulates genes at the end of exponential growth phase. PlcR regulates about 45 genes that encode several degradative enzymes, cytotoxins, hemolytic and non-hemolytic enterotoxins and phosphatidylinositol-specific phospholipase C (PI-PLC). These proteins are involved in food supply, cell protection and environmental sensing. The secretome of genes controlled by PlcR during the early stationary phase is about 80% (Agaisse *et al*., 1999; Gohar 2002, 2008). Salamitou *et al*. (2000) observed a drastic reduction in virulence in *plcR* mutants of *B. thuringiensis* and *B. cereus* strains in insect and mouse infection models. PlcR is activated by PapR peptide, encode by *papR* gene, located downstream the *plcR* gene. Reduction in virulence was also identified in a *papR* mutant (Slamti and Lereclus, 2002). Then, in this QS system PlcR is the regulatory protein and PapR is the cognate signaling peptide. PapR is produced, exported and processed in the extracellular medium. The minimal active form of PapR is mainly the ADLPFEF heptapeptide, located in the carboxy-terminal end of the peptide (Declerck *et al*., 2007; Bouillaut *et al*., 2008). The presence of a putative signal for secretion suggests that PapR propeptide is exported by the general secretion Sec pathway. PapR extracellular maturation depends on NprB, a metalloprotease regulated by PlcR and other unknown proteases (Pomerantsev *et al*., 2009; Slamti, unpublished results). The mature PapR peptide is reimported to the cytoplasm via the oligopeptide permease Opp (Gominet *et al*., 2001). Finally, inside the bacterium, PapR interacts with PlcR promoting allosteric changes, allowing its binding to PlcR boxes (TATGNANNNNANCATA) (Slamti and Lereclus, 2002) (Figure 24). The molecular activation mechanism of PlcR is due to PapR binding in the TPR domain of PlcR, inducing an allosteric change in HTH domains. This changes form a drastic

kink in the helix linking the two domains of PlcR dimer in the two half sites of PlcR box (Grenha *et al*., 2013).

A PlcR box was identified in the promoter regions of *plcR* and *papR* genes, evidencing that their transcription is autoregulated (Lereclus *et al*., 1996; Agaisse *et al*., 1999). Also, *plcR* transcription is negatively regulated by Spo0A (Lereclus *et al*., 2000), thus in a sporulation-specific medium, the expression of PlcR regulon is completely prevented. The global regulator CodY is also involved in PlcR control (Frenzel *et al*., 2012; Lindbäck *et al*., 2012). It was shown that CodY activates the expression of the PlcR regulon via production of the Opp proteins required for reimporting PapR (Slamti *et al*., 2016).

The PlcR/PapR system is strain-specific. Four specificity groups were determined by differences in a few residues of the signaling peptide and of the response regulator (Slamti and Lereclus, 2005; Bouillaut *et al*., 2008). The PlcR regulon of a strain can be activated by its cognate signaling peptide, which belongs to the same specificity group. However, the PlcR regulon is not, or is poorly, activated by the signaling peptide produced by other specificity groups. This characteristic suggests co-evolution of PlcR and PapR, which may reflect specificity in ecological niches (Slamti *et al*., 2014).

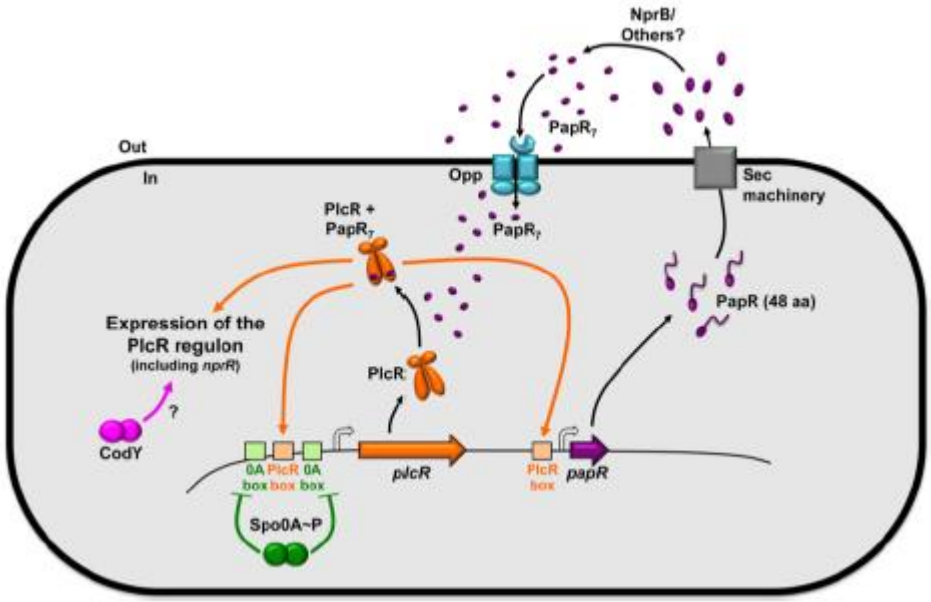

Figure 24. Schematic representation of the QS system of *B. cereus* group, PlcR/PapR. Mechanism of action is described in the text (Slamti *et al*., 2014).

2.1.3 NprR/NprX

NprR was first identified as the activator of the *nprA* gene encoding the major extracellular protease produced by the bacteria of the *B. cereus* group during sporulation (Chitlaru *et al.*, 2006; Perchat *et al.*, 2011). NprR is activated during sporulation and controls at least 41 genes (Dubois *et al.*, 2012). These genes encode degradative enzymes (proteases, lipases and chitinases) and kusrtakin, a lipopetide involved in biofilm formation (Gélis-Jeanvoine *et al.*, 2016). Unlike PlcR/PapR, the NprR/NprX system is not involved in the virulence of bacteria. However, it was shown that the NprR regulon allows bacteria to survive in insect cadavers. Thus, this property indicates that NprR is required for the necrotrophic lifestyle of *B. thuringiensis* (Dubois *et al.*, 2012). This allows bacteria to survive and possibly sporulate in insect larvae, which may improve their ability to disseminate in the environment.

To act as a transcriptional regulator, NprR must be associated with its cognate peptide NprX.

Like PapR, NprX seems to be exported via the common secretory Sec pathway. The extracellular processing of the NprX pro-peptide (43 amino acids) has not yet been described. The minimal active form of NprX is composed of seven amino acids, located in the central part of the C-terminal region (Perchat *et al.*, 2011). The reimport of mature form of NprX is mediated by several oligopeptide permease systems (Dubois *et al*., 2012). The peptide sequences of NprR and NprX in the *B. cereus* group contain seven pherotypes, which are strain specific and like PlcR/PapR may reflect niche specificities. Together, NprR-NprX forms a tetrameric structure which allows the HTH domain of NprR to bind on specific DNA sequences (Zouhir, *et al*., 2013). The mode of action of the NprR-NprX QS system is illustrated in Figure 25.

The *nprR* and *nprX* genes are co-transcribed (Dubois *et al.*, 2013). The upstream promoter *nprR* is negatively controlled by CodY during the exponential growth phase and is positively controlled by PlcR during the stationary phase. CodY is a global transcriptional regulator of the stationary phase which represses genes related to metabolism in Gram-positive bacteria (Sonenshein, 2005). Its activity depends on cofactors such as branched-chain amino acids and GTP. During the early stationary phase, when nutrients are limited in the medium, the molecules necessary for its activation are scarce and genes regulated by CodY are derepressed. The regulation by CodY suggests a link between the nutrient availability and the expression of the NprR regulon (Dubois *et al.*, 2013). The negative regulation by PlcR at the onset of stationary phase indicate a link between the virulence and necrotrophism (Dubois *et al*., 2013).

Two additional promoters, depending on the sporulation-specific sigma factors σ^H and σ^E, are involved in the expression of *nprX*. These regulators ensure the presence of enough NprX to activate NprR and maintain the expression of the NprR-regulated genes during stationary phase (Dubois *et al.*, 2013).

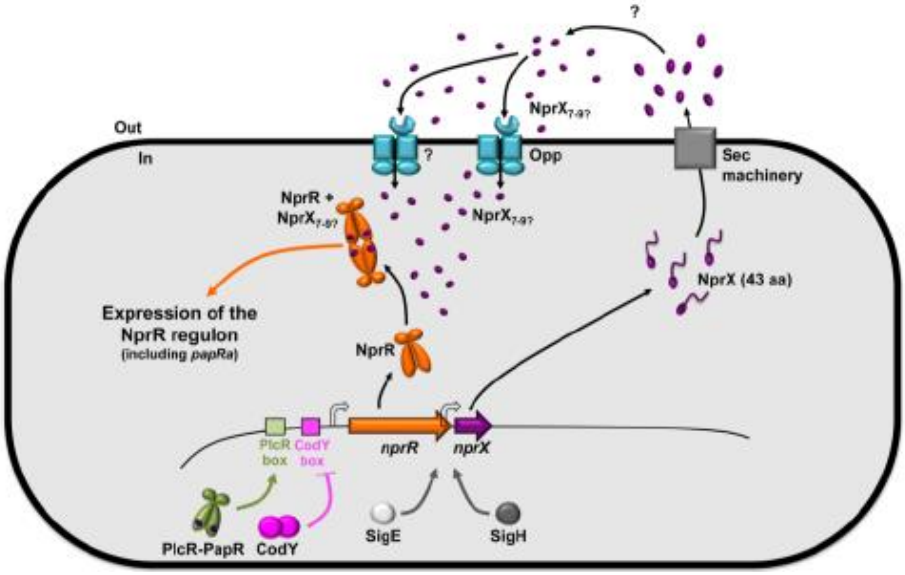

Figure 25. Schematic representation of the QS system of *B. cereus* group, NprR/NprX. Mechanism of action is given in the main text. (Slamti *et al*., 2014).

In summary, NprR acts as a transcription factor when binding to its cognate peptide NprX. In the presence of NprX, NprR adopts a tetrameric conformation, allowing it to bind to specific DNA sequences, regulating genes involved in the survival of these bacteria in insect larvae. However, a new function to NprR was recently described. NprR, in the absence of NprX, adopts a dimer conformation, which negatively controls sporulation. NprR prevents the phosphorylation of Spo0F, inhibiting the phosphorylation cascade (Perchat *et al.*, 2016). This function displays similarities with the Rap proteins which are described below. So, NprR is a bifunctional sensor, a dimeric sporulation inhibitor and tetrameric transcription factor. These two functions provides benefits to bacteria of *B. cereus* group, coordinating cell density, necrotrophism and sporulation events.

2.1.4 Rap/Phr system

Rap proteins consists of phosphatases and have been extensively studied in *B. subtilis*, which encodes 11 chromosomal genes (RapA to RapK) (Kunst *et al*., 1997). Several processes have been described as being controlled by Rap proteins. In *B.* subtilis, studies described 11 Rap proteins (RapA-RapK). RapA, RapB, RapE, RapI and RapJ control sporulation. RapC and RapF regulate competence. RapH acts by controlling both pathways. RapI controls the transfer of a transposon, ICEBs1. RapG inhibits the response regulator DegU, preventing protease production. Others Rap proteins have also been described controlling biofilm formation. The last two Rap proteins, RapD and RapK, have no described function (Perego *et al.*, 1994, Perego *et al.*, 1996; Auchtung, Lee and Grossman, 2006; Bongiorni *et al*., 2005; Parashar, Jeffrey; Neiditch, 2013; Perego, 2013).

Rap proteins are composed of two principal domains. The C-terminal domain consists of TPRs, which form two antiparallel α-helices, recognizing and binding to the response regulator (RR) and the signaling peptide (Core and Perego, 2003, Parashar, Jeffrey; Neiditch, 2013). A second domain (N-terminal) folds as a 3-helix bundle and interacts with its targets, in most cases a transcription factor (Parashar *et al*., 2011). Rap proteins can be organized in two subgroups depending of their action mechanism. The first group binds to different RR, inhibiting its DNA recognition, thus impairing the expression of RR dependent genes (Baker *et al*., 2011, Parashar *et al*., 2011). The second group affects gene expression by dephosphorylating RR (Perego and Hoch, 1996).

Rap proteins' activity is inhibited by their cognate peptides Phr. Eight of the 11 Rap proteins described in *B. subtilis* have their own Phr. However, cross-talk between different systems is possible (Auchtung, Lee and Grossman, 2006; Bongiorni *et al*., 2005; Parashar, Jeffrey and Neiditch, 2013; Perego, 2013; Perego and Hoch, 1996). Phr-encoding genes are located downstream from *rap* genes and are generally co-transcribed (Perego and Brannigan, 2001). However, in most cases the *phr* gene has a secondary promoter controlled by the alternative sigma factor, σ^H. This promoter ensures the presence of sufficient Phr to inhibit Rap activity during the stationary phase (McQuade, Comella and Grossman, 2001; Pottathil and Lazazzera, 2003). Phr is produced as a pro-peptide of approximately 40 amino acids composed of a hydrophobic N-terminal portion and a hydrophilic C-terminal. These domains are separated by a putative type signal peptidase I cleavage site (Perego, Glaser and Hoch, 1996). The

hydrophobic domain and the cleavage site are necessary to export the molecule via the Sec pathway. Once in the extracellular medium, the Phr peptides (generally 19 amino acids) are processed in *B. subtilis* by three members of the subtilisin family of proteases, subtilisin, Epr and Vpr (Lanigan-Gerdes *et al*., 2007). Subsequently, the mature Phr peptides are reimported into the cytoplasm of the bacterium by Opp systems (Lazazzera *et al*., 1997, Perego, 1997). In *B. subtilis*, the active form is composed of the last five amino acids in the C-terminal (Rudner *et al*., 1991, Ishikawa, Core and Perego, 2002), which bind to Rap proteins, inhibiting their activity (McQuade, Comella and Grossman, 2001).

Sporulation and biofilm formation are under Spo0A control and thus depend on its phosphorylation level (Hamon and Lazazzera, 2001, Fujita, González-Pastor and Losick, 2005). Sporulation is initiated by the detection of different external signals, including cell density and starvation (Ireton *et al*., 1993). A multiple histidine kinase complex is autophosphorylated and then the phosphoryl group is transferred to Spo0F. Spo0F~P transfers the phosphoryl groups to Spo0B, and finally to Spo0A, the master transcriptional regulator for the start of sporulation (Burbulys, Trach and Hock, 1991). Biofilm formation is repressed by the master regulator SinR (Kearns *et al*., 2005) and a secondary protein, AbrB (Strauch *et al*., 1990), and both are necessary to inhibit matrix biosynthesis. Spo0A~P negatively regulates a*brB* and positively *sinI*, an antagonist of SinR. Then Spo0A~P triggers biofilm formation (Gaur, Dubnau and Smith, 1986, Hamon and Lazazzera, 2001). Rap proteins dephosphorylate Spo0F, decreasing the concentration of Spo0A-P, which inhibits sporulation and biofilm formation (Perego and Hoch, 1996). With increasing of cellular density, Phr cognate peptide reach the necessary level to inhibit Rap protein, this allows the formation of Spo0A~P, which at certain level trigger sporulation (Ishikawa, Core and Perego, 2002).

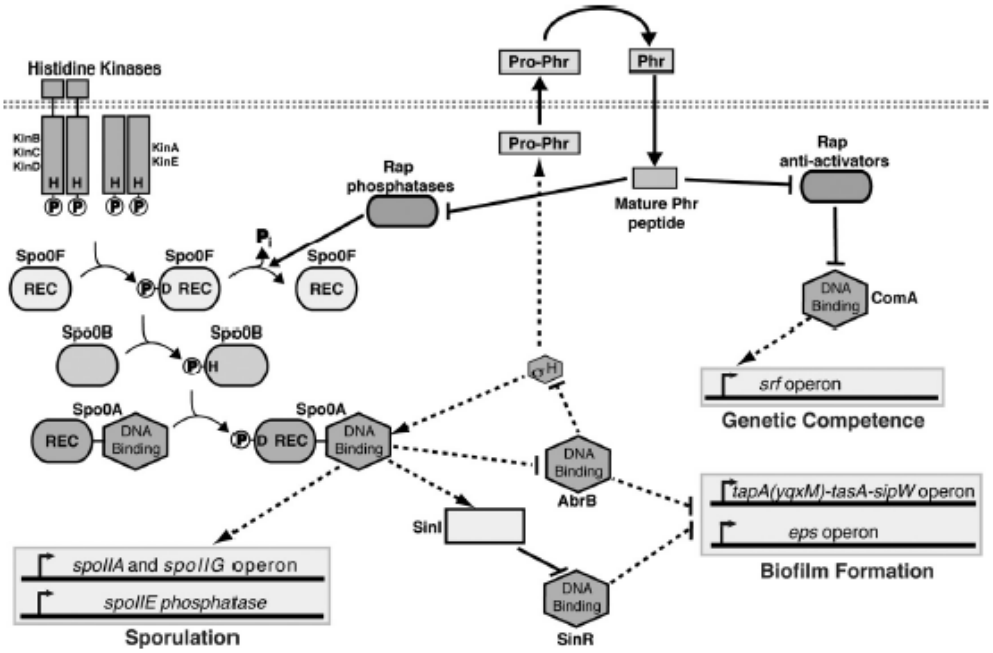

Figure 26. The control of sporulation and biofilm formation by Rap-Phr systems. The phosphorelay is activated by five histidine kinase which transfers the phosphoryl group to Spo0F. Then, Spo0B, a phosphotransferase, is an intermediated protein that transfers the phosphoryl to Spo0A (Spo0A-P), the response regulator and transcription factor. Spo0A-P triggers sporulation and biofilm formation. Some Rap proteins control the sporulation initiation by the capacity to dephosphorylate Spo0A~P, inhibiting the transfer of phosphoryl group to Spo0F. In turns, Phr is encoded and to be active has to be externalized, process and re-imported. Phr active peptide interacts with Rap protein inhibiting its activity so derepressing these processes. Arrows and perpendiculars lines: positive or negative effect, respectively. Solid lines: regulation by protein-protein interaction, and dashed lines: regulation at the level of gene transcription. REC, receiver domain; H, histidine; D, aspartic acid; P, phosphoryl group (Parashar *et al*., 2013).

3 *B. thuringiensis* infectious lifecycle in insect larvae

The ecology of *B. thuringiensis* and its lifecycles have been discussed with two main viewpoints: *B. thuringiensis* as an insect pathogen or as a saprophytic soil bacterium. Several studies indicate that *B. thuringiensis* can be isolated from insects, nematodes, earthworms and terrestrial crustaceans (Chaufaux, *et al*., 1997; Hendriksen and Hansen, 2002; Swiecicka and Mahillon, 2006; Visôtto *et al*., 2009). *B. thuringiensis* can be considered a true pathogen, due to a

set of virulence genes involved in infection (Agaisse *et al*., 1999; Salamitou *et al*., 2000). In addition, *B. thuringiensis* displays all characteristics to be classified as an insect pathogen: (i) occupies the same niche of its host, (ii) persists in the host, (iii) overcomes the host's defenses, and (iv) survives and colonizes its tissues and/or affects its physiology (Raymond *et al.*, 2010). Here the stages of infection and their regulation by quorum sensing systems are described.

