PRISCILLA DE FREITAS CARDOSO

DIVERSITY AND FUNCTIONAL ANALYSIS OF RAP-PHR SYSTEMS FROM *BACILLUS CEREUS* GROUP

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Tese apresentada ao Programa de Pós–Graduação em Genética e Biologia Molecular, da Universidade Estadual de Londrina e à École doctorale Agriculture, alimentation, biologie, environnement et santé (ABIES) da AgroParisTech / Université Paris-Saclay, como requisito para a obtenção do título de Doutora.

Orientadora: Profa. Dra. Gislayne Fernandes Lemes Trindade Vilas-Boas

Co-Orientador: Dr. Didier Lereclus

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Diversity and functional analysis of Rap-Phr systems from *Bacillus cereus* group

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Thèse de doctorat

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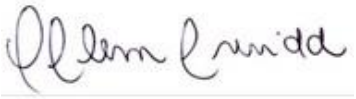

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RESUMO

O grupo *Bacillus cereus* é formado por oito espécies de bactérias Gram positivas esporulantes que podem colonizar diversos nichos ecológicos. As espécies mais importantes do grupo são *B. cereus*, bactéria ubíqua do solo e patógeno oportunista; *B. thuringiensis*, entomopatógeno amplamente utilizado como biopesticida; e *B. anthracis*, agente etiológico do antraz. Embora apresentem fenótipos diferentes, essas espécies são próximas geneticamente e seus principais fatores de virulência são codificados por plasmídeos. O ciclo infeccioso de *B. thuringiensis* na larva de inseto é regulado pela ativação consecutiva de sistemas de *quorum sensing* da família RNPP. Dentre eles, o sistema Rap-Phr foi amplamente estudado em *B. subtilis*, porém apenas pontualmente explorado nas espécies do grupo *B. cereus*. Os sistemas Rap-Phr regulam vários processos fisiológicos bacterianos, inclusive a esporulação. O objetivo deste estudo foi analisar os sistemas Rap-Phr no grupo *B. cereus*, com intuito de conhecer sua distribuição, localização e diversidade a fim de obter um panorama desses sistemas neste grupo. Além disso, o possível envolvimento desses sistemas no controle do processo de esporulação foi predito com base nos dados estruturais descritos para RapH de *B. subtilis*. Genes *rap*, sempre associados a um gene *phr*, estão presentes em todas as 49 linhagens estudadas com uma média de seis alelos *rap-phr* por linhagem e 30% dos sistemas estão localizados em plasmídeos. As linhagens de *B. thuringiensis* possuem seis vezes mais sistemas Rap-Phr plasmidiais do que as linhagens de *B. cereus*. Ademais, linhagens filogeneticamente próximas apresentam um perfil similar de genes *rap-phr*. Um terço das proteínas Rap foram preditas como inibidoras da esporulação e estas proteínas estão preferencialmente localizadas em plasmídeos e, portanto, em linhagens de *B. thuringiensis*. A predição foi parcialmente validada por ensaios de esporulação sugerindo que os resíduos identificados pelo envolvimento na atividade de fosfatase em *B. subtilis* são conservados no grupo *B. cereus*, porém não são suficientes para predizer a função sobre a esporulação. Em seguida, o sistema Rap63-Phr63 codificado pelo plasmídeo pAW63 da linhagem *B. thuringiensis* HD73 foi caracterizado. A proteína Rap inibe moderadamente a esporulação e retarda a expressão de genes regulados por Spo0A. Rap63 é inibida por seu peptídeo cognato Phr63, cuja forma madura corresponde à extremidade carboxi-terminal do pro-peptídeo. Ensaios de esporulação em larvas de inseto sugerem uma atividade sinérgica dos sistemas Rap63-Phr63 e Rap8-Phr8 (do plasmídeo pHT8_1 da linhagem *B. thuringiensis* HD73) sobre a esporulação. Apesar da similaridade entre Phr63 e Phr8 não foi observado *cross-talk* entre os dois sistemas, confirmando sua especificidade. Desta forma, o conjunto dos resultados demonstra a grande diversidade dos sistemas Rap-Phr no grupo *B. cereus* e destaca o impacto de sistemas plasmidiais no desenvolvimento destas bactérias. Consequentemente, reforça a importância dos plasmídeos na adaptação e sobrevivência dessas espécies, particularmente em *B. thuringiensis*.

Palavras-chave: *Bacillus thuringiensis*. Esporulação. *Quorum sensing*. Família RNPP. Plasmídeo.

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ABSTRACT

The *Bacillus cereus* group of Gram positive spore forming bacteria is comprised by eight species that are able to colonize several ecological niches. The most important species are *B. cereus*, a ubiquitous soil bacterium and an opportunistic pathogen; *B. thuringiensis*, an entomopathogen widely used as biopesticide; and *B. anthracis*, the causative agent of anthrax. Even if they present different phenotypes, they are genetic closely related and their main virulence factors are encoded on plasmids. The infectious cycle of *B. thuringiensis* in the insect larvae is regulated by the sequential activation of quorum sensing systems from the RNPP family. Among them, the Rap-Phr was extensively studied in *B. subtilis* but just punctually in *B. cereus* group species. The Rap-Phr systems were shown to regulate various bacterial processes, including the sporulation. The objective of this study was to analyze the Rap-Phr systems in the *B. cereus* group, regarding their distribution, location and diversity to achieve an overview of these systems in these bacteria. Moreover, their possible involvement in the control of the sporulation process was predicted based on structural data described for RapH in *B. subtilis*. The *rap* genes, always associated with a *phr* gene, were present in all 49 studied strains with an average of six *rap-phr* genes per strain and 30% were located on plasmids. Comparison among *B. cereus* and *B. thuringiensis* strains revealed that the last one harbors six-fold more plasmid *rap-phr* system then the former. Moreover, phylogenetic closer strains possess a similar profile of *rap-phr* genes. Interestingly, 32% of the Rap proteins were predicted to inhibit sporulation and these proteins were preferentially located on plasmids and therefore in *B. thuringiensis* strains. This prediction was partially validated by sporulation efficiency assays suggesting that residues identified in *B. subtilis* as involved in the phosphatase activity are conserved but not sufficient to predict the sporulation function. Then, the plasmid-borne Rap63-Phr63 system from pAW63 plasmid of *B. thuringiensis* HD73 strain was further studied. The Rap63 protein moderately inhibits the sporulation and delays the expression of Spo0A-regulated genes. Rap63 is counteracted by its cognate Phr63 peptide, which mature form corresponds to the C-terminal end of the pro-peptide. Sporulation assays in insect larvae suggest a synergistic activity of Rap63-Phr63 and Rap8-Phr8 (from pHT8_1 of *B. thuringiensis* HD73 strain) systems on sporulation efficiency. Despite the similarities of Phr63 and Phr8 no cross-talk was found between these two systems, confirming their specificity. Altogether, these results reveal the high diversity of the Rap-Phr systems in the *B. cereus* group and highlight the relevance of the plasmid-borne systems to cell development. Therefore, the results demonstrated the importance of the plasmids in the adaptation and the survival of these bacteria, especially for *B. thuringiensis*.

Key words: *Bacillus thuringiensis*. Sporulation. Quorum sensing. RNPP family. Plasmid.

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RÉSUMÉ

Le groupe *Bacillus cereus* est composé de huit espèces de bactéries à Gram positif sporulantes qui peuvent coloniser plusieurs niches écologiques. Les espèces les plus importantes sont *B. cereus*, une bactérie ubiquitaire du sol et un pathogène opportuniste; *B. thuringiensis*, un entomopathogène très utilisé comme biopesticide; et *B. anthracis* l'agent de la maladie du charbon. Bien que ces espèces présentent différents phénotypes, elles sont étroitement liées génétiquement et leurs facteurs de virulences principaux sont portés par des plasmides. Le cycle infectieux de *B. thuringiensis* dans la larve d'insecte est régulé par l'activation séquentielle de systèmes de quorum sensing de la famille RNPP. Parmi eux, les systèmes Rap-Phr, caractérisés chez *B. subtilis*, ont très peu été étudiés dans le groupe *B. cereus*. Ces systèmes régulent divers processus bactériens importants dont la sporulation. L'objectif de cette étude est d'analyser les systèmes Rap-Phr dans le groupe *B. cereus*, pour connaitre leur distribution, leur localisation et leur diversité afin d'obtenir une vue globale de ces systèmes chez ces bactéries. De plus, leur possible implication dans la régulation du processus de sporulation a été prédite sur la base de données structurales décrites chez RapH de *B. subtilis*. Les gènes *rap*, toujours associés à un gène *phr*, sont présents dans toutes les souches étudiées avec une moyenne de six gènes *rap-phr* par souche et avec 30% de ces systèmes qui sont portés par des plasmides. Les souches de *B. thuringiensis* portent six fois plus de systèmes Rap-Phr plasmidiques que les souches de *B. cereus*. Par ailleurs, les souches phylogénétiquement proches possèdent un profil de gènes *rap-phr* similaire. Un tiers des protéines Rap sont prédites pour inhiber la sporulation et ces protéines sont préférentiellement localisées sur les plasmides et donc plus fréquemment présentes chez *B. thuringiensis* que chez *B. cereus*. Cette prédiction a été partiellement validée par des tests de sporulation suggérant que les résidus impliqués dans cette activité chez *B. subtilis* sont conservés mais insuffisants pour prédire cette fonction. Le système Rap63-Phr63 porté par le plasmide pAW63 de la souche *B. thuringiensis* HD73 a ensuite été caractérisé. La protéine Rap63 a un effet modéré sur la sporulation et retarde l'expression des gènes régulés par Spo0A. La Rap63 est inhibée par son peptide Phr63, dont la forme mature correspond à l'extrémité C-terminale du pro-peptide. Les résultats de sporulation dans l'insecte suggèrent une activité synergique des systèmes Rap63-Phr63 et Rap8-Phr8 (porté par le pHT8_1) dans la régulation de la sporulation. Malgré la similarité entre les Phr63 et Phr8 aucun cross-talk n'a pu être mis en évidence, ce qui confirme la spécificité de ces systèmes de communication cellulaire. L'ensemble de ces résultats démontre la grande diversité des systèmes Rap-Phr dans le groupe *B. cereus* et souligne l'impact des systèmes plasmidiques dans le développement de ces bactéries. Par conséquent, les plasmides sont des éléments importants pour l'adaptation et la survie de ces bactéries et particulièrement pour *B. thuringiensis*.

Mots-clés: *Bacillus thuringiensis.* Sporulation. Quorum sensing. Famille RNPP. Plasmide.

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1 INTRODUCTION

1.1 *Bacillus cereus* group

1.1.1 The *Bacillus* genus

Bacteria from the *Bacillus* genus are Gram positive, rod shaped, aerobic or facultative anaerobic, spore forming species (Figure 1). These spores are dormant cellular structures resistant to many adverse conditions, such as heat, radiation, disinfectants and desiccation. *Bacillus* bacteria can use a wide variety of nutrient sources which allows their adaptation to a broad range of ecological niches. They are ubiquitous in nature, mainly in soil, but can be also found in water, food and clinical specimens, and show medical, industrial, economic and historical relevance (Logan & De Vos, 2009).

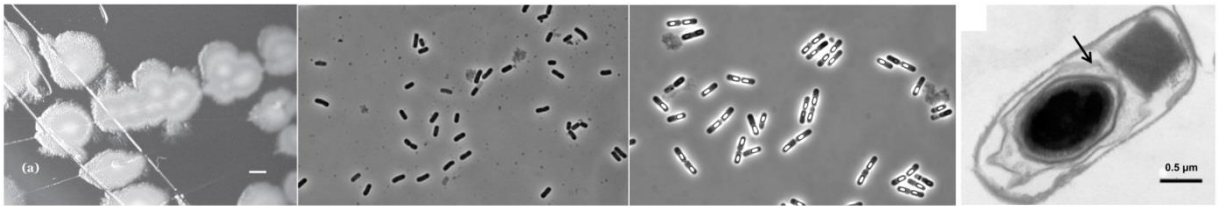

Figure 1. Bacteria from the *Bacillus* genus. A) Colonies of *B. anthracis* on blood agar (Logan & De Vos, 2009); B) Vegetative cells of *B. subtilis*; C) Sporulating cells of *B. cereus*; D) Sporulating cell of *B. thuringiensis* containing the spore and the parasporal crystal (Deng et al., 2014).

Bacillus genus is one of the best characterized genera and is highly represented in genomic database with almost 4000 genome sequences (complete or draft) deposited in the GenBank database until March 2019. Strains with medical or biotechnological interest are the most represented (Hernández-González et al., 2018). Different phylogenetic analyses allow to separate this genus into several groups, including the two remarking well established groups: *Bacillus subtilis* group and *Bacillus cereus* group (Alcaraz et al., 2010; Bhandari et al., 2013; Hernández-González et al., 2018; Maughan & Van der Auwera, 2011; Schmidt et al., 2011).

B. subtilis group includes *B. subtilis*, *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, *Bacillus pumilus*, *Bacillus atrophaeus*, and *Bacillus mojaviensis*. *B. subtilis* is the type species of *Bacillus* genus and Gram positive bacteria. *B. subtilis* strain 168 is the model strain and has been extensively used to investigate important

microbiological characteristics as spore structure and the sporulation process. Furthermore, *B. subtilis* and its close relatives have great biotechnology relevance and are frequently used for industrial production of several components as enzymes and antibiotics (Kunst et al., 1997; Logan & De Vos, 2009).

The *B. cereus* group is a very diverse group, which is actually composed by eight species: *Bacillus cereus* (*sensu stricto*), *Bacillus thuringiensis*, *Bacillus anthracis*, *Bacillus mycoides*, *Bacillus pseudomycoides*, *Bacillus weihenstephanensis*, *Bacillus cytotoxicus* and *Bacillus toyonensis*. This group shows the largest genome, the higher number of proteins predicted and the lower GC content within the *Bacillus* genus (Alcaraz et al., 2010; Anderson et al., 2005). However, the *B. cereus* group composition is often modified and some novel species have been proposed: *Bacillus gaemokensis** (Jung et al., 2010), *Bacillus manliponensis** (Jung et al., 2011), *Bacillus bingmayongensis** (Liu et al., 2014a), *Bacillus wiedmannii* (Miller et al., 2016), *Bacillus paranthracis*, *Bacillus pacificus*, *Bacillus tropicus*, *Bacillus albus*, *Bacillus mobilis*, *Bacillus luti*, *Bacillus proteolyticus*, *Bacillus nitratireducens* and *Bacillus paramycoides* (Liu et al., 2017) (species names with * are not validly published yet). The validation of all these bacteria at the species level will totalize 21 species in the *B. cereus* group.

1.1.2 The *Bacillus cereus* group species

Bacteria from the *B. cereus* group were described in the end of the 19th century or on the beginning of the 20th century and show a long history of medical or biotechnological relevance (Logan & De Vos, 2009; Sanchis, 2011; Vilas-Bôas et al., 2007). *B. cereus sensu stricto* is a ubiquitous bacterium and some strains are considered as an opportunistic pathogen that have been involved in food poisoning outbreaks with emetic and diarrheic symptoms, pneumonia, and endophthalmitis (Logan & De Vos, 2009). *B. thuringiensis* is an entomopathogenic bacterium used as alternative insect pest control for more than 60 years (Sanchis, 2011). *B. anthracis* is the etiological agent of anthrax (Logan & De Vos, 2009). *B. mycoides* is characterized by the rhizoidal morphology of its colonies, lack of motility, and have been considered as a plant growth-promoting bacterium (Logan & De Vos, 2009). *B. pseucomycoides* is isolated mainly from soil and distinguishable from *B. mycoides* by their composition of fatty acid (Nakamura, 1998). *B. weihenstephanensis* comprise

psychrotolerant strains that represent potential risk of food poisoning (Lapidus et al., 2008; Lechner et al., 1998). *B. cytotoxicus* is also associated to severe food poisoning outbreaks and produces a high amount of cytotoxin (Guinebretiere et al., 2013) and *B. toyonensis* is used as probiotic in animal nutrition (Jiménez et al., 2013). Even if the group presents overall phenotypical heterogeneity, these different species may be genetically closely related, essentially at the chromosomal level.

B. cereus group species have a large range of ecological lifestyle, varying according to the species, toxins or virulence factor content and the host (Figure 2) (Ceuppens et al., 2013; Jensen et al., 2003).

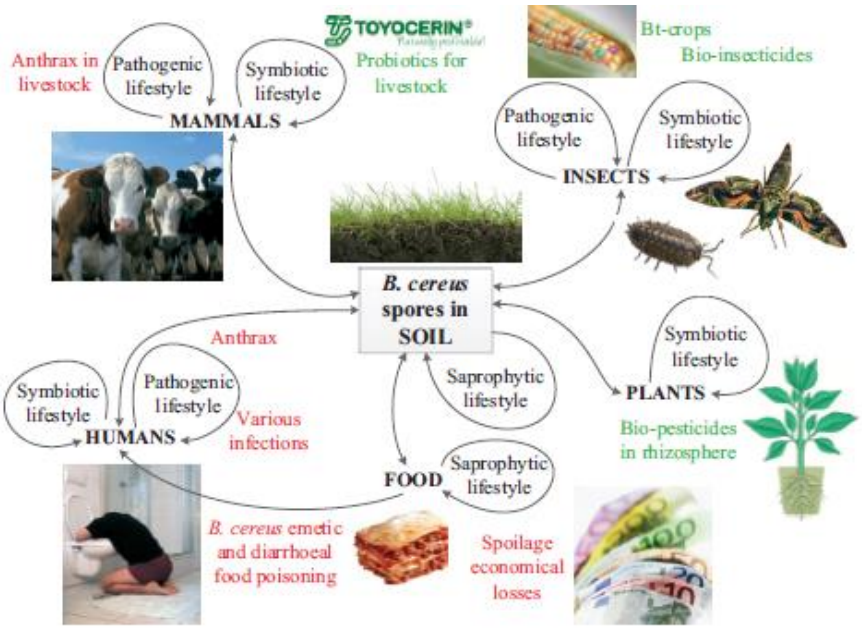

Figure 2. Ecology of *B. cereus* group bacteria. Species of the group could develop different lifestyles and colonize different hosts in a symbiotic or pathogenic way (Ceuppens et al., 2013).

As the soil is the primarily environmental reservoir of *B. cereus* group spores, the common ancestor of these species was suggested to be a soil bacterium. However, genetic content analyses of the *B. cereus* group genomes contradict this hypothesis, since they encode a lower number of genes related to carbohydrate catabolic pathways, numerous in soil bacteria such as *B. subtilis* (Alcaraz et al., 2010; Ivanova et al., 2003; Raymond, 2017). Moreover, genomes of this group are rich in genes encoding enzymes involved in peptide and amino acids processing. This nutrient source preference could suggest that the intestine of invertebrates was the habitat of the common ancestor of the group (Ivanova et al., 2003). *B. cereus*

(*sensu stricto*) is described as a soil saprophyte and an opportunistic vertebrate pathogen, causing foodborne outbreaks and other diseases in humans (Ceuppens et al., 2013; Jensen et al., 2003). Formerly, *B. thuringiensis* was also considered as a soil saprophytic with incidental insecticide activity. Nowadays, *B. thuringiensis* is considered as a specialized invertebrate pathogen that could use plants as vectors for transmission (Monnerat et al., 2009; Raymond, 2017; Raymond & Federici, 2017; Raymond et al., 2010a) and the soil as reservoir (Raymond et al., 2010b). On the other hand, *B. anthracis* is an obligate vertebrate pathogen that is unable to grow in soil (Ceuppens et al., 2013; Jensen et al., 2003).

The three most frequently studied and well characterized species of the *B. cereus* group are *B. cereus* (*sensu stricto*), *B. thuringiensis*, and *B. anthracis* that will be further describe below.

1.1.2.1 Bacillus cereus sensu stricto

Bacillus cereus sensu stricto is a common soil bacterium, where it can complete its saprophytic lifestyle besides colonizing invertebrate guts as symbionts. Some strains of this species are also involved in various opportunistic and nosocomial systemic or local infections, especially in immunocompromised patients, such as periodontitis (Helgason et al., 2000a), pneumonia resembling inhalation anthrax (Hoffmaster et al., 2006) and endophthalmitis (Callegan et al., 2003). Furthermore, *B. cereus* strains are implicated in two types of food-poisoning syndromes: emetic and diarrheic types. The ability of *B. cereus* strains to induce disease is highly variable, depending on a wide variety of factors, including the bacteria dose, the presence of toxin genes and their level of expression and cellular form of bacteria (vegetative cell or spore). In addition, the physiology of the host and the properties of the food, as nutritional composition, processing methods and storage conditions are also important (Ceuppens et al., 2013).

The emetic syndrome is caused by cereulide toxin (Figure 3), a small non-ribosomal synthesized dodecadepsipeptide non antigenic, highly resistant to heat treatments, extreme pH values and proteolysis. The cereulide synthetase gene cluster (*cesHPTABCD*) is carried by a pXO1-like plasmid (270 kb) and its expression does not depend of sporulation (Ehling-Schulz et al., 2006; Hoton et al., 2005). The emetic illness is the result of the ingestion of preformed cereulide toxins, produced by

bacteria in food and not degraded by food processing or gastrointestinal environment. Consequently, the ingestion of viable bacteria or spores is not required to emetic food-poisoning. This type of disease is usually related to food products with high starch content, such as rice, pasta, and mashed potatoes (although the majority of emetic *B. cereus* strains are unable to hydrolyze starch) and, occasionally pasteurized cream or milk pudding (Ceuppens et al., 2013; Jensen et al., 2003; Stenfors Arnesen et al., 2008; Vilas-Bôas et al., 2007).

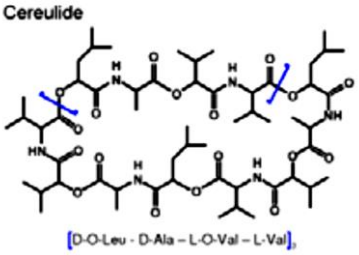

Figure 3. Chemical structure of the cereulide toxin (Ehling-Schulz et al., 2006).

The diarrheic syndrome is the consequence of the production of enterotoxins with hemolytic and pore-forming cytotoxic activity as haemolysin BL (Hbl), nonhaemolytic enterotoxin (Nhe) and cytotoxin K (CytK). Their activities are enhanced by other virulence factors that are not directly cytotoxic, such as phospholipases C, cereolysin and sphingomyelinase. In contrast to the cereulide toxin and spores, enterotoxins and the vegetative cells are inactivated by the gastrointestinal passage. Thus, the diarrheal food poisoning is the consequence of the ingestion of spores and their subsequent germination to vegetative cells with the production of toxins in the small intestine of the host. Most exotoxin genes related with diarrheic syndrome are located on the chromosome and are regulated by the pleiotropic activator PlcR, which regulates the transcription of virulence genes in bacteria of the *B. cereus* group (further description below). Diarrheic cases caused by *B. cereus* strains occur with a wide variety of food commodities. As the food heat-treatments (such as pasteurization) are not able to efficiently kill them, spores of *B. cereus* are found in food of plant or animal origin (Ceuppens et al., 2013; Jensen et al., 2003; Stenfors Arnesen et al., 2008; Vilas-Bôas et al., 2007).

Taken together, both types of foodborne illness related to *B. cereus* correspond to an important number of outbreaks worldwide. Between 2007 and 2014, the European Food and Safety Authority (EFSA) reported 413 cases where *B.*

cereus was the causative agent, totalizing more than 6,600 human cases (EFSA, 2016). Moreover, in France, the *B. cereus* cases correspond to 16% of foodborne outbreaks with confirmed pathogenic agent or 28% from the cases with a suspected cause in 2017 (Santé Publique France, 2019). Regarding foodborne outbreaks occurred in Brazil, *B. cereus* was described to cause 2.3% of outbreaks that the causative agents were laboratorial confirmed from 2009 to 2018 (Ministério da Saúde, 2018). Furthermore, the Centers for Disease Control and Prevention from the USA reported that *B. cereus* is suspect to an average of 37.6 outbreaks per year, affecting more than 1,000 people (Food and Drug Administration, 2012). Despite the expressive number, the number of cases may be underestimated because of unreported or misdiagnosed events; for example, in 64% of the Brazilian outbreaks in the last ten years the cause was not identified.

1.1.2.2 Bacillus thuringiensis

Bacillus thuringiensis strains differ from *B. cereus sensu stricto* by the production of parasporal crystalline proteins (Figure 1D) during the stationary or sporulation phase of the growth cycle. These proteins are toxic to a wide range of insect larvae, mainly belonging to the orders Lepidoptera (butterflies and moths), Diptera (flies) and Coleoptera (beetles), but also to other insect orders (Hymenoptera, Homoptera, Orthoptera, and Mallophaga) and even some nematodes, mites, and protozoa (Deng et al., 2014; Logan & De Vos, 2009; Schnepf et al., 1998; Vilas-Bôas et al., 2007).

Crystal inclusions may display different forms, such as bi-pyramidal, cuboid, spherical or ovoid, and could represent up to 30% of the dry weight of the sporangia (De Souza et al., 1993). The crystal proteins are also known as δ-endotoxins and consist predominantly of Cry proteins. These proteins are encoded by *cry* genes, mainly located on large conjugative plasmids and frequently associated with transposable elements such as insertion sequences and transposons, which facilitate their transfer to other cells (Schnepf et al., 1998; Vilas-Bôas et al., 2007).

Cry toxins have their own nomenclature and classification based on amino acid identity, which allows related toxins with similar host specificity to be ranked together (Crickmore et al., 1998, 2018). These proteins are composed by three domains: Domain I (perforating domain) is involved in the insertion of the toxin in the

cellular membrane and pore formation in the intestinal epithelium of the susceptible insect; Domain II (central or middle domain) is involved in toxin-receptor interactions; and Domain III (galactose-binding domain) also participate in receptor binding and pore formation (Palma et al., 2014; Schnepf et al., 1998). The mode of action of Cry proteins is variable, depending on the type of Cry toxin and susceptible insect. The classical and first described mode of action (Figure 4, top panel) starts with the ingestion and solubilization of the crystal in the alkaline midgut of insect larvae, followed by proteolytic activation of protoxins that are able to bind to specific receptors (such as cadherins) on the apical brush border cells of the midgut. The insertion of the toxin in the membrane cell gives rise to the formation of pores permeable to inorganic ions and small molecules, resulting in leakage intracellular contents and cell lyses. The release of cellular content could activate the germination of spores and consequent septicemia, followed by death of the insect (Bravo et al., 2007; Palma et al., 2014; Soberón et al., 2009).