3.1 Stages of infectious lifecycle

Insect hosts have a wide variety of defense systems against pathogens. These defenses include physical (external cuticle, microbiota and peritrophic membrane) and chemical barriers (pH, proteases and antimicrobial peptides) to prevent infection (Vallet-Gely *et al*., 2008; Raymond *et al*., 2010). To invade and colonize the host, *Bt* displays a wide range of molecular and biochemical resources, which allow bacteria to develop virulence. They include the production of toxins, degradative enzymes and antimicrobials. In addition, *Bt* promotes peristalsis and feeding reduction, which decrease elimination of *Bt* cells from the intestine. The resistance to antimicrobials and digestive enzymes produced by the host and prevention of phagocytosis allow survival and colonization.

The infectious cycle of *B. thuringiensis* can be divided into four sequential stages: toxemia, virulence, necrotrophism and sporulation. Toxemia is characterized by the presence of toxins in the host body. In *B. thuringiensis*, toxemia is caused mainly by insecticidal crystal proteins. Ingestion of the Cry toxins produced by *B. thuringiensis* alone, without spores, is generally sufficient to for insect larvae to develop symptoms of toxemia. The digestive syndrome, related to toxemia, allows spore germination and multiplication of vegetative cells, and allows the bacteria to gain access to the hemolymph. Toxemia is generally followed by septicemia, which can be the cause of the death.

The next stage of the infectious cycle of *Bt* is virulence, characterized by the expression of genes encoding several virulence factors such as phospholipases, collagenases, proteases, hemolysins, and toxins (Gohar *et al*., 2002, Slamti *et al*., 2004, Gohar *et al*., 2008). All these proteins are regulated by the transcriptional regulator, PlcR. Ninety percent of the PlcR-regulated genes encode proteins that are exported or located in the cell wall, evidencing that this phase is essential to colonize the host tissues. Moreover, PlcR also controls the production of antibacterial peptides and drug efflux transporters, which may help bacterial cells evade the host defenses and

also protect the cell from competition from other gut bacteria (Gohar *et al*., 2008, Zhou *et al*., 2014).

In the necrotrophism stage, *B. thuringiensis* uses the cadaver as an environment to multiply and to produce spores and toxins (Dubois *et al*., 2012). After the insect death, NprR, the major transcriptional regulators of necrotrophism, is activated and controls the expression of genes encoding proteins involved in survival of *B. thuringiensis* in the insect cadaver. NprR controls expression of several genes, which can be classified in four functional groups, genes encoding: (i) proteins involved in stress resistance, (ii) oligopeptide permease, which mediate the import of small peptides into the cell, (iii) nonribosomal peptide synthesis (NRPS), involved in the synthesis of lipopeptides, and (iv) degradative enzymes (metalloproteases, chitinases and esterases) and proteins that can bind to organic material (Dubois *et al*., 2012). These studies suggest that the degradative enzymes allow *B. thuringiensis* to use the content of the host. Thus, the bacteria display a necrotrophic lifestyle until sporulation. In addition, chitinases may facilitate the spore and toxin release into the environment by degradation of chitin, a component of the insect cuticle. The NRPS system is involved in the synthesis of kurstakin, a secreted lipopeptide involved in biofilm formation (Gélis-Jeanvoine *et al.*, 2016).

A recent study showed that NprR also controls sporulation in the absence of its cognate peptide NprX. The apo form of NprR prevents the phosphorylation of Spo0F, inhibiting phosphorelay and leading to the phosphorylation of Spo0A. Therefore, this function of NprR inhibits the expression of genes involved in sporulation. From a physiological point of view, the double function of NprR can be explained as follows: (i) the inhibition of sporulation allows maintaining the activity of PlcR and consequently of the virulence state in insect larvae; and (ii) after insect death, the bacterial cell density increases and results in the formation of the NprR-NprX complex, activating the transcription of genes involved in the necrotrophic lifestyle. Moreover, NprR-NprX adopts a tetrameric conformation, which is not able to bind to Spo0F, thus triggering the sporulation process. This causes NprR/NprX to connect the necrotrophism to sporulation during the infectious cycle of *B. thuringiensis*, thus ensuring survival and dissemination of bacteria during host infection (Perchat *et al*., 2016).

Finally, the last system is Rap/Phr, which controls sporulation, an essential process for bacterial persistence and dissemination. The sequential activation of these quorum sensing systems allows the regulation of several processes, ensuring the coordination of gene expression

in a bacterial population. All these QS systems allow efficient invasion, colonization, survival and sporulation of bacteria in insect larvae.

3.2 Sequential activation of quorum sensing systems

The complete lifecycle of *B. thuringiensis* in insect larvae is controlled by three quorum sensing systems, PlcR-PapR, NprR-NprX and Rap-Phr (Figure 26). PlcR is activated in the early infection stage by its cognate peptide PapR. This system regulates the transcription of virulence factors that result in the death of the insect (Slamti and Lereclus, 2002). PlcR is also involved in activation of PlcRa, a PlcR-like protein not described in this review yet. PlcRa is activated by the heptapetide CSIPYEY, located in the internal region of the carboxy-terminal end of PapRa (Huillet *et al*., 2012). This system controls the transcription of genes involved in regulation and synthesis of cysteine and in peroxide stress resistance. Although not yet tested in insect models, this system may be involved in resistance to stresses during the infectious cycle. In addition, PlcR/PapR activates *nprR* and *nprX* transcription, which triggers the development of the necrotrophic lifestyle and allows the bacteria to survive in the insect larvae (Dubois *et al*., 2016).

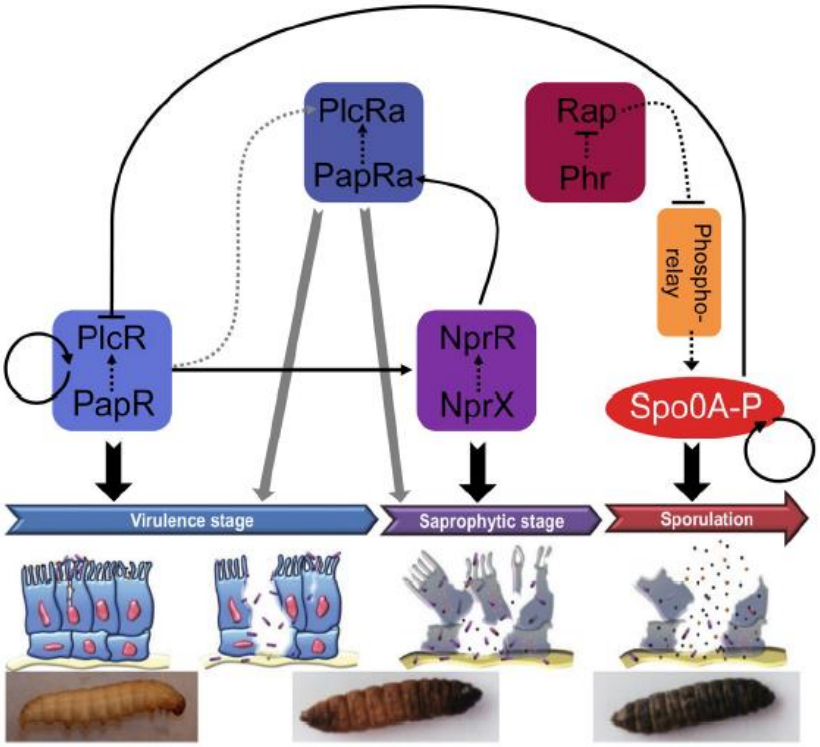

Figure 27. Sequential activation of the QS systems of *B. thuringiensis* pathogenic lifecycle in the insect. Solid lines: transcriptional effect; dotted lines: peptide- or protein-protein interaction; grey lines: putative effect. Arrows and blunt lines: positive or negative effect, respectively. The bottom pictures are *G. mellonella* 6th instar larvae, the model insect used for the infection experiments. Description of the figure is given in the main text (Slamti *et al*., 2014).

4 Plasmids and quorum sensing

4.1 Plasmid-borne quorum sensing systems

Plasmids have a significant role in the dynamics and evolution of the bacterial genome due the gain and/or loss of genes, which force bacterial genomes to evolve (Wiedenbeck and Cohan, 2011). These elements are responsible for the origins of bacterial diversity (Ochman *et al*., 2000). This diversity is due to the presence of several genes that encode important metabolic functions for the host cell, such as antibiotic resistance, degradative enzymes and cofactors for methane utilization (Gogarten, Doolittle and Lawrence, 2002; O'Brien, 2002). Furthermore, these elements harbor genes involved in coordination of important cellular processes (QS systems) (Bongiorni, *et al.*, 2006; Johnson and Nola, 2009). Therefore, plasmids provide physiological capabilities and consequently the ability of bacteria to invade a new niche or to improve their fitness in their current niche (Cohan and Koeppel, 2008; Yano et al., 2016). As described above, the *B. cereus* group has great diversity of plasmid content (Lereclus *et al.*, 1982), and a large part of the disease and host specificity of *B. cereus* species is due to these elements (Rasko, *et al*., 2005).

An important group of genes found in plasmids is those involved in regulation of the quorum sensing process. The lifecycle of *Bt* in the insect host is controlled by quorum sensing systems, as described above. *In silico* analyses located several Rap-Phr systems encoded by plasmids of *B. thuringiensis* (Slamti *et al*., 2014, Cardoso, unpublished results). Plasmid-borne *rap-phr* systems were already described in other *Bacillus* species as controlling important functions of bacterial development. In *B. subtilis*, the Rap-Phr60 system is encoded by the pTA1060 plasmid and regulates production of extracellular proteases, sporulation and biofilm formation through the Spo0A pathway and competence via the ComA pathway (Koetje *et al.*, 2003; Boguslawski *et al.*, 2015). The pBS32 plasmid harbors the RapP-PhrP system, which controls biofilm architecture

and sporulation by modulating the rate of Spo0A-P through the dephosphorylation of Spo0F (Parashar *et al.*, 2013). The Rap-PhrLS20 system coordinates conjugation of the plasmid pLS20 with population density (Singh *et al*., 2013). The Rap-PhrBAX0205 system is encoded by the pXO1 virulence plasmid of *B. anthracis* and controls sporulation (Bongiorni *et al*., 2006). RapQ-PhrQ systems in pBSG3 plasmid from *B. amyloliquefaciens* are able to control sporulation and genetic competence in the heterologous host *B. subtilis* (Yang *et al*., 2015). In addition, Rap-Phr systems have also been identified in plasmids of other bacterial species, such as pPZZ84 in *B. pumilus* (Zhang *et al*., 2010) and pFL5 and pFL7 in *B. licheniformis* (Parini, *et al*., 2004). However, the function of these systems remains unknown.

Furthermore, studies have verified that extrachromosomal genetic material of this species represents a higher portion of total genome when compared with *B. cereus* and *B. anthracis*. This may be due the ability of *B. thuringiensis* strains to adapt and develop their lifecycle in invertebrate hosts (Patiño-Navarrete and Sanchis, 2016). Thus, plasmids seem to participate directly in genome evolution of *B. thuringiensis* in its ecological niche. The exposure of bacteria in different environments allows contact with other plasmids, which contributes to bacterial evolution. In this way, *B. cereus* species compose an interesting group to investigate the relation between the plasmid content and the different hosts colonized by these bacteria (Jackson *et al*., 2011). Therefore, special attention should be given to investigate these elements in *B. cereus* species.

II- OBJECTIVES

The main purpose of this study is investigating the role of two plasmids in the fitness and the control of important process of *B. cereus* and *B. thuringiensis* strains, in its ecological niche. In order to evaluated this role we used two plasmids, pHT73 and pHT8_1, from *B. thuringiensis* serovar *kurstaki* KT0 and *B. thuringiensis* serovar *kurstaki* HD73, which are the genetically identical strains. First, the pHT73 plasmid harboring the *cry*1Ac gene (coding to Cry lepidopteran-active toxin) was used to evaluate its role in the fitness of *B. cereus* and *B. thuringiensis* strains in *Anticarsia gemmatalis* larvae. Second, the *rap-phr* quorum sensing system of pHT8_1 plasmid was characterized *in vitro* and *in vivo* in *G. mellonella* larvae.

III- ARTICLE 1

Plasmid and chromosomal genetic background affect the fitness of *Bacillus cereus* and *Bacillus thuringiensis* strains in insect cadaver

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ABSTRACT

Bacillus cereus and *Bacillus thuringiensis* are highly related species, and the presence of plasmid genes (*cry* genes) encoding parasporal crystals in *B. thuringiensis* is the unique distinctive trait between them. The plasmids are generally involved the adaptation of the bacteria to their ecological niche. In order to understand the ecological role of a plasmid harboring a *cry* gene, we assayed its effect on the growth fitness of nine strains (two *B. thuringiensis* and seven *B. cereus*). The plasmid pHT73 carrying a *cry1Ac* gene encoding a toxin active lepidopteran insects was used in this study. We conducted assays with three different plasmid contents for each strain (plasmidless, harboring a pHT73-Em^R plasmid and harboring a pHT73Δ*cry*1Ac plasmid deficient for the production Cry toxin), totaling 27 genotypes. Insect larvae were fed with spores of individual strains. Then, spores and vegetative cells of each strain were monitored at four times in *Anticarsia gemmatalis* larvae. The two *B. thuringiensis* and seven *B. cereus* strains were classified into five groups according to the bacterial fitness. In these groups, the two plasmids positively or negatively affected bacterial fitness compared to the plasmidless strain. Altogether, our results indicate that *B. cereus* group strains must have a suitable genetic background to display a high fitness allowing optimal multiplication and dissemination of the bacterial population within insect larvae.

INTRODUCTION

The *Bacillus cereus* group, also known as *B. cereus sensu lato*, consists of Gram-positive, rod-shaped, spore-forming aerobic bacteria widespread in natural environments. Currently, this group comprises eleven closely related species (Liu *et al.*, 2015), although *B. anthracis*, *B. cereus*, and *B. thuringiensis*, are the best-known species because they have a significant impact on human health, agriculture, and food industry (Vilas-Bôas *et al.*, 2007).

The classification of new isolates, namely as *B. anthracis*, *B. cereus* or *B. thuringiensis* is based on 16S rRNA -sequencing and phenotypic characteristics, such as the presence of capsule (virulence plasmids pXO1 and pXO2) in *B. anthracis*, production of the Cry toxin (plasmids harboring *cry* genes) in *B. thuringiensis*, or the ability to cause food contamination by the production enterotoxins and emetic toxins in *B. cereus*. Nevertheless, the taxonomy of the *B. cereus* group remains controversial and has been extensively studied using various typing methods (Helgason *et al.*, 2000, Vilas-Bôas *et al.*, 2002). Recently, Liu *et al.*, (2015) conducted a large-scale study using whole-genome sequences to investigate the taxonomic status of the group. The latter authors demonstrated that *B. cereus* and *B. thuringiensis* strains are widely distributed in several clusters into the group, although many strains have been located into three closely related clusters belonging to a same clade in the tree, indicating a common ancestor. The authors also showed the wide distribution of pXO plasmids and *cry* genes and suggested a little correlation among plasmids and phylogenetic position of the host bacteria.

Conjugation is the main process of plasmid dispersion. Several studies have described the transmission of plasmids harboring *cry* genes between *B. cereus* group species in the culture media, soil microcosms, infected insect larvae and foods (Gonzalez *et al.*, 1982, Battisti *et al.*, 1985, Reddy *et al.*, 1987, Hu *et al.*, 2004, Van de Auwera *et al.*, 2007, Santos *et al.*, 2010). Furthermore, multiplication of *B. thuringiensis* strains in insects has been described (Aly *et al*., 1985a, b; Jarrett & Stephenson 1990; Vilas-Bôas *et al*., 1998; Takatsuka & Kunimi 2000; Thomas *et al.*, 2000; 2001, 2002, Suzuki *et al*., 2004).

Santos *et al.* (2010) substantiated that high-performance multiplication of recipient cells is not required for the occurrence of conjugation among *B. thuringiensis* strains or between *B. thuringiensis* and *B. cereus* strains. Thus, many papers show conjugation inside Bacillus genus and studies of the timing of conjugation are few (Singh *et al.*, 2013). Moreover, environmental interactions that regulate the occurrence of conjugation are little known.

The *B. cereus* group species appear to have lifestyle closely dependent on its host specificity. Usually, *B. thuringiensis* has been prospected in invertebrates, *B. cereus sensu stricto* in food and humans, and *B. anthracis* in animals, although all have the soil as a common natural reservoir. These hosts are considered a source of nutrients and a mean for population dispersal and plasmid exchange. Therefore, studies focusing on the behavior of bacteria belonging to the *B. cereus* group, in these different ecological niches may be useful auxiliary tools, in addition to genome analysis, for determining the ecology and taxonomy within this bacterial group.

In this study we investigated the effects of the presence of the pHT73 plasmid, originally identified in *B. thuringiensis* var. *kurstaki* KT0, and its mutant pHT73Δ*cry*1A, in which the *cry1*Ac gene present in the wild-plasmid was deleted, in the multiplication and development of *B. cereus* and *B. thuringiensis* strains. The experiments were conducted using larvae of the lepidopteran *Anticarsia gemmatalis*, a soybean caterpillar, which are known as hotspots for the exchange of genetic material and development of *B. thuringiensis* (Vilas-Bôas *et al.*, 1998, Suzuki *et al.*, 2004).

MATERIALS AND METHODS

Bacterial strains, plasmids and culture conditions

The bacterial strains used in the present study are described in Table 1. *B. thuringiensis* var. *kurstaki* KT0 pHT73-Em^R harbors the 75 kb resident plasmid pHT73 which carries the *cry*1Ac gene tagged with an *ermC* gene conferring erythromycin resistance (Vilas-Bôas *et al.*, 1998). *B. thuringiensis* var. *kurstaki* KT0 pHT73Δ*cry*1A harbors the pHT73 plasmid deleted for *cry*1Ac gene and tagged with an *tetR* gene conferring tetracycline resistance. *B. thuringiensis* var. *kurstaki* KT0 plasmidless strain was obtained by plasmid cure of the original *B. thuringiensis* var. *kurstaki* KT0 strain.

B. thuringiensis var. *thuringiensis* 407-1 *and B. cereus* strains harboring pHT73-Em^R plasmid were obtained by conjugation by Santos *et al.* (2010). *B. thuringiensis* 407-1 and *B. cereus* strains carrying pHT73Δ*cry*1Ac plasmid were obtained by mating experiments using *B. thuringiensis* var. *kurstaki* KT0 pHT73Δ*cry*1Ac as donor, as previously described by Santos *et al.* 2010.

Hence, considering plasmid content, this study employs three genotypes for each bacterial strain (Table 1): i) absence of pHT73 plasmid (plasmidless strains); ii) presence of

pHT73-Em^R plasmid; and iii) presence of pHT73Δ*cry*1Ac plasmid.