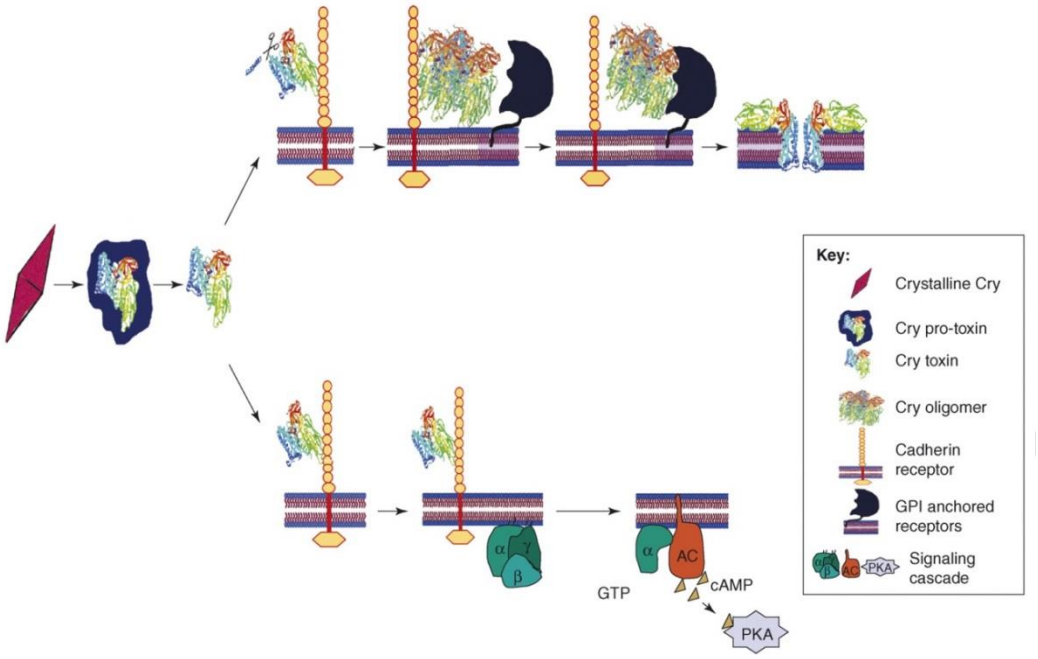

Figure 4. Mode of action of Cry proteins. Initially, the crystal is solubilized and activated in the insect gut. Following steps could arise in two different mechanism: the pore formation model (top) or the signal transduction model (bottom) (Bravo & Soberón, 2008).

Other proposed action mechanism of Cry toxins is the signal transduction model (Figure 4, bottom panel). In this model the mechanism begins in the same way that the pore formation model with solubilization and proteolytic activation. However, the interaction with a specific receptor, named cadherin, activates a Mg-dependent

signal cascade, via G protein, intracellular adenylyl cyclase (cAMP) and protein kinase, activating cellular process that result in cell death (Soberón et al., 2009; Zhang et al., 2006).

In addition to the Cry proteins, some *B. thuringiensis* strains also produce parasporal cytotoxic (Cyt) proteins, with cytolytic activity predominantly against dipteran larvae (Bravo et al., 2007). Furthermore, *B. thuringiensis* bacteria may produce other insecticidal proteins, such as vegetative insecticidal protein (Vip) and secreted insecticidal protein (Sip) (Palma et al., 2014). Highly toxic *B. thuringiensis* strains contain higher amount of insecticidal-toxicity related genes and higher expression of these virulence genes than the less virulent strains (Zhu et al., 2015). Moreover, the accumulation of multiple virulence factors, that acts against a specific insect order, allows *B. thuringiensis* strains to have an host specialization, which is valuable to increase pathogenicity and overcome insect resistance or expand insecticidal spectrum (Zheng et al., 2017).

Due to its insecticidal properties and its extremely specific mechanism of action, *B. thuringiensis* has been used as biopesticides for over half century as alternative or supplement to synthetic chemical pesticides (Raymond & Federici, 2017; Vilas-Bôas et al., 2007). *B. thuringiensis* products are widely used in the control of insect pests in agricultural areas and forest management, as well as for the control of mosquito disease vectors. Furthermore, in the last decades, *cry* genes have been introduced in plants, developing the so-called Bt transgenic crops, resistant to target insects (Bravo & Soberón, 2008; Schnepf et al., 1998).

1.1.2.3 Bacillus anthracis

Bacillus anthracis is the etiological agent of anthrax, an acute disease that affects mammals, primarily herbivores. Anthrax in human is rare, despite of the human susceptibility to natural infections. *B. anthracis* is also a potential agent for bioterrorism and has an important role in the history of microbiology. This bacterium was used by Robert Koch for the development of Koch postulates, which relates infectious agent and specific disease and also for the development of the first antibacterial vaccine by Pasteur (Logan & De Vos, 2009; Vilas-Bôas et al., 2007).

There are three forms of anthrax disease, defined by the route of infection: cutaneous (most common of human infections), gastrointestinal or inhalational. All

forms may progress to the systemic disease. The disease begins with the entrance of the spores in the body. Afterwards, spores are phagocytosed by macrophages, which carry them to lymph nodes, where they germinate and multiply in vegetative form, producing toxins and capsule (Logan & De Vos, 2009; Vilas-Bôas et al., 2007).

The main virulence factors of *B. anthracis* are the tripartite toxins and the capsule, encoded by genes located in the large extra chromosomal plasmids pXO1 (182 kb) and pXO2 (95kb) and required for *B. anthracis* full virulence. The plasmid pXO1 carries genes *pag*, *lef*, and *cya* that encode protective antigen (PA), lethal factor (LF) and edema factor (EF), respectively. These genes are organized within a 44.8-kb pathogenic island, flanked by insertion sequences. None of these proteins are toxic separately but active in binary combination: lethal toxin (PA and LF) and edema toxin (PA and EF). The role of PA component is to bind to the host cellular receptor whereas LF and EF are the catalytic fraction of the toxins (Liu et al., 2014b; Okinaka et al., 1999).

The other characteristic virulence factor of *B. anthracis* is the poly-D-γ-glutamic acid capsule which confers resistance to phagocytosis. The genes involved in the capsule biosynthesis (operon *capBCADE*) are carried on the plasmid pXO2. This capsule enables the vegetative bacteria to evade the host immune system, leading to systemic infection (Kolstø et al., 2009; Van der Auwera et al., 2005).

Toxins and capsule production is regulated by the transcriptional activator AtxA, located in pXO1. AtxA is functionally incompatible with the pleiotropic regulator PlcR. The acquisition of pXO1 plasmid, harboring the *atxA* gene is suggested to have led to the selection for the *plcR* nonsense mutation found in *B. anthracis* strains (Kolstø et al., 2009; Mignot et al., 2002).

Bacillus anthracis can be differentiated from *B. cereus* and *B. thuringiensis* through some phenotypic and genotypic features. The microbiological and biochemical traits that characterize the *B. anthracis* isolates are non-hemolytic, non-motile, penicillin-sensitive, susceptible to γ-phage and the poly-D-γ-glutamic acid capsule (Kolstø et al., 2009; Rasko et al., 2005). Concerning genomic traits, *B. anthracis* have the particular nonsense point mutation in the *plcR* gene and four chromosome regions unique to *B. anthracis* that correspond to lambda prophages (Kolstø et al., 2009).

1.1.3 Genome & taxonomy

B. cereus, B. thuringiensis and *B. anthracis* were initially classified in different species using phenotypical characteristics. However, initial findings in the molecular genetic era, such as DNA-DNA hybridization or 16S rDNA sequencing and multi-locus enzyme electrophoresis (MLEE) suggested to class the three species as a unique species (Vilas-Bôas et al., 2007). Analyses performed with more than 200 strains by MLEE and chromosomal genes sequences indicated that *B. anthracis* seemed to be genetically indistinguishable from *B. cereus* and *B. thuringiensis*, since *B. cereus* and *B. thuringiensis* strains were scattered throughout the dendrogram branches, also close to *B. anthracis* strain (Helgason et al., 2000b). Thereafter, the development of sequencing technology has contributed to the debate of genetic diversity and taxonomic structure of the *B. cereus* group.

Different MultiLocus Sequence Typing (MLST) schemes for the *B. cereus* group were developed by comparing partial sequences of different chromosomal housekeeping genes for phylogenetic analyses of bacterial population (Helgason et al., 2004; Ko et al., 2004; Priest et al., 2004; Sorokin et al., 2006; Tourasse et al., 2006). Their results lead to two phylogenetic grouping interpretations in which strains were allocated into three clades or seven groups (Figure 5, Table 1).

Table 1. Phylogenetic classification of the *B. cereus* group species

Clade	Group	Species	Growth temperature	Features
3	**I**	*B. pseudomycoides*	10°C to 43°C	
	II	*B. cereus* *B. thuringiensis*	7°C to 40°C	
1	**III**	*B. anthracis* *B. cereus* *B. thuringiensis*	15°C to 45°C	Mainly pathogenic to mammals Anthrax Cereulide strains Periodontitis
2	**IV**	*B. thuringiensis* *B. cereus*	10°C to 45°C	Environmental strains Pathogenic to invertebrates Strains used in biopesticide products
3	**V**	*B. cereus* *B. thuringiensis* *B. toyonensis*	8°C to 40°C	
	VI	*B. weihenstephanensis* *B. mycoides* *B. thuringiensis*	5°C to 37°C	Psychrotrophic strains Isolated from soil and food
	VII	*B. cytotoxicus*	15°C to 53°C	Some strains display cytotoxicity

(Ceuppens et al., 2013; Guinebretière et al., 2008; Patiño-Navarrete & Sanchis, 2017; Tourasse et al., 2011).

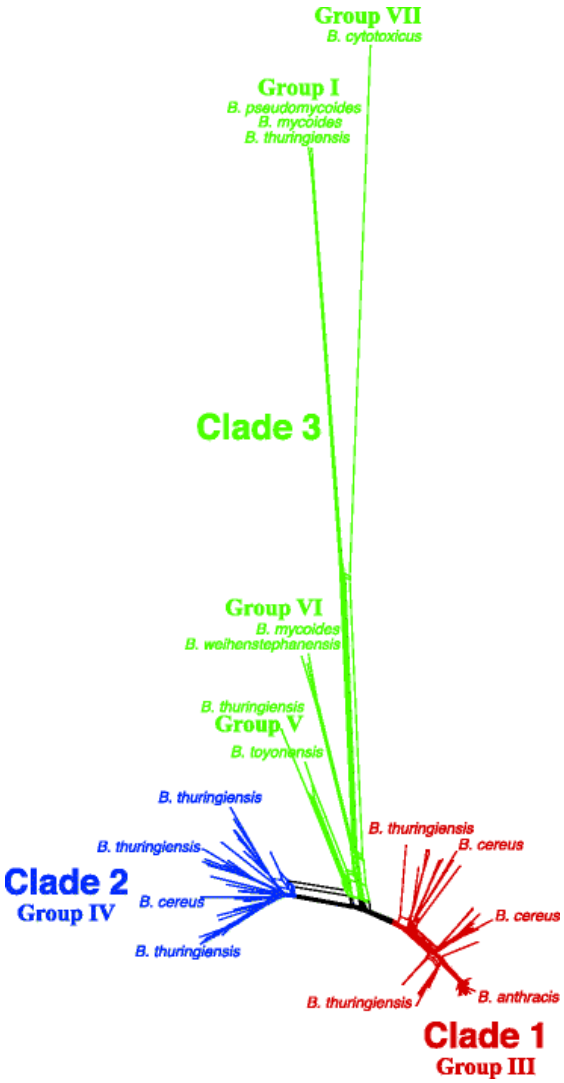

Figure 5. Phylogeny of *B. cereus* group bacteria. Different analyses lead to two interpretations of genetic structure classification, clustering strains in three Clades or seven Groups (Bazinet, 2017).

The three clade categorization was first described by Priest et al. (2004). Clade 1 (Figure 5, red) was mainly composed by *B. cereus* strains (as emetic cluster), all *B. anthracis* strains and some *B. thuringiensis* strains; Clade 2 (Figure 5, blue) consisted predominantly by *B. thuringiensis* strains (*kurstaki*, *sotto*, *thuringiensis*, *tolworthi* subspecies) with some *B. cereus* isolates; Clade 3 (Figure 5, green) gathered together all other species and *B. cereus* and *B. thuringiensis* isolates, forming a more heterogeneous clade. Using seven housekeeping alleles, this MLST scheme unveiled that the *B. cereus* group was largely clonal, with evidence that recombination occurs mainly among bacteria of a given clade than between strains from different clade (Didelot et al., 2009; Okinaka & Keim, 2016; Priest et al., 2004; Zwick et al., 2012).

The seven groups classification described by Guinebretière et al. (2008) was developed taken into account the ecological diversification of the group by using molecular and phenotypic data (Figure 5, Table 1). Group I consisted of the mesophilic *B. pseudomycoides* strains; psychrotolerant isolates of *B. cereus* and *B. thuringiensis* were positioned in Group II; Group III contained isolates related to mammal pathogenesis of *B. anthracis*, *B. cereus* and rarely *B. thuringiensis*; entomopathogenic *B. thuringiensis* and environmental *B. cereus* strains were assembled in Group IV, both Groups III and IV were composed by mesophilic bacteria; Group V was composed by intermediate isolates of *B. cereus*, *B. thuringiensis* and *B. toyonensis*; Group VI included psychrotolerant *B. weihenstephanensis*, *B. mycoides* and *B. thuringiensis* strains; and *B. cytotoxicus* strains were included in Group VII (Guinebretière et al., 2008; Patiño-Navarrete & Sanchis, 2017; Tourasse et al., 2011).

The advancement of whole genome sequencing has reported the genomic analyses to a new statement. For the study of population structure the up to date evaluation is the pan-genome phylogeny, in which genes were divided into three categories: genes shared by all isolates (core genome), partially shared genes, *i.e.* non-core genes present in several strains (accessory genes) and strain-specific genes (unique genes) (Bazinet, 2017; Tettelin et al., 2005). The first attempt to build a *B. cereus* group pan-genome identified around 3,000 core genes and the pan-genome was estimated to be about 20-25,000 protein-coding genes (Lapidus et al., 2008). Considering 45 genome sequences of *B. cereus* group strains, Zwick et al. (2012) found around 1,750 core and 2,150 accessory genes. As new isolates are sampled, the number of core genes reduces and the number of unique genes, and consequently the whole pan-genome, increase, which means that the *B. cereus* pan-genome is still open. The most recent study based on 114 complete genomes, recognized a pan-genome of 60,000 genes and almost 600 core genes (1% of the total pan-genome) suggesting that the environmentally diversity and adaptability of bacteria from this group may be due to the abundance of strain specific genes (about 50% of the pan-genome). Moreover, the phylogenetic analyses based on the pan-genome was largely concordant with the MLST's interpretations (Bazinet, 2017).

The overall evaluation of the *B. cereus* group population structure, with all these methods, indicates that *B. anthracis* strains have a monophyletic origin. *B.*

anthracis have been characterized as a clonal species, with very low genetic diversity, as a result of its life cycle. The acute nature of anthrax infections led to a rapid period as vegetative cells, a reproductive stage that allows genetic variation (mutation, genetic transfer…) and a long period as dormant spores in the environment (Helgason et al., 2000b; Kolstø et al., 2009; Rasko et al., 2005). *B. thuringiensis* was considered as polyphyletic species having been found in more than one group of the tree. Additionally, this species was also considered as paraphyletic, since clades containing *B. thuringiensis* are also composed of *B. cereus*. *B. thuringiensis* could not be distinguished from *B. cereus* if the bacteria lost the plasmid containing *cry* genes, showing the great relevance of plasmids for these species (Raymond, 2017).

Another example of taxonomic mixed species is the recently proposition to reclassify *B. weihenstephanensis* strains as later heterotypic synonym of *B. mycoides* based on the inability to distinguish both species by signature sequences of 16S rDNA, *cspA* genes, digital DNA–DNA hybridization and average nucleotide identity values (Liu et al., 2018). Even if the authors do not consider the determinant characteristic of *B. mycoides* strains, their rhizoidal format of colony, this new classification is already used in NCBI Genome Database.

After all, the taxonomy of *B. cereus* group is still in debate. Currently species definitions within the group consider in advance their phenotypes (virulence, physiology and morphology) than the phylogenetic relatedness. New taxonomic organizations of the group have been suggested, considering a polyphasic taxonomic approach, which includes genotypic and phenotypic characterization. For instance, one method determined the different taxa using the whole-genome sequence-based Genome BLAST Distance Phylogeny (GBDP) approach. This approach divided the group into 30 clusters, each representing independent species (Liu et al., 2015). Another proposition suggests a new taxonomic rearrangement, but in a more moderate view of splitting the group. Taking into account the three major clades, it clearly separates strains responsible for acute vertebrate infections from entomopathogenic lineages (Raymond, 2017).

1.1.4 Plasmids

Plasmids play an important role in the ecology of *B. cereus* group species. The main virulence factors of *B. anthracis*, *B. thuringiensis* and *B. cereus* are encoded by genes located on plasmids. *B. anthracis* is mainly characterized by the presence of the plasmids pXO1 and pXO2, which carries the toxin genes and capsule biosynthesis genes, respectively. Strains are characterized as *B. thuringiensis* when produce δ-endotoxins, the primary factor for its entomopathogenic features, encoded by *cry* genes predominantly harbored in plasmids. Moreover, B. cereus strains that causes the emetic syndrome harbor the pCER270 plasmid, that contain the genetic apparatus to the production of the cereulide toxin (Rasko et al., 2005; Vilas-Bôas et al., 2007).

The *B. cereus* group strains possess a wide range of plasmids, especially in *B. thuringiensis* strains (Patiño-Navarrete & Sanchis, 2017; Zhu et al., 2015), varying in number (up to 14 different plasmids) and size (from 2kb to 500kb). Considering that each plasmid could be present in multiple copies, the total amount of plasmid DNA (sum of plasmid sizes by its copy number) could be greater than the chromosomal DNA amount (Zhong et al., 2011). Frequently the copy numbers of a plasmid is inversely proportional to plasmid size, such as *B. thuringiensis* YBT-1520 strain plasmid pBMB8513 (8,5 kb), identical to the pHT8_1 from *B. thuringiensis* HD73 (Fazion et al., 2018), with approximately 75 copies and pBMB293 (293 kb) with only 3 copies (Zhong et al., 2011).

The presence of mobile genetic elements (MGE) in the genome of *B. cereus* group bacteria allows the occurrence of genetic exchanges between chromosomes and plasmids. As consequence, chromosomes and plasmids share genes of all functional categories, differing in proportion and in regulatory components (Zheng et al., 2015). The presence of mobile elements and plasmids promote horizontal gene transfer among different strains, improving diversification and adaptation to different conditions.

The main question is that the genes encoding the specific traits used to taxonomic definition are carried on plasmids, which could be lost or transferred to bacteria that belong to other species of the group (Santos et al., 2010). However, genomic analyses linking chromosomal properties with plasmid content evidenced the co-evolutionary interactions of both replicons and that this association resulted in

proliferation of successful plasmid and chromosome combinations and that are important to adaptation of specialized pathogens to characteristic niches (Méric et al., 2018; Zheng et al., 2017).

1.2 Quorum sensing systems – RNPP Family

For a long time, bacteria had been described as simple single-cell organisms that live in an autonomous and individual manner. Findings in the last 30 years have changed this concept and nowadays, the multicellularity social behavior is considered as a general trait of bacteria species (Shapiro, 1988, 1998). One of the remarks that leads to this change of thinking was the complex architecture of colonies from some species, such as *B. subtilis* (Aguilar et al., 2007). Multicellular cooperation and labor division provide countless benefits for bacteria, such as more effective access to resources and protection against antagonists or environmental variations.

To coordinate their multicellular functions, bacteria perform a cell-cell communication known as quorum sensing. This communication allows bacteria to coordinate their gene expression and behavior with population density by producing, releasing, detecting and responding to autoinducers (AIs), small extracellular signaling molecules. The concentration of AIs in the bacterial environment increases in relation to the bacterial population density until it reaches a threshold level that is detected by cells and result in modification on gene expression. Quorum sensing enable bacteria to regulate key processes such as bioluminescence, sporulation, competence, antibiotic production, biofilm formation, production of virulence factors and gene transfer (Miller & Bassler, 2001; Rutherford & Bassler, 2012; Waters & Bassler, 2005). In pathogenic bacteria for example, release of virulence factors when in a small number of cells may not be relevant because bacteria could be detected and destroyed by the immunity system of the host. Thus, it is more beneficial for these bacteria to activate the expression of the virulence genes in conditions where the population density can efficiently attack the host and run over the immune system (Shapiro, 1998).

1.2.1 Quorum sensing in Gram negative and Gram positive bacteria

The quorum sensing systems were first described in the Gram negative bioluminescent marine bacterium *Vibrio fischeri*. This bacterium colonizes the light organ of the Hawaiian squid *Euprymna scolopes*, which is rich in nutrients. Inside the light organ, the bacteria are able to grow and reach a high population density, resulting in activation of expression of the luciferase operon (*luxCDABE*) by quorum sensing. This light provides a counter-illumination to the host, avoiding shadow formation and, consequently, predator's evasion. When the bacteria are in the ocean, outside the light organ, the autoinducers released by them are spread in water and are not able to activate the luciferase expression. This circuit is controlled by the LuxI-LuxR system (Figure 6). LuxI synthesize the autoinducer, an acyl-homoserine lactone (AHL) and LuxR is the cytoplasmic receptor that acts as a transcriptional activator. The LuxR-AHL complex binds to the *luxICDABE* promoter and activates its transcription (Miller & Bassler, 2001; Ng & Bassler, 2009; Papenfort & Bassler, 2016; Waters & Bassler, 2005).

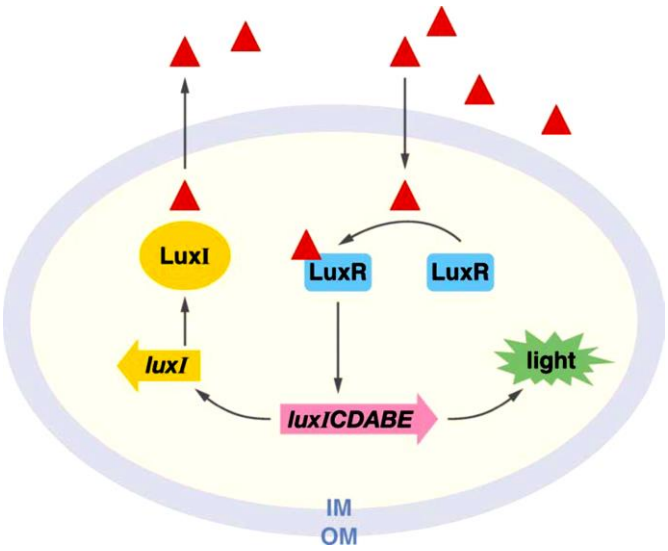

Figure 6. Signaling circuit of LuxI-LuxR quorum sensing system in *V. fisheri*. Red triangles: autoinducer (produced by LuxI); IM: inner membrane; OM: outer membrane (Waters & Bassler, 2005).

The LuxI-LuxR system is the paradigm for most Gram negative quorum sensing. The Gram negative autoinducers are generally AHL (Figure 7), composed by a core N-acylated homoserine-lactone ring carrying acyl chains of 4-18 carbon in length with occasional modifications or unsaturated double bonds. The AHL molecules are able to diffuse through the membrane, resulting in equal intra- and

extracellular concentrations. When the autoinducer achieves a threshold concentration it binds to a LuxR-like receptor and activate gene expression, whose products allow bacteria to react to a given stimuli (Miller & Bassler, 2001; Ng & Bassler, 2009; Papenfort & Bassler, 2016; Waters & Bassler, 2005).

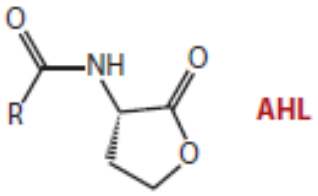

Figure 7. Core molecule of acyl-homoserine lactone autoinducers (Ng & Bassler, 2009).

Gram positive bacteria use oligopeptides as autoinducers. These signaling molecules are encoded as a precursor with a signal sequence for secretion. Unlike the AHL autoinducers, membrane is not permeable to oligopeptides that need to be actively exported. In the extracellular environment, the peptides can also undergo processing and modifications to acquire their active form. These pheromones are detected by two different mechanisms (Figure 8): (i) by binding to receptors in the membrane surface (middle panel) or (ii) by being re-imported within the bacteria to act over cytoplasmic receptors (left panel) (Hoch, 2000; Kalamara et al., 2018; Perego & Hoch, 2002; Rutherford & Bassler, 2012).

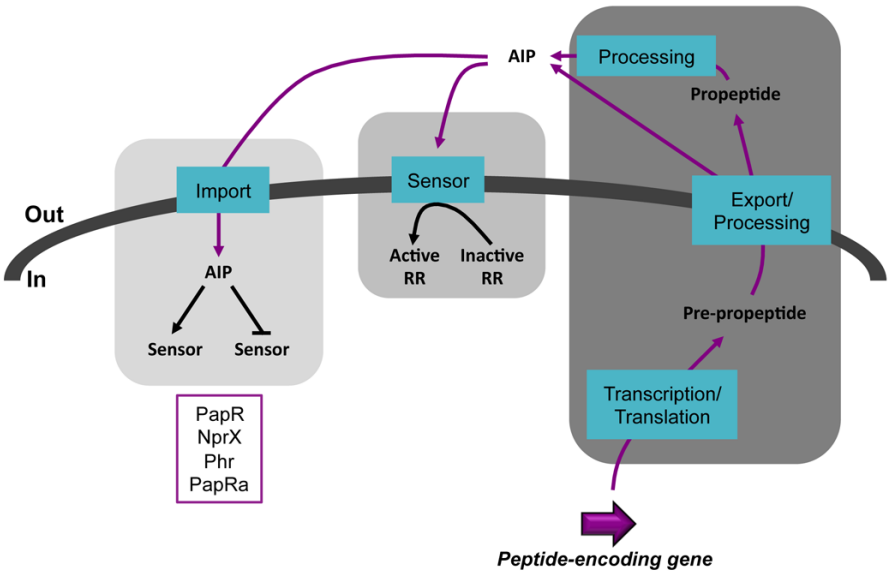

Figure 8. Quorum sensing in Gram positive bacteria. The signaling molecules of both types of systems are autoinducer peptides (AIP). AIPs could be detected by membrane receptors (middle panel – two components system) or imported to act intracellularly (left panel - RNPP family). Dark grey panel: common steps for both mechanisms. RR: response regulator (Slamti et al., 2014).

The first mechanism is known as two component signal transduction systems, composed by a membrane histidine kinase sensor and a cytoplasmic response regulator (Figure 9). The oligopeptide is recognized by the sensor domain (Signal Input) of the histidine kinase in the extracellular surface of the membrane and activates the autophosphorylation of the catalytic domain of this protein (Autokinase). The phosphoryl group is, then, transferred to the regulatory domain of the cytoplasmic response regulator (Regulator). The phosphorylation of the response regulator activates the effector domain of this protein (Output), generally a DNA binding domain that allows it to function as a transcription factor, regulating gene expression and consequently, the bacterial response to that specific signal.

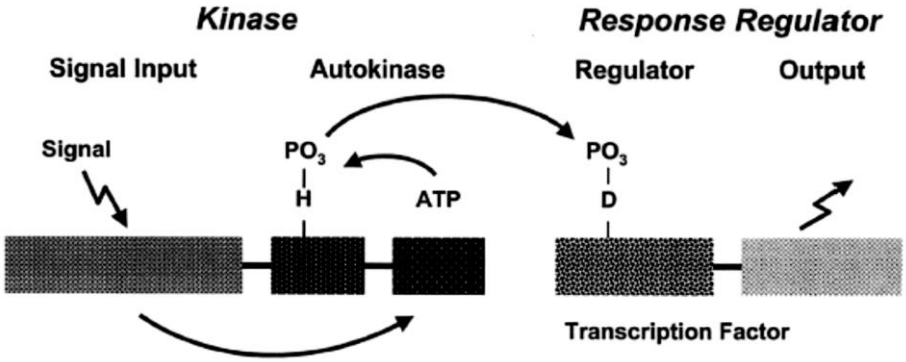

Figure 9. The two-component signal transduction system. Detailed description is found in the text (Perego & Hoch, 2002).