B. thuringiensis and *B. cereus* strains were cultured in Bacto-Peptone agar medium (BP, Lecadet *et al.*, 1980), at 30°C for 72 h. The medium was supplemented with 100 μg·mL^{-1} erythromycin and/or 10 μg·mL^{-1} tetracycline and/or 200 μg·mL^{-1} streptomycin and/or 100 μg mL^{-1} of rifampicin when appropriate.

The degree of sporulation of the cultures was observed by phase-contrast microscopy and subsequent heat treatment during 20 min at 80°C. The cultures were then harvested by centrifugation (3000 × *g*, 15 min, 4 °C), and the pellets were washed twice and suspended in 3 mL sterile saline solution. The spores count of each culture was determined by serial dilution. In all assays, bacterial cultures were freshly prepared.

General infection of *Anticarsia gemmatalis* larvae

Our study comprised the assessment of spore germination capacity, vegetative cell multiplication and sporulation were studied in third-instar larvae of the *A. gemmatalis*, a soybean caterpillar, which was bred at the rearing unit of Empresa Brasileira de Pesquisa Agropecuária EMBRAPA-Soja, Londrina/PR, Brazil. The experiments were carried out by free ingestion on soybean leaves (*Glicine max*, Fabaceae.) in 128-well bioassay trays (BIO-BA-128®-Pitman, NJ, USA). Forty larvae remained in individual cells, and each one fed from a soybean leaf disc containing 10 μL of a sporulated heat-treated culture (80 °C for 20 minutes) of each bacterial strain (Table 1) containing 10^9 spores of each strain. The complete trays were kept in BOD incubators with temperature (25-27 ºC), photoperiod (16/8 h light/dark) and humidity (80%) controlled.

The count of the experimental time initiated after *A. gemmatalis* larvae ingested the whole content of the foliar leaf disc. Approximately 40 h after the start of the assays, we detected the death of larvae fed with foliar leaf disc containing bacterial spores and crystals solution, i.e., larvae that received a foliar disc containing bacterial strain harboring the pHT73-Em^R plasmid. At that stage, larvae that fed on foliar disc containing strains non-producers of Cry toxin were mechanically killed and maintained at 30 °C. Counts of spores and vegetative cells of each bacterial strain were analyzed in four different time points. T1 = 36 h and T2 = 6 h before the death of larvae, T3 = 24 h and T4 = 69 h after the death of larvae.

For all experiments, ten larvae were crushed in each time point, following serial dilution,

and plating onto LB agar supplemented with appropriate antibiotics, according to each strain to determine the amount/number of spores and vegetative cells. For the spores count, the larvae dilutions underwent heat-treatment (80 °C for 20 min) and plated onto LB agar supplemented with appropriate antibiotics. In parallel control experiments, fifteen larvae were similarly fed with soy leaf discs with water.

Statistical analysis

The experiment was set up as a factorial design using ten replicates per treatment and consisting of one dependent variable (the number of CFU) and three independent variables:

× Strains, with nine different strains (Table 1);

× Plasmid content, with three different contents: (1) plasmidless, (2) presence of pHT73-Em^R, (3) presence of pHT73Δ*cry*1Ac;

× Time-points, with four points: T1=36h and T2=6h before larvae died, and T3=24h and T4=69h after larvae died.

Statistical analysis was carried out with the log-transformed data using the Analysis of Variance (ANOVA). Means of the treatments were tested for the level of significance at 0.05 by Tukey's test. All statistical analyses were performed using the Assistat software (http://www.assistat.com/indexi.html).

RESULTS AND DISCUSSION

***B. cereus* and *B. thuringiensis* strains have a broad range of multiplication efficiency**

We conducted a performance-based evaluation comparing plasmidless to plasmid-carrying strains. Thus, the multiplication of vegetative cells and sporulation of nine plasmidless strains, including two *B. thuringiensis* and seven *B. cereus* (Table 1), were studied using the soybean caterpillar *A. gemmatalis* as a host. Afterwards of larvae autoinfection with each heat-treated bacterial culture, the subsequent bacterial development in larvae carcasses was following lasting up to 69 h after larvae dead.

Although readily isolated from soil, the *Bacillus thuringiensis* is unable to multiply effectively in either bulk soil or water (Ohana, Margalit and Barak, 1987; Yara *et al.*, 1997; Thomas *et al.*, 2000, 2001; Vilas-Bôas *et al.*, 2000; Ferreira *et al.*, 2003; Raymond *et al.*, 2010a). Furthermore, conjugation involving *B. cereus* group species has often been described in broth

culture and in insect larvae using plasmids harboring the *cry* genes (Gonzalez *et al.*, 1982, Battisti *et al.*, 1985, Reddy *et al.*, 1987, Hu *et al.*, 2004, Santos *et al.*, 2010). Also, there is enough evidence that insects are the optimal environment for exchanging genetic material (Jarrett & Stephenson 1990; Vilas-Bôas *et al.*, 1998; Takatsuka & Kunimi 2000; Thomas *et al.*, 2000; 2001; 2002; Suzuki *et al.*, 2004; Santos *et al.*, 2010). Nonetheless, Santos *et al.* (2010) substantiated that there is no positive association between the bacterial multiplication efficiency of the recipient strains and conjugation ability in the infected insects for the used strains.

CFU counting of all plasmidless strains in T1 ranged from 10^5 to 10^7, although the larvae were fed with 10^9 spores. All plasmidless strains showed a significant vegetative cell multiplication between T2 and T3. However, between T3 and T4 some strains did not show vegetative cell multiplication (Table 2). *B. cereus* 433 and MADM 1279R showed 1000-fold increase of CFU between T1 and T4, while most of the strains showed 100-fold increase. In T3, the highest CFU values (10^8) were showed by *B. cereus* D14430, 433, MADM 1279R and 569. The highest CFU of the assays among all plasmidless strains were showed by *B. cereus* D14430 and 433 in T4 (10^9), while all the other strains remained with 10^8 (Table 2). Results of the counting of spores revealed a decrease in spore numbers between T1 and T2 ($P < 0.05$) in *B. thuringiensis* KT0 and 407-1, *B. cereus* 569 and D14430 strains. The remaining strains did not show significant changes in spore numbers between these times. All strains exhibited the formation of new spores after the larval death. Most strains showed a significant increase in spore numbers between T2 and T3 ($P < 0.05$) (*B. thuringiensis* KT0, *B. cereus* strains 388, 433, 569, D14430 and MADM 1279R). However, *B. thuringiensis* 407-1, *B. cereus* ATCC 10987 and ATCC 14579 showed significant increase in spore numbers only between T3 and T4 ($P < 0.05$), indicating a delay in the sporulation cycle.

Some authors observed a decrease in the spore number in living insects (Salamitou *et al.*, 2000, Aly, 1985, Aly *et al*., 1985) and supported their explanations with different reasons, such as spore germination, killing of germinated spores, and transit elimination. The results also demonstrated that the extent of the experiment time was sufficient to show the onset of sporulation of the newly formed vegetative cells for all strains, but not enough to reach 100% due to the advanced disintegration stage of the larval cadavers.

There are few studies on the ability of *B. thuringiensis* to multiply in insects, either using

individual strains separately (Milutinovic *et al.*, 2015, Thomas *et al.*, 2001, Takatsuka & Kunimi 2000) or comparatively between different strains (Suzuki *et al.*, 2004, Thomas *et al.*, 2000, 2002). First and foremost, the results of this study displayed vegetative cell multiplications of all plasmidless strains assayed in *A. gemmatalis* larvae (two *B. thuringiensis* and seven *B. cereus*) but with variable efficiency. The multiplication of these same strains (except for *B. thuringiensis* KT0 plasmidless) in carcasses of *B. mori* was previously analyzed by Santos *et al.* (2010). Only *B. thuringiensis* 407 and *B. cereus* 569, D14430 and 433 could grew on this host, but showing variable fitness as well.

The results of these studies demonstrate that fitness differences among strains in different insects result from of a combination of factors, including the gut microbiota of the insects, the number of bacterial spores, and the origin of the spores, the genetic background of the bacterial strain, besides the influence of host-pathogen interaction. The set of results from Santos *et al*. (2010) and those presented herein showed that among the strains that cannot kill the insect, *B. cereus* strains 569, D14430 and 433 are the strains that exhibit the highest fitness for using both *A. gemmatalis* and *B. mori* insects as niche.

The pHT73 plasmid and *cry*1Ac gene can modulate the efficiency of *B. cereus* group strains to colonize *A. gemmatalis*

The pHT73 is one of the best known conjugative plasmids of *B. thuringiensis* (Vilas-Bôas, *et al.* 1998). The complete sequence of the pHT73 (77351 bp) is available in the GeneBank (https://www.ncbi.nlm.nih.gov/genbank/) under the ID CP004070.1. Interestingly, in addition to the *cry*1Ac gene that encodes the Cry lepidopteran-active toxin, another 73 open reading frames were annotated on its entire nucleotide sequence; of these, 27% encodes a hypothetical protein, 14.8% transposases, 10.8% putative exported protein, and 5.4% transcriptional regulator. Additionally, proteins involved in conjugation, sporulation, and chromosome segregation are also present in the pHT73 nucleotide sequence (Supplementary Table 1).

The *cry* genes impact on the host specificity is widely recognized for *B. thuringiensis* strains. Once the Cry toxin kills the insect, its corpse becomes a conducive environment conducive for bacterial development, presenting variable rates of cell multiplication. Previously studies have suggested that the presence of plasmids can modulate the ecological behavior of *B.*

cereus and *B. thuringiensis* (Hoffmaster *et al.*, 2008; Raymond *et al.*, 2010b).

However, the effect of the presence or absence of a plasmid carrying a *cry* gene, has never been investigated among strains of the *B. cereus* group. Thus, the main objective of this work was to analyze the influence of the presence of a plasmid on the multiplication efficiency of bacterial strains in insect larvae, in order to determine whether this effect is attributable either to the presence of *cry* genes or to the uncharacterized plasmid genes.

CFU for the four timepoints from the three genotypes of each strain was evaluated individually by the analysis of variance ($P < 0.05$) and compared as follows: i) plasmidless *versus* presence of pHT73Δ*cry*1Ac, and ii) plasmidless *versus* presence of pHT73 (Table 3). Thus, the first comparison allows investigating the effects of the presence of pHT73Δ*cry*1Ac on the multiplication efficiency of each strain, since the presence of pHT73Δ*cry*1Ac plasmid is the only difference between each pair of genotypes. The second comparison allows assessing the effects of the presence of the pHT73 plasmid, and/or of the *cry*1Ac gene on the multiplication efficiency of each strain.

Data shown in Table 3 and illustrated in Figure 1A demonstrate that the strains harboring pHT73Δ*cry*1Ac compared to the plasmidless strains showed: a) a significant reduction of CFU counts in *B. cereus* 433, 569, D14430 and MADM 1279R strains; b) a significant increase in CFU in *B. thuringiensis* KT0, *B. cereus* 388 and 10987 strains; c) no significant changes in CFU in *B. thuringiensis* 407-1 and *B. cereus* ATCC 14579 strains.

Similarly, according to the data in Table 3 and Figure 1B, strains harboring pHT73-Em^R plasmid showed: a) significant decrease in CFU in *B. cereus* 433 and 569 strains; b) significant increase in CFU in *B. thuringiensis* KT0 and 407-1, *B. cereus* MADM 1279R, 388, 10987, 14579 strains; c) no significant changes in CFU in *B. cereus* D14430 strain.

Subsequently, a qualitative comparison between Figure 1A and 1B allowed us to assemble the strains whose genotypes displayed the same growth fitness in *A. gemmatalis*. The first cluster includes *B. cereus* 433 and 569. For these strains, both plasmids (pHT73-Em^R and pHT73Δ*cry*1Ac) are a burden to these bacteria, and the fitness of the genotypes harboring the plasmids is worse than plasmidless strains.

The second cluster, is composed only by *B. cereus* D14430, for which, the pHT73Δ*cry*1Ac plasmid was a burden when compared with plasmidless strain. However, when D14430 harbor pHT73-Em^R, the plasmid is not a burden anymore, since the strain harboring this

plasmid showed a similar multiplication efficiency compared to the plasmidless strain. Then, the *cry*1Ac gene must promote genetic interactions that neutralize the negative effect of the pHT73Δ*cry*1Ac plasmid.

The third cluster formed by *B. cereus* MADM1279 showed similarity but not the same fitness compared to the second cluster. The pHT73Δ*cry*1Ac plasmid was a burden when compared with the plasmidless strain. However, the fitness of the strain harboring pHT73-Em^R plasmid becomes better than that of the plasmidless strain. Then, in this cluster, it is the *cry* gene that promotes the increase in the bacterial multiplication efficiency.

The fourth cluster includes *B. thuringiensis* 407 and *B. cereus* 14579, which do not present any significant differences between the multiplication efficiency of the strain harboring pHT73Δ*cry*1Ac and plasmidless strains. However, the presence of pHT73 harboring the *cry*1Ac gene allowed an improvement in the multiplication efficiency. Thus, pHT73Δ*cry*1Ac does not worsen the strain fitness when compared to plasmidless strains, and pHT73-Em^R improves the fitness of the strains by the presence of *cry*1Ac gene.

The last cluster includes *B. thuringiensis* KT0, *B. cereus* 388, and 10987 strains. Concerning these strains, both plasmids improve the fitness of the bacteria when compared to plasmidless strains.

The outcomes of this study showed that the presence of both the plasmid and *cry* gene do not display a unique pattern of influence on the strain fitness of the used host. Thus, we suggest that the presence of the *cry*1Ac gene improves fitness to colonize *A. gemmatalis* in three strains (third and fourth clusters). On the other hand, both plasmids worse the fitness of two strains (first group). The other clusters (second and fifth) exhibited an intermediary behavior.

Consequently, the interaction with other plasmid-borne and chromosomal genes is of fundamental importance to define bacterial fitness when a plasmid is received.

CONCLUSION

B. cereus strains can opportunistically germinate and multiply in an insect carcass. However, the findings of this study indicate that strains that do not have a suitable genetic background may not have a high fitness level in this insect, i.e., multiply, survive in this niche and disperse its population. Thus, for a strain of *B. cereus* group, getting a pathogenicity plasmid is not enough to effectively increase bacterial population, i.e., colonize the host. Likewise, losing

a pathogenic plasmid does not necessarily mean losing their ability to efficiently multiply in the host, despite being unable to kill it.

Thence, rather than constituting a driving force in bacterial evolution or being solely involved in the dissemination of important traits, namely antibiotic resistance, virulence determinants, and metabolic pathways, the transfer of plasmids has a significant role in the adaptation of strains to environmental niches. Thus, we suggest that plasmids carrying pathogenicity factors are tools used by bacteria in search of the niche most advantageous to their genome.

Bacterial populations are dynamic, and their evolutionary processes are underway. In this study, we identified behavioral diversity in relation to its host, which may suggest a mosaic or speciation gradient in progress. The many strains of *B. cereus* and *B. thuringiensis* are at different evolutionary times. Indeed, in the insect gut environment, the plasmids can be involved in genetic exchanges among bacteria and the occurrence of subsequent selection, either improving or worsening bacterial fitness, favoring their evolution. Cohan, 1994, substantiates that speciation in bacteria primarily requires ecological divergence, whereas in highly sexual eukaryotes it requires both reproductive and ecological divergence".

The findings of this study strongly suggest that several genes may be involved, improving or worsening *B. cereus* / *B. thuringiensis* host specificity, including the *cry* gene, genes of the plasmid that harbor the *cry* gene, plasmid-borne and chromosomal genes. We propose that further researches should be undertaken to define how this set of genes modulate/regulate/stimulate the host specificity, as well as determine whether other *B. cereus* group members share this system.

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TABLES AND FIGURES

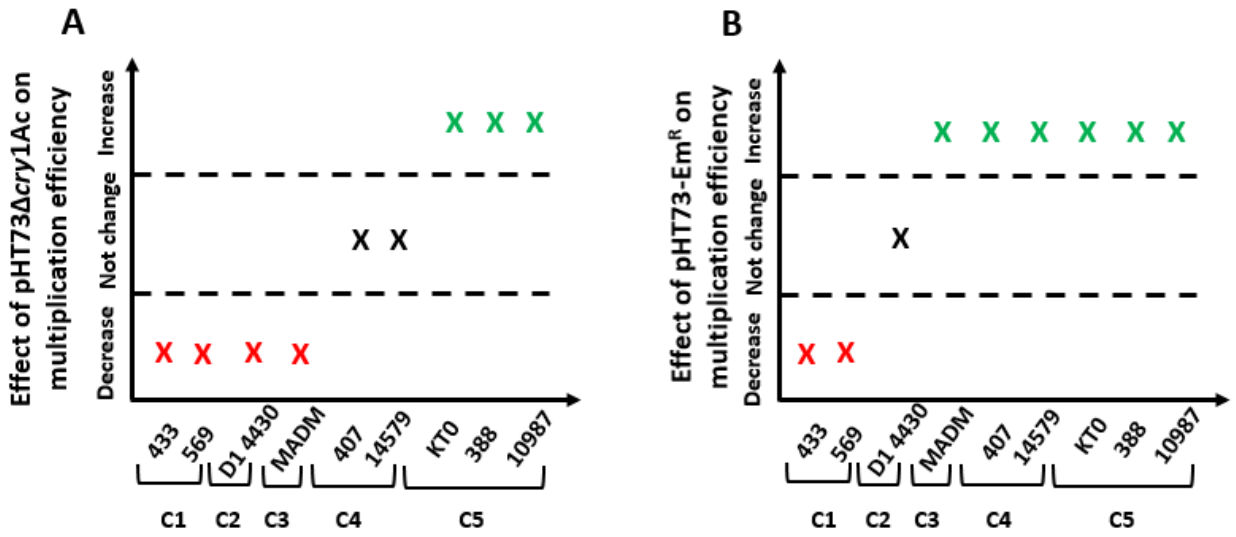

Figure 1. Influence of the presence of pHT73Δ*cry*1Ac and pHT73-Em^R plasmids on the multiplication efficiency of *B. cereus* and *B. thuringiensis* strains compared to the plasmidless strains in *A. gemmatalis* larvae. Significant level, using ANOVA factorial design, was obtained by comparison with the CFU means of the four times, as follow: (A): strains harboring pHT73Δ*cry*1Ac *versus* plasmidless strains; (B): strains harboring pHT73-Em^R plasmid *versus* plasmidless strains. C1-C5: Assembling of the strains whose genotypes displayed the same growth fitness.

Table 1. Properties of *B. thuringiensis* and *B. cereus* strains used in this study.