The signal transduction system for competence in *B. subtilis*, ComP-ComA system (or ComXQPA system), is an example of two-component systems (Figure 10).

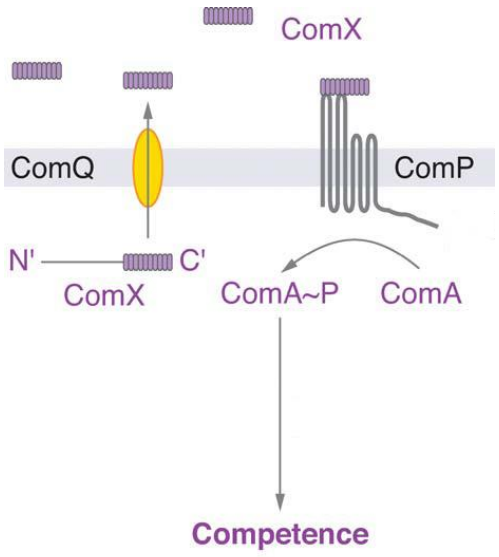

Figure 10. Competence quorum sensing system of *B. subtilis*. Detailed description is found in the text (Waters & Bassler, 2005).

In this pathway, the autoinducer precursor of ComX is secreted and processed by ComQ. The ComX active decapeptide binds to the ComP membrane bound sensor histine kinase resulting in its autophosphorylation. ComP transfers the phosphate to the response regulator ComA enabling it to bind to DNA and activating expression of genes related to bacterial competence, and also production of surfactin (Kalamara et al., 2018; Lazazzera, 2000; Miller & Bassler, 2001).

The two-component systems may have evolved to a more elaborated signal transduction pathway: the phosphorelay, also activated by the transfer of phosphoryl groups. The phosphorelay is a cascade of phosphate transfer, with additional components (domains or proteins) that allow a precise modulation with several points for regulation. The most known bacterial representative is the sporulation phosphorelay (Figure 11), well studied in *B. subtilis*.

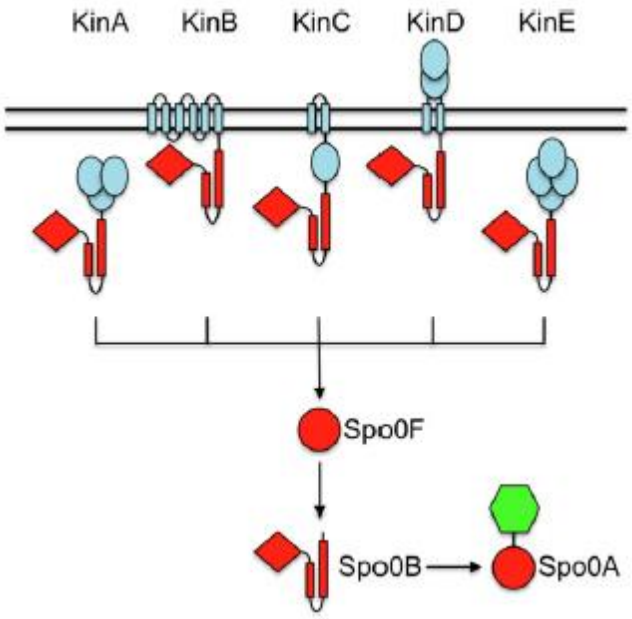

Figure 11. Sporulation phosphorelay. Transfer of phosphoryl groups are indicated by arrows. Signal are detected by kinases that autophosphorylate and transfer the phosphoryl groups to the response regulator Spo0A through two phosphotransfer proteins, Spo0F and Spo0B (Wu et al., 2013).

Various signals, as starvation, activate this pathway by five kinases - named KinA to KinE; KinA and KinE are located at the cytoplasm while KinB, KinC and KinD are membrane-spanning proteins. These kinases phosphorylate the intermediate response regulator Spo0F, the phosphate is sequentially transferred to the phosphotransferase Spo0B and then to the major response regulator of sporulation, Spo0A, that needs to be phosphorylate to be active. Additionally, this cascade is also

regulated by two phosphatase families: Rap and Spo0E, which dephosphorylate Spo0F and Spo0A, respectively. The higher complexity of this signal transduction pathway enables the bacteria to accurately regulate the cell fate, as the sporulation, which is a complex and irreversible process (Burbulys et al., 1991; Hoch, 2000; Jiang et al., 2000b; Perego & Hoch, 2002; Trach et al., 1991).

1.2.2 RNPP family

The second quorum sensing mechanism of Gram positive bacteria is based on the direct binding of the oligopeptide to the cytoplasmic response regulator in the responder cell, particularly in bacteria from the Firmicutes phylum. These systems belong to the RNPP family – named from the key regulator members – Rap, NprR, PlcR and PrgX. Even if these proteins regulate various processes in different bacterial species, they share two main features: the intracellular interaction with a linear processed oligopeptide (Phr, NprX, PapR, cCF10, respectively) that is re-imported by oligopeptide permeases (Opp), and a similar structure of the regulators, which contain tetratricopeptide repeat (TPR) motifs (Figure 12) (Declerck et al., 2007; Perez-Pascual et al., 2016; Rocha-Estrada et al., 2010).

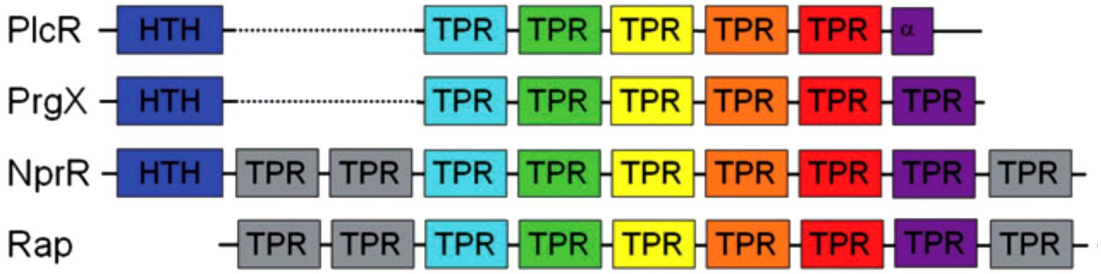

Figure 12. Structure of RNPP protein. Regulators of the RNPP family contain multiple TPR repeats. TPR: tetratricopeptide repeat; HTH: helix-turn-helix, a DNA binding-domain (Perchat et al., 2011).

The TPR domain is a structural motif present in a wide range of proteins, identified in diverse organisms from bacteria to humans and involved in different biological processes such as cell cycle regulation, transcriptional control and protein folding. The TPR motifs comprise three to sixteen tandem repeats of degenerated sequences of 34 amino acids residues and adopt a structural arrangement of two-antiparallel α-helices. This arrangement is essential to its role in mediating protein-protein or protein-peptide interactions or in assembling multiprotein complex (D'Andrea & Regan, 2003).

In addition to these main features, the genes coding for the precursor of the signaling oligopeptide is generally located downstream from genes coding for the response regulator and can be found on the chromosome or plasmids. When the oligopeptide binds to its cognate receptor it induces a conformational change, activating or inhibiting its activity. Except from the Rap phosphatase, the RNPP regulators possess a helix-turn-helix (HTH) DNA binding domain in the N-terminal region (Figure 12), allowing them to act as transcriptional factors. The similar characteristics of the RNPP systems suggest that they derive from a same ancestor (Figure 13) (Declerck et al., 2007; Do & Kumaraswami, 2016; Perchat et al., 2016b; Perez-Pascual et al., 2016; Rocha-Estrada et al., 2010).

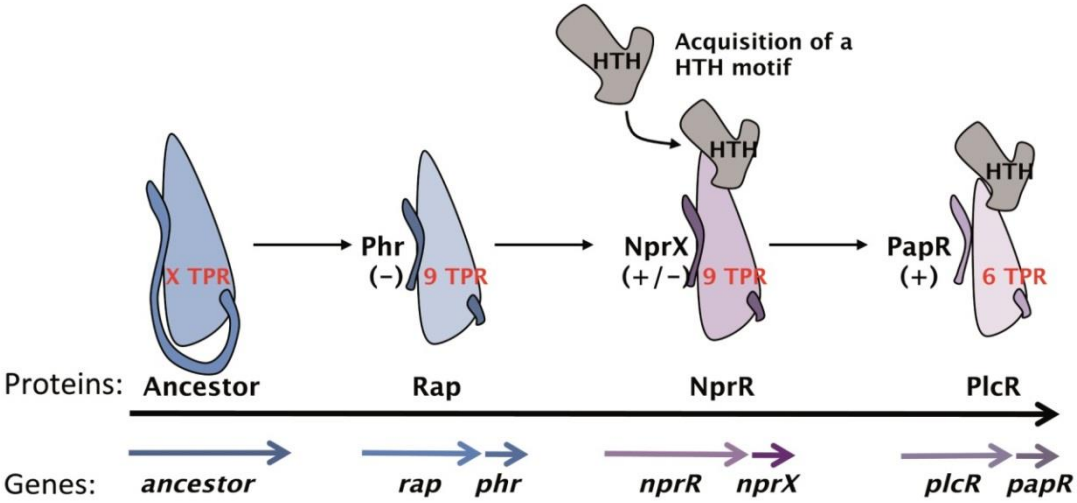

Figure 13. Evolution of RNPP regulators. The disposition of all *Bacillus* RNPP systems in signaling cassettes suggest that the ancestor system was encoded by a unique gene that was separated during evolutionary time. Another evolutionary process was the acquisition of the HTH motif, which enable the regulators to directly control the expression of genes related to its pathway. nTPR: number of TPR repeats; +/-: if the signaling molecule inhibits (-) or activates (+) the regulator activity: HTH: DNA-binding domain (Perchat et al., 2016b).

1.2.2.1 Rap-Phr systems

Response regulator aspartate phosphatases (Rap) are found in genome of *Bacillus* species. These proteins were extensively studied in *B. subtilis*, in which they were shown to regulate sporulation, competence, transfer of genetic mobile elements (plasmids or insertion sequence), production of extracellular proteases and biofilm formation (Perego, 2013). The main described roles of Rap are in sporulation and competence with two distinct modes of action: (i) as phosphatase, by dephosphorylating the intermediate response regulator of the phosphorelay, Spo0F,

thus inhibiting indirectly the phosphorylation of Spo0A and consequently, the sporulation process (Perego et al., 1994); or (ii) by binding to a cytoplasmic regulator, such as the ComA response regulator, in which Rap binds to the HTH DNA-binding domain, inhibiting the transcriptional activator activity of ComA over genes from its regulon, related to competence (Bongiorni et al., 2005; Core & Perego, 2003). The Rap proteins are inhibited by the Phr oligopeptide (Perego & Brannigan, 2001; Perego & Hoch, 1996). In the *B. cereus* group species, Rap-Phr systems were already shown to regulate sporulation in *B. anthracis* (Bongiorni et al., 2006) and *B. thuringiensis* (Fazion et al., 2018).

Rap proteins are structurally organized in an N-terminal three helix bundle and a C-terminal domain containing TPR motifs (six canonical and one non-canonical). Phr binding induces a conformational change where the entire protein consists of one single domain containing nine TPR-like folds (Figure 12) (Parashar et al., 2011; Perego, 2013). Due to the lack of the HTH domain (Figure 12) and the highly diversity among the RNPP regulators, Rap proteins are suggested as the most ancestral system of the RNPP family (Figure 13) (Declerck et al., 2007).

1.2.2.2 NprR-NprX system

In bacteria from the *B. cereus* group, the neutral protease regulator (NprR) was primarily described to regulate the expression of the metalloprotease NprA (also known as NprB or Npr599), which is the main constituent of the secretome of these bacteria during the stationary phase in sporulation medium (Chitlaru et al., 2006; Perchat et al., 2011). NprR is active as a transcriptional regulator in the presence of its cognate oligopeptide NprX (Perchat et al., 2011). The gene encoding the NprX precursor is located downstream from the *nprR* gene, and both genes are co-transcribed. The expression from the *nprR* promoter is repressed by the global regulator CodY during the exponential growth and activated by the PlcR quorum sensor (described below) at the onset of the stationary phase. Moreover, the transcription of the *nprX* gene is also independently activated by two promoters, located upstream the *nprX* gene, within the *nprR* gene and related to the sporulation specific sigma factors σ^H and σ^E. These independent expressions result in higher concentration of active NprX in the late exponential phase. Differently from some

RNPP systems, the transcription of the *nprR-nprX* cassette is not auto-regulated (Dubois et al., 2013).

In fact, NprR is a bifunctional regulator (Figure 14), depending on the presence of NprX that modify its conformational structure (Perchat et al., 2016a; Zouhir et al., 2013).

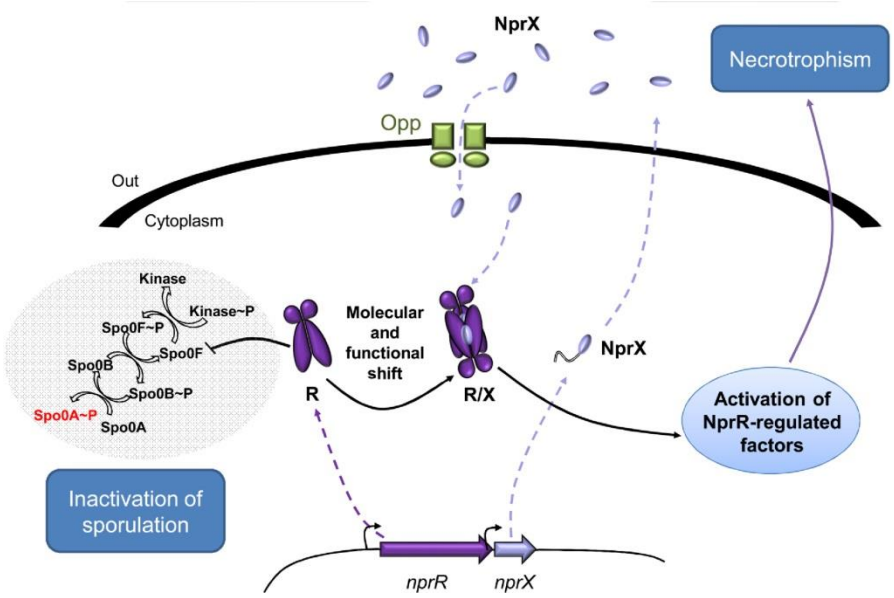

Figure 14. Bifunctional activity of NprR. When in its dimeric form, NprR (R) inhibits the sporulation phosphorelay. Binding of the NprX signaling peptide (R/X), activates the transcriptional regulator function of NprR. The switch between the two forms is regulated in a quorum sensing manner (Perchat et al., 2016a).

In the absence of NprX, NprR is in a dimeric form structurally closed to Rap protein, with an additional HTH domain (Figure 12). NprR inhibit the initiation of the sporulation process by binding and dephosphorylating Spo0F from the sporulation phosphorelay, in a Rap phosphatase-like manner. Residues described to be involved in the activity of Rap over Spo0F are conserved in NprR. The binding of the NprX switches the structure of NprR from the dimeric to the tetrameric form and activates its function of transcriptional regulator (Perchat et al., 2016a). The NprR regulon includes the *nprA* gene and at least 40 other genes, some of which encoding degradative enzymes (proteases, lipases and chitinases). The main role of the NprR regulon is the survival of *B. thuringiensis* in the insect host cadaver (Dubois et al., 2012). Thus, the NprR-NprX system seems to play a role in the tight control of the transition between the necrotrophic lifestyle and sporulation (Perchat et al., 2016a, 2016b). Furthermore, due to its bifunctional characteristic, the NprR regulator is thought to be the evolutionary intermediate of the RNPP family proteins, between the

Rap phosphatase and the transcriptional regulators PlcR and PrgX (Figure 13) (Declerck et al., 2007; Perchat et al., 2016b).

The active form of NprX is located within the C-terminal portion of the 43 amino acids precursor peptide, and a heptapeptide is suggested to be its active form. Phylogenetic analyses revealed a coevolution of the two cognate components of this system and that NprR-NprX and housekeeping genes have a different evolutionary history. In addition, seven different NprR-NprX clusters were detected, which correspond to different pherotypes. Comparison between groups indicates that these pherotypes are strain-specific with the possibility of cross-talk between some groups (Perchat et al., 2011).

1.2.2.3 PlcR-PapR system

The phospholipase C regulator (PlcR) was first described to positively regulate the expression of phosphatidyl inositol-specific phospholipase C (PI-PLC). The expression of this regulator starts at the end of vegetative growth and reaches the peak two hours after the onset of stationary phase (Lereclus et al., 1996). PlcR is a 34 kDa pleiotropic regulator and the PlcR regulon contains at least 45 genes that represent 80% of the secretome of *B. cereus* or *B. thuringiensis* at the onset of the stationary phase (Agaisse et al., 1999; Gohar et al., 2008, 2002). Genes activated by PlcR have in their promoter region a PlcR-box, a highly conserved palindromic sequence, which is the binding site of the PlcR-PapR complex (Agaisse et al., 1999). The most strongly PlcR-induced genes encode for enterotoxins (*hbl* and *nhe*), cytotoxins (*cytK*) and hemolysins (*clO*) (Gohar et al., 2008). The PlcR regulon is involved in the virulence with three main functions: (i) food supply, with phospholipases, proteases and toxins; (ii) cell protection, such as bacteriocins, toxins, transporters and cell wall biogenesis; and (iii) environment sensing, as two-component sensors or chemotaxis proteins (Gohar et al., 2008). Genes belonging to the PlcR regulon are spread in the bacterial chromosome, not forming a pathogenic island (Agaisse et al., 1999).

PlcR is activated by the signaling peptide PapR (from Peptide activating PlcR) (Figure 15) (Slamti & Lereclus, 2002). PapR active form is a heptapeptide and its precursor peptide (48 amino acids) is encoded by the *papR* gene located 70bp downstream from the *plcR* gene and belongs to the PlcR regulon (Bouillaut et al.,

2008; Slamti & Lereclus, 2002). The PlcR-PapR system positively regulates its own transcription (Lereclus et al., 1996). The expression of *plcR-papR* is also negatively controlled by Spo0A, leading to the absence of expression of this system in sporulation-specific medium (Lereclus et al., 2000). This system is indirectly controlled by CodY, as this regulator controls the transcription of genes encoding for the Opp permease required for the re-import of the PapR oligopeptide (Gominet et al., 2001; Slamti et al., 2016).

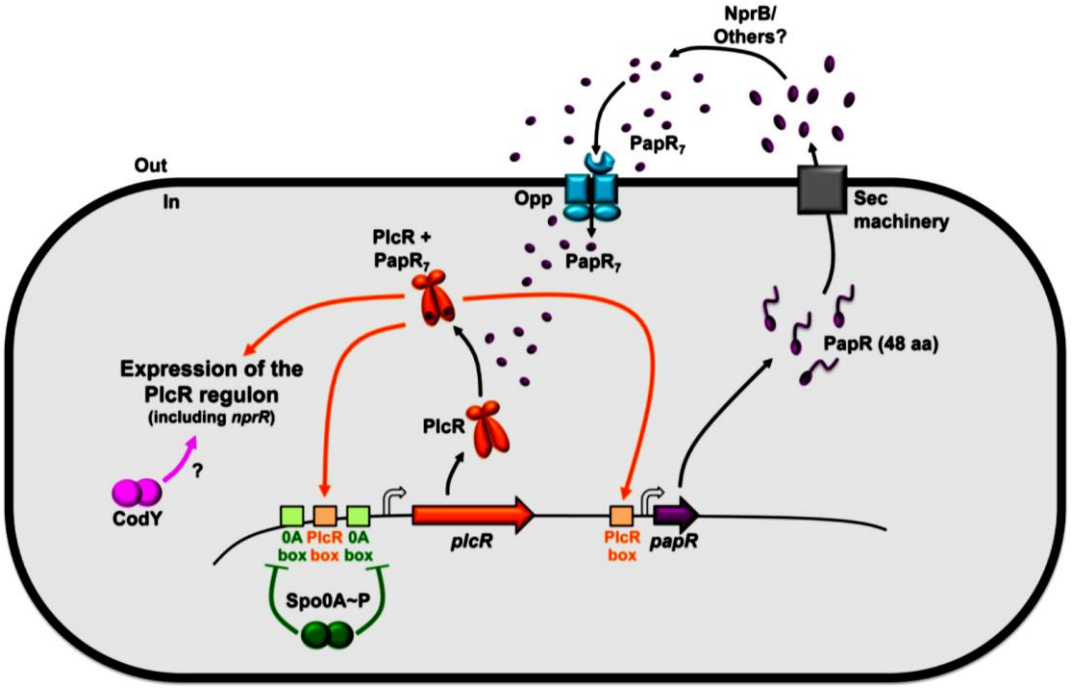

Figure 15. The PlcR-PapR quorum sensing system. PapR is transcribed in a premature form of 48 amino acids that is secreted, processed and re-imported as a heptapeptide. Inside the cell, the PapR7 binds to PlcR and activates the transcription of virulence factor genes that belong to the PlcR regulon (Slamti et al., 2014).

The PlcR structure is composed by an N-terminal HTH DNA binding domain and a regulatory domain that consist of five degenerated TPR motifs and a capping helix at the C-terminal end of the protein (Figure 12) (Declerck et al., 2007). PlcR is a dimer and the binding of PapR result in a slight conformational change of the regulatory domain, enabling PlcR to bind to DNA (Declerck et al., 2007). PlcR-PapR system seems to be specific from species of the *B. cereus* group. However, as already cited, *plcR* genes from *B. anthracis* strains bear a nonsense mutation and, consequently this regulator is inactive in this species (Agaisse et al., 1999). This singular variation could explain some phenotypic differences between *B. anthracis* strains from *B. cereus* or *B. thuringiensis*, such as the absence of hemolysis.

Similarly to the NprR-NprX systems, PlcR-PapR exhibit strain-specific properties, with four distinct pherotypes, in which a given PlcR is more strongly activated by its cognate PapR than heterologous molecules (Bouillaut et al., 2008; Slamti & Lereclus, 2005). Moreover, *plcR* and *papR* genes seem to have co-evolved but have a different evolutionary history when compared to the housekeeping genes (Ko et al., 2004; Slamti & Lereclus, 2005).

The PlcR, and consequently its regulon, play an important role in virulence and thus in adaptation of bacteria from the *B. cereus* group to its host environment. Due to the ability of *B. cereus* group strains, especially *B. cereus sensu stricto*, to cause human infections, new studies rely on mechanisms to disrupt bacterial quorum-sensing by interfering in cell-cell communication to affect their capacity to produce virulence factors, a processes called quorum quenching (Waters & Bassler, 2005). Recently, Yehuda et al. (2018) design synthetic PapR peptide analogs that prevent the activation of PlcR by endogenous PapR, leading to the loss of virulence factors production.

1.2.2.4. PrgX & cCF10/iCF10

PrgX is a sex pheromone receptor of *Enterococcus faecalis*, which is a commensal bacterium but also act as opportunistic human pathogen, associated to nosocomial infections (Bae et al., 2000; Perez-Pascual et al., 2016). The *prgX* gene is encoded in the tetracycline resistant plasmid pCF10 and participates in the transcription regulation of conjugative transfer gene (*prgQ* operon), which controls the conjugation capacity of pCF10. The repressor activity of PrgX is controlled by two antagonistic peptides (Figure 16): the inhibitor iCF10, located in the plasmid pCF10, and the activator cCF10, encode by a chromosomal gene (Do & Kumaraswami, 2016). Both cCF10 and iCF10 are heptapeptides corresponding to the C-terminal end of their precursor lipoprotein (Mori et al., 1988; Nakayama et al., 1994). Moreover, both peptides bind to the same pocket but induce different conformations in C-terminal part of PrgX. The two PrgX-peptide complexes adopt a tetrameric form that induce a particular conformation (Figure 16) (Chen et al., 2017). When iCF10 binds to PrgX regulator, they attach to two sites of the DNA sequence and form a loop that prevents activity of RNA polymerase on the *prgQ* operon. By contrast, when bonded to cCF10, this repression is disrupted and the transcription of conjugation

genes could take place (Neiditch et al., 2017). Donor and recipient cells express cCF10, which is neutralized by iCF10 in cells containing pCF10 plasmid (Rocha-Estrada et al., 2010). When the density of recipient cells increases, concentration of cCF10 overcome iCF10 concentration and release the transcription of conjugation genes repressed by PrgX, enabling conjugation.

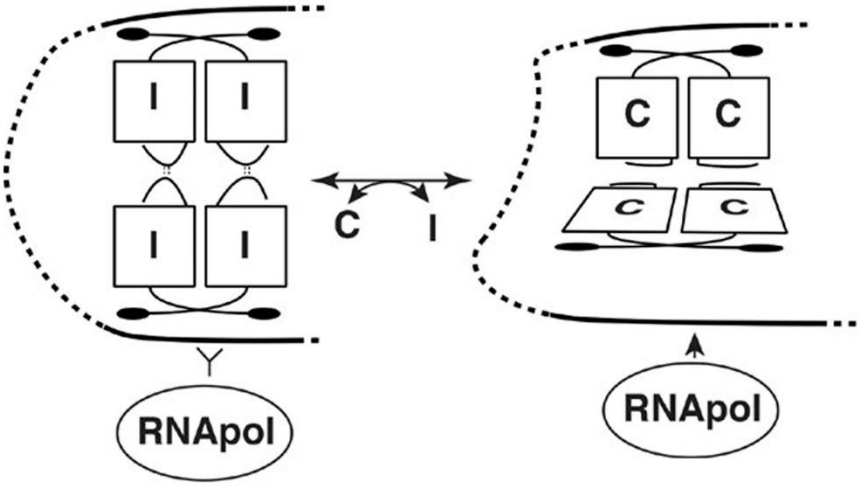

Figure 16. PrgX is regulated by two types of signaling peptides. When bind to the plasmid-encoded iCF10 (i), PrgX binds to DNA, inhibiting the access of RNA polymerase to the conjugation genes. Alternatively, when PrgX is bound to cCF10 (c), the regulator achieves another conformation that liberates the transcription of the *prgQ* operon (adapted from Chen et al., 2017).

1.2.2.5 Expansion of the RNPP family

New findings in Gram positive bacteria quorum sensing systems had led to the description of new RNPP-like systems, as the Rgg-SHP system from Streptococci, given rise to the new RRNPP family (Monnet & Gardan, 2015; Parashar et al., 2015). Rgg (regulator gene of glucosyltransferase) transcription factors are widespread in Firmicutes, but it is particularly associated to a signaling peptide in *Streptococcus* species. Rgg proteins represents a large family of receptors that regulate genes with diverse functions, such as commensalism and production of virulence factors, and are regulated by small hydrophobic peptides (SHP) (Neiditch et al., 2017; Parashar et al., 2015; Perez-Pascual et al., 2016). Rgg protein, as well as PrgX, do not contain detectable TPR motif but adopt TPR-like folds (Neiditch et al., 2017). The ComR-XIP system, initially described as member of the Rgg family, was shown to be another new member of the RNPP family (Talagas et al., 2016). ComR is a transcription

factor that positively controls competence in Streptococci and is directly activated by XIP (the active form of the ComS peptide).