Strain	Relevant characteristics	Microbiological markers, plasmid content (reference)		
		Absence of pHT73	Presence of pHT73-Em^R	Presence of pHT73Δ*cry*1Ac
B. thuringiensis var. *kurstaki* KT0	Lepidopteran toxin-active strain, originally harboring pHT73 plasmid containing *cry1Ac* gene	$Sm^{R\,(1)}$	Cry^+ $Em^{R\,(2)}$	Cry^- $Tet^{R\,(7)}$
B. thuringiensis var. *thuringiensis* 407-1	Acrystalliferous brown pigment-producing strain	Sm^R Pig^+ $Cry^{-\,(2)}$	Sm^R Pig^+ Cry^+ $Em^{R\,(3)}$	Sm^R Pig^+ Cry^-, $Tet^{R\,(7)}$
B. cereus D1 4430	Diarrheal strain producer strain isolated from pea soup sample.	$Sm^{R\,(4)}$	Sm^R Cry^+ $Em^{R\,(3)}$	Sm^R Cry^- $Tet^{R\,(7)}$
B. cereus 569	Strain isolated of soil sample from Chile	$Sm^{R\,(4)}$	Sm^R Cry^+ $Em^{R\,(3)}$	Sm^R Cry^- $Tet^{R\,(7)}$
B. cereus 388	Strain isolated of soil sample from Indonesia	$Rif^{R\,(4)}$	Rif^R Cry^+ $Em^{R\,(3)}$	Rif^R Cry^- $Tet^{R\,(7)}$
B. cereus 433	Strain isolated of soil sample from South Korea	$Rif^{R\,(4)}$	Rif^R Cry^+ $Em^{R\,(3)}$	Rif^R Cry^- $Tet^{R\,(7)}$
B. cereus ATCC 14579	Type strain,	$Sm^{R\,(5)}$	Sm^R Cry^+ $Em^{R\,(3)}$	Sm^R Cry^- $Tet^{R\,(7)}$
B. cereus ATCC 10987	Type strain, originally isolated from a non-lethal dairy (cheese spoilage in Canada in 1930)	$Sm^{R\,(5)}$	Sm^R Cry^+ $Em^{R\,(3)}$	Sm^R Cry^- $Tet^{R\,(7)}$
B. cereus MADM 1279R	Strain isolated from sugar candy	$Rif^{R\,(6)}$	Rif^R Cry^+ $Em^{R\,(3)}$	Rif^R Cry^- $Tet^{R\,(7)}$

[a] Cry^+: produces insecticidal crystal; Cry^-: does not produce insecticidal crystal; Pig^+: produces a brown pigment; Em^R: erythromycin resistant; Sm^R: streptomycin resistant; Rif^R: rifampicin resistant. All strains harboring pHT73Δ*cry*1A were obtained in this study. All strains harboring pHT73-Em^R were obtained by Santos *et al.*, (2010), with exception of *B. thuringiensis* var. *kurstaki* KT0 pHT73-Em^R, which was obtained by Vilas-Bôas *et al.*, 1998.

[1] Lereclus *et al*., (1983).

[2] Vilas-Bôas *et al.* (1998).

[3] Santos *et al.* (2010).

[4] INRA-Génétique Microbienne et Environnement, Guyancourt/France.

[5] American Type Culture Collection, Rockville, MD, EUA.

[6] National Environmental Research Institute, Roskilde/Denmark.

[7] This study.

Table 2. ANOVA factorial design analysis contrasting *B. cereus* and *B. thuringiensis* plasmidless strains with time, using CFU counts from ten independent repetitions.

Strain	CFU means			
	T1	T2	T3	T4
B. thuringiensis var. *kurstaki* KT0	1.7×10^6 cdC	1.0×10^6 deC	2.6×10^7 cB	1.3×10^8 cA
B. thuringiensis var. *thuringiensis* 407	5.3×10^6 bC	2.4×10^6 bcD	5.2×10^7 cB	2.3×10^8 cA
B. cereus D1 4430	$1,3 \times 10^7$ aC	5.1×10^6 abD	3.7×10^8 aB	8.1×10^9 aA
B. cereus 569	3.0×10^6 bcB	5.0×10^6 abB	2.5×10^8 abA	2.8×10^8 cA
B. cereus 388	2.7×10^6 bcC	2.2×10^6 bcdC	1.4×10^8 bB	7.0×10^8 bA
B. cereus 433	1.3×10^6 CdC	2.0×10^5 fD	3.7×10^8 aB	9.6×10^9 aA
B. cereus ATCC 14579	5.3×10^5 eC	6.6×10^5 eC	2.7×10^7 cA	8.5×10^6 dB
B. cereus ATCC 10987	1.6×10^6 cdD	5.8×10^6 aC	5.5×10^7 cB	1.8×10^8 cA
B. cereus MADM 1279R	7.8×10^5 deB	1.1×10^6 cdeB	3.0×10^8 abA	1.9×10^8 cA

Minimal Significant Difference (DMS) for columns: 0.23×10^1; DMS for lines: 0.2×10^1. Lowercase letters are used to compare the strains at each time while capital letters are used to compare the effect of the time in each strain individually. Means followed by the same letter did not show significant differences at $P < 0.05$ Tukey's Test. T1 = 36 h and T2 = 6 h before the death of larvae, T3 = 24 h and T4 = 69 h after the death of larvae.

Table 3. ANOVA factorial design analysis contrasting *B. cereus* and *B. thuringiensis* CFU counts with plasmid content using data of four times with ten independent repetitions.

Strains	CFU means		
	Plasmidless	Presence of pHT73Δ*cry*1A	Presence of pHT73-Em^R
B. thuringiensis var. *kurstaki* KT0	8.9×10^5 dC	9.7×10^6 dB	7.8×10^7 bA
B. thuringiensis var. *thuringiensis* 407-1	1.4×10^7 cB	2.0×10^7 abcAB	2.8×10^7 cdA
B. cereus 433	4.7×10^7 bA	2.6×10^7 aB	2.4×10^7 cdB
B. cereus 569	1.8×10^8 aA	1.0×10^7 cdC	1.8×10^7 deB
B. cereus D1 4430	3.7×10^8 aA	1.2×10^7 bcdB	3.6×10^8 aA
B. cereus MADM 1279R	1.2×10^7 cB	3.9×10^6 eC	6.9×10^7 bA
B. cereus 388	1.5×10^7 cB	3.1×10^7 aA	4.7×10^7 bcA
B. cereus ATCC 10987	7.5×10^6 dB	2.2×10^7 abA	3.2×10^7 cdA
B. cereus ATCC 14579	1.4×10^6 dB	2.0×10^6 eB	9.5×10^6 eA

Minimal Significant Difference (MSD) for columns: 0.2×10^1; MSD for lines: 0.17×10^1. Lowercase letters are used to compare the strains considering each genotype individually, while capital letters are used to compare the effect of the three plasmid genotypes in each strain individually. Means followed by the same letter did not show significant differences at $P < 0.05$ Tukey's Test.

SUPPLEMENTARY DATA

Table S1. CDSs of pHT73: homologies and comparison using BLASTp and Phyre[2].

CDS	Locus Tag	Size (aa)	NCBI annotation	BLASTp	Phyre[2] (Confiance) (Identity %)
1	HD73_6001[1]	250	ATP-binding protein IstB	Insertion sequence IS232 ATP-binding protein/ Transposase (Bt)	
2	HD73_6002	431	Transposase ItsA, IS232B	Transposase (Bc group)	
3	HD73_6003	83	Hypothetical protein	Hypothetical protein (Bc group)	1- Glucocorticoid receptor-like (DNA-binding domain) (25,7) (50%) 2- Transcription regulator/protein binding (20,7) (70%)
4	HD73_6004	1178	Pesticidal crystal protein Cry1Ac	Cry1Ac	
5	HD73_6005	318	N-acetylmuramoyl-L-alanine amidase	N-acetylmuramoyl-L-alanine amidase (Bc group)	
6	HD73_6006	286	a+/H+ antiporter NapA-like protein	Potassium transporter (Bt)	Transport protein (100) (20%)
7	HD73_6007	478	Transposase for insertion sequence element IS231B	IS4 family transposase (Bc group)	Ribonuclease H-like motif (100) (11%)
8	HD73_6008	431	Transposase ItsA, IS232B	Transposase IstA (*Bt*)	
9	HD73_6009[1]	250	ATP-binding protein IstB	Insertion sequence IS232 ATP-binding protein (*Bt*)	
10	HD73_6010	207	DNA recombinase	Recombinase (*Bt*)	
11	HD73_6011	1023	Transposase	Transposase (Bt)	

12	HD73_6012	135	Hypothetical protein	Transposase (Bt)	1-YgfY-like (39,4) (10%) 2- Myosin phosphatase inhibitor (26) (19%)
13	HD73_6013	478	Transposase for insertion sequence element IS231B	IS4 family transposase (Bc group)	
14	HD73_6014 [2, 3]	987	Transposase TnpA	DDE transposase (Bc group)	Protein binding (69,7) (10%)
15	HD73_6015 [2, 3]	284	TnP I recombinase	TnP resolvase *Bc*	Recombination (100) (25%)
16	HD73_6016	478	Transposase for insertion sequence element IS231B	IS4 family transposase (Bc group)	
17	HD73_6017	37	Hypothetical protein	Hypothetical protein (Bc group)	DNA/RNA-binding 3-helical bundle (14,8) (46%)
18	HD73_6018	350	Transposase	Transposase for insertion sequence element IS231F, partial (*Bc*)	
19	HD73_6019	40	Hypothetical protein	Hypothetical protein (Bt)	Structural genomics, unknown function (13,2) (47%)
20	HD73_6020	435	Putative reverse transcriptase	Group II intron reverse transcriptase/maturase (Bt)	
21	HD73_6021	77	Hypothetical protein	Transposase for insertion sequence element IS231F, partial (Bm) (100%)	1- Ferritin-like (46,5) (47) 2- De novo protein (29) (45%)
22	HD73_6022	38	Hypothetical protein	Hypothetical protein	1 – Replication (18,1) (58) 2 - Electron transport

					(11) (100) 3 - Single transmembrane helix (11) (100)
23	HD73_6023	409	Tnp166	IS110 family transposase (Bc group)	Transferase (98,5) (14%)
24	HD73_6024	311	DNA integration/recombination/inversion protein	Integrase (Bc group)	1-Recombination (100) (17%) 2-hydrolase, ligase/dna (100) (14%)
25	HD73_6025	69	Hypothetical protein	Hypothetical protein (Bc)	1- Plant protein (21,3) (25%) 2- Ligand binding protein (19,4) (35%) 3- GUN4-like (19,2) (35%)
26	HD73_6026	142	Hypothetical protein	Hypothetical protein (Bt)	Structural protein (63,8) (20%)
27	HD73_6027	397	Hypothetical protein	Hypothetical protein	1-Unknown function (100) (19%) 2-Structural protein (100) (17%)
28	HD73_6028	431	Transposase ItsA	Transposase ItsA	
29	HD73_6029	250	ATP-binding protein IstB	Insertion sequence IS232 ATP-binding protein IstB	
30	HD73_6031	312	Hypothetical protein	Hypothetical protein/ Hidrolase (Bc group)	1- Transcription regulator (89,2) (24%) 2- DNA/RNA-binding (87,8) (17%)
31	HD73_6032	325	Conserved protein of unknown function	Hypothetical protein	1-Unknown function (24) (100%) 2-Hydrolase (16) (100%)

32	HD73_6033	452	TCS histidine kinase-like protein; Sporulation kinase	ATP blinding protein (Bc group)	Transferase (100) (19%)
33	HD73_6034	190	Transcriptional regulator	Transcriptional regulator LuxR (*Bt*)	
34	HD73_6035	63	Transcriptional regulator	Transcriptional regulator (*Bc*)	
35	HD73_6036	50	Hypothetical protein	Hypothetical protein	1- Transcription (29,2) (46%) 2- RuvA C-terminal domain-like (25,9) (56%)
36	HD73_6037	130	Transcriptional regulator	Transcriptional regulator (Bc group)	
37	HD73_6038	79	Transcriptional regulator	Transcriptional regulator (Bc group)	
38	HD73_6039	84	Hypothetical protein	Hypothetical protein	1- Beta-Grasp (ubiquitin-like) (25,6) (10%) 2- De novo protein (25) (30%)
39	HD73_6040	182	Putative membrane protein	Hypothetical protein	1- Hydrolase (37,8) (13%) 2- Sugar binding protein (28,6) (21%)
40	HD73_6041	402	S-layer domain-containing protein	Hypothetical protein (Bc group)	1- Structural protein (100) (32%) 2- Hydrolase (64,3) (15%)
41	HD73_6042	849	Putative exported protein	Hypothetical protein (Bc group)	1- Cell adhesion (90,9) (23%) 2- Structural genomics,

					unknown function (89,9) (24%)
42	HD73_6043	140	Putative exported protein	Hypothetical protein	Signaling protein, cell adhesion (27,4) (57%)
43	HD73_6044	264	Putative exported protein	Putative exported protein (Bt)	
44	HD73_6045	271	SAF domain protein	Hypothetical protein	1- Hydrolase (100) (13%) 2- Oxidoreductase (100) (16%) 3- P-loop containing nucleoside triphosphate hydrolases (100) (20%)
45	HD73_6046	485	Putative NTPase involved in conjugation and in Type II secretion machinery	Conjugation transfer protein (Bc group)	Hydrolase (100) (21%)
46	HD73_6047	315	GSPII-F domain involved in Type II secretion machinery	Hypothetical protein (Bc)	Transport protein (99,1) (17%)
47	HD73_6048	297	Putative membrane protein	Hypothetical protein (Bc) (98%)	1- Transport protein (98,6) (11%) 2- Protein transport (97,9) (15%) 3- Membrane protein (97) (11%)
48	HD73_6049	397	Transposase IS116-110-902 family	IS110 family transposase (Bc group)	
49	HD73_6050	88	Putative exported protein	Hypothetical protein (Bc group)	1- Lambda repressor-like DNA-binding

					domains (36,8) (53%) 2- DNA binding protein (35,5) (15%) 3- Lambda repressor-like DNA-binding domains (34,7) (53%)
50	HD73_6051	128	Putative exported protein	Hypothetical protein	Hydrolase (43,8) (29%)
51	HD73_6052	65	Hypothetical protein	Hypothetical protein (Bc group)	Hormone receptor (32,3) (60%)
52	HD73_6053	43	Hypothetical protein	Hypothetical protein	1- Toxin (19,5) (50%) 2- Unknown function (9,2) (41%)
53	HD73_6054	122	Putative conjugative protein TrsK-like protein	Hypothetical protein (Bc group)	1- P-loop containing nucleoside triphosphate hydrolases (97,7) (8%) 2- Single transmembrane helix (32,5) (44%)
54	HD73_6055	57	Hypothetical protein	Hypothetical protein (Bt)	1- BSD domain-like (42,3) (27%) 2- Structural protein (38,8) (57%) 3- Integral membrane protein (11,9) (50%) 4- Gene regulation (11,7) (62%)
55	HD73_6056	45	Hypothetical protein	Hypothetical protein (Bt)	Gene regulation (95,7) (65%)
56	HD73_6057	105	Hypothetical protein	Hypothetical protein (Bc group)	1- Hormone/growth factor (27,4) (41%)

					2- Single-stranded right-handed beta-helix (26,9) (47%)
57	HD73_6058	85	Hypothetical protein	Hypothetical protein (Bc group)	1- Ligase (47,8) (14%) 2- Transferase (39,2) (45%)
58	HD73_6059	63	Hypothetical protein	Hypothetical protein (Bc group)	Structural genomics, unknown function (23,4) (33%)
59	HD73_6060	63	Hypothetical protein	Hypothetical protein (Bc group)	1- Transferase (25,4) (39%) 2- Membrane protein (25,4) (39%)
60	HD73_6061	103	Hypothetical protein	Hypothetical protein (Bc group)	1- Structural genomics, unknown function (71) (21%) 2- Transferase (63,2) (15%)
61	HD73_6062	59	Hypothetical protein	Hypothetical protein (Bc group)	de novo protein (37) (34,8%)
62	HD73_6063	966	Hypothetical protein	Hypothetical protein (Bt)	1- Hydrolase (100) (18%) 2- P-loop containing nucleoside triphosphate hydrolases (100) (16%)
63	HD73_6064	277	Hypothetical protein	Hypothetical protein (Bc group)	1- DNA/RNA-binding 3-helical bundle (96,5) (11%) 2- Transcription regulator (95,5)

					(21%)
64	HD73_6065	74	Hypothetical protein	Hypothetical protein (Bt)	1- Structural genomics, unknown function (36,5) (35%) 2- Capping enzyme (21,7) (50%) 3- Hydrolase, transferase (15,4) (75%)
65	HD73_6066	312	Putative exported protein	Transposase	1- Transport protein (100) (22%) 2- Unknown function (98,2) (17%)
66	HD73_6067	81	Hypothetical protein	Hypothetical protein	1- Long alpha-hairpin (17,2) (64%) 2- Ribosomal protein (13,3) (34%) 3- Toxins' membrane translocation domains (11,6) (23%)
67	HD73_6068	177	Putative ATPase involved in chromosome segregation	Conjugal Transfer protein (Bc group)	1- Immunoglobulin-like beta-sandwich (64) (36%) 2- Oxygen transport/protein binding (47,9) (33%)
68	HD73_6069	862	Putative ATP/GTP binding protein; DNA segregation ATPase	DNA recombination protein	Hydrolase (100) (21%)
69	HD73_6070	171	Putative exported protein	Hypothetical protein	1- Transport protein (58,2) (25%) 2- Hydrolase regulator

					(57,8) (22%)
70	HD73_6071	1197	Putative membrane protein	Hypothetical protein (Bc group)	Transport protein (96,3) (19%)
71	HD73_6072	369	Peptidoglycan-specific endopeptidase	Peptidase M23 (Bc group)	
72	HD73_6073	419	Transposase IS116-110-902 family	Transposase family (Bt)	
73	HD73_6074	193	Putative exported protein	Hypothetical protein (Bc group)	1- Hydrolase (94,2) (14%) 2- Transport protein (88,8) (13%) 3- Protein binding (83,9) (14%)
74	HD73_6075	58	Hypothetical protein	Hypothetical protein (Bc group)	1- Cell adhesion (25) (18%) 2- Toxin, hydrolase (17,4) (35%) 3- Membrane protein (16,6) (64%)

[1] Menou *et al*., 1990.
[2] Lereclus *et al*., 1986.
[3] Lereclus and Mahillon, 1988.

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IV- ARTICLE 2

A plasmid-borne Rap-Phr system regulates sporulation of *Bacillus thuringiensis* in insect larvae

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Running title: Rap-Phr controls sporulation in *Bacillus thuringiensis*

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Abstract

The entomopathogen *Bacillus thuringiensis* species harbour numerous plasmids essentially studied regarding to their involvement in pathogenicity, as Cry-plasmids. The lifecycle of *B. thuringiensis* in the insect host is regulated by the sequential activation of quorum sensing systems to kill, survive and sporulate. In this study, we characterize a new quorum sensing system belonging to the Rap-Phr family. The Rap8-Phr8 system is borne by the pHT8_1 plasmid, a small cryptic plasmid from the *B. thuringiensis* var. *kurstaki* HD73 strain. Our results demonstrate that the Rap8 protein inhibits sporulation and biofilm formation through the Spo0A pathway. The Rap8 activity is inhibited by the mature Phr8 heptapeptide YAHGKDI. The key residues specific for the Rap phosphatase activity are conserved in Rap8 suggesting a common mechanism inhibition on the phosphorelay. Interestingly, we show that the Rap8-Phr8 system is specifically required for regulating sporulation of *B. thuringiensis* in insect larvae. This system may allow the bacteria to exert a tight control of the sporulation process in the host cadaver for optimizing the multiplication, the survival and the dissemination of the bacteria. The pHT8_1 is a mobilizable plasmid which can provide advantages for the adaptation and the evolution of *B. thuringiensis* in its ecological niche.

Keywords: Bacillus, insect, quorum sensing, plasmid, sporulation, Rap phosphatase

Introduction

The entomopathogen bacterium *Bacillus thuringiensis* is used as alternative insect pest control for more than 60 years (Sanchis, 2011). Its insecticidal activity is mainly due to the production of toxins, named Cry and Cyt toxins, forming a crystal during sporulation (Deng *et al.*, 2014). Moreover, the production of various adaptation and virulence factors contribute to the infection and to the development of the bacteria in the insect host which appears as the primary means of reproduction of *B. thuringiensis* (Raymond *et al.*, 2010). Most of these factors are exported cytotoxins and degradatives enzymes which allow the bacteria to kill the insect larvae and to survive in their cadavers. The expression of the genes coding for these factors is activated during stationary phase and sporulation by the activation of two quorum sensing (QS) systems PlcR-PapR and NprR-NprX (Agaisse *et al*., 1999; Gohar *et al.*, 2008; Perchat *et al.*, 2011; Dubois *et al*., 2016). QS is a cell-cell communication mechanism which coordinates gene expression to cell density by secreted signaling peptides (Perego and Hoch, 1996; Lazazzera *et al*., 1997). This process provides to the bacteria the ability to communicate and to change behavior in response to conditions and perturbations of the environment.

Most of the gram-positive QS systems belong to the RNPP family, named according to their effective regulators Rap, NprR, PlcR and PrgX (Declerck *et al.*, 2007). These QS systems imply a secreted signaling peptide and a cytoplasmic regulator. In *B. thuringiensis,* the infectious cycle is divided in different stages: virulence, survival and sporulation (Slamti *et al.*, 2014). Initially, PlcR-PapR, activates genes involved mainly in virulence causing insect death (Slamti and Lereclus, 2002). Then, NprR-NprX activates genes needed to the necrotrophism lifestyle allowing the bacteria to survive in the insect cadaver (Dubois *et al.*, 2012). Moreover, this QS system is also required to connect necrotrophism to the initiation of the sporulation (Perchat *et al.*, 2016).

The sporulation has been widely studied in *Bacillus subtilis* (Sonenshein, 2000). This complex differentiation pathway is triggered by the phosphorylation state of Spo0A, the key regulator controlling early sporulation gene expression (Burbulys *et al.*, 1991). Phosphorylation of Spo0A is directed by the phosphorelay consisting in five histidine kinases (KinA to KinE) and the Spo0F and Spo0B phosphotransferase proteins (Jiang *et al.*, 2000). In response to starvation signals, kinases are autophosphorylated. The phosphoryl group is transferred from kinases to Spo0F (Spo0F-P) then to Spo0B (Spo0B-P). At the end, Spo0A is phosphorylated to generate Spo0A-P (Burbulys *et al.*, 1991). Biofilm formation is also regulated by the phosphorylation state of Spo0A in *B. subtilis* as in *B. thuringiensis* (Hamon and Lazazzera, 2001; Fagerlund *et al.*, 2014). Spo0A-P activates the transcription of *sinI* which encodes the antagonist of SinR (Kearns *et al.*, 2005) and represses a*brB* expression (Strauch *et al.*, 1990). In *B. subtilis* and *B. thuringiensis*, both SinR and AbrB are repressors of biofilm formation by inhibiting the transcription of genes involved in matrix biosynthesis (Hamon and Lazazzera, 2001, Kearns *et al.*, 2005, Majed *et al.*, 2016).

The rate of Spo0A-P is modulated by Rap phosphatases which dephosphorylate Spo0F-P (Perego and Hoch, 1996). A Rap phosphatase is inhibited by its cognate Phr that restores the activity of the phosphorelay and allows the phosphorylation of Spo0A (Ishikawa *et al.*, 2002). Rap proteins are structured in TPR domains which mediate interactions with Spo0F-P or with the Phr inhibitor peptide (Parashar *et al.*, 2011; Gallego del Sol and Marina, 2013). Phr-encoding genes are located downstream from the *rap* genes and are co-transcribed (Perego and Brannigan, 2001). However, the *phr* genes generally have a secondary promoter controlled by the alternative σ^H factor (McQuade *et al.*, 2001). Phr is produced as a pro-peptide composed by an N-terminal part with a signal peptide necessary to its secretion and a C-terminal part including the mature Phr peptide (Pottathil and Lazazzera, 2003). After being outside processed, the active Phr is

reimported *via* oligopeptide permease system (Opp) within the bacteria (Perego, 1997). Then, the mature Phr binds to the Rap protein resulting in the inhibition of Rap activity.

Rap proteins have been defined as response regulator aspartate phosphatase and are involved in various bacterial processes. The *rap-phr* genes can be located both on the chromosome and on the plasmid. Recently, several studies have reported the regulation of important processes by plasmid-borne Rap-Phr systems in *Bacillus* species. In *B. subtilis*, several plasmid-borne Rap-Phr systems have been described. The Rap-Phr60 system borne by the pTA1060 regulates the production of extracellular proteases, sporulation and biofilm formation through the Spo0A pathway and competence *via* the ComA pathway (Koetje *et al.,* 2003; Boguslawski *et al.*, 2015). The pBS32 plasmid encodes the RapP-PhrP system that controls biofilm architecture and sporulation by modulating the rate of Spo0A-P through the dephosphorylation of Spo0F (Parashar *et al.*, 2013b). The Rap-PhrLS20 system coordinates conjugation of plasmid pLS20 with population density (Singh *et al.*, 2013). In other *Bacillus* species, a Rap-Phr system present in the *B. anthracis* pXO1 virulence plasmid was shown to control sporulation (Bongiorni *et al.*, 2006) and the RapQ-PhrQ systems in pBSG3 plasmid from *B. amyloliquefaciens* is able to control sporulation and genetic competence in the heterologous host *B. subtilis* (Yang *et al.*, 2015).

B. thuringiensis species exhibit a complex plasmid profile, varying in size (2-80 MDa) and number (1-17) (Lereclus *et al*., 1982). The small plasmids are generally described as cryptic plasmids with no identified functions excepting genes for maintenance (Andrup *et al.*, 2003; Lereclus and Arantes, 1992). However, some studies demonstrated that these plasmids may provide genes with important metabolic functions (Hoflack *et al.*, 1997; Rasko *et al.*, 2005). The genome sequence of *B. thuringiensis* serovar *kurstaki* HD73 strain (Liu *et al.*, 2013) reveals the presence of eight Rap-Phr systems, five chromosomal and three in different plasmids (pAW63,

pHT77, pHT8_1). In this study, we characterize the Rap8-Phr8 system harbored by the pHT8_1 plasmid. We present several evidences that this Rap-Phr system is a regulator of sporulation and biofilm formation *in vitro*. Moreover, we show the involvement of this Rap-Phr system in the regulation of the sporulation process in the insect, thus demonstrating the essential role of this plasmid-borne QS system when the bacteria grow and develop in naturalistic conditions.

Results

General description of the pHT8_1

The pHT8_1 of the *B. thuringiensis* strain HD73 is a small plasmid with a size of 8513 bp and a G + C content of 30.79 %. Blast analyses shown that the same plasmid is found in seven other *B. thuringiensis* strains (*Bt* Bc601, *Bt* YWC2-8, *Bt* YC-10, *Bt* serovar *kurstaki* strain HD1, *Bt* 407, *Bt* serovar *chinensis* CT-43, *Bt* serovar *kurstaki* strain YBT-1520).

The pHT8_1 harbors 10 putative open reading frames (ORFs) described in Table 1. This plasmid belongs to the rolling circle plasmid family with a Rep protein of 348 amino acids and a double-strand origin located 182 bp upstream from the start codon of the *rep* gene. The Rep protein displayed similarities with pC194 Rep family with the three characteristic conserved domains, I-FLTLTTPN, II-NPHFHVLIA and III-MAKYSGKDSD (Gros, *et* al., 1987; Ilyina and Koonin, 1992). The Mob protein present in pHT8_1 is classified into Mob_V family, which is defined by two conserved motifs, I-H-x2-R, III- HxDE(xn)phxh (Garcillán-Barcia *et al.*, 2009). The presence of a Mob protein suggests that the pHT8_1 plasmid is mobilizable.

The *orf* (HD73_8505) encodes a protein showing similarities with the Rap proteins (25% identity with RapH of *B. subtilis* and 45% identity with RapBAX0205 of *B. anthracis*) and was designated as *rap8*. We hypothesized that the downstream sequence (HD73_8506) was a *phr* gene and was designated as *phr8*. The amino acid sequence of Rap8 was used to predict the

protein structure and to verify the similarities with others Rap proteins by using the tool Phyre[2] (http://www.sbg.bio.ic.ac.uk/~phyre2/html/page.cgi?id=index). The predict structure of this protein showed similarities with the structure of RapH of *B. subtilis* (PDB ID 3Q15). Moreover, all the key residues involved in RapH-Spo0F interaction are conserved in Rap8 sequence (Fig. S1), in particular the Q47 residue required for the dephosphorylation of Spo0F-P (Parashar *et al.*, 2011).

Transcriptional analysis of the *rap* and *phr* genes

In *B. subtilis*, the *rap* and *phr* genes are often co-transcribed from a promoter upstream from the *rap* gene (McQuade *et al.*, 2001). The coding sequence of the *phr8* gene overlapped the *rap8* gene on 4 nucleotides suggesting that the two genes are co-transcribed (Table 1). We performed an RT-PCR analysis on RNA extracted 3 hours after the onset of stationary phase from HD73 wild type strain culture in HCT, a medium optimized for sporulation (Lereclus, *et al.*, 1982). We used primers overlapping the *rap8* and the *phr8* genes (Fig. 1A). The RT-PCR results showed that the *rap8* and *phr8* genes are co-transcribed (Fig. 1B). However, *phr* genes are generally also transcribed from a secondary independent promoter (McQuade *et al.*, 2001).

In order to study the kinetics of *rap8* and *phr8* expression and to determine whether *phr8* is transcribed from discrete promoter, the promoter regions of these two genes were fused separately to the *lacZ* reporter gene in pHT304-18'Z. The resulting plasmids (pHT-P_{rap}'Z and pHT-P_{phr}'Z) were transformed in the HD73 strain and β-galactosidase activities were measured in HCT medium at 37°C (Fig. 1C and 1D). The P*rap'lacZ* expression started 1 h after the onset of the stationary phase (t1) and increased up to t3 while the P*phr'lacZ* expression started at t2 and continued at least until t4. To determine whether *rap8* expression depends on sporulation, the plasmid pHT-P_{rap}'Z was transformed in the HD73 *spo0A*-deficient strain (Fig. 1C). The kinetics

of P*rap'lacZ* expression in the HD73 *spo0A*-deficient strain was similar to that in the wild-type strain until t2, but activation is prolonged in the Δ*spo0A* (Fig. 1C). This result indicates that *rap8* transcription occurs during sporulation but is independent on sporulation-specific sigma factors. In the 413-bp region upstream from *phr8* and used to construct the plasmid pHT-P_{phr}'Z, we identified a DNA sequence similar to the consensus – 10 box recognized by the sporulation-specific σ^E factor. This sequence (CATACAAG) is located at 118 pb upstream from the start codon. We assessed the P*phr'lacZ* expression in a HD73 *sigE*-deficient strain and no β-galactosidase activity was recovered (Fig. 1D). This result indicates that, in addition to the transcription from the *rap8* promoter, the *phr8* gene is transcribed from an additional promoter depending on the sporulation-specific sigma factor σ^E.

Rap8 inhibits sporulation and biofilm formation

In order to determine the role of Rap8 in sporulation and biofilm formation we expressed *rap8*, *rap/phr8* and *phr8* genes in the multicopy vector pHT315*xyl* under a xylose inducible promoter. These plasmids were designated pHT-*xyl*R, pHT-*xyl*RP and pHT-*xyl*P, respectively. They were introduced in the strain HD73 and the sporulation rates were determined after 48 h of growth in HCT medium, in the presence of xylose at 30°C (Figure 2A and Table S3). The HD73-*xyl* strain harbouring the empty plasmid pHT315*xyl* sporulated efficiently (83.31 ± 2.82%) while the HD73-*xyl*R strain has a sporulation-deficient phenotype (0.24 ± 0.07%) with a heat-resistant spore production 1500-fold fewer compared to the control strain. In contrast, the HD73-*xyl*RP and HD73-*xylP* strains displayed a sporulation efficiency similar to the control strain. These results demonstrated that Rap8 inhibits the sporulation and that the Phr8 peptide inhibits Rap8 activity, restoring the sporulation process.

Biofilm formation was measured in glass tubes after 48 h of static growth at 30°C in HCT medium in the presence of xylose. The HD73-*xyl* strain forms a biofilm at the air-liquid interface composed by a dense ring and a thick pellicle with an OD_{600} of 1.69 ± 0.12 (Figure 2B). The HD73-*xyl*R strain presented a strong inhibition of the biofilm formation with a very thick ring and an OD_{600} 7-fold less than the control strain (0.26 ± 0.03). Interestingly, co-expression of Rap8 and Phr8 in the HD73-*xyl*RP strain restores the Rap8 phenotype and forms a denser biofilm (3.95 ± 0.39), supporting the hypothesis that the Phr8 peptide inhibits Rap8 activity. Moreover, the HD73-*xyl*P strain forms a biofilm similar to the control strain (1.86 ± 0.35).

Altogether, our results demonstrated that the plasmid-borne Rap8-Phr8 system is involved in the regulation of the sporulation and biofilm formation processes. This system seems to function as a typical Rap-Phr system in which the Rap activity is inhibited by the Phr peptide.

Determination of the Phr peptide active form

The Rap activity is inhibited by the binding of its cognate mature Phr peptides (Ishikawa *et al.*, 2002). In *B. subtilis*, this active peptide generally corresponds to the five amino acids in C-terminal part of the peptide sequence and has an amino acid positively charged in second position (Pottathil and Lazazzera, 2003). To characterize the Phr8 peptide active form, we constructed the pHT-*xyl*RP3’ plasmid expressing the Rap8 and the Phr8 peptide truncated for the last six C-terminal amino acids AHGKDI including the putative mature Phr. The sporulation rate of the strain HD73-*xyl*RP3’ was drastically lower than the sporulation of the control strain (Fig. 3B and Table S3). This phenotype is similar to that obtained with the HD73-*xyl*R strain which did not produce the Phr8 peptide, suggesting that the carboxy-terminal end of Phr8 is required to generate an active peptide.

To determine the active form of Phr8, we used the synthetic peptides Phr8-6, Phr8-7 and Phr8-8 consisting in the sequences AHGKDI, YAHGKDI and QYAHGKDI, respectively (Fig. 3A). We measured the sporulation efficiency of the HD73-*xyl*R strain after addition of xylose and synthetic peptides at the onset of the stationary phase (t0). The sporulation defective phenotype caused by Rap8 was only restored when the bacterial cells were cultured with addition of 50 µM of Phr8-7 (Figure 3B and Table S3). Thus, the mature signaling peptide inhibiting Rap8 activity corresponds to the last seven C-terminal amino acids of Phr8.

Rap8 prevents expression of Spo0A-regulated genes

The commitment to sporulation is regulated by Spo0A which is the major regulator of sporulation (Sonenshein, 2000). The activity of Spo0A is modulated by the phosphorelay that allows the accumulation of Spo0A-P until the triggering of sporulation (Fujita *et al.*, 2005). To determine whether Rap8 affects the phosphorylation state of Spo0A, we measured the activity of the *spoIIE* promoter, positively controlled by Spo0A-P in *B. thuringiensis* (Perchat *et al.*, 2016). The *spoIIE* promoter (P_{spoIIE}) was fused to the *yfp* fluorescent reporter gene and cloned in the plasmids pHT315*xyl*, pHT-*xyl*R and pHT-*xyl*RP. The resulting plasmids (pHT-*xyl*-P_{spoIIE}'*yfp*, pHT-*xyl*R-P_{spoIIE}'*yfp* and pHT-*xyl*RP-P_{spoIIE}'*yfp*, respectively) were transformed into the HD73 strain and YFP fluorescence was measured with a microplate reader after growth in HCT medium in the presence of xylose (Fig. 4). The expression of the P_{spoIIE}'*yfp* transcriptional fusion is transiently activated 2 hours after the onset of stationary phase in the strain carrying the plasmid pHT-*xyl*-P_{spoIIE}'*yfp*. In sharp contrast, activation of *spoIIE* expression was not detected in the HD73 strain expressing Rap8 (strain harbouring pHT-xylR-P_{spoIIE}'*yfp*). However, *spoIIE* expression was restored in the strain expressing both Rap8 and Phr8 (strain harbouring pHT-*xyl*RP-P_{spoIIE}'*yfp*) and in the strain carrying pHT-*xyl*R-P_{spoIIE}'*yfp* after addition of the mature

Phr8-7 peptide to the culture medium. These results show that Rap8 prevents the transcription of a Spo0A-regulated gene and that the heptapeptide YAHGKDI is sufficient to abolish this negative effect. Therefore, these results strongly suggest that the activity of Rap8 on sporulation and biofilm formation is through the Spo0A pathway, probably by an action on the sporulation phosphorelay.

The Rap8-Phr8 system controls sporulation in cadavers of infected insects

We have shown that overexpression of the *rap8* gene under the control of xylose inducible promoter negatively affects biofilm formation and sporulation of the HD73 strain (Fig. 2). To determine the role of the Rap8-Phr8 QS system from a physiological point of view, we constructed deficient *rap8-phr8* and *phr8* mutant strains (designated ΔRP and ΔP, respectively). We first tested the sporulation of these mutant strains *in vitro* in HCT medium. Contrary to the results obtained by overexpression, the *phr8* deletion did not affect sporulation (Fig. 5A and Table S3). Several studies described the insect larvae as the ecological niche of *Bt*, where the infectious cycle can be completed (Raymond *et al.*, 2010; Slamti *et al.*, 2014). Thus, we evaluated the role of these mutant strains on sporulation in the larvae of the lepidopteran insect *Galleria mellonella*. Insect larvae were infected by intrahemocoelic injection of 2×10^4 vegetative bacteria and the sporulation efficiency was measured in dead larvae after 96 h at 30°C (Fig. 5B and Table S3). The ΔRP mutant strain sporulated as efficiently as the wild type strain. Interestingly, sporulation was significantly lower in the ΔP mutant strain than the wild type and the ΔRP mutant strains ($p < 0.01$). These results demonstrated that the Rap8 from the pHT8_1 plasmid acts as a negative regulator of the sporulation pathway in the cadaver of insect larvae.