1.2.2.6 Regulation of the infectious cycle in B. thuringiensis

The three RNPP systems present in the *B. cereus* group – PlcR, NprR and Rap – have been shown to regulate the infectious cycle of *B. thuringiensis* in the insect model *Galleria mellonella*. These quorum sensing systems are sequentially activated during the lifestyle of *B. thuringiensis* throughout the different phases of infectious process, namely: pathogenic, necrotrophic and spore formation (Figure 17) (Slamti et al., 2014).

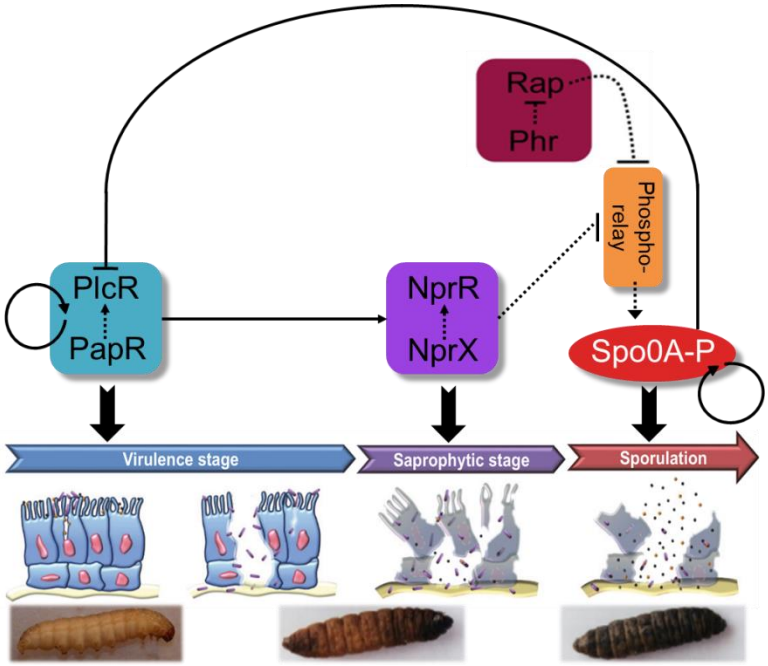

Figure 17. The infectious cycle of *B. thuringiensis* in the insect is regulated by quorum sensing systems. In larvae of *G. mellonella*, the infectious process of *B. thuringiensis* was shown to be controlled by the sequential activity of PlcR-PapR; NprR-NprX and Rap-Phr. Solid lines: transcriptional regulation; Dotted lines: peptide- or protein-protein interaction (modified from Slamti et al., 2014).

In the insect host, their expression is preceded by the activity of the major insecticidal toxins, Cry δ-endotoxins, and the subsequent lysis of midgut epithelial cells that promotes bacterial growth. At the onset of stationary phase, the PlcR-PapR quorum sensing systems is activated, promoting the production of extracellular virulence factors. During the time that the PlcR regulon is maintained, the dimeric form of NprR and the Rap prevent the initiation of sporulation. After the host death, at the late stationary phase of bacterial growth and with higher population density, the

concentration of NprX is sufficient to bind to NprR and switch its conformation to the tetrameric form. This rearrangement inhibits its activity on the phosphorelay and stimulates the production of proteases and consequent exploitation of the host cadaver nutrients. This switch of NprR together with the Phr inhibition of Rap phosphatases enables the commitment to sporulation. Therefore, the tightly control of the bacterial lifestyle by the sequentially activation of these quorum sensing systems, allow the co-ordination between the bacterial population density and the different phases of the infectious cycle.

1.3 Rap-Phr Systems

1.3.1 Sporulation in *Bacillus*

Spores play an important role in survival and dispersion of *Bacillus* species and the sporulation process was well studied in *B. subtilis*. The capacity of bacterium to sense its own metabolic state and the environmental changes contribute to regulate the commitment to sporulation. This decision is driven by the major sporulation response regulator Spo0A that is active when it reaches a phosphorylation threshold (Burbulys et al., 1991; Fujita & Losick, 2005). The phosphorylation state of Spo0A is controlled by the phosphorelay (Figure 11) which is a signal transduction system allowing the bacteria to sense different types of environmental signals: the cell density by Rap-Phr quorum sensing systems and nutrient limitation (such as source of carbon, nitrogen and phosphorus) and other environmental stresses sensed by kinases (Sonenshein, 2000). In *B. subtilis*, there are five different kinases related to the phosphorelay (KinA to KinE) and they are all able to phosphorylate Spo0F (the intermediate response regulator). KinA and KinB are the most active of these sensor kinases, being required to initiate sporulation, and seem to depend on signals present on early stationary phase (Jiang et al., 2000b). Whereas KinC and KinD may respond to signals present during growth. Moreover, KinC and KinD were shown to control biofilm formation, while KinE did not have a sufficient effect to lead bacteria to sporulation (Aguilar et al., 2010; Fujita & Losick, 2005; Wu et al., 2013). Spo0F~P is used as source of phosphate by the phosphotransferase Spo0B to phosphorylate the Spo0A transcription factor (Burbulys et al., 1991).

The Spo0A regulon comprise at least 121 genes, responding to a low or a high threshold of Spo0A~P, that are repressed or activated (Fujita et al., 2005; Molle et al., 2003). This dose-dependent response is related to the binding constant of the DNA binding domain of Spo0A~P to the 0A box at the promoter region of target genes (Fujita et al., 2005). Spo0A~P indirectly auto regulate its own transcription by the activation of the stationary phase sigma factor σ^H, which direct transcription of *spo0A* gene and other sporulation related genes, as *spo0F*, *spoIIAA-AB-sigF* and some *phr* (Britton et al., 2002). Genes regulated by low level of Spo0A~P show a high binding constant or are under indirect control of Spo0A, *via* repression of *abrB* gene. AbrB is a pleiotropic transcription repressor and its inhibition by Spo0A release the expression of genes related to the stationary phase and early stage of sporulation. Low level of Spo0A~P regulates genes that are related to other processes during stationary development, such as cannibalism, competence and biofilm formation. During the time that the activity of kinases and phosphatases (Rap and Spo0E) are counter-balanced, Spo0A~P are maintained at low levels (Sonenshein, 2000). Some factors, as the activity of Phr peptides inhibiting Rap phosphatases, the increase of kinases activity and the expression induced by σ^H, allow to accumulate Spo0A~P up to a critical threshold leading to commitment of sporulation. Thus, increasing amount of Spo0A~P controls the expression of several genes directly involved in the sporulation process, such as *spoIIA*, *spoIIE* and *spoIIG* (Fujita et al., 2005; Fujita & Losick, 2005).

Sporulation begins with an asymmetrical cell division triggered by Spo0A~P. This asymmetrical division originates two distinct cell types, genetically identical but with different fates: a larger mother cell and a smaller forespore (also known as prespore). The sporulation process is driven by differential and sequential activation of sigma factors (Figure 18), subunits of RNA polymerase, that direct transcription of different set of genes in the two bacterial compartments. These sigma factors are activated at specific times and in a particular compartment allowing changes in gene regulation to be coupled with morphological states. Just after the septum formation (asymmetric division) the sigma factor σ^F is activated in the forespore and shortly after the σ^E become active in the mother cell. σ^E is encoded in a pro-σ^E precursor form and its proteolytic activation is controlled by proteins regulated by σ^F. σ^E and σ^F regulate the expression of genes involved in the engulfment of the forespore in the

mother cell, a phagocytic-like process that results in a double membrane forespore included in the mother cell cytosol. After the engulfment, σ^G is activated in the forespore, the *sigG* gene is under control of σ^F and its sigma factor product regulate the transcription of genes related to the protection of chromosome and the arrangement of the spore for germination, when in an appropriate condition. σ^G also induces the cleavage of the pro-σ^K (regulated by σ^E) in the mother cell. σ^E and σ^K are required for the synthesis of the cortex (a peptidoglycan layer) and the coat (a proteinaceous layer) that encase the mature spore. σ^K also regulate genes which products lead to the mother cell lysis and delivery of the mature spores to the environment (Fimlaid & Shen, 2015; Kroos et al., 1999; Piggot & Hilbert, 2004; Tan & Ramamurthi, 2014).

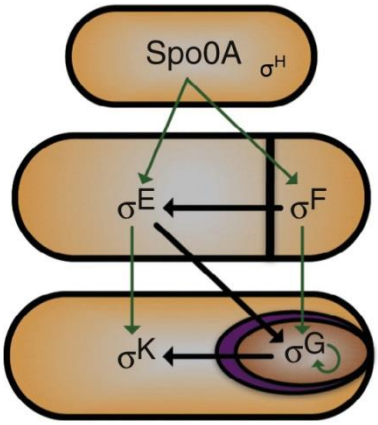

Figure 18. Sequential and alternative activation of sporulation-specific sigma factors control the spore formation process. The crisscross activity of these sigma factors allows the tight regulation between morphogenesis and gene expression. Green arrows: transcriptional regulation; Black arrows: post-translational activation. Complete description is found in the text (modified from Fimlaid & Shen, 2015).

Although analogous sporulation process are described in other species of *Bacillus* and genes involved in sporulation was initially expected to be found among the core genes in their pan genome, a study on *Bacillus* genus found a great intraspecific and interspecific variation in these genes (Alcaraz et al., 2010). From the 185 sporulation related genes from *B. subtilis* considered in this pan genomic analysis, only 52 were categorized as core genes. Among them were found the major regulators of sporulation, required for temporal and spatial regulation of this process (*spo0A*, genes coding for sporulation sigma factors, *spoIIE*…). It is in agreement with Stephenson & Hoch (2002) showing that the main components of the phosphorelay - Spo0F, Spo0B and Spo0A – are highly similar between *B. subtilis* and *B. anthracis*.

In contrast, the divergence could be found, for example, in the number of sensor histidine kinases: five in *B. subtilis* against nine in *B. anthracis*, and the sensor domain are not well conserved (Brunsing et al., 2005). Another important difference concerns Rap-Phr systems, which shows divergence in sequence and in number. *rap-phr* systems were described to be present in all strains of *B. subtilis* and *B. cereus* group species and also in more distant *Bacillus* species such as *Bacillus halodurans* and *Bacillus clausii* (Even-Tov et al., 2016). Phylogenetic analysis of Rap sequences from *B. subtilis* and *B. cereus* groups showed that Rap proteins of each group clustered separately (Figure 19B) (Even-Tov et al., 2016). Moreover, in the pan genomic study, *rap* genes from the *B. cereus* group species were not sufficiently conserved to be recognized as core genes, using *B. subtilis* genome as reference (Alcaraz et al., 2010). In addition to sequence divergence, the difference in number of these quorum sensing systems have already been described for these two most important groups of *Bacillus* genus: *B. subtilis* group strains possess a higher number of *rap* genes (11 ± 2) than the *B. cereus* group strains (6 ± 3) (Figure 19A) (Anderson et al., 2005; Even-Tov et al., 2016). These results suggest that the regulatory components of the phosphorelay cascade may be species or strain-specific and have separately evolved to respond to particular environments (Alcaraz et al., 2010).

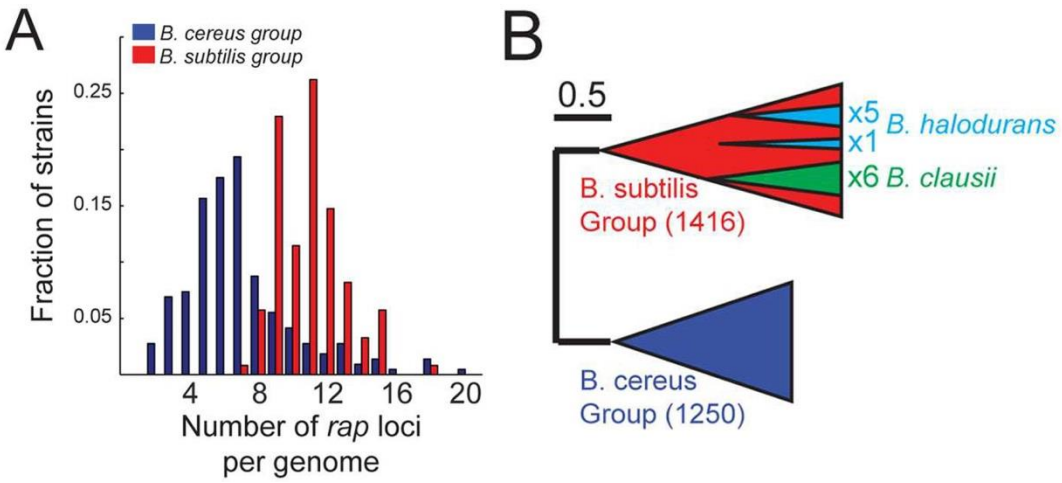

Figure 19. Differences between *B. cereus* and *B. subtilis* groups concerning number and sequence of *rap* genes. A) Distribution of *rap* genes among strain genomes. Most part of *B. cereus* group strains possess from four to eight *rap* genes, while the majority of *B. subtilis* strains have at least nine *rap* genes. B) Phylogenetic tree of Rap proteins. Sequences of regulators from *B. subtilis* and *B. cereus* groups cluster separately (Even-Tov et al., 2016).

1.3.2 Rap-Phr systems in *B. subtilis*

The first studied Rap protein was described as a phosphatase that acts on the sporulation phosphorelay by dephosphorylating Spo0F. This activity gives its name: response regulator aspartate phosphatase (Perego et al., 1994). Rap relevance as phosphatase relies on the fact that it represents some additional regulatory points to the signal transduction system that regulates the sporulation, and contribute to the phosphorelay complexity (Perego & Brannigan, 2001). Thereafter, it was shown that some Rap proteins regulate other developmental pathway of the stationary phase, such as the competence (ability to DNA uptake) *via* the ComA response regulator. The release of the *B. subtilis* complete genome sequence reveals that its type strain (str. 168) possesses 11 *rap* genes (*rapA* to *rapK*) and eight of which are followed by a downstream short coding sequence, the *phr* gene (Kunst et al., 1997). The *phr* genes slightly overlap the *rap* gene sequences forming transcript units: the *rap-phr* signaling cassettes (Perego & Hoch, 1996). The mature form of the Phr peptide inhibits its cognate Rap activity, excepted for RapB, RapD and RapJ that are orphans. However, the PhrC, also known as competence and sporulation factor (CSF), is able to inhibit RapB and RapJ phosphatases (Parashar et al., 2013a; Perego, 1997).

RapA, RapB, RapE, RapI and RapJ regulates sporulation by specifically acting on Spo0F (Figure 20, Table 2) (Jiang et al., 2000a; Parashar et al., 2011; Perego et al., 1994; Singh et al., 2013). Likewise, RapC, RapD, RapF and RapK inhibit competence by binding to the response regulator ComA and preventing its functions as transcription factor (Auchtung et al., 2006; Bongiorni et al., 2005; Ogura & Fujita, 2007; Solomon et al., 1996). RapH is the unique protein of the Rap family that was demonstrated to have dual specificity on Spo0F and on ComA (Smits et al., 2007). RapH was shown to play a role in the temporal separation of competence and sporulation pathways which are mutually exclusive. Thus, RapH prevents sporulation initiation in competent cells and later enables the cell to escape from the competent state to enter into the sporulation pathway (Smits et al., 2007). In addition, the other *B. subtilis* chromosomal Rap proteins were shown to act on other bacterial pathways. RapG regulates negatively the activity of the response regulator DegU which activates the expression of extracellular alkaline protease encoded by *aprE* (Ogura et al., 2003). RapI is encoded on the mobile genetic element ICE*Bs*1 which is an

integrative and conjugative element (ICE) (Auchtung et al., 2005). These conjugative transposons are able to excise, transfer to recipient cells through conjugation and integrate to the chromosome. RapI regulate the ICE*Bs*1 expression, excision and transfer *via* the inhibition of the immunity repressor ImmR activity. When the cell is surrounded by bacteria that also harbor ICE*Bs*1, RapI is inhibited by the PhrI produced by these bacteria, preventing the expression of ICEBs1 when there are no potential recipient cells (Auchtung et al., 2005).

Table 2. Rap-Phr systems already functionally analyzed.

Rap	Phr active	Species/replicon	Target Pathway	Reference
RapA	ARNQT	*B. subtilis* chromosome	Sporulation	(Perego et al., 1994; Perego & Hoch, 1996)
RapB	PhrC	*B. subtilis* chromosome	Sporulation	(Perego, 1997; Perego et al., 1994)
RapC	ERGMT	*B. subtilis* chromosome	Competence	(Auchtung et al., 2006; Lazazzera et al., 1999; Solomon et al., 1996)
RapD	-	*B. subtilis* chromosome	Competence	(Ogura & Fujita, 2007)
RapE	SRNVT	*B. subtilis* chromosome	Sporulation	(Jiang et al., 2000a)
RapF	QRGMI	*B. subtilis* chromosome	Competence	(Auchtung et al., 2006; Bongiorni et al., 2005)
RapG	EKMIG	*B. subtilis* chromosome	Protease production	(Hayashi et al., 2006; Ogura et al., 2003)
RapH	TDRNTT	*B. subtilis* chromosome	Competence; Sporulation	(Hayashi et al., 2006; Mirouze et al., 2011; Smits et al., 2007)
RapI	ADRVGA	*B. subtilis* chromosome	Sporulation; Transfer of ICE*Bs*1	(Auchtung et al., 2005; Mirouze et al., 2011; Singh et al., 2013)
RapJ	PhrC	*B. subtilis* chromosome	Sporulation	(Parashar et al., 2013a, 2011)
RapK	ERPVG	*B. subtilis* chromosome	Competence	(Auchtung et al., 2006)
Rap60	SRNAT or ASRNAT	*B. subtilis* pTA1060	Protease production	(Boguslawski et al., 2015; Koetje et al., 2003)
RapQ	SRNAT	*B. amyloliquefaciens* pBSG3	Competence; Surfactin production; Sporulation	(Qiao et al., 2011; Yang et al., 2015)
RapP	RapP is insensitive to PhrP	*B. subtilis* pBS32	Biofilm formation; Sporulation ; Competence	(Omer Bendori et al., 2015; Parashar et al., 2013b)
Rap$_{LS20}$	QKGMY	*B. subtilis* pLS20	Conjugation	(Rösch & Graumann, 2015; Singh et al., 2013)
BXA0205	GHTGG	*B. anthracis* pXO1	Sporulation	(Bongiorni et al., 2006)
BA3790	Not defined	*B. anthracis* chromosome	Sporulation	(Bongiorni et al., 2006)
Rap8	YAHGKDI	*B. thuringiensis* pHT8_1	Sporulation; Biofilm formation	(Fazion et al., 2018)

PhrC (ERGMT) was demonstrated to regulate the non-cognate RapB and RapJ proteins. (Kalamara et al., 2018; Neiditch et al., 2017; Pottathil & Lazazzera, 2003).

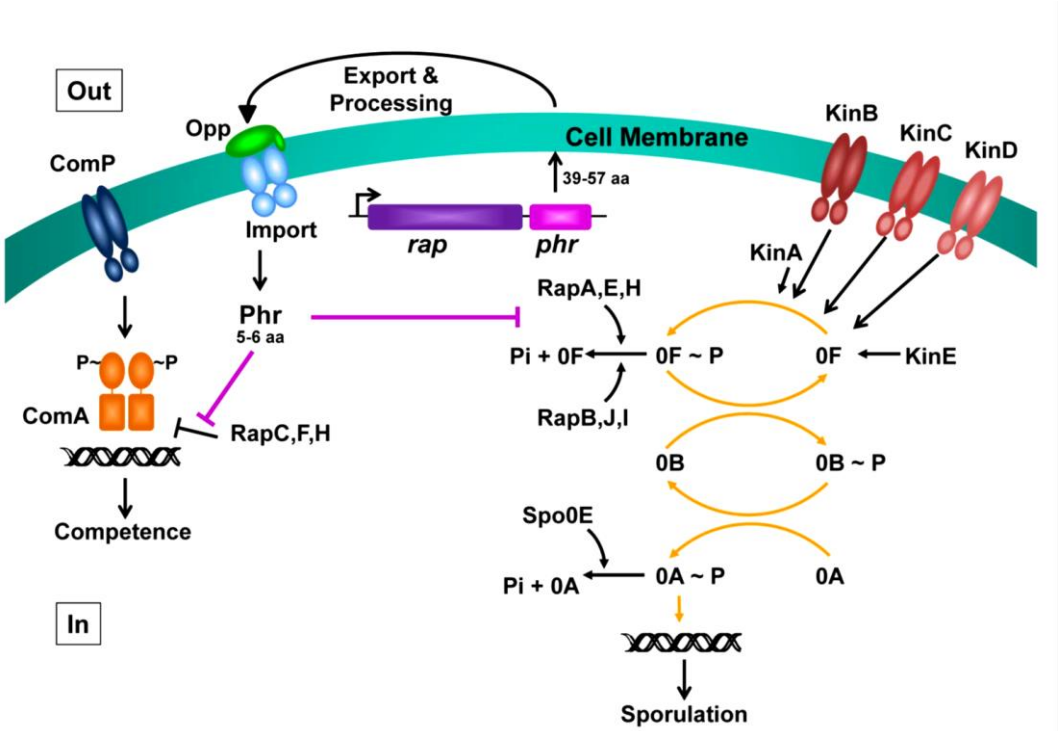

Figure 20. Activity of chromosomal Rap-Phr systems from *B. subtilis*. The schematic representation illustrates the two main process regulated by Rap-Phr systems, competence (left side) and sporulation (right side). RapD and RapK were also demonstrated to have a role in the competence regulation. Complete description is found in the text (Perego, 2013).

The involvement of multiple Rap-Phr systems in a given developmental processes (sporulation or competence) could represent different environmental cues that are integrated for a strict regulation of bacterial development (Auchtung et al., 2006; Mirouze et al., 2011). Analogous Rap-Phr systems could act synergistically, being all required for effective regulation of sporulation or competence. For example, PhrC, PhrF and PhrK activity was shown to happen in different times during growth and in a distinct level but all of them are indispensable to the full expression of ComA regulated genes (Auchtung et al., 2006). Alternatively, this redundancy could promote communication of subpopulations that form an isogenic community based on spatiotemporal structure, namely in biofilms (Bischofs et al., 2009).

1.3.3 Rap characteristics

The transcription of the *rap* genes (coupled to *phr* genes) is regulated by different factors. Expression of *rapA*, *rapC*, *rapE* and *rapF* genes is controlled by ComA (Perego et al., 1996, 1994). ComA~P may activates expression of sporulation-

Rap proteins in order to prevent commitment to sporulation in bacteria that are on a competent state (Perego et al., 1994). Whereas, the occurrence of a ComA-box in the promoter region of competence-Rap proteins may serve as an auto regulatory circuit modulating ComA activity (Bongiorni et al., 2005). *rapB* expression is under control of AbrB and is induced in vegetative growth (Perego et al., 1994). AbrB also inhibits the transcription of *rapI* (Auchtung et al., 2005). *rapC* is repressed by CodY and *rapK* is suggested to be indirectly activated by Spo0A (Auchtung et al., 2006). Moreover, the transcription factor RghR represses the transcription of *rapD*, *rapG* and *rapH* (Hayashi et al., 2006; Ogura & Fujita, 2007). Hence, the expression of Rap proteins is generally regulated by conditions antithetical to the sporulation process (Perego & Hoch, 1996).

The chromosomal Rap proteins of *B. subtilis* are about 380 amino acids long and share around 45% of identity among their sequence and this high level of homology indicates a similar overall structure arrangement (Diaz et al., 2012; Perego, 2013). Rap proteins were generally found to be dimers in their native state (Bongiorni et al., 2005; Ishikawa et al., 2002; Parashar et al., 2011). For example, RapA forms a stable complex with Spo0F in which a RapA dimer is associated with 2 molecules of Spo0F (Figure 21A) (Ishikawa et al., 2002). In contrast, RapF was found as a monomer, alone or in complex with ComA (Baker & Neiditch, 2011).

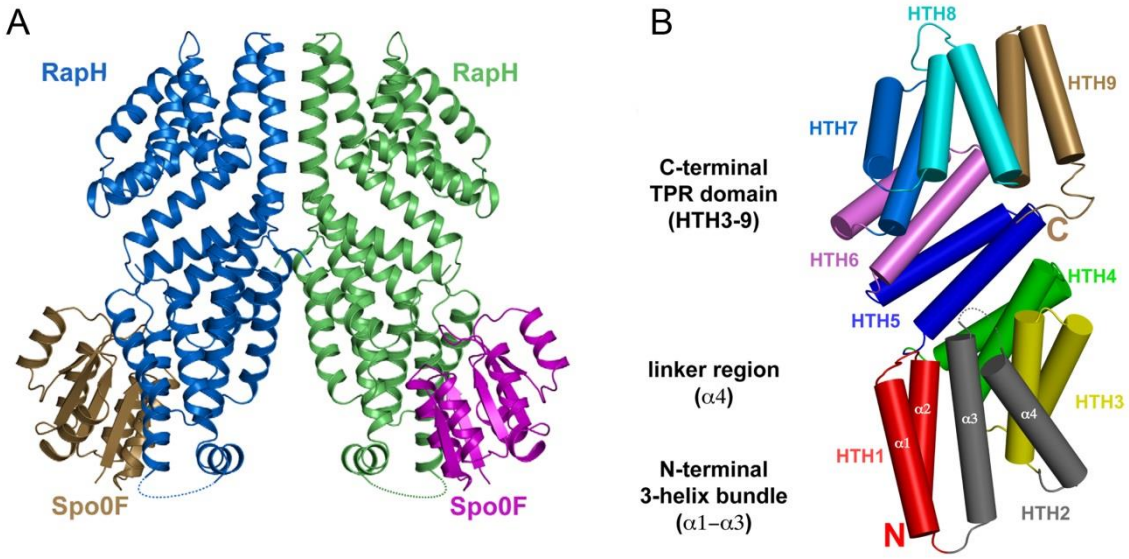

Figure 21. Structure of Rap proteins. A) Rap proteins are normally found in a dimeric form. In this representation a RapH dimer interact with two molecules of Spo0F (Parashar et al., 2011); B) Schematic representation of RapI crystal structure. Cylinders: α-helices; N: amino terminal end; C: carboxyl terminal end; HTH: TPR-like helix-turn-helix (Parashar et al., 2013a).

Early insights about Rap protein structures had recognize TPR motifs in the protein sequences (Perego & Brannigan, 2001). The TPR repeats are described to mediate protein-protein interaction and the six putative motifs of the Rap proteins were predicted to shape in a right-handed super helical structure forming a suitable binding environment to the Phr peptide (Perego & Brannigan, 2001). Later X-ray crystal structure of Rap proteins alone and in complex with Spo0F (RapH-Spo0F), ComA (RapF-ComA) or with Phr (RapF-PhrF or RapJ-PhrC) had contributed to the comprehension about Rap structure and interaction mechanisms (Baker & Neiditch, 2011; Gallego del Sol & Marina, 2013; Parashar et al., 2013a, 2011). The Rap proteins are formed by an N-terminal 3-helix bundle separated from the C-terminal TPR domain by a flexible linker and a short helix (Figure 21B) (Parashar et al., 2011). The TPR domain is composed of 6 genuine TPR folds and a helix-turn-helix TPR-like fold that is located between the fifth and sixth TPR motifs.