Discussion

Plasmids are major factors of variability in bacteria. While plasmid replication, horizontal transfer and maintenance were extensively studied, the plasmid ecology and their role in the environment of bacterial communities are poorly understood (Smalla, *et al*., 2015). In the *B. cereus* group, the large-sized plasmids are the most studied, whereas small plasmids are frequently considered as cryptic plasmids. In this study, we characterized the Rap8-Phr8 QS system harboured by the pHT8_1 plasmid in the *B. thuringiensis* var. *kurstaki* HD73 strain. We showed that Rap8 prevents sporulation and biofilm formation and that its activity is inhibited by the Phr8 peptide. Sporulation and biofilm are two important processes that confer high resistance and dissemination capacity to bacteria (Majed *et al.*, 2016). Further plasmid-borne Rap-Phr systems have been described to regulate sporulation *in vitro* as Rap60 in *B. subtilis* (Boguslawski *et al.*, 2015), RapBAX0205 in *B. anthracis* (Bongiorni *et al.*, 2006) and RapQ in *B. amyloliquefaciens* (Yang *et al.*, 2015) or biofilm formation as RapP in *B. subtilis* (Parashar *et al.*, 2013b). Here we demonstrated the *in vivo* involvement of a Rap-Phr system in the sporulation control in insect larvae, the ecological niche of the *B. thuringiensis* bacteria (Raymond *et al.*, 2010; Slamti *et al.*, 2014).

The transcriptional regulator Spo0A activates the expression of early sporulation genes leading to the initiation of sporulation process in Bacilli (Hilbert and Piggot, 2004). Furthermore, Spo0A is also involved in biofilm formation (Hamon and Lazazzera, 2001; Fagerlund *et al.*, 2014) through the repression of *abrB* transcription (Hamon *et al.*, 2004) and the activation of *sinI* transcription which produces the antagonist of SinR, the master repressor of biofilm formation (Chu *et al.*, 2006). We demonstrated that the inhibition of sporulation and biofilm formation by Rap8 is mediated through a Spo0A-dependent pathway: in the absence of Phr8, the Rap8 protein represses the expression of the *spoIIE* gene, which is positively regulated by Spo0A~P in

B. subtilis (York *et al.*, 1992) and in *B. thuringiensis* (Perchat *et al.*, 2016). Rap8 might impair the sporulation process by dephosphorylating Spo0F-P as described for other Rap proteins (Perego, 2013; Bongiorni *et al.*, 2006; Parashar, *et al.*, 2013a). This hypothesis is supported by the conservation, in Rap8, of the key residues identified in RapH and needed to the binding and the dephosphorylation of Spo0F-P (Parashar *et al.*, 2011).

The Rap8 activity is inhibited by its cognate signaling peptide Phr8. We demonstrated that the active form of Phr8 is an heptapeptide located in C-terminal part of the peptide sequence. In *B. subtilis,* Phr active peptides generally consist in pentapeptides located in the C-terminal end or within the C-terminal domain (Perego and Brannigan, 2001; Solomon *et al.*, 1996). Interestingly, in *B. thuringiensis* and *B. cereus,* the signaling peptides controlling the activity of the quorum sensors PlcR and NprR are heptapeptides (Bouillaut *et al.*, 2008; Perchat *et al.*, 2011). It would be interesting to determine whether this size difference is species-specific and what are the reasons for such a specificity. The Phr8 pro-peptide sequence (48 AA) contains two typical domains, a hydrophobic N-terminal and a hydrophilic C-terminal, separated by a putative signal peptidase cleavage site identified by SignalP server between the position 32 and 33 (HDA-KE). The hydrophobic domain and the cleavage site are necessary to the peptide export *via* Sec pathway, a common secretory system (Schatz and Beckwith, 1990). The presence of an alanine residue before the N-terminus of the active Phr peptides is necessary for the maturation process in *B. subtilis* (Stephenson *et al.*, 2003) and the cleavage requires several peptidases such as subtilisin, Epr and Vpr excepted for PhrE (Lanigan-Gerdes *et al.*, 2007). No alanine residue was found upstream from the Phr8 heptapeptide suggesting that this peptide is processed in a different way. Then, to inhibit Rap activity, the mature Phr peptides should to be reimported into the cytoplasm of the bacteria. The signaling peptides from the RNPP family have been found to be uptake inside the bacteria by the oligopeptide permease Opp system (Leonard *et al.*, 1996;

Perego, 1997; Gominet *et al.*, 2001). In *B. thuringiensis*, five complete oligopeptide permease systems were found (Slamti *et al.*, 2016), but only one has been characterized for the uptake of PapR (Gominet *et al.*, 2001).

Our transcriptional analysis show that the *rap8-phr8* genes are co-transcribed from a promoter upstream from *rap8*. The transcription from this promoter is activated at the onset of sporulation when bacteria are grown in a sporulation-specific medium (HCT). The *phr8* gene is co-transcribed with *rap8*, but also transcribed from another promoter located in the *rap8* coding sequence and activated by the early mother-cell-specific σ^E factor. In sporulation medium, *phr8* transcription from the σ^E-dependent promoter starts two hours after the onset of the stationary phase and allows Phr8 production while *rap8* transcription is reduced or shut off. In *B. subtilis*, the *phr* genes generally have a secondary σ^H promoter enhancing the accumulation of Phr peptide and consequently triggering sporulation (McQuade *et al.*, 2001). In insect larvae, at low bacterial density, Rap8 protein probably dephosphorylates Spo0F-P to maintain a low Spo0A-P concentration and prevent sporulation initiation. After the insect death, the cell density increases and Phr8 accumulates and inhibits Rap8 activity leading to reach a high Spo0A~P threshold needed to initiate the transcription of sporulation genes. In the same time, *rap8* transcription is reduced and σ^E-dependent expression of *phr8* boosts the production of Phr8, thus preventing Rap8 activity and generating a high Spo0A-P concentration. This allows bacteria to achieve the sporulation process in the host cadaver to survive and disseminate.

Thus, the pHT8_1 plasmid is an effective element regulating the development of *B. thuringiensis* in its ecological niche. The broad distribution of this plasmid in various *B. thuringiensis* strains may reflect its importance for the development and the adaption of these bacteria in insects. Others cryptic plasmids harbouring Rap-Phr system have been described in *Bacillus* genus. The Rap-Phr60 from the *B. subtilis* pTA1060 plasmid is involved in sporulation,

biofilm formation, competence and protease production (Koetje *et al.*, 2003; Boguslawski *et al.*, 2015). The Rap-PhrQ system from the *B. amyloliquefaciens* pBSG3 plasmid controls sporulation, competence and surfactin production (Yang *et al.*, 2015). However, these systems were evaluated *in vitro* without considering the ecological niche of each species. Others uncharacterized Rap-Phr systems have been also identified in the *B. pumilus* pPZZ84 plasmid (Zhang *et al.*, 2010) and the *B. licheniformis* pFL5 and pFL7 plasmids (Parini *et al.*, 2004) without functional studies.

B. thuringiensis has a riche plasmid profile (Lereclus *et al.*, 1982) and these plasmids may provide important indications to understand the ecology and the evolution of the bacteria. Within the *B. cereus* group, plasmids were mainly studied for their contribution to the bacterial pathogenicity through the production of insecticidal toxins in *B. thuringiensis* (Deng *et al.*, 2014), of the anthrax toxins in *B. anthracis* (Mock and Mignot, 2003) and of the cereulide toxin in the emetic *B. cereus* strains (Ehling-Schulz *et al.*, 2006). In this work we report, for the first time, that a plasmid-borne Rap-Phr system regulates the sporulation process in naturalistic conditions. This quorum sensing system controls the last stage of bacteria lifecycle in the insect larvae. Thus, a particular attention must be turned to plasmids, especially the small cryptic plasmids, which might harbor important genes related to metabolic or adaptive functions and consequently to the bacterial fitness. However, complementary studies should be done to characterize the functions of other genes harbored by the pHT8_1 and to show a direct involvement of this small plasmid in the adaptation and evolution of the bacteria.

Experimental procedures

Bacterial strains and growth conditions

Bacillus thuringiensis (*Bt*) strains used in this study are all derivatives of the parental acrystalliferous (Cry^-) *Bacillus thuringiensis* var. *kurstaki* HD73 strain cured of the plasmid

pHT73 carrying the *cry1Ac* gene (Wilcks *et al.*, 1998). *Escherichia coli* K-12 strain TG1 was used as host for the construction of plasmids and cloning experiments. *E. coli* strain ET12567 (Stratagene, La Jolla, CA, USA) was used to prepare plasmid DNA for *Bacillus* electroporation (Dam- Dcm-). *E. coli* and *Bt* strains were grown in Luria Bertani (LB) medium or in HCT, a sporulation-specific medium (Lecadet *et al.*, 1980), at 37°C or 30°C. *E. coli* strains were transformed by thermal shock and *Bt* by electroporation (Lereclus *et al.*, 1989). *B. thuringiensis* var. *kurstaki* HD73-Δ*spo0A* mutant (Yang *et al.*, 2012) and *B. thuringiensis* var. *kurstaki* HD73-Δ*sigE* mutant (Yang *et al.*, 2012) were used to promoter expression experiments. Appropriate antibiotic were used at the following concentrations: ampicillin 100 µg ml^{-1} for *E. coli*; erythromycin 10 µg ml^{-1} and kanamycine 200 µg ml^{-1} for *Bt*. Bacteria with the Lac^{+} phenotype were identified on LB plates containing 100 µg ml^{-1} Xgal. In *Bacillus*, *xylA* promoter was induced in culture medium containing 20 mM of xylose.

DNA manipulations

Chromosomal DNA was extracted from *Bt* cells using the Puregene Yeast/Bact. Kit B (QIAgen, France). Plasmid DNA was extracted from *E. coli* by a standard alkaline lyses procedure using QIAprep spin columns (QIAgen, France). PCRs were performed in an Applied Biosystems 2720 Thermal cycler (Applied Biosystem, USA) with Phusion High-Fidelity or Taq DNA Polymerase (New England Biolabs). Amplified fragments were purified using the QIAquick PCR purification Kit (QIAgen, France). Oligonucleotides (Table S1) were synthesized by Sigma-Proligo (France). Digested DNA fragments were separated on 1% agarose gels and purified from gels using the QIAquick gel extraction kit (QIAgen, France). Restriction enzymes and T4 DNA ligase (New England Biolabs, USA) were used in accordance with the

manufacturer's recommendations. Nucleotide sequences were determined by GATC Biotech (Konstanz, Deutschland).

Plasmid constructions

The high copy number plasmid pHT315-P*xylA* was used to produce Rap8 and Phr8 under xylose-inducible promoter (Grandvalet *et al.*, 2001). Transcriptional fusions for *rap8* and *phr8* promoter regions were constructed in pHT304.18'Z (Arantes and Lereclus, 1991). The plasmid pMAD (Arnaud *et al.*, 2004) was used for homologous recombination. All the constructed plasmids used in this study are described in Table S2.

Construction of the *B. thuringiensis* mutant strains

The plasmids pMADΩ*rap8/phr*8::*aphA3* and pMADΩ*phr*8::*aphA3* were used to disrupt *rap8/phr8* and *phr8* genes, respectively. The *rap8/ph8r* and *phr8* genes were replaced with the *aphA3* gene with its own propoter by homologous recombination (Lereclus *et al*., 1992). The recombinants strains, designated ΔRP and ΔP respectively, present a Lac- phenotype and were resistant to kanamycine and sensible to erythromycin.

Sporulation assays

HCT sporulation medium was used to determine sporulation efficiency. The cultures were grown at 30°C for 48 hours in the presence of xylose 20 mM, and then serial dilutions were plated in triplicate before and after heat treatment during 12 minutes at 80°C. The sporulation percentage was calculated as 100 x ratio between heat-resistant spores ml^{-1} and total colony-forming units ml^{-1}. All experiments were repeated at least three times and mean values were calculated.

Biofilm assays

The ability to form biofilms in air-liquid surface was evaluated in glass tubes in HCT medium at 30°C for 48 hours in the presence of xylose 20 mM, as previously described (Fagerlund *et al.*, 2014). All experiments were repeated at least three times and mean values were calculated.

Use of synthetic peptides

Three Phr peptides were synthesized, purified and identified by mass spectrophotometry with a degree of purity superior to 95 % by GenScript (USA). To determine the active peptide, cells were cultured at 37°C in HCT medium until t1 (one hour after the onset of stationary phase). The culture was then divided into three aliquots and each synthetic peptide was added to one aliquot at a final concentration of 50 µM. The sporulation efficiency was measured as previously described. All experiments were repeated at least three times and mean values were calculated.

RT-PCR analysis of the *rap/phr8_1* locus

B. thuringiensis HD73 strain was grown with shaking at 175 rpm, in HCT medium at 37°C until t3. RNA extraction and primer extension were performed as described previously (Gélis-Jeanvoine *et al.*, 2016). RNA was reverse transcribed into cDNA with the iScript cDNA synthesis kit (Bio-Rad, France) using the provided random primers. PCR reactions were performed with overlapping oligonucleotides (RT1 and RT2) (Table S1) on DNA (positive control), RNA (negative control) and cDNA.

β-*Galactosidase* assays

β-Galactosidase activity was measured during growth in HCT medium at 37°C with shaking at 175 rpm. The assays were performed as described previously (Perchat *et al.*, 2011). Specific

activities are expressed in units of β-galactosidase per milligram of protein (Miller units). The assays were independently repeated three times and mean values were calculated.

Fluorescence assays

Fluorescence was measured during growth in HCT medium at 37°C with shaking at 175 rpm. The xylose and synthetic peptides were added at the onset of the stationary phase (t0). Cells from 500 µl of growing culture were harvested and fixed as previously described (Fagerlund *et al.*, 2014). YFP fluorescence from the *spoIIE* promoter was recovered at 535 nm after excitation at 485 nm using the Infinite 200 PRO microplate reader (Tecan, Switzerland). The HD73-pHT315*xyl* strain was used as auto-fluorescence control. Data were collected with i-Control software (Tecan, Switzerland) and results are expressed in arbitrary units per OD_{600} unit. The assays were independently repeated three times and mean values were calculated.

Insect larvae experiments

G. mellonella larvae were infected with intrahemocoelic injection of 2×10^4 vegetative cells as previously described (Salamitou *et al.*, 2000). Infected larvae were kept at 30°C and 24 h after infection surviving insects were eliminated. After 96 h, 3 pairs of dead larvae were transferred to 3 different 14 ml tubes, crushed and homogenized in 10 ml of physiologic water (0,9% NaCl). Serial dilutions were plated before and after heat treatment 12 min at 80°C, and the sporulation percentage were calculated (100x heat-resistant spores/total cells). At least three independently replicates were performed for each strain. The data were analyzed by using a student T-test ($p \leq 0.05$).

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Tables and figures

Table 1 Putative open reading frames from the pHT8_1 plasmid annotated in NCBI database sequences (NC_020241.1 and CP004074.1).

ORF/ gene name	Position (bp)	Size of the putative protein (aa)	Annotation	Accession number
HD73_8502	330-593	87	Hypothetical protein	WP_000874967.1
HD73_8503	867-1094	75	DNA-binding transcriptional regulator, XRE family	WP_000869869.1
HD73_8505	1566-2645	359	Rap	WP_001102658.1
HD73_8506	2642-2788	48	Phr	AGE81756.1
HD73_8507	2899-3489	196	Hypothetical membrane protein	WP_002029249.1
HD73_8508	3473-3679	68	Hypothetical protein	WP_001160161.1
HD73_8512	4772-4957	61	Hypothetical protein	WP_001138309.1

HD73_8513	5052-6257	401	Recombinase Mob superfamily	WP_000033305.1
HD73_8515	6727-7773	348	Rep superfamily	WP_001085408.1
HD73_8516	7777-7974	65	Hypothetical protein	WP_000861196.1

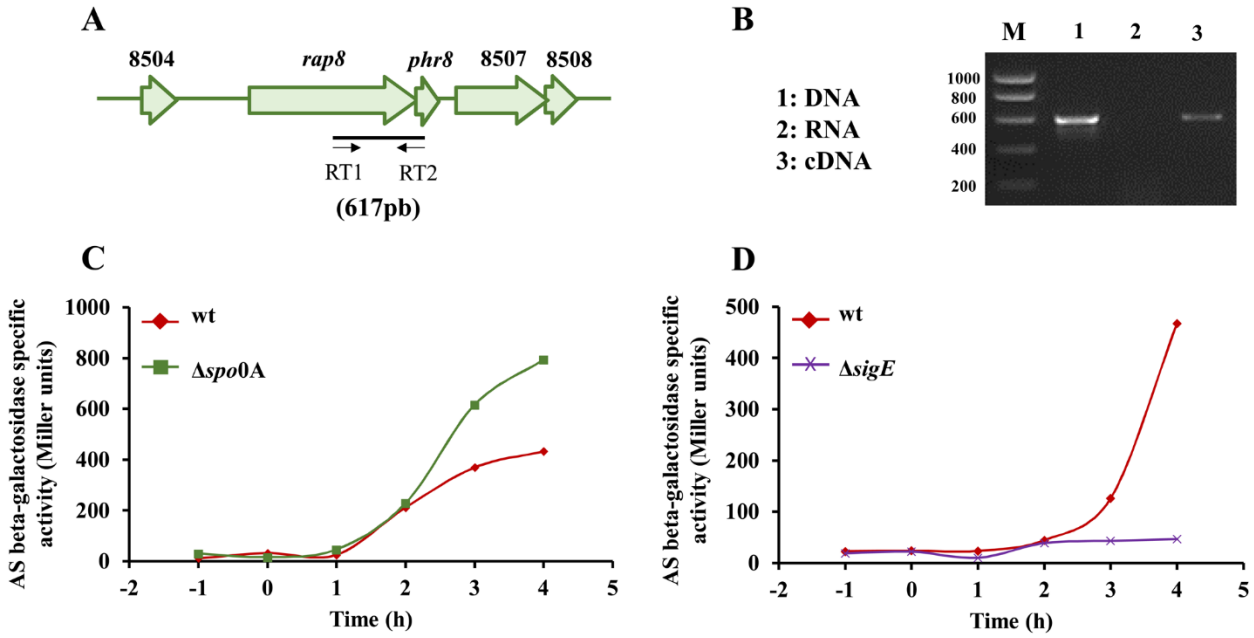

Fig. 1 Characterization of the *rap8-phr8* transcription unit. (A) Schematic representation of the *rap8-phr8* locus of the pHT8_1 plasmid. The fragment, with its respective size, amplified in the RT-PCR experiment is represented below the locus. **(B)** RT-PCR experiments. Total RNA was extracted from a t3 culture in HCT medium at 37°C and 175 rpm. DNA (positive control, lane 1), RNA (negative control, lane 2) and cDNA (lane 3) were used as a template for PCR experiments. PCR products were separated on 1% agarose gel. (M) Molecular weight marker Smartladder small fragments (Eurogentec). **(C)** Kinetics of *rap8-phr8* expression. β-Galactosidase activity of the HD73 wild-type and HD73 Δ*spo0A* mutant strains carrying the pHT-P*rap'lacZ*. The bacteria were grown at 37°C in HCT medium. Time zero was defined as the onset of the stationary phase. **(D)** Kinetics of *phr8* expression. β-Galactosidase activity of the

HD73 wild-type and HD73 Δ*sigE* mutant strains carrying the pHT-P*phr'lacZ*. The bacteria were grown at 37°C in HCT medium. Time zero was defined as the onset of the stationary phase.