The N-terminal module of Rap proteins contains the determinants for substrate binding and catalytic activity (Diaz et al., 2012). In fact, the enzymatic active site for dephosphorylation of Spo0F~P is a glutamine (Q47) located within the 3-helix bundle (Parashar et al., 2011). Besides the catalytic role, binding of Rap to Spo0F also inhibits the transfer of phosphate to and from the response regulator by steric hindrance limiting the access of other phosphorelay components, kinases and Spo0B, to the aspartyl phosphate active site of Spo0F (Parashar et al., 2011). Regarding Rap interaction with Spo0F, some residues of the TPR domain also play a role in placing both proteins in a correct position to enable their interaction. Alignment of sporulation-Rap sequences showed that the residues required for Rap activity on Spo0F were conserved among them. This conservation is to such an extent that the sporulation activity of a given Rap could be predicted using this data (Parashar et al., 2011).

Initially, the regulation of ComA by the Rap protein was supposed to be based on its phosphatase activity. Then, it was demonstrated that the competence-Rap proteins did not dephosphorylate ComA~P but bind to the DNA-binding domain of the response regulator to prevent its activity as transcription factor (Baker & Neiditch, 2011; Core & Perego, 2003). Even if the N-terminal of Rap proteins is the responsible module for both response regulators, Spo0F and ComA bind to distinct

non-overlapping sites within the 3-helix bundle (Baker & Neiditch, 2011; Diaz et al., 2012; Parashar et al., 2011).

The Phr oligopeptide was shown to bind to the Rap protein in an extend conformation along the groove of the TPR domain, specifically to the region from the TPR3 to TPR5 (Diaz et al., 2012). Binding of Phr induces a constriction in Rap structure which is propagated from the TPR domains to the 3-helix bundle region disrupting the association of Rap protein with ComA or Spo0F (Gallego del Sol & Marina, 2013; Parashar et al., 2013a). In this rearranged structure, the Rap protein is a single continuous super helical structure formed by 9 TPR-like folds (Figures 12 and 21B) (Parashar et al., 2013a). Therefore, the inhibitor peptide and the target substrate interact with Rap at distinct sites. The N-terminal part is generally the effector domain related to the activity on the target response regulator, while the C-terminal part is the regulatory domain involved in the binding module of the quorum sensor (Diaz et al., 2012; Gallego del Sol & Marina, 2013; Reizer et al., 1997).

1.3.4 Phr: sequence and maturation

Aside from being transcriptionally regulated by the promoter upstream from the *rap* gene, most of *phr* genes are also independently transcribed from a promoter located inside *rap* gene and upstream from the *phr* gene (Figure 22A) (McQuade et al., 2001).

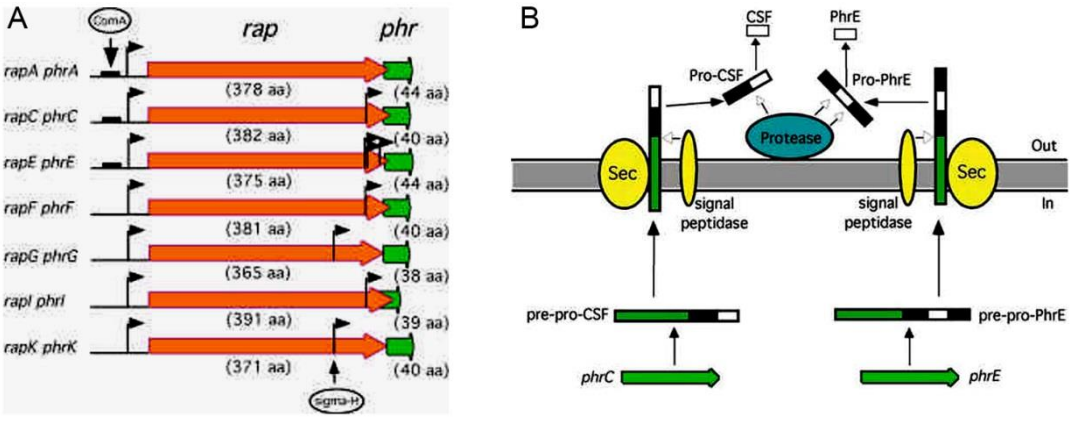

Figure 22. Transcription regulation and maturation of Phr signaling molecules. A) Chromosome *rap-phr* signaling cassettes of *B. subtilis*. *phr*-orphan *rap* genes are not represented; likewise *phrA*, expression of *phrH* gene is not regulated by the sigma factor σ^H. Black arrows: transcriptional start sites. (Pottathil & Lazazzera, 2003) B) Phr molecules are transcribed in a premature form, which is secreted through the Sec export system and then by proteases. An additional protease cleavage seems to occur in Phr peptides that the active form is found inside the C-terminal region (Pottathil & Lazazzera, 2003).

phrC, *phrE*, *phrF*, *phrG*, *phrI* and *phrK* genes are controlled by the alternative sigma factor σ^H which may ensure a higher expression of the signaling molecules during the early stationary phase (McQuade et al., 2001). For instance, Spo0A~P induces the *sigH* transcription which promote the production of sufficient Phr peptide to prevent activity of Rap phosphatase on the phosphorelay after sporulation had begun.

Unlike Rap sequences, Phr precursors show little homology in amino acid sequences but a similar structure. Phr sequences are arranged in a hydrophobic N-terminal part linked to an hydrophilic C-terminal part by a potential signal peptidase cleavage site (Perego & Hoch, 1996). To achieve its active form, the Phr precursor undergoes two maturation processes (Figure 22B). Primarily, the pre-proPhr is probably processed by a SecA dependent system for export (Pottathil & Lazazzera, 2003). However, the five known *B. subtilis* peptidases were not indispensable to this activity (Stephenson et al., 2003). The second cleavage occurs extracellularly to release the mature oligopeptide from the proPhr peptide. In *B. subtilis*, this step is performed by the redundant proteases subtilisin, Epr and Vpr that are widespread in bacteria (Lanigan-Gerdes et al., 2007). The five residues that precede the mature peptide (cleavage site) were shown to be important to cleavage reaction and relatively conserved in *B. subtilis* Phr sequences (Lanigan-Gerdes et al., 2008). Nonetheless, all the Phr peptides do not seem to be processed in the same manner. Indeed, the mature form can be found in the C-terminal end of the precursor peptide (PhrA, PhrC, PhrG, PhrI, and PhrF) or within the C-terminal region (PhrE, PhrH, and PhrK) (Jiang et al., 2000a; Pottathil & Lazazzera, 2003). In the latter case, the proteases from the subtilisin family are not able to process these signaling peptides, at least not alone, as two cleavage reactions are required.

After the maturation, the Phr oligopeptide is reimported into the bacterial cell by oligopeptide permeases, as the other signaling peptides of the RNPP family. The active Phr were firstly described to be pentapeptides (Perego, 1997) but lately, it was demonstrated that these inhibitors could also have six amino acids long, as in the case of PhrH and PhrI (Mirouze et al., 2011). Altogether, these characteristics difficult the prediction of the active signaling peptide (Pottathil & Lazazzera, 2003). However, the active Phr have a conserved positively charged amino acid (arginine, histidine or lysine) in the second position when it is a pentapeptide. This export-import circuit is

typical of the oligopeptides way of life in cell-cell communication. In addition to its role as quorum sensors, this circuit is also supposed to be a mechanism to regulate the timing of the phosphatase activity (Mirouze et al., 2011; Perego, 1997).

Except the regulation of RapB and RapJ by PhrC, the Phr oligopeptides act specifically on their cognate Rap proteins (Pottathil & Lazazzera, 2003). Indeed, no cross reactivity was shown among RapA-PhrA and RapC-PhrC neither between these systems and RapE-PhrE (Jiang, Grau, et al., 2000; Perego, 1997). Therefore, it is suggested that both components had co-evolved and the presence of multiple Rap-Phr systems in *B. subtilis* was due to recent gene duplication event (Perego, 2013; Pottathil & Lazazzera, 2003; Reizer et al., 1997).

1.3.5 Plasmid-borne Rap-Phr (QS) systems

Plasmids and other genetic mobile elements (such as ICE) represent important elements for genes spreading and diversification. Furthermore, *rap-phr* genes were found in various plasmids and some of them were further evaluated with functional studies which have shown the relevance of these plasmid Rap-Phr systems for bacterial development.

The first described plasmid Rap-Phr system from *B. subtilis* was the Rap60-Phr60, harbor on plasmid pTA1060. This system regulate the production of extracellular proteolytic enzymes (Koetje et al., 2003). The quorum sensing control of the production of protease allows the bacteria to better exploit the nutrients. When in just a small number of bacteria, in which there are sufficient resources to their development, Rap60 inhibit protease production, since it is not required. Once in a high cell density, when the nutrients were already depleted, the Phr60 binds to Rap60 and activates expression of the extracellular protease to improve energy sources. Lately, the Rap60-Phr60 couple was demonstrated to also control the phosphorelay and ComA activity by a non-canonical mechanisms, resulting in modification of diverse process, such as sporulation, cannibalism, biofilm formation and genetic competence (Boguslawski et al., 2015). In addition to the usual role as phosphatase on Spo0F, Rap60 inhibit the autophosphorylation of kinase KinA. Concerning ComA, Rap60 inhibits its activity as transcription regulator by forming a ternary complex with ComA and the DNA promoter region of its target.

Encoded by plasmid pBSG3 from *B. amyloliquefaciens*, the RapQ-PhrQ system shows great similarity with Rap60-Phr60. In fact, RapQ presents 64% of identity to Rap60 sequence and was also described to inhibit ComA activity through the establishment of a ternary complex (Qiao et al., 2011; Yang et al., 2015). When expressed in a heterologous host (*B. subtilis* OKB105), RapQ-PhrQ was shown to control protease production, sporulation and genetic competence.

The RapP-PhrP from *B. subtilis* NCIB3610 plasmid pBS32 regulates biofilm formation, sporulation and competence (Parashar et al., 2013b). Biofilm formation were shown to be also regulated by sporulation-Rap proteins, *via* low level Spo0A~P activity through AbrB and SinI-SinR. Generally, most *B. subtilis* studies were based in domesticated plasmid-free strains that had lose their ability to form dense biofilms, then this post exponential process was not evaluated in *B. subtilis* chromosomal Rap-Phr systems. However, considering the role of Spo0A in biofilm formation all Rap proteins with sporulation effect would also regulate biofilm. RapP inhibits Spo0F and an additional target seems to be required for its strong effect on biofilm (Omer Bendori et al., 2015). In contrast to all known Rap-Phr system, RapP is not inhibited by its cognate PhrP, due to a rare mutation in the RapP sequence. Moreover, the catalytic residue of RapP related to phosphatase activity is a glutamate instead of the well conserved glutamine (Q47).

In another case of plasmid-borne Rap-Phr system, Rap_{LS20}-Phr_{LS20} controls the mobility of its conjugative plasmid pLS20 from *B. subtilis natto* strain IFO3335 (Rösch & Graumann, 2015; Singh et al., 2013). This control occurs in the same manner that of the ICE*Bs*1 regulation by RapI-PhrI. The expression of genes essential for the plasmid conjugation is controlled by the master repressor Rco_{LS20}. Rap_{LS20} act as conjugation antirepressor over Rco_{LS20}, binding to its DNA-binding domain (Rösch & Graumann, 2015). When surrounded by recipient cells that do not express Phr_{LS20}, Rap_{LS20} inhibit Rco_{LS20} and the conjugation apparatus is expressed. When the majority of the population carries the pLS20 plasmid, hence the concentration of the anti-antirepressor Phr is high and the conjugation is inhibited (Singh et al., 2013).

Regarding the *B. cereus* group, the genome of *B. anthracis* bear six *rap-phr* modules, five on chromosome and one in the pathogenic plasmid pXO1 (Bongiorni et al., 2006). These quorum sensing systems were identified using the *B. subtilis* RapA

as query in a search through the *B. anthracis* A2012 genome. The six identified systems share, in average, 24% identical residues with RapA. One chromosomal (BA3790) and the plasmid (BXA0205) Rap proteins were shown to inhibit sporulation *via* phosphatase activity on Spo0F (Bongiorni et al., 2006). The C-terminal end pentapeptide of BXA0205Phr was able to counteract its cognate Rap activity; however, it was not possible to outline the active form of the Phr Ba3791. A strict regulation of sporulation is especially relevant to the pathogenicity of *B. anthracis* bacteria (Bongiorni et al., 2006; Brunsing et al., 2005). When inside the host, it is valuable to maintain the vegetative state that is able to produce toxins and escape from the immunity system. Thus, during growth in the blood stream, the Phr produced are dissipated and not able to neutralize Rap phosphatase, inhibiting commitment to sporulation and maintaining cell growth. However, once the infection is established in an organ, bacteria proliferate; increasing cellular density and, consequently, the amount of Phr molecules. Thus, together with the decrease of nutrient availability, Phr peptides are able to inhibit Rap proteins and bacteria sporulate.

Another plasmid-borne Rap-Phr system studied in the *B. cereus* group was the Rap8-Phr8, encoded by the small cryptic plasmid pHT8_1 from *B. thuringiensis* var. *kurstaki* HD73 strain (Fazion et al., 2018). Rap8 acts on the phosphorelay inhibiting sporulation and biofilm formation. The mature form of Phr8 is the C-terminal end heptapeptide of the precursor Phr peptide, similarly to the other RNPP family signaling peptides from the *B. cereus* group. Moreover, this Rap-Phr system was demonstrated to regulate sporulation in the insect larvae, the ecological niche of *B. thuringiensis*. Likewise in *B. anthracis*, the Rap8-Phr8 system may allow a precise control of the sporulation process, improving the bacterial survival and dissemination.

2 OBJECTIVES

The main objective of this study was to perform an overview of the Rap-Phr quorum sensing systems in the *B. cereus* group concerning their number, their location and their diversity. Moreover, we aimed to predict the activity of Rap proteins on sporulation by using *in silico* analyzes based on data described for RapH in *B. subtilis*. Thereafter, the validation of prediction was made by functional analysis. The second objective of this study was the characterization of the plasmid-borne Rap63-Phr63 system in relation to the sporulation function. The determination of the active Phr form and the possible interaction with the co-existent Rap8-Phr8 in the *B. thuringiensis* HD73 strain were also investigated.

3 ARTICLE 1

Diversity of the Rap-Phr quorum sensing systems in the *Bacillus cereus* group

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Running title: Rap-Phr in the *B. cereus* group

Keywords: Rap-Phr, *Bacillus cereus* group, RNPP, sporulation, phosphatase

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Abstract

Bacteria of the *Bacillus cereus* group colonize several ecological niches and infect different hosts. *Bacillus cereus*, a ubiquitous species causing food poisoning, *Bacillus thuringiensis*, an entomopathogen, and *Bacillus anthracis*, which is highly pathogenic to mammals, are the most important species of this group. These species are closely related genetically, and their specific toxins are encoded by plasmids. The infectious cycle of *B. thuringiensis* in its insect host is regulated by quorum sensing systems from the RNPP family. Among them, the Rap-Phr systems, which are well-described in *Bacillus subtilis*, regulate essential processes, such as sporulation. Given the importance of these systems, we performed a global *in silico* analysis to investigate their prevalence, distribution, diversity and their role in sporulation in *B. cereus* group species. The *rap-phr* genes were identified in all selected strains with 30% located on plasmids, predominantly in *B. thuringiensis*. Despite a high variability in their sequences, there is a remarkable association between closely related strains and their Rap-Phr profile. Based on the key residues involved in RapH phosphatase activity, we predicted that 32% of the Rap proteins could regulate sporulation by preventing the phosphorylation of Spo0F. These Rap are preferentially located on plasmids and mostly related to *B. thuringiensis*. The predictions were partially validated by *in vivo* sporulation experiments suggesting that the residues linked to the phosphatase function are necessary but not sufficient to predict this activity. The wide distribution and diversity of Rap-Phr systems could strictly control the commitment to sporulation and then improve the adaptation capacities of the bacteria to environmental changes.

Introduction

Several bacterial processes are regulated by quorum sensing, a cell-cell communication that enables bacteria to regulate their fate with regard to the population density. The Rap proteins and their cognate Phr peptide inhibitors are quorum sensing systems present in the *Bacillus cereus* group but not extensively studied in these bacteria. The *B. cereus* group comprises at least seven species (*Bacillus cereus sensu stricto*, *Bacillus thuringiensis*, *Bacillus anthracis*, *Bacillus weihenstephanensis*, *Bacillus mycoides*, *Bacillus pseudomycoides* and *Bacillus*

cytotoxicus) of rod-shaped, spore-forming, Gram-positive bacteria that are found in diverse ecological niches and able to colonize different hosts (Liu et al. 2015). Due to the complex phylogeny of the group, as phylogenetic clades are polyphyletics and species are paraphyletics, its taxonomy continues to be debated (Bazinet 2017; Guinebretière et al. 2008; Helgason et al. 2000; Liu et al. 2015; Raymond, 2017; Tourasse et al. 2011).

The three main species of the *B. cereus* group have a significant impact on human activity. *B. cereus* is a ubiquitous and opportunistic bacterium and includes strains that cause food poisoning with vomiting or diarrhea and severe local infections, such as endophthalmitis or periodontitis (Callegan et al. 2003; Ehling-schulz et al. 2006; Stenfors Arnesen et al. 2008). *B. thuringiensis* is the world's most used biopesticide due to its production of insecticidal toxins (designated as Cry proteins) specifically pathogenic to a wide range of insects (Schnepf et al. 1998). *B. anthracis* is a mammal pathogen, including humans, and is the causative agent of anthrax (Liu et al. 2014). Although phenotypically different, these species are closely related genetically (Rasko et al. 2005), and the species determinants are encoded by plasmid genes (Vilas Boas et al. 2007). For *B. cereus*, the enzymatic complex involved in cereulide (emetic toxin) synthesis is encoded by pCER270 (Ehling-Schulz et al. 2006). Strains are identified as *B. thuringiensis* if they produce a crystal inclusion during sporulation due to the presence of plasmids carrying genes encoding Cry toxins, generally active against insects or nematodes (Deng et al. 2014; Schnepf et al., 1998). The high toxicity of *B. anthracis* is due to toxins and its capsule, which are encoded by genes located on the plasmids pXO1 and pXO2, respectively (Kolstø et al. 2009).

Several microorganisms behaviors, such as biofilm formation, sporulation, motility, genetic exchange (competence and conjugation), and virulence factor production, are regulated by quorum sensing (QS), a cell-cell communication process that allows bacteria and eukaryotic microorganisms to coordinate their biological processes based on the population density (Polke and Jacobsen 2017; Rutherford and Bassler 2012). In Gram-positive bacteria, this communication is done by signaling oligopeptides that are recognized by cognate regulators, such as the QS systems of the RNPP family (from Rap, NprR, PlcR, and PrgX) (Declerck et al. 2007). These regulators are formed by tetratricopeptide repeat (TPR) domains that are

structural motifs of degenerated residues that mediate protein-protein and protein-peptide interactions (D'Andrea and Regan 2003). The activity of these cytoplasmic regulators is activated (NprR and PlcR) or inhibited (Rap) by secreted, matured, and re-imported peptides that function as signaling molecules (Perchat et al. 2011, Perego and Hoch 1996; Pottathil and Lazazzera 2003; Slamti and Lereclus 2002). The genes encoding these signaling peptides are located directly downstream from the coding sequence of their cognate RNPP regulator, and the two genes are transcribed in the same orientation (Declerck et al. 2007). Except for the Rap proteins, RNPP regulators have an HTH (helix-turn-helix) DNA-binding domain, allowing them to function as transcriptional regulators (Declerck et al. 2007). During the infectious cycle of *B. thuringiensis* in insect larvae, three QS systems are successively activated (Slamti et al. 2014): i) PlcR-PapR regulates the virulence stage by controlling the expression of virulence genes; ii) NprR-NprX regulates the necrotrophic stage, allowing bacteria to survive and to sporulate in the insect cadaver; and iii) Rap-Phr regulates the initiation of the sporulation process.

Sporulation is essential for survival and dispersion of a wide variety of organisms (Huang and Hull 2017). In *Bacillus subtilis*, this differentiation process is regulated by a complex pathway (Sonenshein 2000), in which Spo0A is the major regulator of sporulation that must be phosphorylated to be active. External signals, such as starvation, are detected by different sporulation kinases (KinA to KinE), which phosphorylate the Spo0F response regulator (Burbulys et al. 1991). The phosphoryl group is then transferred through the phosphorelay from Spo0F to the phosphotransferase Spo0B, and then to Spo0A (Jiang et al. 2000a). Certain Rap proteins indirectly inhibit the phosphorylation of Spo0A by dephosphorylating Spo0F and thus impair the initiation of sporulation (Perego and Hoch 1996).

Eleven *rap* genes (from *rapA* to *rapK*) were identified on the chromosome of the *B. subtilis* 168 strain. Functional studies have shown that RapA, RapB, RapE, RapH, RapI, and RapJ can dephosphorylate Spo0F (Jiang et al. 2000b; Parashar et al. 2013a; Parashar et al. 2011; Perego et al. 1996; Smits et al. 2007). RapC, RapD, RapF, RapH, and RapK regulate competence by inhibiting ComA (Auchtung et al. 2006; Bongiorni et al. 2005; Core and Perego 2003; Ogura and Fujita 2007; Smits et al. 2007), RapG regulates extracellular protease production by inhibiting DegU (Ogura et al. 2003), and RapI also regulates the mobility of the ICBs1 genetic

element (Auchtung et al. 2005). *rap-phr* genes have also been identified in *B. subtilis* plasmids. These plasmid systems are involved in the regulation of proteolytic enzyme production (Koetje et al. 2003), sporulation, competence, biofilm formation (Parashar et al. 2013b), and plasmid conjugation (Singh et al. 2013). The activities of Rap proteins are inhibited by their cognate Phr peptides. Phr-encoding genes are located downstream from the *rap* genes and are generally co-transcribed, although many *phr* genes have a secondary promoter (Perego and Brannigan 2001; McQuade et al. 2001). The pro-Phr are secreted and processed in the extracellular environment. The mature Phr are then internalized within the bacterial cells by oligopeptide permeases and bind to Rap proteins to inhibit their activity (Perego 1997).

The Rap-Phr systems are also present in bacteria of the *B. cereus* group. Bongiorni et al. (2006) have identified five *rap* genes in the *B. anthracis* A2012 strain, among which only two were shown to inhibit sporulation. More recently, Fazion et al. (2018) characterized the Rap-Phr system from a small plasmid (pHT8_1) of the *B. thuringiensis* HD73 strain and demonstrated its involvement in the regulation of sporulation in insect larvae.

In this study, we performed an overview of the Rap-Phr systems in the *B. cereus* group, including their identification, distribution, and prediction of their sporulation activity. We show that the Rap-Phr systems are widespread in all strains, in both chromosomes and plasmids, and with great sequence variability. A comparison between the *B. cereus* and *B. thuringiensis* strains showed that plasmid Rap-Phr systems are more frequently present in *B. thuringiensis* than in *B. cereus*. One-third of the Rap proteins were predicted to have a sporulation function and these Rap proteins are preferentially located on plasmids and, therefore, are mainly present in *B. thuringiensis*.

Materials and Methods

Bacterial genomes

The *B. cereus* group strains with a complete genome sequence available in the NCBI Genome database (http://www.ncbi.nlm.nih.gov/genome/) on April 2015 were selected. The species classification was considered as found in the database in the moment of data collection. The main available features (chromosome size and GC

content, MLST sequence type, size and number of plasmids, and the proportion of the total genome they represent) of the genomes from the 49 selected strains are presented in Table 1 and Online Resource Table 1.

Construction of the *rap-phr* database

Each selected genome sequence was screened for the presence of *rap* genes. Three strategies were used: i) using ‘rap’ and ‘response regulator aspartate phosphatase’ as keywords; ii) using each identified sequence for a sequence similarity search by BLASTn against all selected genomes and; iii) using all chromosomal Rap protein sequences from the *B.* subtilis 168 strain as a query for a tBLASTn alignment with *B. cereus* group genomes. All protein sequences were analyzed using InterProScan (http://www.ebi.ac.uk/interpro/sequence-search) and SMART (http://smart.embl-heidelberg.de) servers for the identification of domains and motifs (Jones et al. 2014; Letunic et al. 2015). The detection of TPR domains and the absence of HTH DNA-binding domains were used as the main criteria to validate putative sequences as Rap proteins. Sequences shorter than 333 amino acids or showing uncharacteristic domains for Rap proteins were excluded.

Identification of *phr* genes was performed considering the gene organization: i) short open reading frames (encoding 35 to 120 aa) and location, ii) overlapping the *rap* gene (up to 10 bp) at the 3′ terminal end or located immediately downstream (up to 100 bp) from the *rap* gene, and iii) transcription from the same DNA strand as the *rap* gene. When a *phr* gene was not identified by this strategy, the downstream region of *rap* was scanned for small open reading frames (ORFs) checking for putative unannotated *phr* using the VectorNTI software (Invitrogen).The identified systems were numbered according to their location (first the chromosomal genes, then the plasmid ones). The chromosomal genes were numbered according to their order from the replication origin.

A 20kb region around the *rap* genes (10kb upstream and 10Kb downstream) was analyzed using the ISfinder database (Siguier et al. 2006) to verify the presence of mobile elements within these genomic regions.

Rap proteins clustering

Phylogenetic trees were constructed with Rap protein sequences according to the location of their genes in the genome: i) total (all sequences), ii) chromosome, and iii) plasmids. The DAMBE program (Xia 2013) was used to gather sequences with 100% identity. Unique Rap sequences were aligned using the MUSCLE algorithm, and MEGA 6 (Tamura et al. 2013) was used to build phylogenetic trees by the Neighbor-Joining method with the best model corrections for each alignment. RapH from *B. subtilis* 168 was used as outgroup in the Total tree, which was visualized on iTOL - Interactive Tree Of Life (Letunic and Bork 2019). For Rap clustering delimitation, a value of 0.8 was used as the cut-off using the average distance of the number of amino acid substitutions for chromosomal and plasmid trees. These trees were visualized on MEGA 6. For the Phr peptide, identical sequences recognition was done as described for the Rap proteins.

Multilocus sequencing type (MLST) tree

According to the scheme of Tourasse et al. (2006), sequences of MLST housekeeping genes of all selected genomes were obtained from the 'University of Oslo's *Bacillus cereus* group MultiLocus and MultiData Typing website (http://mlstoslo.uio.no)'. Sequences of *adk*, *glpT*, *glpF*, *panC*, *pycA*, *ccpA*, and *pta* genes were downloaded already concatenated. Alignment and phylogenetic tree development were performed as described for Rap proteins.

Plasmids construction and growth conditions

To assess the effect of Rap proteins on sporulation, seven plasmid *rap* genes (*rap6*-BtHD1, *rap8*-BtHD1, *rap10*-BtHD1, *rap6*-Bt407, *rap7*-Bt407, *rap8*-Bt407 and *rap7*-BtHD73) and three chromosomal *rap* genes (*rap1*-BcATCC14579, *rap2*-BcATCC14579 and *rap5*-BtHD73) were cloned in the plasmid pHT315-P_{xylA}, a multi-copy vector with xylose-inducible promoter (Grandvalet et al. 2001). All genes were amplified by PCR using primers listed in Online Resource Table 2 and ligated to the plasmid pHT315-P_{xylA} using the appropriate restriction sites. For cloning steps, these plasmids were transformed in *Escherichia coli* K-12 strain TG1 and then in the Dam-Dcm- *E. coli* strain ET12567 (Stratagene, La Jolla, CA, USA) by thermal shock.