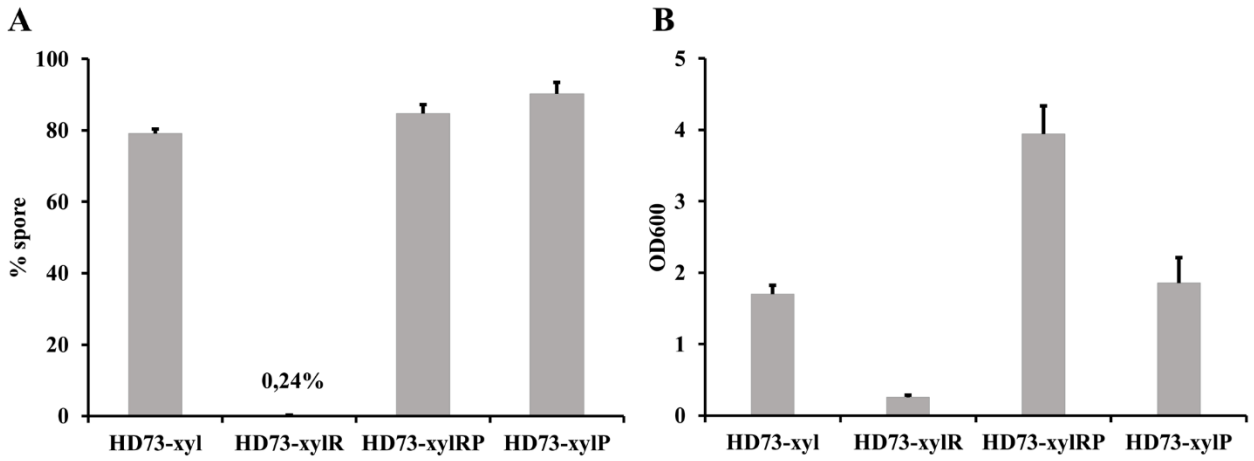

Fig. 2 Rap8 prevents sporulation and biofilm formation. (A) Sporulation efficiency of the HD73 control (HD73-*xyl*) strain and expressing Rap8 (HD73-*xyl*R), Rap8-Phr8 (HD73-*xyl*RP), Phr8 (HD73-*xyl*P) strains. The percentages of spores were calculated as 100 × the ratio between heat-resistant spores ml^{-1} and viable cells ml^{-1}. The viable cells and heat-resistant spores were counted after 2 days in HCT medium at 30°C. Error bars: Standard Error of the Mean (SEM). The experimental values are given in S3 Table. **(B)** Biofilm formation by the HD73 control *(xyl*) strain and expressing Rap8 *(xyl*R), Rap8-Phr8 *(xyl*RP), Phr8 *(xyl*P) strains. Biofilm production was assessed in glass tubes after 48h of static growth in HCT medium. Error bars: Standard Error of the Mean (SEM).

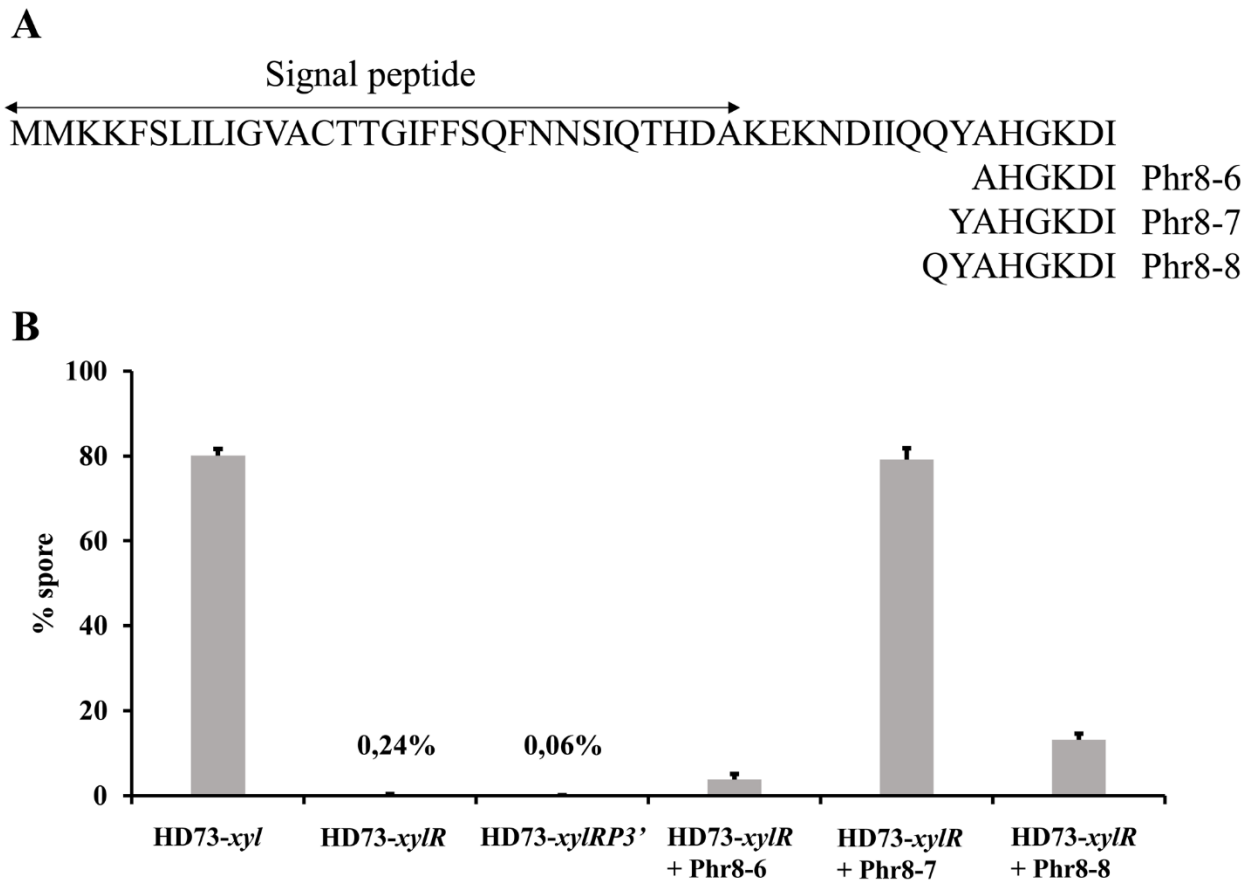

Fig. 3 Characterization of the signaling peptide Phr. (A) Amino acid sequence of the Phr8 peptide and of the synthetic peptides used for complementation experiments. The double arrow indicates the N-terminal putative signal peptide sequence. The potential cleavage site was predicted with the SignalP 4.1 program (http://www.cbs.dtu.dk/services/SignalP). **(B)** Sporulation efficiency of the HD73 control (HD73-*xyl*) strain and expressing Rap8 (HD73-*xyl*R), Rap8-Phr8 truncated for 6 last C-terminal amino acids (HD73-*xyl*RP3') strains. The strain expressing Rap8 (HD73-*xyl*R) was complemented with the synthetic peptides Phr8-6, Phr8-7 and Phr8-8 added independently 1 hour after the onset of the stationary phase at 50 µM final concentrations. The viable cells and heat-resistant spores were counted after 2 days in HCT medium at 30°C. Error bars: Standard Error of the Mean (SEM).

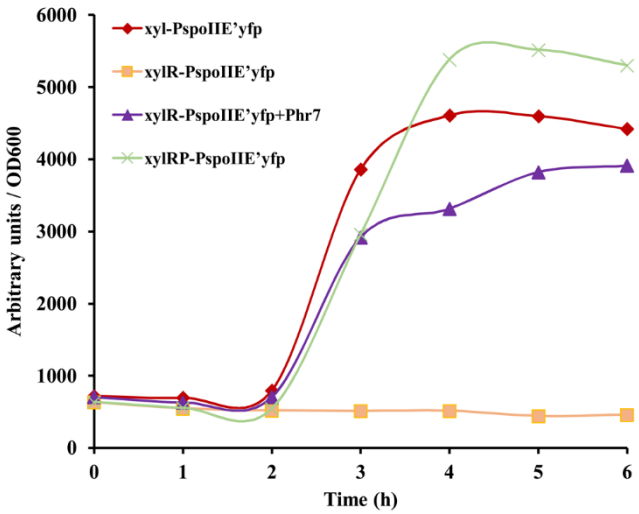

Fig. 4 Rap8 prevents expression of Spo0A-regulated genes. Kinetics of *spoIIE* expression. YFP activity of the HD73 wild type strain carrying the pHT-*xyl*-P_{spoIIE}'*yfp* (xyl-PspoIIE'yfp), the pHT-*xyl*R-P_{spoIIE}'*yfp* (*xyl*R-PspoIIE'yfp), pHT-*xyl*RP-P_{spoIIE}'*yfp* (*xyl*RP-spoIIE'yfp) and in the HD73 harbouring the pHT-*xyl*R-P_{spoIIE}'*yfp* complemented with the Phr8-7 synthetic peptide (*xyl*R-PspoIIE'yfp+Phr7). The YFP fluorescence was measured during growth in HCT medium at 30°C. Time zero was defined as the onset of the stationary phase. The results are expressed in arbitrary units per OD_{600} unit.

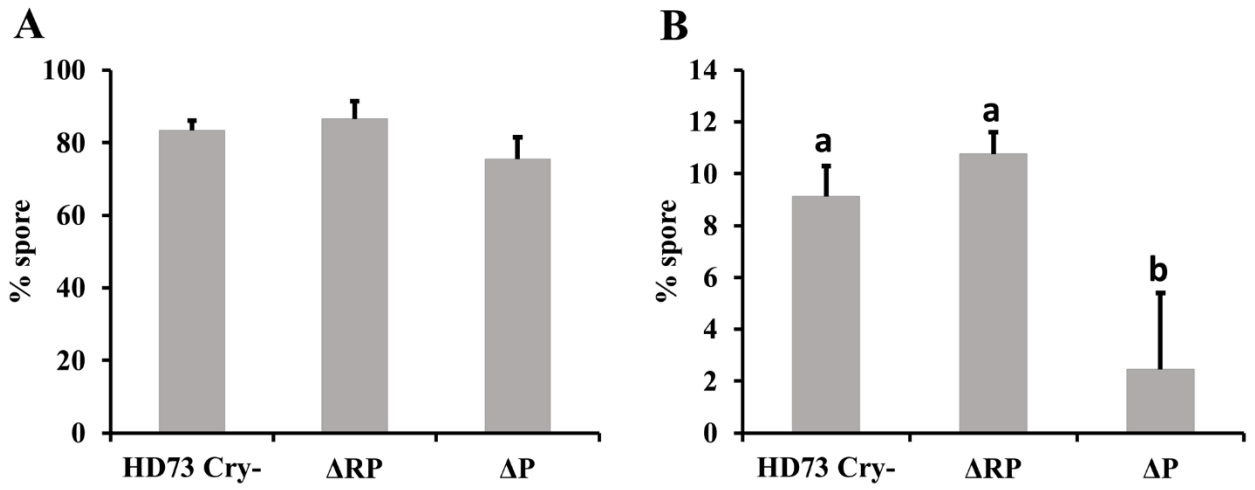

Fig. 5 Rap8-Phr8 system controls sporulation in cadavers of infected insects. (A) Sporulation efficiency of the HD73 wild type, Δ*rap8-phr8* (ΔRP) and Δ*phr8* (ΔP) mutants strains *in vitro.* The viable cells and heat-resistant spores were counted after 2 days in HCT medium at 30°C. Error bars: Standard Error of the Mean (SEM). **(B)** Sporulation efficiency of the HD73 wild type, Δ*rap8-phr8* (ΔRP) and Δ*phr8* (ΔP) mutants strains *in vivo*. The viable cells and heat-resistant spores were counted after 4 days in dead *G. mellonella* larvae at 30 °C. Error bars: Standard deviation. Bars with different letters (a, b) indicate significant differences in their mean values ($P < 0.01$).

SUPPLEMENTARY MATERIAL

TABLE S1 Primers used in this study.

Name	Sequence	Restriction site
Rap8505-F	CGCGGATCCGGAAAAGGAGAAGATTCTATGAATG	*Bam*HI
Rap8505-R	CCCAAGCTTTCATCATTTCAATGACTCCCTTTC	*Hind*III
Phr8505-F	CGCGGATCCGAAAGGGAGTCATTGAAATGATG	*Bam*HI
Phr8505-R	CCCAAGCTTGCTTTTAAATATCTTTTCCATGTGC	*Hind*III
Amont1-F	CATGCCATGGGCTTTGTAATTTGTTAGGAATGCC	*Nco*I
Amont1-R	CGGGGTACCAGAATCTTCTCCTTTTCCAAAATTATC	*Kpn*I
Amont2-F	CATGCCATGGCGAGCGCAACACATTATTAAAGC	*Nco*I
Amont2-R	CGGGGTACCTCATTTCAATGACTCCCTTTCTAATAAG	*Kpn*I
Aval-F	CCGCGCATGCAAGCAAATTCCAACAATTAGCAATCG	*Sph*I
Aval-R	CCGGAATTCGAAACGAGGAAAAATTAACTTGTAAACC	*Eco*RI
Prom8505-F	CCCAAGCTTGTATGAATATGGTTTGAATGTGGTAAATG	*Hind*III
Prom8505-R	CGCGGATCCAGAATCTTCTCCTTTTCCAAAATTATC	*Bam*HI
Prom8505Phr-F	CCCAAGCTTGCAGATCAAAATCTTTCTGAACTTGC	*Hind*III
Prom8505Phr-R	CGCGGATCCTTCAATGACTCCCTTTCTAATAAGTTTC	*Bam*HI
Phr8505-R3	CCCAAGCTTAATATTGTTGAATAATATCATTTTTTTCTTTAGC	*Hind*III
RT1	GATCTGTGAGAAGTTGGATGTTGAG	
RT2	GTTGCGCTCGAATTTCTTGATAC	
P*spoIIE*-F	AACTGCAGCTGGCTAGAGCGTACGG	*Pst*I
P*spoIIE*-R	CGTCTAGAGCTAAAAATGCTAGCGGC	*Xba*I
xylRout3’	GGAATGTCCTCCATTGTGATTGATC	
PU-EcoRI	CGGAATTCGCCAGGGTTTTCCCAGTCACGAC	*Eco*RI
YFP-R	CGGAATTCTTATTTGTATAGTTCATCCATGC	*Eco*RI

TABLE S2 Plasmids constructed for this study.

Plasmid	Characteristics
pHT-*xyl*R	Wild-type *rap8* gene was amplified using primers Rap8505-F/Rap8505-R and HD73- chromosomal DNA as template, and inserted between the *Bam*HI and *Hind*III sites of pHT*315xyl*.
pHT-*xyl*RP	Wild-type *rap8/phr8* genes were amplified using primers Rap8505-F/Phr8505-R and HD73- chromosomal DNA as template, and inserted between the *Bam*HI and *Hind*III sites of pHT*315xyl*.
pHT-*xyl*P	Wild-type *phr8* gene was amplified using primers Phr8505-F/Phr8505-R and HD73- chromosomal DNA as template, and inserted between the *Bam*HI and *Hind*III sites of pHT*315xyl*.
pHT-*xyl*RP3'	Wild-type *rap8* gene and *phr8* gene truncated for the last 6 codons were amplified using primers Rap8505-F/Phr8505-R3 and HD73- chromosomal DNA as template, and inserted between the *Bam*HI and *Hind*III sites of pHT*315xyl*.
pHT-P_{spoIIE}'*yfp*	The promoter region of the *spoIIE* gene was amplified using primers P*spoIIE*-F/ P*spoIIE*-R and HD73- chromosomal DNA as template, and inserted between the *Pst*I and *Xba*I sites of pHT304-18Ω*yfp* plasmid (Fagerlund et al., 2014). This results in the creation of a transcriptional fusion between *spoIIE* promoter and *yfp* gene.
pHT-*xyl*-P_{spoIIE}'*yfp*	The fragment P*spoIIE'yfp* was amplified using primers PU-*Eco*RI /YFP-R and pHT304-18ΩP*spoIIE'yfp* as template. The fragment was inserted in the *Eco*RI site of pHT315*xyl*. The cloning orientation was verified by PCR with primers P*spoIIE*-F/xylRout3' to avoid influence *xylR* promoter.
pHT-*xyl*R-P_{spoIIE}'*yfp*	The fragment P*spoIIE'yfp* was amplified using primers PU-*Eco*RI /YFP-R and pHT304-18ΩP*spoIIE'yfp* as template. The fragment was inserted in the *Eco*RI site of the pHT-*xyl*R plasmid. The cloning orientation was verified by PCR with primers P*spoIIE*-F/xylRout3' to avoid influence *xylR* promoter.
pHT-*xyl*RP-P_{spoIIE}'*yfp*	The fragment P*spoIIE'yfp* was amplified using primers PU-*Eco*RI /YFP-R and pHT304-18ΩP*spoIIE'yfp* as template. The fragment was inserted in the *Eco*RI site of the pHT-*xyl*RP plasmid. The cloning orientation was verified by PCR with primers P*spoIIE*-F/xylRout3' to avoid influence *xylR* promoter.
pHT-P_{rap}'Z	The promoter region of the *rap8* gene was amplified using primers Prom8505-F/Prom8505-R and HD73-

	chromosomal DNA as template, and inserted between the *Hind*III and *Bam*HI sites of pHT304-18'Z. This results in the creation of a transcriptional fusion between *rap8* promoter and *lacZ* gene.
pHT-P_{phr}'Z	The promoter region of the *phr8* gene was amplified using primers Prom8505Phr-F/Prom8505Phr-R and HD73- chromosomal DNA as template, and inserted between the *Hind*III and *Bam*HI sites of pHT304-18'Z. This results in the creation of a transcriptional fusion between *phr8* promoter and *lacZ* gene.
pMADΩ*rap8/phr*8::*aphA3*	5' and 3' regions of *rap8-phr8* genes were amplified using primers Amont1-F/ Amont1-R and Aval-F/Aval-R, respectively, and HD73- chromosomal DNA as template. The 5' end was purified as an *Nco*I/*Kpn*I and the 3' end as a *Sph*I and *Eco*RI. The kanamycine resistance gene (*aphA3*) was purified as a *Kpn*I/*Sph*I fragment from pDG783 (Guérout-Fleury *et al.*, 1995) and inserted with the 5' and 3' parts of *rap8/phr8* between the *Nco*I and *Eco*RI sites of pMAD.
pMADΩ*phr*8::*aphA3*	5' and 3' regions of *phr8* gene were amplified using primers Amont2-F/ Amont2-R and Aval-F/Aval-R, respectively, and HD73- chromosomal DNA as template. The 5' end was purified as an *Nco*I/*Kpn*I and the 3' end as a *Sph*I and *Eco*RI. The kanamycine resistance gene (*aphA3*) was purified as a *Kpn*I/*Sph*I fragment from pDG783 (Guérout-Fleury *et al.*, 1995) and inserted with the 5' and 3' parts of *phr* between the *Nco*I and *Eco*RI sites of pMAD.