Finally, each constructed plasmid was transformed by electroporation (Lereclus et al. 1989) in the acrystalliferous (Cry-) *B. thuringiensis* var. *kurstaki* HD73 strain (Wilcks et al. 1998). Luria Bertani (LB) medium was used to cultivate *E. coli* and *B. thuringiensis* at 37°C for DNA preparation. The medium HCT was used to optimize the sporulation of *B. thuringiensis* (Lereclus et al. 1982). Antibiotics were used at the following concentration: ampicillin 100 µg/mL for *E. coli* and erythromycin 10 µg/mL for *B. thuringiensis*.

DNA manipulation

Genomic DNA from the three *B. thuringiensis* strains (HD-1, Bt407, and HD73) and the *B. cereus* strain (ATCC14579) was extracted using the Puregene Yeast/Bact. Kit B (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, USA) and oligonucleotides (Online Resource Table 2) were synthesized by Eurofins Genomics (Germany). The QIAquick PCR Purification Kit (Qiagen, France) was used to purify the amplified DNA fragments that were subsequently treated with appropriated restriction enzymes (New England Biolabs). Digested DNA fragments were separated on 1% agarose gels and purified from gels using the QIAquick gel extraction kit (Qiagen, France). T4 DNA ligase and restriction enzymes were used following the manufacturer's recommendations (New England Biolabs). *E. coli* plasmid DNA extractions were performed using the QIAprep Spin Miniprep Kit (Qiagen, France). DNA sequencing was carried out by GATC Biotech (Konstanz, Germany).

Sporulation assay

The sporulation efficiency of *B. thuringiensis* HD73 strain expressing *rap* genes was determined in the sporulation-specific medium HCT supplemented with 20 mM of xylose at the beginning of stationary growth phase. After 48 h of growth at 30°C, serial dilutions were plated before and after heat treatment for 12 min at 80°C. The sporulation percentage was calculated as 100 × the ratio between heat-resistant spores per milliliter and total viable cells per milliliter. All experiments were repeated at least three times, and the mean values (± standard error of the mean) were calculated.

Statistical analyses

The appropriate statistical test for each data was performed in GraphPad InStat Software version 3.05. Comparisons between *B. cereus* and *B. thuringiensis* means were analyzed with t-test while when contingence table were used to confront both species, the Fisher's Exact test was used. The data obtained with sporulation assay was analyzed by using One-way Analysis of Variance (ANOVA) followed by Tukey-Kramer Multiple Comparisons Test ($P<0.01$).

Results

Genomic overview of the *B. cereus* group strains

To study the occurrence, the prevalence and the distribution of Rap-Phr systems in the *B. cereus* group, those genome sequences with complete assembly level available in NCBI Genome section until April 2015 were selected. Most of these genomes belong to *B. cereus*, *B. thuringiensis* or *B. anthracis*, considering the relevance of these species. All *B. cereus* and *B. thuringiensis* available strains were selected. However, as *B. anthracis* is a clonal species (Helgason et al. 2000; Rasko et al. 2005), only one representative genome sequence (Ames Ancestor strain) was selected, even if around 30 genomes of this species were available. Moreover, our preliminary results revealed identical Rap-Phr profiles in all the *B. anthracis* isolates, thus confirming the clonal aspect of the *B. anthracis* strains (data not shown). For the other species (*B. mycoides*, *B. pseudomycoides*, *B. weihenstephanensis*, and *B. cytotoxicus*), only one genome for each species was chosen. When the same strain was sequenced twice, only one was selected. Following these criteria, 49 genomes were used in this study (Table 1).

The GC content of these genomes was around 35%, with minor differences among the strains. In the *B. cereus* strains, the chromosomal size ranged from 5.08 to 5.46 Mb, while in *B. thuringiensis* the variation was from 5.21 to 6 Mb. All the other species showed chromosomes larger than 5 Mb, except *B. cytotoxicus* NVH 391-98, which was 4.09 Mb (Table 1). The analysis of the plasmid content of the 49 selected strains revealed a total of 197 plasmids with a size ranging from 2.1 to 502 Kb (Online Resource Table 3). These plasmids can provide a significant increase in genome size (Table 1). In *B. cereus*, the average increase of the genome size is 5%

with a maximum of 12% in the strain *B. cereus* 03BB108. In *B. thuringiensis*, the average increase of the genome size was 12%, with a maximum of 24% in the *B. thuringiensis* MC28 and *B. thuringiensis* IS5056 strains.

rap-phr genes distribution

In the 49 genomes sequences, 302 *rap* genes were identified (Online Resource Data 1 and Online Resource Table 4) whose 144 (47.7%) were correctly annotated as 'response regulator aspartate phosphatase' or 'rap'. A *phr* gene was identified downstream from all *rap* genes, but 31 of them were not annotated as ORFs (Online Resource Table 4). The *rap* and *phr* genes were always located in the same DNA strand with a slight overlapping (usually 1 or 4 nucleotides). The average size was 1099 bp for the *rap* genes (from 1032 to 1185 bp) and 166 bp for the *phr* genes (from 120 to 330 bp).

From two to 16 *rap-phr* genes were identified in all strains (Fig. 1a, Online Resource Table 4). Two to eight chromosomal *rap-phr* systems were found by strain, representing 70% of all the identified *rap-phr* genes (Fig. 1b). Plasmid *rap-phr* genes were found in 27 of the 49 strains and on 65 of the 197 plasmids. Some strains harbor a large number of plasmid *rap-phr* genes, up to 12 plasmid systems for the *B. thuringiensis* strain IS 5056 (Online Resource Table 3). The size of plasmids harboring *rap-phr* genes varied from 6.88 to 502 kb. However, the occurrence of these genes is most common (70%) in plasmids larger than 70 kb (Online Resource Table 3). Also, large plasmids (> 200 kb) might contain several *rap-phr* genes, up to five, such as the pBMB422 plasmid (422.7 kb) of the *B. thuringiensis* YBT-1520 strain (Online Resources Table 3 and 4).

The average number of chromosomal *rap-phr* genes is similar between *B. cereus* and *B. thuringiensis* (4.04 and 4.45, respectively). However, the average number of plasmid *rap-phr* genes is 6-fold higher in *B. thuringiensis* than in *B. cereus* (3.6 and 0.6, respectively; P=0.001). The percentage of plasmids harboring *rap-phr* genes is higher in *B. thuringiensis* than in *B. cereus* (38.6% *versus* 22.0%, respectively, P=0.03). Moreover, *B. thuringiensis* strains show a similar amount of chromosomal and plasmid *rap-phr* genes, while in the *B. cereus* strains the number of chromosomal *rap-phr* genes is almost 7 times higher (P<0.001) than plasmid genes (Table 2).

As the genome of *B. cereus* group strains are rich in mobile and repeated elements (Kolstø et al. 2009), the presence of these elements in the vicinity of *rap-phr* genes was analyzed. A mobile element was found in the vicinity of 48% of all the *rap* genes, corresponding to 39% of the chromosomal genes and 68% of the plasmid ones (Online Resource Data 1). The prevalence of mobile elements in the 20-kb region around *rap-phr* chromosomal genes is similar between *B. cereus* and *B. thuringiensis* (Table 2). However, these elements were found in the vicinity of 36% of plasmid *rap* genes from *B. cereus* against 72% of *B. thuringiensis* ones ($P<0.01$).

Protein clustering

Among the 302 Rap and Phr proteins initially identified, we distinguished 192 different Rap protein sequences and 152 different Phr pro-peptide sequences, corresponding to 63.5% and 50.3% of all sequences, respectively. Rap protein sequences with 100% of identity were found in several strains from different species (Fig. 2). The identical sequences are always identified on different strains and on the same type of replicon (chromosome or plasmid), except for the chromosomal Rap1 from the *B. cereus* 03BB87 strain, which is identical to the plasmid Rap3 of the pBCX01 plasmid from the *B. cereus* G9241 strain. To investigate this unique case, a 10-kb region around the *rap*1 gene from the *B. cereus* 03BB87 strain was analyzed with BLASTn. By this approach, we detected 99–100% identity to the pXO1 and pXO1-like plasmids. A BLAST alignment between the pXO1 plasmid and the entire *B. cereus* 03BB87 chromosome highlighted a region corresponding to 3% of the chromosome with 98% of coverage and 99% of identity with the plasmid. Moreover, the *B. cereus* 03BB87 strain also carries a pXO1-like plasmid, the pBCX01, sharing 97% identity with pXO1 with less than 10% of coverage (data not shown). This might be due to a mistake in the genome assembly or to the integration of DNA regions from the pBCX01 plasmid in the chromosome.

Three phylogenetic trees were constructed using Rap protein sequences, according to the location of their encoding genes in the genome (all sequences, chromosome or plasmid sequences) (Fig. 2 and Online Resource Fig. 1). These trees were built by the Neighbor-Joining method with corrections based on the Jones-Taylor-Thornton matrix model that enables the assessment of the overall divergence among Rap proteins. The phylogenetic tree with all sequences showed a

high number of branches with a dispersion of plasmid sequences in several branches. Closely related Rap sequences are encoded by similar kind of plasmids distributed in eight major sets (Fig. 2, sets A-H). Set A comprises *rap* genes harbored by an identical 8.5 kb plasmid present in different strains and set B by plasmids higher than 70 kb. Set C includes pXO1-like plasmids; set D plasmids larger than 200 kb, while plasmids from set E are smaller than 20 kb. Set F includes plasmids around 70 kb and set G plasmids larger than 200 kb that can also harbor Rap sequences from set B. The set H is the most versatile since it comprises two distinct subsets: one with plasmids larger than 400 kb and another with plasmids ranging from 75 to 235 kb.

The chromosomal tree is divided into 12 Rap groups (Online Resource Fig. 1a). Some groups are composed of several sequences, such as Group C6 which includes 38 Rap sequences. However, some groups have few Rap sequences, such as Group C11 with Rap4 from *B. anthracis* Ames Ancestor and Rap5 from *B. cytotoxicus* NHV391-98. Also, Group C10 is formed by three identical sequences from two *B. cereus* emetic strains (*B. cereus* AH187 and *B. cereus* NC7401) and from BcQ1. The plasmid tree is separated into nine Rap groups (Online Resource Fig. 1b). Likewise, in the chromosomal tree, many groups are composed of several sequences, such as Group P2 with 26 Rap proteins. However, Group P4 and Group P5 are composed of unique sequences and Group P6 by two identical sequences.

Phr peptides

In silico determination of the mature Phr sequences is complex due to the high variability of these sequences within the *B. cereus* group. Indeed, the mature Phr already described from the *B. cereus* group revealed some differences in size and location within the pro-peptide. The BXA0205Phr from the pXO1 plasmid (Phr5-*B. anthracis*) is a pentapeptide while the Phr8 from the pHT8_1 plasmid (Phr8-*B. thuringiensis* HD73) is an heptapeptide but both are located in the C-terminal end. Moreover, the active form of the BA3791Phr from *B. anthracis* (Phr3-*B. anthracis*) is located within the C-terminal region of its precursor, but its exact sequence was not determined (Bongiorni et al. 2006; Fazion et al. 2018). However, all these mature peptides present a positively charged residue, the typical feature of Phr active form (Pottathil and Lazazzera 2003). The Phr sequences from the *B. cereus* group present

a great variability in their amino acid sequences and in their sizes (Online Resources Data 1). The mature Phr peptides from the *B. cereus* group described above were sought among the Phr sequences in our database. While the mature Phr8-*B. thuringiensis* HD73 (YAHGKDI) was identified only on identical Phr sequences, the Phr5-*B. anthracis* (GHTGG) was found in several sequences. A great number of longer Phr possess the GDTGG/GDGGG/GETGG repetition sequences duplication described by Even Tov et al. (2016). These sequences were defined as the putative autoinducer sequences although they are not generally associated with a positively charged residue. However, the Phr peptides containing these repetitions also bear an ARPDY sequence, which could be the active form.

The relationship between MLST phylogenetic tree and Rap distribution

The 49 selected strains are distributed into six of the seven phylogenetic clusters determined by Guinebretière et al. (2008). These clusters, established from the *Bacillus cereus* group MultiLocus and MultiData Typing website (Tourasse et al. 2011), are supported by recent results of a pangenomic study of this clade (Bazinet 2017). Moreover, the MLST data are still effective in discriminating variation of biology, ecology and host association among this group strains (Raymond and Federici 2017). The cluster III, including *B. anthracis* strains, emetic strains, and other pathogenic strains (mainly composed by *B. cereus* strains), and the cluster IV with *B. cereus* and *B. thuringiensis* strains from diverse environmental sources (mainly formed by *B. thuringiensis* strains) are the more extensively represented (Fig. 3). The Rap profile of each strain was analyzed in relation to the MLST tree of the *B. cereus* group. We observed that any Rap group was not present in all strains and that a Rap group was not exclusively related to an MLST cluster. None of the strains has more than five different Rap chromosomal groups, and a same Rap group can be present more than once in the same strain. As expected, the Cluster IV (mainly composed of *B. thuringiensis* strains) has a higher number of plasmid Rap. Additionally, phylogenetically related strains show a similar Rap profile both for chromosomal and plasmid groups. For example, the closely related strains *B. thuringiensis* Bt407 Cry^-, *B. thuringiensis* CT-43, and *B. thuringiensis* IS5056 show an identical chromosomal profile and similar plasmid profile. These three strains might derive from a same

parental strain, for example the *B. thuringiensis* strain 407 Cry^+ from the serotype 1 (Lereclus et al. 1989).

Sporulation activity prediction

Considering the importance of sporulation for the survival and dispersion of *Bacillus* and the role of some Rap-Phr systems in this process, we aimed to predict the activity of Rap proteins from the *B. cereus* group on sporulation. The RapH residues E45, D46, Q47, L50, F58, L96, D134, E137, and Y175 have been described to be involved in the binding and the dephosphorylation of Spo0F in *B. subtilis* (Parashar et al. 2011). First, we used the sequences of Rap proteins interacting with Spo0F to define a consensual sequence of residues potentially involved in the sporulation process (Online Resource Table 5). Next, the Rap protein sequences of each chromosomal and plasmid groups were separately aligned with the RapH sequence, and the presence of the nine key residues was examined (Online Resource Table 5). Depending on the presence of these residues, the Rap proteins from the *B. cereus* group were classified as Spo+ (predicted phosphatase activity on Spo0F) or Spo- (no predicted phosphatase activity) (Online Resource Table 4).

This analysis showed that 97 of the 302 Rap proteins display a Spo+ profile (32%) and the predicted Rap Spo+ are more frequently found in plasmids (65% of plasmid Rap) than in chromosomes (18% of chromosomal Rap) (Fig. 4a). However, there is no correlation between Rap groups and predicted sporulation function because there are groups with only Spo- or Spo+ Rap proteins, as well as mixed groups. Most of the chromosomal groups were exclusively Spo- and the plasmid groups are mainly mixed (Online Resource Table 5). Interestingly, the amount of Rap Spo+ is significantly higher ($P<0.01$) in *B. thuringiensis* than in *B. cereus* (40% and 26%, respectively) (Fig. 4a). Ten *B. cereus* strains (42%) do not harbor chromosomal Rap Spo+, including four strains that do not have any Rap Spo+ at all (Online Resource Table 4). The *B. thuringiensis* HD-1 strain, widely used as a biopesticide against lepidopteran insects, has nine Rap Spo+. In sharp contrast, five *B. thuringiensis* strains do not have chromosomal Rap Spo+, and the nematicidal *B. thuringiensis* YBT1518 strain is the only one *B. thuringiensis* to have no predicted Rap Spo+ at all.

To validate the *in silico* prediction, ten Rap proteins representative of various plasmid or chromosomal groups with a predicted Spo+ (seven Rap) or Spo- (three Rap) activity were selected to study their effect on sporulation (Online Resource Table 5). The corresponding *rap* genes were expressed under the xylose-inducible promoter P_{xylA} in the *B. thuringiensis* HD73 strain, and the sporulation efficiency was measured after 48 h at 30°C in a sporulation-specific medium (HCT). The three Rap Spo- (*rap6*-BtHD1, *rap6*-Bt407, and *rap7*-BtHD73) did not inhibit the sporulation efficiency compared to the control strain, confirming their prediction. Among the Rap Spo+ analyzed, the expression of *rap8*-Bt407, *rap10*-BtHD1, *rap7*-Bt407, and *rap5*-HD73 strongly prevent the sporulation ($P<0.001$) while the expression of *rap1*-BcATCC14579, and *rap2*-BcATCC14579 slightly inhibited the sporulation efficiency compared to the control strain ($P<0.01$) (Fig. 4b, Online Resource Table 6). Hence, the sporulation results allow us to confirm the predicted phenotype for nine of the ten tested Rap. However, the *rap8*-BtHD1, predicted Rap Spo+ does not display a role in sporulation in our *B. thuringiensis* HD73 model strain in this growth condition.

Discussion

Despite the importance of the Rap-Phr systems in the regulation of various essential pathways, they have been poorly studied in the bacteria of the *B. cereus* group (Bongiorni et al. 2006; Fazion et al. 2018; Slamti et al. 2014). Here we provide a complete and detailed overview of these systems in this group, concerning their prevalence, sequence diversity, relevant association to plasmids and their role in sporulation. We show that the *rap* genes are widespread in all the studied strains of the *B. cereus* group and that a putative *phr* gene is always present immediately downstream from all *rap* genes. The *rap-phr* genes are always encoded on the same DNA strand but in different transcription frames, a characteristic of the RNPP family (Declerck et al. 2007). Genes coding for the Phr peptides were diverse in size and the occurrence of *phr* genes two times longer than the average could be explained by the duplication of the region coding for the mature signaling peptide, important for the evolutionary diversification of Rap-Phr specificity (Even-Tov et al. 2016). The *rap-phr* genes are located in all chromosomes and numerous plasmids. The total number of chromosome *rap-phr* genes is similar between *B. cereus* and *B. thuringiensis* species. However, chromosome size seems unrelated to the amount of chromosomal

rap-phr genes as the *B. cytotoxicus* NVH 391-98 strain (distantly related to other strains of the *B. cereus* group) presents the smallest chromosome and the largest number of chromosomal *rap-phr* genes.

A global analysis of *rap* genes in the genus *Bacillus* showed that species from the *B. subtilis* group contain 11 (± 2) *rap* genes in opposition to six (± 3) for the *B. cereus* group species (Even-Tov et al. 2016). In agreement with this study, we found a similar number of *rap* genes in the *B. cereus* group (6.2 ± 3.2). Another difference between the two *Bacillus* groups is related to the *phr* gene occurrence. While 27% of the *rap* genes from *B. subtilis* strain 168 do not have an associated *phr* gene (Perego 2013), a common pattern for the *B. subtilis* group (Even-Tov et al. 2016), a putative *phr* gene is always located downstream from all identified *rap* genes in the *B. cereus* group. Interestingly, Rap proteins from the *B. subtilis* and *B. cereus* species constitute two independent clusters, suggesting that the diversification of the Rap sequences occurred after the evolutionary separation of the two bacterial groups (Even-Tov et al. 2016). After this separation, Rap-Phr systems from the *B. cereus* group might have been subjected to genetic variations that also evolved this quorum sensing system to the other RNPP family systems, like PlcR-PapR and NprR-NprX. The role of these QS systems in the production of extracellular proteases or sporulation corresponds to functions performed by some Rap-Phr systems of *B. subtilis* (Auchtung et al. 2006; Ogura et al. 2003). Interestingly, NprR, which presents a Rap-like structure combined with an HTH DNA-binding domain, was suggested to be the evolutionary intermediate between Rap proteins and the other regulators of the RNPP family (Perchat et al. 2016a). This hypothesis could also explain the difference in the number of *rap-phr* genes between *B. subtilis* and *B. cereus*.

Our analysis revealed that 30% of the identified *rap-phr* genes were plasmid-born. The plasmid *rap-phr* genes have been described in diverse *Bacillus* species (Koetje et al. 2003; Parashar et al. 2013b; Singh et al. 2013; Yang et al. 2015), including the *B. cereus* group (Bongiorni et al. 2006; Chao et al. 2007; Fazion et al. 2018; Slamti et al. 2014; Van der Auwera et al. 2005). These plasmid *rap-phr* genes are carried by a wide range of plasmids but are mainly located in conjugative plasmids greater than 70 kb and are more abundant in *B. thuringiensis* than in *B. cereus*. This difference is not only the consequence of a higher number of plasmids

in *B. thuringiensis*, which has only twice as many plasmids as *B. cereus* and six-times as many *rap-phr* plasmid genes. Moreover, this difference might be explained by the higher presence of mobile elements close to plasmid *rap* genes in *B. thuringiensis* than in *B. cereus*. We have shown that plasmids larger than 200 kb can host multiple *rap-phr* genes, and it is also remarkable that *rap-phr* genes were found on virulence plasmids, such as pXO1 in *B. anthracis* and pCER270 in *B. cereus*, as well as in some Cry plasmids in *B. thuringiensis*. However, we did not find specific *rap-phr* genes associated to the Cry plasmids. The multiplicity of the *rap-phr* genes in the *B. cereus* group creates appropriate conditions for their diversity and evolution, as demonstrated by Even-Tov et al. (2016). Thus, different *rap-phr* genes can respond to various signals and be regulated differently at transcriptional level.

Identical Rap protein sequences are located on the same type of replicon: i) in the chromosome as a consequence of common ancestor, or ii) in plasmids as a consequence of conjugation events. The proportion of identical Phr is higher than that of identical Rap. This characteristic might allow the Phr peptides to act cooperatively on various Rap proteins from different strains. The mature Phr can be located inside or at the C-terminal extremity of the pro-peptide (Pottathil and Lazazzera 2003), hampering the identification of the active form of some Phr. Due to this difficulty and to the wide variability of pro-Phr sequences, the Phr phylogenetic tree was not estimated and the evolutive correlation between Rap and Phr was not determined. Nevertheless, this coevolution was described for the NprR-NprX and PlcR-PapR systems (Perchat et al. 2011; Slamti and Lereclus, 2005).

The correlation between the Rap-Phr system distribution and the MLST tree based on housekeeping genes revealed that closely related strains harbor a similar Rap-Phr system pattern, suggesting a similar evolutionary history of both genetic characters. This correlation was not observed for the PlcR and NprR regulators from the RNPP family (Ko et al. 2004; Perchat et al. 2011). Also, the closest strains have a similar profile, even for plasmid genes, suggesting a beneficial association of particular plasmid-chromosome combinations that leads to the maintenance and propagation of these proficient combinations (Méric et al. 2018).

One-third of the Rap proteins of the *B. cereus* group are predicted to have phosphatase activity on Spo0F. However, some strains did not have any predicted Rap Spo+ (four *B. cereus* strains, one *B thuringiensis* strain, *B. mycoides* and *B.*

pseucomycoides). This absence of Rap Spo+ could be compensated by NprR, which has a Rap-like activity on the sporulation phosphorelay and which is conserved in all the strains of the *B. cereus* group (Perchat et al. 2016b). In these strains, Rap proteins might have undergone genetic variations leading to the loss of sporulation function and the acquisition of new undetermined functions. Interestingly, Rap proteins predicted to regulate sporulation are mainly encoded by plasmid genes and, therefore, are more abundant in *B. thuringiensis*. These plasmid Rap-Phr systems could help *B. thuringiensis* to adapt and survive in its complex ecological niche, the insect. Moreover, many Rap-Phr plasmid systems are located on cryptic plasmids less than 16 kb in size. These plasmid genes could regulate different beneficial functions leading to the maintenance of these plasmids in the bacterial cell. Recently, the plasmid pHT8_1 from the *B. thuringiensis* HD73 strain has been characterized, and the role of its Rap-Phr system in the regulation of the sporulation process in insect larvae has been demonstrated (Fazion et al. 2018).

Sporulation assays validated the *in silico* prediction except for one Spo+ Rap. The residues involved in RapH-Spo0F interaction are highly conserved in *B. subtilis* and *B. cereus,* and small differences are sufficient to lose this activity. However, these key residues are relatively well-conserved in Rap proteins that do not regulate the sporulation pathway, suggesting that the Rap activity on sporulation was the ancestral role of these proteins (Even-Tov et al. 2016). Thus, the presence of these residues is a good indicator but is not sufficient to predict the sporulation activity. The role of the Rap Spo- remains the main unsolved question. Given the large amount of plasmid Rap-Phr and of the role of these systems in plasmid conjugation in *B. subtilis* (Singh et al., 2013), it will be interesting to study this phenotype.

Cell-cell communication systems enable a fine regulation of important processes in bacteria. Indeed, some Rap-Phr systems regulate sporulation that allows bacteria to adapt, survive and disseminate. This work highlights the importance of Rap-Phr systems linked to genetic mobile elements in the *B. cereus* group, especially in *B. thuringiensis*. This location on mobile elements could increase the spreading of these genes in bacteria of the *B. cereus* group.

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Tables

Table 1. Main features of the 49 selected genomes from the *B. cereus* group strains.

	Strain (named as in NCBI database)	Release date	Assembly	Chromosome		Plasmids			STs (MLST)
				Size (Mb)	%GC	Nb	Size (Mb)	% ↑ genome	
B. cereus	ATCC 10987	2002	GCA_000008005.1	5.22	35.6	1	0.21	4%	2
	ATCC 14579	2003	GCA_000007825.1	5.41	35.3	1	0.01	0%	33
	E33L	2004	GCA_000011625.1	5.30	35.4	5	0.55	10%	57
	AH187	2008	GCA_000021225.1	5.27	35.6	4	0.33	6%	3
	B4264	2008	GCA_000021205.1	5.42	35.3	0	-	-	-2
	G9842	2008	GCA_000021305.1	5.39	35.3	2	0.35	6%	120
	AH820	2008	GCA_000021785.1	5.3	35.4	3	0.28	5%	39
	Q1	2009	GCA_000013065.1	5.21	35.6	2	0.29	6%	40
	03BB102	2009	GCA_000022505.1	5.27	35.4	1	0.18	3%	122
	anthracis CI	2010	GCA_000143605.1	5.2	35.4	3	0.28	5%	153
	NC7401	2011	GCA_000283675.1	5.2	35.6	5	0.33	6%	3
	F837/76	2011	GCA_000239195.1	5.22	35.4	2	0.07	1%	182
	FRI-35	2012	GCA_000292415.1	5.08	35.6	4	0.3	6%	188
	FT9	2014	GCA_000724585.1	5.22	35.5	0	-	-	191
	03BB87	2014	GCA_000789315.1	5.46	35.3	2	0.26	5%	58
	D17	2015	GCA_000832385.1	5.38	35.4	1	0.21	4%	179
	FM1	2015	GCA_000832525.1	5.3	35.5	1	0.40	8%	186
	3a	2015	GCA_000832765.1	5.27	35.4	3	0.37	7%	124
	G9241	2015	GCA_000832805.1	5.27	35.4	3	0.45	9%	58
	ATCC 4342	2015	GCA_000832845.1	5.27	35.4	1	0.04	1%	4
	03BB108	2015	GCA_000832865.1	5.34	35.3	7	0.73	14%	119

	Strain (named as in NCBI database)	Release date	Assembly	Chromosome		Plasmids			STs (MLST)
				Size (Mb)	%GC	Nb	Size (Mb)	% ↑ genome	
	Al Hakam *	2015	GCA_000832885.1	5,23	35,8	6	0.45	9%	173
	S2-8	2015	GCA_000835185.1	5.27	35.4	2	0.37	7%	124
	FORC_005	2015	GCA_000978375.1	5.35	35.3	0	-	-	187
	Average			5.29	35.4	2.5	0.31	6%	
B. thuringiensis	*konkukian* 97-27	2004	GCA_000008505.1	5.24	35.4	1	0.07	1%	59
	Al Hakam	2006	GCA_000015065.1	5.26	35.4	1	0.06	1%	89
	BMB171	2010	GCA_000092165.1	5.33	35.3	1	0.31	6%	152
	finitimus YBT-020	2011	GCA_000190515.1	5.36	35.5	2	0.33	6%	155
	chinensis CT-43	2011	GCA_000193355.1	5.49	35.4	10	0.66	12%	44
	HD-771	2012	GCA_000292455.1	5.89	35.2	8	0.56	10%	75
	HD-789	2012	GCA_000292705.1	5.5	35.3	6	0.84	15%	136
	MC28	2012	GCA_000300475.1	5.41	35.4	7	1.28	24%	231
	Bt407	2012	GCA_000306745.1	5.5	35.4	9	0.65	12%	44
	kurstaki HD73	2013	GCA_000338755.1	5.65	35.3	7	0.27	5%	115
	thuringiensis IS5056	2013	GCA_000341665.1	5.49	35.4	14	1.3	24%	44
	YBT-1518	2013	GCA_000497525.2	6	35.4	6	0.68	11%	261
	kurstaki YBT-1520	2014	GCA_000688795.1	5.6	35.3	11	0.98	18%	115
	kurstaki HD-1	2014	GCA_000717535.1	5.63	35.3	13	1.13	20%	115
	galleriae HD-29	2014	GCA_000803665.1	5.7	35.3	10	1.04	18%	211
	HD1011	2015	GCA_000832485.1	5.23	35.5	4	0.86	16%	71
	HD571	2015	GCA_000832825.1	5.26	35.4	1	0.06	1%	89
	HD682	2015	GCA_000832925.1	5.21	35.5	3	0.08	2%	212
	HD1002	2015	GCA_000835025.1	5.49	35.3	7	1.08	20%	136
	morrisoni BGSC 4AA1	2015	GCA_000940785.1	5.65	35.3	6	0.53	9%	112
	Average			5.49	35.4	6.4	0.64	12%	
	B. anthracis 'Ames Ancestor' A2084	2004	GCA_000008445.1	5.23	35.4	2	0.27	5%	1
	B. weihenstephanensis KBAB4 #	2007	GCA_000018825.1	5.26	35.6	4	0.61	12%	118
	B. mycoides ATCC 6462	2015	GCA_000832605.1	5.26	35.5	3	0.38	7%	133
	B. pseudomycoides DSM 12442	2012	GCA_000161455.1	5.78	35.4	-	-	-	132
	B. cytotoxicus NVH 391-98	2007	GCA_000017425.1	4.09	35.9	1	0.01	0%	117
	***B. cereus group* Average**			5.35	35.4	4.1	0.46	9%	

STs (Sequence Types) data was obtained from the University of Oslo's *Bacillus cereus* group MultiLocus and MultiData Typing website (http://mlstoslo.uio.no). Plasmid 'Nb' is the sum of different plasmids sequenced for each strain and '% ↑ genome' is how much all these plasmids increase the genome size of that strain (in relation to chromosome size alone). * This strain was first annotated as *B. cereus* (Johnson et al. 2015), and is now classified as *B. pseudomycoides* (strain BTZ). # Nowadays, *B. weihenstephanensis* strains are considered as heterotypic synonym of *B. mycoides* (Liu et al. 2018).

Table 2. Comparison between *B. cereus* and *B. thuringiensis* concerning the Rap-Phr systems.

	B. cereus	*B. thuringiensis*	Significance
Number of strains	24	20	
Number of plasmids	59	127	
Number of chromosome *rap-phr* genes[1]	97 (4.0/strain)	89 (4.5/strain)	ns
Number of plasmid *rap-phr* genes[2]	14 (0.6/strain)	72 (3.6/strain)	***
Chromosomal/plasmid *rap-phr* genes ratio[3]	6.9	1.2	***
Plasmids with *rap-phr* genes[3]	13 (22%)	49 (38%)	*
Chromosomal *rap* genes with nearby mobile elements[1]	36 (37%)	35 (39%)	ns
Plasmid *rap* genes with nearby mobile elements[4]	5 (36%)	52 (72%)	**

Statistical analyses: [1]Unpaired t test; [2]Mann-Whitney test; [3] Fisher's Exact test; [4] unpaired t test with Welch correction. ns: not significant; *P < 0.05; **P < 0.01; ***P ≤ 0.001.

Figures

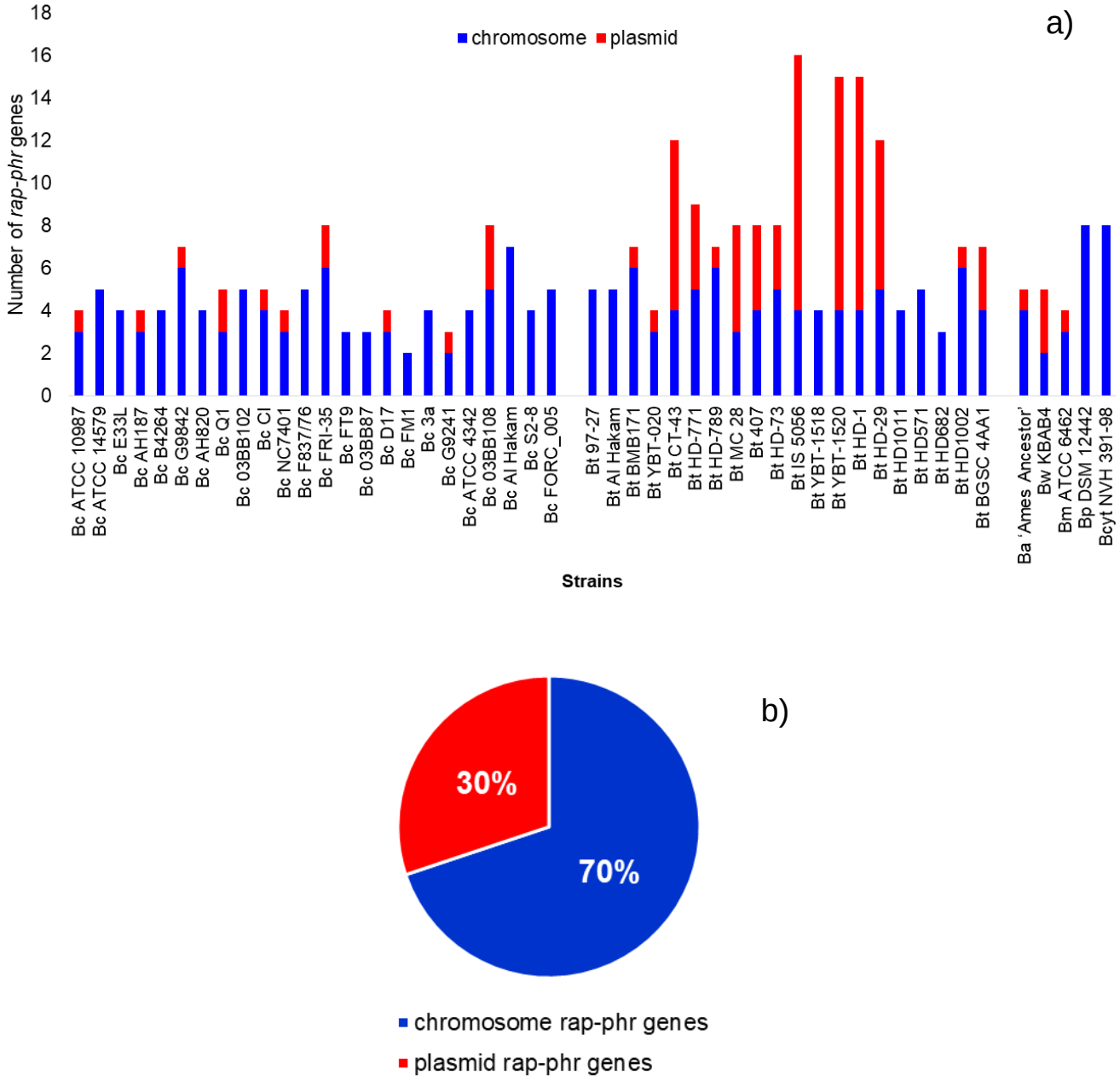
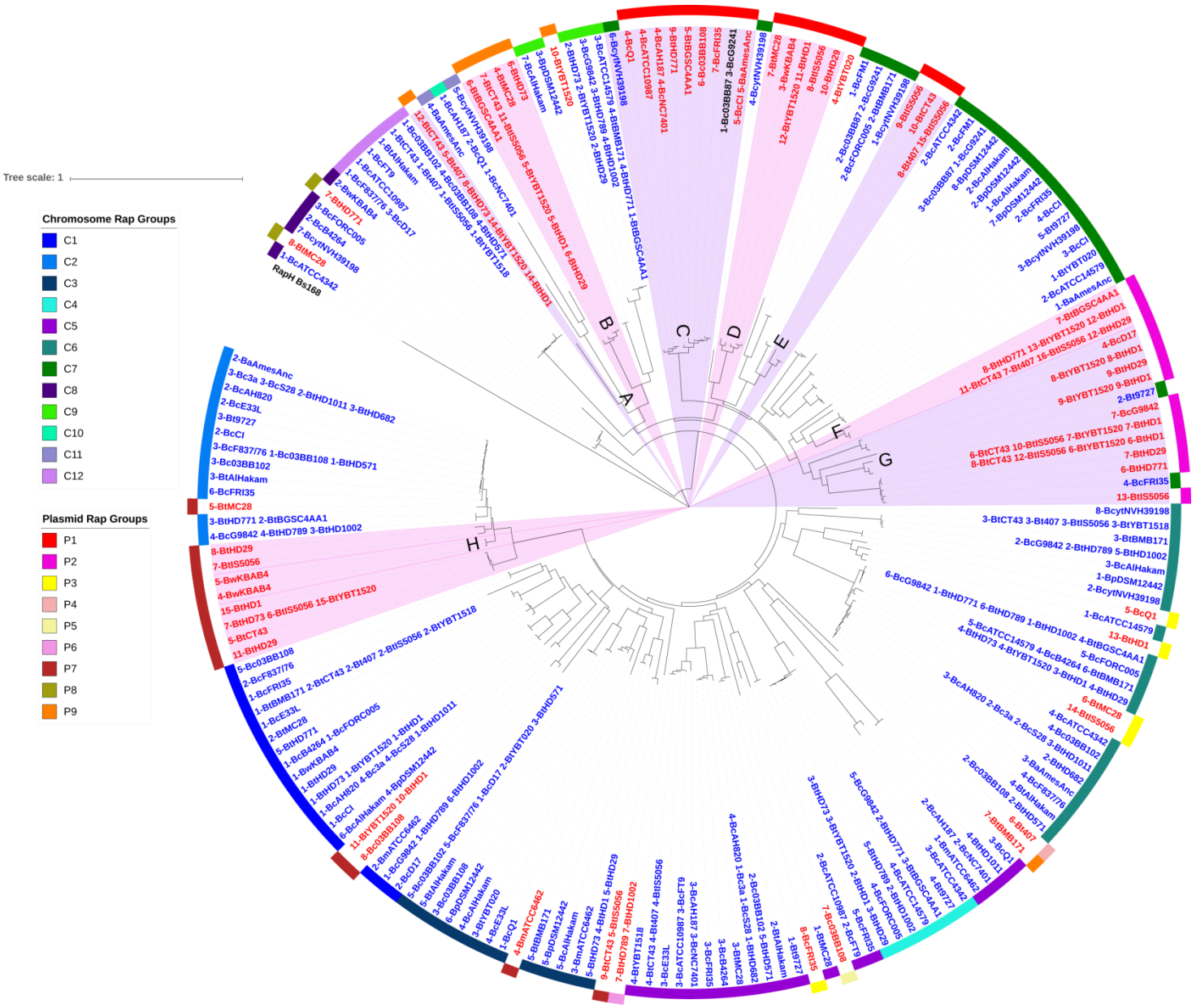


Fig. 1 Distribution of the identified *rap-phr* genes in the *B. cereus* group. a) The number of *rap-phr* genes by strain with the chromosomal systems in blue and the plasmid systems in red. b) Percentage of the *rap-phr* genes by replicon with chromosomes in blue and plasmids in red.

Fig. 2 Phylogenetic tree of all Rap protein sequences (plasmid and chromosome sequences) inferred by Neighbor-Joining method (conducted in MEGA6) and visualized by iTOL (Letunic and Bork 2019). RapH from *B. subtilis* 168 was used as outgroup. The evolutionary distances were computed using the JTT matrix-based method and are in the units of the number of amino acid substitutions per site. Chromosome-encoded proteins are in blue and plasmid-encoded proteins are in red. Sets A to H represent related Rap proteins associated to the same kind of plasmid. Chromosome and plasmid groups are indicated in the two outer circles.



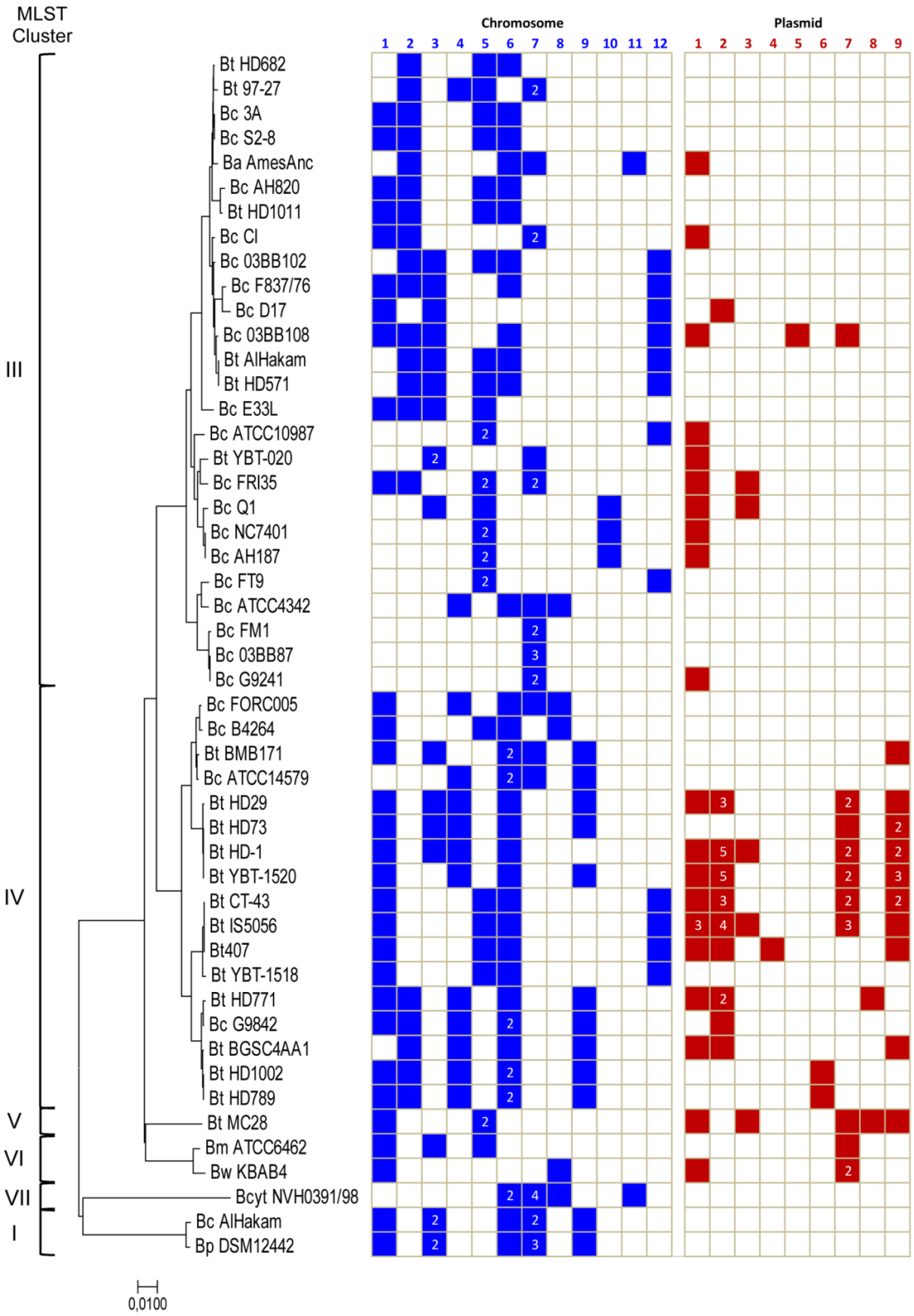


Fig. 3 Distribution of the chromosomal and plasmid Rap-Phr systems groups in relation to the MLST phylogenetic tree (*Bacillus cereus* group MultiLocus and MultiData Typing website -http://mlstoslo.uio.no). MLST clusters proposed by Guinebretière et al. (2008) are showed on the left. Chromosomal systems are in blue, and plasmid systems are in red. Numbers inside the boxes specify how many times (if > 1) that group is found in each strain.

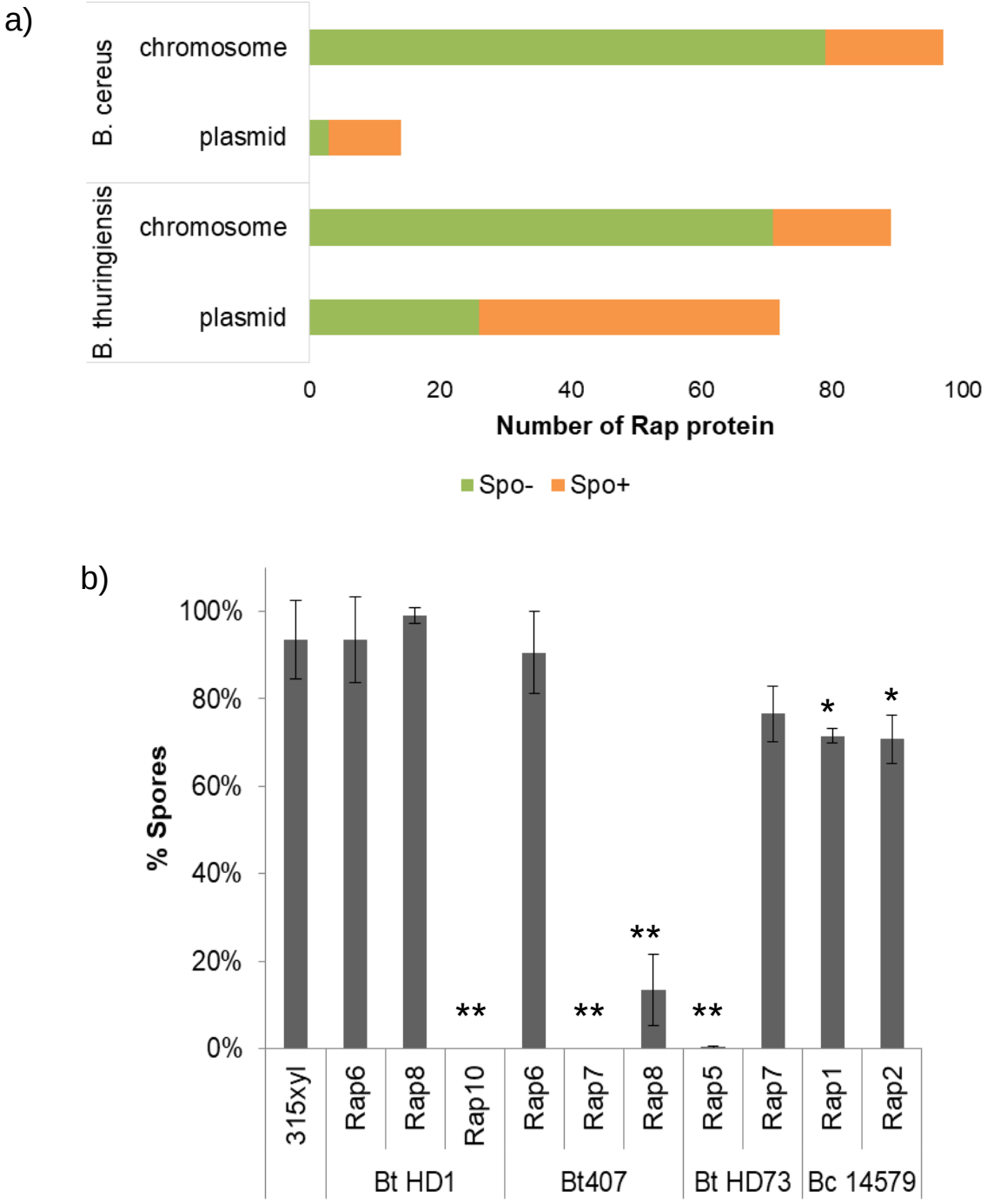


Fig. 4 Activity of Rap from *B. cereus* group in sporulation. a) Distribution of the Rap protein predicted as Spo+ (orange) or Spo- (green), difference between species are statistically significant (P < 0.01 – Fisher's Exact test); b) Efficiency of sporulation, calculated as 100 × the ratio between heat-resistant spores per milliliter and total viable cells per milliliter. The data were analyzed by using One-way Analysis of Variance (ANOVA) followed by Tukey-Kramer Multiple Comparisons Test (P<0.01).

* (P < 0.01) and ** (P < 0.001): % of spores is statistically different from control (315xyl).

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Online Resources

Online Resource - Data 1 Rap-Phr database

Online Resources - Table 1. Source and main features of the selected strains.

Strain	Source / Main Feature
B. cereus ATCC 10987	Isolated from dairy products
B. cereus ATCC 14579	*Bacillus cereus* type strain. Isolated from environment
B. cereus E33L	Isolated from the carcass of a dead zebra
B. cereus AH187	Associated with emetic food poisoning outbreak; reference emetic toxin/cereulide producer. Also known as strain F4810/72
B. cereus B4264	Isolated from a case of fatal pneumonia in a male patient
B. cereus G9842	Isolated from stool samples from patients with diarrhea
B. cereus AH820	Isolated from periodontitis
B. cereus Q1	Non-pathogenic strain isolated from a deep-subsurface oil reservoir
B. cereus 03BB102	Isolated from a fatal case of pneumonia
B. cereus biovar *anthracis* str. CI	Isolated from a chimpanzee died with anthrax-like symptoms
B. cereus NC7401	Isolated from emetic food poisoning outbreak, cereulide producer
B. cereus F837/76	Isolated from prostate wound infection
B. cereus FRI-35	Unknown data
B. cereus FT9	Isolated from a hot spring
B. cereus 03BB87	Isolated from a fatal case of pneumonia
B. cereus D17	Isolated from gastroenteritis cases with diarrhoeal symptoms
B. cereus FM1	Isolated from heavy metal contaminated agricultural soil
B. cereus 3a	Unknown data
B. cereus G9241	Isolated from a case of pneumonia ressembling anthrax
B. cereus ATCC 4342	Isolated from dairy products
B. cereus 03BB108	Isolate from settled dust at the Bc 03BB102 case's worksite
B. cereus str. Al Hakam **B. pseudomycoides* BTZ	The strain identified as *B. cereus* Al. Hakam (then *B. thuringiensis*, *B. mycoides)* later determined to be *B. pseudomycoides* strain BTZ of unknown provenance
B. cereus S2-8	Isolated from soil
B. cereus FORC_005	Isolated from food products
B. thuringiensis serovar *konkukian* str. 97-27	Isolated from a case of human tissue necrosis
B. thuringiensis str. Al Hakam	Isolated from at a suspected bioweapons facility
B. thuringiensis BMB171	Acrystalliferous mutant of strain YBT-1463, subsp. *kurstaki*, toxic to Lepidoptera
B. thuringiensis serovar *finitimus* YBT-020	Parasporal crystals proteins are located between the exosporium and the spore coat and remain adhering to the spore after sporulation

B. thuringiensis serovar *chinensis* CT-43	Toxic to lepidopteran and dipteran insects
B. thuringiensis HD-771	Serovar *sotto*
B. thuringiensis HD-789	Serovar *israelensis* toxic dipteran insects
B. thuringiensis MC28	Serovar *sichuansis*, toxic to lepidopteran and dipteran insects
B. thuringiensis Bt407 Cry-	Isolated as a lepidopteran-active strain, a Cry$^-$ strain was obtained by curing the plasmid harbouring the *cry* genes
B. thuringiensis serovar *kurstaki* str. HD73	Toxic to lepidopteran insects, also designated as KT0 strain
B. thuringiensis serovar *thuringiensis* str. IS5056	Toxic to lepidopteran insects
B. thuringiensis YBT-1518	Toxic to nematodes
B. thuringiensis serovar *kurstaki* str. YBT-1520	Widely used for *Bt* biopesticide production (Mianfeng®) in China
B. thuringiensis serovar *kurstaki* str. HD-1	The original strain used in the microbial insecticide Dipel®. U.S. reference standard strain for toxicity evaluation of commercial *B.thuringiensis* formulations
B. thuringiensis serovar *galleriae* HD-29	Toxic to lepidopteran insects
B. thuringiensis HD1011	Serovar *pondicheriensis*
B. thuringiensis HD571	Serovar *kyushuensis*
B. thuringiensis HD682	Serovar *finitimus*
B. thuringiensis HD1002	Serovar *israelensis* toxic dipteran insects
B. thuringiensis serovar *morrisoni* BGSC 4AA1	Biovar *tenebrionis*. Toxic to colepteran insects
B. anthracis str. 'Ames Ancestor' A2084	Isolated from a dead female heifer. Progenitor of all the Ames strains used as research tools in laboratories around the world
B. weihenstephanensis KBAB4	Psychrophilic strain isolated from forest soil
B. mycoides ATCC 6462	Soil isolate. *B. mycoides* type strain
B. pseudomycoides DSM 12442	Soil isolate. *B. pseudomycoides* type strain
B. cytotoxicus NVH 391-98	Identified as a cause of a severe food poisoning outbreak, isolated as a contaminant of vegetable puree

Table 2. Primers used in this study.

Primer	Sequence	Restriction site
Rap6HD1-F	CGC**GGATCC**AATTGGTATTGAGGGGGTAGATTATG	*BamHI*
Rap6HD1-R	CCC**AAGCTT**CTATTTTTTTCATTTTAAAGCTCCTTCC	*HindIII*
Rap8HD1-F	CCC**AAGCTT**GCCTTTTTCATTTTAATGCCTCC	*HindIII*
Rap8HD1-R	CGC**GGATCC**GGATGGGGGAATATATAATGATGG	*BamHI*
Rap10HD1-F	CCC**AAGCTT**GATTTTTTTCATTATTTTAACGCTCC	*HindIII*
Rap10HD1-R	CGC**GGATCC**GGAAAAGGGGAAGATTCTATGAATG	*BamHI*
Rap6-Bt407-F	CGC**GGATCC**GGGAAGAGGGGATTTTATGAACAC	*BamHI*
Rap6-Bt407-R	AA**CTGCAG**AATTTTTTCATCACTGTAACGCTCC	*PstI*
Rap7-Bt407-F	AA**CTGCAG**TTTTCTTCATTTTAATGCCTCCTTATC	*PstI*
Rap7-Bt407-R	CGC**GGATCC**AGATGAGGAGGGATTTTATGTATACAGC	*BamHI*
Rap8-Bt407-F	AA**CTGCAG**GATTGTTTTTTTCATTTTAAAGCCC	*PstI*
Rap8-Bt407-R	CGC**GGATCC**TCGTAAAAAAGGGGGCTATTTTG	*BamHI*
Rap5-BtHD73-F	AA**CTGCAG**CTTTAACTTTTTCATCACACTAACGCC	*PstI*
Rap5-Bt HD73-R	CGC**GGATCC**GAAAGAAGGAAGTGATTTTATGAATGC	*BamHI*
Rap7061-For	CGC**GGATCC**GAAAGAAGGGGGATATTATGAGC	*BamHI*
Rap7061-Rev	CCC**AAGCTT**TCATTATTTCAACGCCCCTTTTC	*HindIII*
Rap1-Bc14579-F	CGC**GGATCC**GAGGAGGACGTAGTGATATGAGCAC	*BamHI*
Rap1-Bc14579-R	AA**CTGCAG**CAAGCTTCTTCATTATTTTAACGCC	*PstI*
Rap2-Bc14579-F	CGC**GGATCC**GGAGGGGTTTCATGATTACATCTAC	*BamHI*
Rap2-Bc14579-F	AA**CTGCAG**CCACTTTTTTCATTTTAAAGCCC	*PstI*

Rap7061 corresponds to Rap7 – Bt HD73. Nucleotides in bold represent the restriction enzyme sites.