TABLE S3 Efficiency of sporulation of *B. thuringiensis* HD73 derivative strains.

Strain	Medium	Viable cells	Heat-resistent spore	% spore
HD73 Cry$^-$	HCT	5.46E+08 (±4.52E+07)	4.54E+08 (±3.89E+07)	83.31 (±2.82)
HD73-*xyl*	HCT	3.93E+08 (±1.85E+07)	3.12E+08 (±1.73E+07)	79.09 (±1.29)
HD73-*xyl*R	HCT	6.70E+07 (±4.67E+06)	1.66E+05 (±4.65E+04)	0.24 (±0.07)
HD73-*xyl*RP	HCT	3.28E+08 (±8.58E+06)	2.77E+08 (±8.16E+06)	84.75 (±2.40)
HD73-*xyl*P	HCT	3.74E+08 (±2.12E+07)	3.37E+08 (±2.10E+07)	90.23 (±3.25)
HD73xylRP R3'	HCT	6.79E+07 (±4.26E+06)	4.25E+04 (±1.00E+04)	0.06(±0.01)
HD73-*xyl*R + Phr7	HCT	2.20E+08 (±1.08E+07)	1.74E+08 (±1.27E+07)	79 (±2.70)
ΔRP	HCT	5.11E+08 (±8.54E+07)	4.43E+08 (±7.92E+07)	87 (±4.92)
ΔP	HCT	5.76E+08 (±8.76E+07)	4.24E+08 (±4.98E+07)	75 (±5.89)
HD73 Cry$^-$	Insect larvae	1.81E+08 (±6.88E+07)	2.10E+07 (±9.69E+06)	9.45 (±1.17)
ΔRP	Insect larvae	1.65E+08 (±5.30E+07)	1.82E+07 (±6.12E+06)	10.77 (±0.84)
ΔP	Insect larvae	1.93E+07 (±5.15E+06)	6.45E+05 (±2.53E+05)	2.55 (±2.95)

The percentages of spores were calculated as 100 × the ratio between heat-resistant spores ml−1 and viable cells ml−1. The viable cells and heat-resistant spores were counted after 2 days in HCT medium at 30°C. Results are given as mean ± standard error of the mean (SEM).

A

```
RapH      1 MSQAIPSSRVGVKINEWYKMIRQFSVPDAEILKAEVEQDIQQMEEDQDLL     50
            |:..:.:.::...:|:||:.||...:..|:.||.::|::|..:||||:||
Rap8      1 MNVQLGNEQITKLLNDWYQEIRAQHIIKAKQLKQKIEKEINNIEEDQNLL     50

RapH     51 IYYSLMCFRHQLMLDYLEPGKTYGNRPTVTELLETIETPQKKLTGLLKYY    100
            :||||:..|::::.:      .|.:|   .:.||.||..::.....|.||
Rap8     51 LYYSLIDLRYKILTN------DYADR---EQSLEKIEQLKEHTNNFLDYY     91

RapH    101 SLFFRGMYEFDQKEYVEAIGYYREAEKELPFVSDDIEKAEFHFKVAEAYY    150
            ..||:||:......|.|...||..|||.|..:.|::|||||::.||..||
Rap8     92 YHFFKGMHAMKTGNYSETQKYYDIAEKLLEEIPDEVEKAEFNYAVATFYY    141

RapH    151 HMKQTHVSMYHILQALDIYQNHPLYSIRTIQSLFVIAGNYDDFKHYDKAL    200
            |..|..::..:..:|...:.....|.|:|......:.......:.:..|.
Rap8    142 HTHQALLATQYANKAKSFFSGKLGYEIKTGACENTLGMACITLREFSVAE    191

RapH    201 PHLEAALELAMDIQNDRFIAISLLNIANSYDRSGDDQMAVEHFQKAAKVS    250
            .:|.:|:........::...|...|:...|......::|:.:..::.:..
Rap8    192 EYLLSAINKFEKHSENKLALIVRYNLGLLYADQNLSELAIRYLLESFENQ    241

RapH    251 REKVPDLLPKVLFGLSWTLCKAGQTQKAFQFIEEGLDHITARSHKFYKEL    300
            .:..     |.:|.|:....|..||.....:||:||:    ..::.||..
Rap8    242 EDDY-----KTMFLLAQEYYKLKQTNTVNTYIEKGLN----VCNQEYKIH    282

RapH    301 FLFLQAVYKETVD---ERKIHDLLSYFEKKNLHAYIEACARSAAAVFESS    347
            ||||||:..:...   |:.:.|.:.||||:||..||:......|..|...
Rap8    283 FLFLQALNNKLSTEQLEKVMLDAIPYFEKQNLWKYIQDYTEELAIRFYEE    332

RapH    348 CHFEQAAAFYRKVLKAQEDILKGECL    373
            .:.:::..::.|..||:.::|:.|.|
Rap8    333 KNKDKSNEYFYKSYKAKRNLLERESL    358
```

B

RapH	Rap8
E45	E45
D46	D46
Q47	Q47
L50	L50
L55	L55
F58	Y60
L96	L88
D134	D125
E137	E128
Y175	Y166

FIG S1 (A) RapH of *B. subtilis* and Rap8 sequences alignment. The numbers indicated above the sequences refer to the RapH sequence. The Q47 residue essential for the dephosphorylation of Spo0F-P is highlighted in red box. * (asterisk) indicates positions which have a single, fully conserved residue, : (colon) indicates conservation between groups of strongly similar properties and . (period) indicates conservation between groups of weakly similar properties. **(B)** Conservation of the key residues involved in Rap-Spo0F interaction between RapH and Rap8.

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V- DISCUSSION AND PERSPECTIVE

Several studies have highlighted the importance of plasmid-borne genes to bacterial adaptation. These elements have been extensively studied in laboratory conditions, but little is known about plasmids influence in their natural hosts. Bacteria from the *B. cereus* group have great variability of plasmid contents, which suggests some questions: What is the role of these elements in the *B. cereus* group? What could be the importance of plasmid-borne genes in the ecological niche of these bacteria? In order to evaluate these questions, we studied two plasmids of the *B. thuringiensis* serovar *kurstaki* HD73 and KT0 strains.

6.1 Role of pHT73 harboring a *cry*1Ac gene in *B. cereus* strains

B. cereus group is composed by species with different phenotypic traits and some of those traits are encoded by plasmids. Moreover, plasmids harbor genes capable to interfere with the bacterial lifecycle. An important point is the capacity of these elements to transfer to other bacteria, facilitating their spread. Vogwill and MacLean (2015) demonstrated that chromosomal mutations carry a larger cost than the acquisition of virulence traits by plasmids. The pHT73 is a conjugative plasmid and some experiments evidenced that high transfer frequencies between *B. cereus* group bacteria occur in insect larvae (Vilas-Bôas, *et al*., 1998; Santos *et al*., 2010), the ecological niche of *B. thuringiensis* strains.

Studies focusing on the behavior of bacteria belonging to the *B. cereus* group with different plasmid content, in insect larvae may be useful auxiliary tools for determining the role of plasmids in this bacterial group. Santos and co-workers (2010) found that *Bt* strains, harboring the pHT73 plasmid, germinated and multiplied more efficiently in infected insect larvae than *Bc* strains, without the pHT73 plasmid. This result raised the question whether is the pHT73 plasmid and/or the *cry*1Ac gene the direct responsible for the high fitness of *Bt* strains. In order to elucidate this question, we carried out experiments with two plasmids, pHT73-Em^R (carrying the *cry*1Ac gene) and pHT73Δ*cry*1Ac (with deletion of *cry*1Ac gene). The fitness of nine bacterial strains, two *Bt* and seven *Bc*, were evaluated in insect larvae. Firstly, conjugation essays were done to obtain the three genotypes for each bacterial strain: plasmidless, harboring pHT73-Em^R and harboring pHT73Δ*cry*1Ac, totalizing 27 strains/genotypes. Comparisons were carried out as

follows: i) plasmidless *versus* presence of pHT73Δ*cry*1Ac, and ii) plasmidless *versus* presence of pHT73, carrying the *cry*1Ac gene. Thus, the first comparison allowed us investigating the effects of the presence of pHT73Δ*cry*1Ac on the multiplication efficiency of each strain. The second comparison allows assessing the effects of the presence of the pHT73 plasmid, and/or of the *cry*1Ac gene on the multiplication efficiency of each strain.

The qualitative comparisons among the strains with different plasmid contents showed that the presence of both the plasmid and *cry* gene do not display a unique pattern of influence on the strain fitness. Summarizing, both plasmids worsen, improved or not changed the fitness of the strains, depending of the genetic background of each strain.

In addition to the *cry*1Ac gene, 73 open reading frames are annotated in the pHT73. These genes encode several hypothetical proteins, putative exported proteins, transposases and transcriptional regulators. Thus, we conclude that the improvement in the fitness of strains harboring the pHT73Δ*cry*1Ac plasmid is also due to the other genes encoded by this plasmid. The transcriptional regulators, for example, can control the expression of plasmid-borne or chromosomal genes. Then, the control of the development and fitness of these bacteria are influenced by the interactions among the plasmid profile and chromosomal genes.

Furthermore, in our study not all the *Bc* strains showed improvement in their fitness when receiving the plasmid, indicating that the genetic background of each strain is also important to generate a positive response when the plasmid is acquired. Other studies have shown the dependence of bacterial background in bacterial fitness and functional analysis has revealed that the tolerance to toxic ions can be higher or lower depending on the host strain (Dziewit *et al*., 2015). Thus, we can conclude that for a given strain of the *B. cereus* group, receiving a pathogenic plasmid is not enough to effectively increase bacterial population and colonize the host. Similarly, the loss of a pathogenic plasmid does not necessarily change the ability to multiply in the host, despite being unable to kill it.

Our results revealed that the interaction with other plasmid-borne and chromosomal genes are of fundamental importance to define bacterial fitness when a plasmid is received. Thence, rather than constituting a driving force in bacterial evolution or being solely involved in the dissemination of important traits, plasmids have a significant role in the adaptation of strains to environmental niches. Thus, we suggest that plasmids carrying pathogenicity factors are tools used by bacteria in search of the niche most advantageous to their genome.

However, other experiments are needed to determine what other genes are involved in improving fitness. For example, RNA-seq experiments could be performed to compare the genetic expression pattern of bacteria harboring or not pHT73-Em^R pHT73Δ*cry*1Ac plasmids in infected larvae. This technique is highly sensitive to detect and quantify of RNA transcripts. The whole transcriptome sequencing could be performed at different time points and the differences in gene expression could indicate which genes are directly affected by the presence of pHT73 plasmid and/or *cry*1Ac gene. Once identified, the candidate chromosomal and plasmid genes could be deleted in order to validate and evaluate their involvement in fitness of insect larvae. Another possibility is to perform real-time PCR. However, to execute this technique some candidate genes must be chosen. The quantification could show differences in their expression according to the three plasmid profiles tested (plasmidless strain, harboring pHT73-Em^R plasmid and harboring pHT73Δ*cry*1Ac plasmid). A last approach (more laborious) could be the construction of different mutants of the pHT73 plasmid, deleting different ORFs and evaluating their fitness in insect larvae. This could identify other genes of pHT73 plasmid involved in improving bacterial fitness.

6.2 Role of the plasmid-borne *rap/phr* system controlling sporulation and biofilm formation *in vitro* and *in vivo*

The *B. thuringiensis* serovar *kurstaki* HD73 carries seven different plasmids. Among them, three encode Rap-Phr systems: pHT8_1, pHT77 and pAW63. The pHT8_1 is a mobilizable plasmid and the two others are conjugative plasmids. Our results revealed that the Rap protein encoded by the pHT8_1 plasmid (Rap8) strongly inhibits sporulation and biofilm formation. Moreover, we demonstrated the influence of this system in naturalistic conditions.

Sporulation and biofilm formation are processes dependent of the master regulator Spo0A. In *B. subtilis*, Rap proteins inhibit these processes by dephosphorylating Spo0F, consequently inhibiting the phosphoryl cascade and preventing the Spo0A-P formation. We suggest that Rap8 has a phosphatase activity because this protein displays several similarities in the amino acid sequence with the phosphatase RapH of *B. subtilis*, able to inhibit sporulation *via* the Spo0A pathway (Parashar *et al*., 2011). In addition, we showed that Rap8 prevents *spoIIE* expression, a Spo0A-dependent gene in *Bt* (Perchat *et al.*, 2016), suggesting a control of the sporulation and the biofilm formation through the Spo0A pathway, as described in *B. subtilis* and in *B. anthracis*

(Bongiorni *et al.*, 2006; Parashar, Jeffrey; Neiditch, 2013; Perego, 2013).

Rap proteins are inhibited by their Phr cognate peptide. Analysis of the amino acid sequence of Phr8 peptide showed a putative signal peptidase cleavage site between the positions 32 and 33 (HDA-KE). Phr8 is probably exported via Sec pathway and extracellularly processed by specific proteases. The mature Phr8 is probably reimported via Opp, as the others signaling peptides from the RNPP family (Leonard *et al.*, 1996; Perego, 1997; Gominet *et al.*, 2001). In the cytoplasm, mature Phr8 peptide inhibits Rap8 phosphatase activity, this was confirmed by the expression of *rap/phr* system in pHT315*xyl*, which restores the sporulation and biofilm formation.

In *B. subtilis*, the active Phr is normally composed by five amino acids located in the C-terminal end (Perego, 2001; Solomon *et al.*, 1996). By using synthetic peptides we determined that the Phr8 active form is formed by the last seven amino acids (YAHGKDI), such as found in others quorum sensing systems of *Bt*, NprX and PapR (Bouillaut *et al.*, 2008; Perchat *et al.*, 2011). The difference found in Phr size between *Bt* and *B. subtilis* raise the question of the species-specificity and of the possible advantages of this.

Contrary to the effect of the expression of Rap8 protein on sporulation and biofilm formation, the mutant of *phr* gene did not display the expected results in both processes. This can be explained by the other Rap/Phr systems encoded by this strain (five chromosomal and two other plasmid-borne), which may also control sporulation and hide the effects of Rap8. Cross-talk between the Rap-Phr systems might also interfere with the Rap8 activity, as already suggested in *B. subtilis* (Koetje et al., 2003). Interestingly, two other Phr peptides display similarities with the active form of Phr8: YAHGETI encoded by the pAW63 plasmid and MDHGEHI encoded by a chromosomal system. Preliminary tests using synthetic peptides showed that the Phr encoded by the pAW63 did not inhibit Rap8 activity. Other experiments, including the Phr encoded by the chromosome, will be needed to evaluate the possibility of cross-talk. Moreover, the culture medium selected for these assays (HCT) is a sporulation-specific medium that promotes rapid and efficient sporulation, so this might hide the mutant effect. So, we tested this mutant in insect larvae, the ecological niche of *Bt*. In this natural condition, the *phr* mutant reduced the sporulation efficiency in *G. mellonella* larvae compared to the *rap/phr* mutant and the wild type strain. This demonstrated that the Rap8-Phr8 system is required to regulate sporulation of *Bt* during its infectious cycle in insect larvae. The infectious *Bt* lifecycle is composed of three stages: virulence, survival and sporulation. The first two stages

are under the control of the PlcR-PapR and NprR-NprX systems, respectively (Slamti and Lereclus, 2002; Dubois *et al*., 2012). Our results complete the full regulation of *Bt* lifecycle in insect larvae by QS systems, demonstrating that sporulation *in vivo* is controlled by a plasmid-borne *rap/phr* system.

In addition, the pHT8_1 plasmid is found in several sequenced bacterial genomes, suggesting high dissemination capacity and its involvement in the adaptation and evolution of *Bt* in insect larvae. However, complementary studies will be needed to determine which genes are indirectly controlled by Rap8. In addition, the other Rap/Phr systems present in this strain could be assayed to obtain an overall view about the processes controlled by these systems.

The QS systems have an important role allowing bacteria to coordinate gene expression, and consequently change their behavior in response to environmental conditions and perturbations (Perego and Hoch, 1996; Lazazzera *et al*., 1997). The QS systems from *Bt* may allow the bacteria to optimize their multiplication, survival, sporulation and dissemination (Slamti *et al*., 2014).

CONCLUSIONS

In this study, we highlight the role of two plasmids, pHT73 and pHT8_1, of *B. thuringiensis*, which encode important traits for bacteria in their ecological niche. In addition, both plasmids are able to disseminate by horizontal gene transfer, facilitating the spread of these genes. Our results show the importance of plasmids from the *B. cereus* group in insects, interfering with their fitness and controlling essential functions, such as sporulation. In addition, our results provide new insights into the *B. thuringiensis* ecology. Indeed, the set of virulence genes encoded by these bacteria and the specialized QS systems controlling all stages of infection in insect larvae in *B. thuringiensis*. Our results emphasize the importance of plasmids in the adaptation of the *Bc* group in insect larvae. Until now, these elements were considered as accessory elements of the bacterial genome, providing some advantages in specific conditions. The energy cost generated by plasmids is unquestionable, but they have several strategies to substantially diminish, and even eliminate, these costs and remain in a bacterial population (Bahl, Hansen and Sørensen, 2009). Analysis of the plasmid contents of the *B. cereus* group can shed light on their real role in bacterial communities. Interestingly, one study demonstrated the altruist behavior of *B. thuringiensis* that harbor plasmids encoding Cry toxins (Raymond and Bonsall, 2013). The Cry

toxins produced improve the fitness of non-producing bacteria. This creates a cooperative community and highlights the importance of plasmids in the *B. cereus* group as a strong driver of evolution, with considerable implications in bacterial community ecology. Our results bring new perspectives about plasmids: these elements are an integral part of the bacterial genome and have active participation in the adaptation and evolution of bacterial populations.

VI- REFERENCES

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