Table 3. List of plasmids from all strains studied.

Strain	Plasmid	%GC	Size (Kb)	Nb of *rap-phr* genes	Special traits
Bc ATCC 10987	pBc10987	33.4	208	1	-
Bc ATCC 14579	pBClin15	38.0	15	-	-
Bc E33L	pE33L5	30.9	5.1	-	-
	pE33L8	31.9	8.2	-	-
	pE33L9	31.0	9.1	-	-
	pE33L54	31.9	53	-	-
	pE33L466	33.1	466	-	-
Bc AH187	pAH187_3	34.9	3.1	-	-
	pAH187_12	31.1	12	-	-
	pAH187_45	35.5	45	-	-
	pAH187_270 (pCER270)	34.2	270	1	Cereulide genes
Bc B4264	-				-
Bc G9842	pG9842_140	32.9	140	-	-
	pG9842_209	30.0	209	1	-
Bc AH820	pAH820_3	34.9	3.1	-	-
	pAH820_10	33.6	11	-	-
	pAH820_272	33.6	272	-	-
Bc Q1	pBc53	35.1	53	-	-
	pBc239	33.5	239	2	-
Bc 03BB102	p03BB102_179	32.2	180	-	anthrax capsule genes anthrax toxins genes
Bc biovar *anthracis* CI	pBAslCI14	37.9	14	-	-
	pCIXO2	33.1	182	-	anthrax capsule genes
	pCIXO1	32.5	94	1	anthrax toxins genes
Bc NC7401	pNC4	34.9	3.1	-	-
	pNC3	34.7	3.9	-	-
	pNC2	34.1	5.4	-	-
	pNC1	36.5	48	-	-
	pNCcld	34.2	270	1	Cereulide genes
Bc F837/76	pF837_10	31.1	10	-	-
	pF837_55	36.2	55	-	-
Bc FRI-35	p04	34.9	3.1	-	-
	p03	30.7	36	-	-
	p02	32.5	41	1	-
	p01	33.4	219	1	-
Bc FT9	-				-
Bc 03BB87	pBCN	36.5	52	-	-
	pBCX01	31.7	209	-	-
Bc D17	plasmid unnamed	33.2	211	1	-
Bc FM1	plasmid unnamed	32.6	403	-	-
Bc 3a	pBFC_1	35.7	7.3	-	-
	pBFC_2	36.6	51	-	-
	pBFC_3	32.7	312	-	-
Bc G9241	pBFH_1	36.5	52	-	-
	pBC210	31.7	209	-	-
	pBCX01	32.6	190	1	anthrax toxins genes
Bc ATCC 4342	pBGM	30.7	37	-	-
Bc 03BB108	pBFI_7	33.0	4.9	-	-
	pBFI_6	31.8	9.8	1	-
	pBFI_2	31.9	239	1	anthrax capsule genes
	pBFI_5	36.1	42	-	-
	pBFI_4	35.5	62	-	-
	pBFI_1	30.8	282	-	-
	pBFI_3	32.5	86	1	-
Bc Al Hakam	pBTZ_5	33.0	4.9	-	-

Strain	Plasmid	%GC	Size (Kb)	Nb of *rap-phr* genes	Special traits
(*Bp BTZ)	pBTZ_6	33.7	4.6	-	-
	pBTZ_4	31.3	12	-	-
	pBTZ_3	32.5	12	-	-
	pBTZ_2	34.0	141	-	-
	pBTZ_1	33.4	274	-	-
Bc S2-8	pBFR_3	36.6	51	-	-
	pBFR_1	35.7	7.3	-	-
	pBFR_2	32.7	312	-	-
Bc FORC_005	-				-
Bt *konkukian* 97-27	pBT9727	32.6	77	-	-
Bt Al Hakam	pALH1	36.2	56	-	-
Bt BMB171	pBMB171	33.3	313	1	-
Bt *finitimus* YBT-020	pBMB28	33.9	139	1	*cry*
	pBMB26	33.1	188	-	*cry*
Bt *chinensis* CT-43	pCT6880	31.8	6.9	1	-
	pCT8252	32.4	8.2	-	-
	pCT8513	30.8	8.5	1	-
	pCT9547	33.1	9.5	-	-
	pCT14	31.5	15	-	-
	pCT51	35.0	51	1	-
	pCT72	32.0	72	1	-
	pCT83	33.2	84	-	-
	pCT127	32.1	128	1	*cry*
	pCT281	33.0	281	3	*cry*
Bt HD-771	p04	32.0	65	1	-
	p08	30.1	8	-	-
	p07	33.5	9	-	-
	p06	35.2	14	-	-
	p01	33.0	171	1	
	p05	36.0	45	-	-
	p03	34.3	70	1	*cry*
	p02	33.2	169	1	
Bt HD-789	p06	36.0	6.8	-	-
	p05	35.3	7.7	-	-
	p04	39.7	15	-	-
	p03	33.1	225	-	*cry*
	p02	36.6	235	1	-
	p01	33.4	350	-	-
Bt MC 28	pMC8	32.1	7.8	-	-
	pMC54	34.7	54	1	-
	pMC95	34.0	95	-	*cry*
	pMC183	32.8	183	1	*cry*
	pMC189	33.4	190	-	*cry*
	pMC319	32.5	320	-	*cry*
	pMC429	32.6	430	3	-
Bt 407 (*Cry*-)	BTB_2p	34.8	2.1	-	-
	BTB_5p	31.6	5.5	-	-
	BTB_6p	31.8	6.9	1	-
	BTB_7p	32.2	7.6	-	-
	BTB_8p	29.7	8.2	-	-
	BTB_9p	30.8	8.5	1	-
	BTB_15p	35.7	151	-	-
	BTB_78p	32.3	78	1	-
	BTB_502p	31.5	502	1	-
Bt *kurstaki* HD-73	pHT7	32.2	7.6	-	-
	pHT8_2	29.7	8.2	-	-
	pHT8_1	30.8	8.5	1	-
	pHT11	31.8	12	-	-

Strain	Plasmid	%GC	Size (Kb)	Nb of *rap-phr* genes	Special traits
	pAW63	33.8	72	1	-
	pHT77	30.8	76	1	-
	pHT73	34.7	77	-	*cry*
Bt *thuringiensis* IS5056	pIS56-6	31.8	6.9	1	-
	pIS56-8	32.4	8.2	-	-
	pIS56-9	33.0	9.7	-	-
	pIS56-11	31.6	11	-	-
	pIS56-15	35.7	15	-	-
	pIS56-16	33.3	16	1	-
	pIS56-39	34.9	40	1	-
	pIS56-63	34.7	64	-	*cry*
	pIS56-68	31.8	69	1	-
	pIS56-85	33.2	85	-	-
	pIS56-107	31.0	107	1	*cry*
	pIS56-233	32.7	234	2	-
	pIS56-285	33.0	285	3	*cry*
	pIS56-328	32.6	328	2	-
Bt YBT-1518	pBMB0228	29.5	18	-	*cry*
	pBMB0229	36.6	45	-	-
	pBMB0230	33.7	49	-	-
	pBMB0231	33.9	146	-	-
	pBMB0232	33.8	172	-	-
	pBMB0233	35.0	241	-	-
Bt *kurstaki* YBT-1520	pBMB2062	34.8	2.1	-	-
	pBMB422	32.6	423	5	-
	pBMB293	33.2	294	3	*cry*
	pBMB53	34.4	54	-	-
	pBMB11	31.8	12	-	-
	pBMB8513	30.8	8.5	1	-
	pBMB8240	29.7	8.2	-	-
	pBMB7921	32.3	7.9	-	-
	pBMB7635	32.2	7.6	-	-
	pBMB94	31.4	95	1	*cry*
	pBMB67	32.4	67	1	-
Bt *kurstaki* HD-1	pBMB46	35.4	47	-	-
	pBMB64	31.9	64	1	-
	pBMB65	34.8	66	-	*cry*
	pBMB74	33.7	74	1	
	pBMB95	31.5	96	1	*cry*
	pBMB299	33.2	300	3	*cry*
	pBMB431	32.7	431	4	-
	pBMB2062	34.8	2.1	-	-
	pBMB7635	32.2	7.6	-	-
	pBMB8240	29.7	8.2	-	-
	pBMB8513	30.9	8.5	1	-
	pBMBLin15	40.1	15	-	-
	pBMB14	31.0	15	-	-
Bt *galleriae* HD-29	pBMB426	32.9	426	3	*cry*
	pBMB267	33.2	267	2	*cry*
	pBMB126	31.7	127	1	*cry*
	pBMB71	32.2	71	1	-
	pBMB55	34.9	55	-	-
	pBMB47	35.5	47	-	-
	pBMBLin15	39.9	15	-	-
	pBMB12	31.5	13	-	-
	pBMB7	33.7	11	-	-
	pBMB8	29.6	8.4	-	-
Bt HD1011	plasmid 3	33.9	82	-	-

Strain	Plasmid	%GC	Size (Kb)	Nb of *rap-phr* genes	Special traits
	plasmid 4	32.5	70	-	-
	plasmid 2	33.4	350	-	-
	plasmid 1	32.5	359	-	-
Bt HD571	pBFQ	36.2	56	-	-
Bt HD682	pBGN_3	36.3	7.2	-	-
	pBGN_2	35.0	56	-	-
	pBGN_1q	30.2	15	-	-
Bt HD1002	plasmid 7	36.0	6.8	-	-
	plasmid 6	35.3	7.7	-	-
	plasmid 5	39.7	15	-	-
	plasmid 3	36.6	235	1	-
	plasmid 2	33.4	350	-	-
	plasmid 1	32.3	359	-	-
	plasmid 4	33.8	107	-	-
Bt *morrisoni* BGSC4AA1	pBMB232	33.7	233	2	-
	pBMB92	34.5	93	-	-
	pBMB76	30.3	77	-	-
	pBMB68	32.2	68	1	-
	pBMB51	29.3	52	-	-
	pBMB48	41.6	4.8	-	-
Ba 'Ames Ancestor'	pXO2	33.0	95	-	anthrax capsule genes
	pXO1	32.5	182	1	anthrax toxins genes
B. *weihenstephanensis* KBAB4	pBWB401	33.7	420	3	-
	pBWB402	33.3	80	-	-
	pBWB403	43.4	60	-	-
	pBWB404	35.4	50	-	-
B. *mycoides* ATCC 6462	pBMX_3	32.6	9.9	-	-
	pBMX_2	31.4	10	-	-
	pBMX_1	33.9	361	1	-
B. *pseudomycoides* DSM 12442	-				-
B. *cytotoxicus* NVH 391-98	pBC9801	30.3	10	-	-

Special traits: toxin cereulide for *B. cereus* strains; gene *cry* for *B. thuringiensis* strains; anthrax toxin components genes: *pag*, *lef*, and *cya* and the polyglutamate capsule operon (*capBCADE*) for *B. anthracis*.

Table 4. Distribution of the *rap-phr* genes by strain, with gene sizes, location in the genome, and DNA strand. Rap proteins highlighted in green were predicted as Spo- and those in orange were predicted as Spo+.

Strains	*rap-phr*	replicon	DNA strand	*rap* genes size (bp)	*rap* genes Location (bp)		*phr* genes size (bp)	*phr* genes Location (bp)
***Bc* ATCC 10987**	1	chromosome	+	1110	1.078.652-1.079.761		138	1.079.758-1.079.895
	2		+	1095	2.886.506-2.887.600		147	2.887.600-2.887.746
	3		+	1095	3.278.356-3.279.450		189	3.279.447-3.279.635
	4	pBc10987	-	1095	130.663-131.757		141	130.526-130.666
***Bc* ATCC 14579**	1	chromosome	+	1110	971.287-972.396	*	177	972.349-972.525
	2		+	1113	1.007.124-1.008.236	*	132	1.008.234-1.008.365
	3		+	1074	2.091.417-2.092.490		126	2.092.487-2.092.612
	4		-	1098	3.457.151-3.458.248		132	3.456.965-3.457.096
	5		+	1116	3.475.275-3.476.390		162	3.476.390-3.476.551
***Bc* E33L**	1	chromosome	+	1095	1.040.836-1.041.930		309	1.041.930-1.042.238
	2		-	1113	2.393.749-2.394.861		159	2.393.591-2.393.749
	3		+	1095	3.345.257-3.346.351		189	3.346.348-3.346.536
	4		+	1095	4.927.987-4.929.081		306	4.929.081-4.929.386
***Bc* AH187**	1	chromosome	+	1095	1.861.946-1.863.040		144	1.863.037-1.863.180
	2		+	1104	3.062.411-3.063.514		288	3.063.511-3.063.798
	3		+	1095	3.279.233-3.280.327		189	3.280.324-3.280.512
	4	pAH187_270	+	1095	204.553-205.647		141	205.644-205.784
***Bc* B4264**	1	chromosome	+	1095	1.030.971-1.032.065		324	1.032.065-1.032.388
	2		+	1011	2.069.164-2.070.174		216	2.070.171-2.070.386
	3		+	1095	3.390.307-3.391.401		189	3.391.398-3.391.586
	4		+	1116	3.409.737-3.410.852		162	3.410.852-3.411.013
***Bc* G9842**	1	chromosome	+	1095	1.009.685-1.010.779		201	1.010.779-1.010.979
	2		-	1095	1.652.488-1.653.582		138	1.652.351-1.652.488
	3		+	1074	2.038.586-2.039.659		126	2.039.656-2.039.781
	4		-	1101	2.364.416-2.365.516		159	2.364.258-2.364.416
	5		-	1095	3.387.052-3.388.146		132	3.386.866-3.386.997
	6		+	1116	3.429.984-3.431.099		162	3.431.099-3.431.260
	7	pG9842_209	-	1095	89.302-90.396		138	89.168-89.305
***Bc* AH820**	1	chromosome	+	1089	1.050.152-1.051.240		204	1.051.240-1.051.443
	2		-	1101	2.410.483-2.411.583		159	2.410.325-2.410.483
	3		+	1116	3.118.600-3.119.715		162	3.119.715-3.119.876
	4		+	1095	3.353.248-3.354.342		189	3.354.339-3.354.527
***Bc* Q1**	1	chromosome	-	1095	581.509-582.603		258	581.252-581.509
	2		+	1095	1.863.400-1.864.494		144	1.864.491-1.864.634
	3		+	1098	3.249.705-3.250.802		189	3.250.805-3.250.993
	4	pBc239	+	1095	101.157-102.251		141	102.248-102.388
	5		-	1119	155.498-156.616		132	155.370-155.501
***Bc* 03BB102**	1	chromosome	+	1110	959.479-960.588		138	960.585-960.722
	2		+	1095	996.508-997.602		246	997.602-997.847
	3		-	1101	2.428.174-2.429.274		159	2.428.016-2.428.174
	4		+	1116	3.109.162-3.110.277		162	3.110.277-3.110.438
	5		+	1095	3.344.266-3.345.360		138	3.345.360-3.345.497
***Bc anthracis* CI**	1	chromosome	+	1089	998.747-999.835		204	999.835-1.000.038
	2		-	1101	2.349.164-2.350.264		159	2.349.006-2.349.164
	3		+	1113	3.262.482-3.263.594	*	135	3.263.592-3.263.726
	4		+	1170	4.218.902-4.220.071		123	4.220.068-4.220.190
	5	pCI-XO1	+	1095	172.539-173.633	*	141	173.631-173.771
***Bc* NC7401**	1	chromosome	+	1095	1.820.769-1.821.863	*	144	1.821.861-1.822.004
	2		+	1104	3.016.626-3.017.729		288	3.017.726-3.018.013
	3		+	1095	3.233.393-3.234.487		189	3.234.484-3.234.672
	4	pNCcId	+	1095	259.831-260.925		141	260.922-261.062
***Bc* F837/76**	1	chromosome	+	1110	951.743-952.852		138	952.849-952.986
	2		+	1095	990.203-991.297		330	991.298-991.627
	3		-	1101	2.389.042-2.390.142		159	2.388.884-2.389.042
	4		+	1116	3.040.092-3.041.207		162	3.041.207-3.041.368
	5		+	1095	3.269.648-3.270.742		138	3.270.742-3.270.879

Strains	*rap-phr*	replicon	DNA strand	*rap* genes			*phr* genes	
				size (bp)	Location (bp)		size (bp)	Location (bp)
***Bc* FRI-35**	**1**	chromosome	-	1095	574.271-575.365		297	573.975-574.271
	2		-	1095	2.543.322-2.544.416		123	2.543.203-2.543.325
	3		-	1095	3.488.656-3.489.750		189	3.488.471-3.488.659
	4		+	1092	3.675.432-3.676.523		135	3.676.520-3.676.654
	5		-	1095	3.853.088-3.854.182		147	3.852.942-3.853.088
	6		+	1101	4.337.601-4.338.701		159	4.338.701-4.338.859
	7	p01	-	1095	185.206-186.300		141	185.069-185.209
	8	p02	+	1092	20.184-21.275	*	156	21.276-21.431
***Bc* FT9**	**1**	chromosome	+	1110	1.078.812-1.079.921	*	138	1.079.919-1.080.056
	2		+	1095	2.886.180-2.887.274	*	147	2.887.275-2.887.421
	3		+	1095	3.277.965-3.279.059		189	3.279.056-3.279.244
***Bc* 03BB87**	**1**	chromosome	+	1095	1.336.604-1.337.698		141	1.337.695-1.337.835
	2		+	1098	2.099.185-2.100.282		135	2.100.279-2.100.413
	3		-	1095	4.372.039-4.373.133		147	4.371.896-4.372.042
***Bc* D17**	**1**	chromosome	-	1095	1.134.325-1.135.419		138	1.134.188-1.134.325
	2		-	1095	3.492.590-3.493.684		237	3.492.354-3.492.590
	3		-	1110	3.531.365-3.532.474		138	3.531.231-3.531.368
	4	plasmid	+	1095	93.598-94.692		132	94.689-94.820
***Bc* FM1**	**1**	chromosome	+	1095	1.656.117-1.657.211		135	1.657.208-1.657.342
	2		-	1095	3.975.590-3.976.684		147	3.975.447-3.975.593
***Bc* 3a**	**1**	chromosome	-	1095	269.856-270.950		189	269.671-269.859
	2		-	1116	513.795-514.910		162	513.634-513.795
	3		+	1101	1.255.166-1.256.266		159	1.256.266-1.256.424
	4		-	1089	2.615.418-2.616.506		204	2.615.215-2.615.418
***Bc* G9241**	**1**	chromosome	-	1095	1.460.144-1.461.238		147	1.460.001-1.460.147
	2		+	1098	4.462.142-4.463.239		135	4.463.236-4.463.370
	3	pBCX01	+	1095	110.992-112.086		141	112.083-112.223
***Bc* ATCC 4342**	**1**	chromosome	-	1101	1.621.503-1.622.603		225	1.621.282-1.621.506
	2		-	1095	1.662.995-1.664.089		147	1.662.852-1.662.998
	3		+	1095	4.532.572-4.533.666		201	4.533.666-4.533.866
	4		-	1116	4.765.608-4.766.723		162	4.765.447-4.765.608
***Bc* 03BB108**	**1**	chromosome	-	1101	1.093.224-1.094.324		159	1.093.066-1.093.224
	2		+	1116	1.790.830-1.791.945		162	1.791.945-1.792.106
	3		+	1095	2.034.019-2.035.113		138	2.035.113-2.035.250
	4		+	1110	5.028.270-5.029.379		138	5.029.376-5.029.513
	5		+	1095	5.064.906-5.066.000		330	5.066.001-5.066.330
	6	pBFI_2	+	1095	64.235-65.329		141	65.326-65.466
	7	pBFI_3	-	1095	16.183-17.277		159	16.025-16.183
	8	pBFI_6	-	1092	5.970-7.061		159	5.812-5.970
***Bc* Al Hakam** **(Bp BTZ)**	**1**	chromosome	+	1095	86.170-87.264		123	87.261-87.383
	2		-	1095	1.008.891-1.009.985		135	1.008.760-1.008.894
	3		-	1095	1.274.174-1.275.268		198	1.273.977-1.274.174
	4		-	1098	3.634.368-3.635.465		294	3.634.075-3.634.368
	5		+	1095	4.021.292-4.022.386		207	4.022.386-4.022.592
	6		+	1089	4.198.170-4.199.258		264	4.199.255-4.199.518
	7		+	1071	4.925.955-4.927.025		129	4.927.031-4.927.159
***Bc* S2-8**	**1**	chromosome	-	1095	814.692-815.786		189	814.507-814.695
	2		-	1116	1.058.634-1.059.749		162	1.058.473-1.058.634
	3		+	1101	1.799.972-1.801.072		159	1.801.072-1.801.230
	4		-	1089	3.160.430-3.161.518		204	3.160.227-3.160.430
***Bc* FORC_005**	**1**	chromosome	+	1095	1.004.690-1.005.784		246	1.005.784-1.006.029
	2		-	1095	1.522.889-1.523.983	*	135	1.522.759-1.522.893
	3		+	1101	2.119.586-2.120.686		216	2.120.683-2.120.898
	4		-	1095	3.422.170-3.423.264		132	3.421.984-3.422.115
	5		+	1116	3.451.258-3.452.373		162	3.452.373-3.452.534
***Bt konkukian* 97-27**	**1**	chromosome	+	1095	1.035.581-1.036.675		303	1.036.675-1.036.977
	2		-	1095	1.670.130-1.671.224	*	138	1.669.997-1.670.134
	3		-	1113	2.394.150-2.395.262	*	159	2.393.993-2.394.151
	4		-	1098	3.342.605-3.343.702	*	132	3.342.420-3.342.551
	5		+	1095	4.277.835-4.278.929	*	123	4.278.927-4.279.049

Strains	*rap-phr*	replicon	DNA strand	*rap* genes			*phr* genes	
				size (bp)	Location (bp)		size (bp)	Location (bp)
***Bt* Al Hakam**	1	chromosome	+	1173	1.005.186-1.006.358		138	1.006.355-1.006.492
	2		+	1146	1.043.675-1.044.820		246	1.044.820-1.045.065
	3		-	1113	2.432.384-2.433.496		159	2.432.226-2.432.384
	4		+	1131	3.105.671-3.106.801		162	3.106.801-3.106.962
	5		+	1149	3.340.908-3.342.056		138	3.342.056-3.342.193
***Bt* BMB171**	1	chromosome	+	1095	1.016.761-1.017.855		246	1.017.855-1.018.100
	2		-	1095	1.514.380-1.515.474		135	1.514.249-1.514.383
	3		-	1095	1.695.243-1.696.337		138	1.695.106-1.695.243
	4		+	1074	2.051.994-2.053.067		126	2.053.064-2.053.189
	5		+	1095	3.375.253-3.376.347		210	3.376.347-3.376.556
	6		+	1116	3.394.769-3.395.884		162	3.395.884-3.396.045
	7	pBMB171	-	1095	159.318-160.412		168	159.151-159.318
***Bt finitimus* YBT-020**	1	chromosome	+	1113	1.161.350-1.162.462		132	1.162.459-1.162.590
	2		+	1095	3.347.380-3.348.474		138	3.348.474-3.348.611
	3		+	1095	4.933.546-4.934.640		285	4.934.640-4.934.924
	4	pBMB28	+	1095	34.091-35.185		138	35.182-35.319
***Bt chinensis* CT-43**	1	chromosome	+	1110	1.008.129-1.009.238		138	1.009.235-1.009.372
	2		+	1095	1.048.768-1.049.862		291	1.049.862-1.050.152
	3		-	1095	1.695.987-1.697.081		138	1.695.850-1.695.987
	4		+	1095	3.444.321-3.445.415		189	3.445.412-3.445.600
	5	pCT127	+	1185	106.034-107.218		159	107.218-107.376
	6	pCT281	+	1095	26.485-27.579		138	27.576-27.713
	7		+	1074	68.056-69.129		135	69.129-69.263
	8		+	1092	206.029-207.120		135	207.117-207.251
	9	pCT51	+	1116	27.117-28.232		246	28.232-28.477
	10	pCT6880	-	1056	5.731-6.786	*	141	5.595-5.735
	11	pCT72	+	1113	49.517-50.629		135	50.626-50.760
	12	pCT8513	+	1080	6.352-7.431		147	7.428-7.574
***Bt* HD-771**	1	chromosome	-	1116	398.289-399.404		162	398.128-398.289
	2		+	1095	441.239-442.333		132	442.388-442.519
	3		+	1101	1.456.930-1.458.030		159	1.458.030-1.458.188
	4		-	1074	1.787.304-1.788.377		126	1.787.182-1.787.307
	5		-	1095	3.073.068-3.074.162		225	3.072.844-3.073.068
	6	p01	-	1095	73.603-74.697		135	73.472-73.606
	7	p03	+	1101	35.763-36.863		246	36.860-37.105
	8	p04	+	1113	31.211-32.323		135	32.320-32.454
	9	p02	-	1095	55.822-56.916		141	55.685-55.825
***Bt* HD-789**	1	chromosome	+	1095	578.988-580.082		201	580.082-580.282
	2		-	1095	1.238.979-1.240.073		138	1.238.842-1.238.979
	3		+	1074	1.600.813-1.601.886		126	1.601.883-1.602.008
	4		-	1101	1.924.381-1.925.481		159	1.924.223-1.924.381
	5		-	1098	2.883.557-2.884.654		132	2.883.371-2.883.502
	6		+	1116	2.926.522-2.927.637		162	2.927.637-2.927.798
	7	p02	+	1098	144.773-145.870		150	145.870-146.019
***Bt* MC 28**	1	chromosome	-	1095	217.830-218.924		159	217.672-217.830
	2		+	1095	247.902-248.996		183	248.996-249.178
	3		+	1095	2.598.048-2.599.142		189	2.599.139-2.599.327
	4	pMC183	-	1089	135.705-136.793		135	135.571-135.705
	5	pMC429	-	1113	20.163-21.275		159	20.005-20.163
	6		-	1131	36.737-37.867		162	36.576-36.737
	7		-	1095	346.932-348.026		129	346.807-346.935
	8	pMC54	+	1137	24.448-25.584		246	25.581-25.826
***Bt* 407**	1	chromosome	+	1110	1.009.952-1.011.061		138	1.011.058-1.011.195
	2		+	1095	1.050.589-1.051.683		291	1.051.683-1.051.973
	3		-	1095	1.703.065-1.704.159		138	1.702.928-1.703.065
	4		+	1095	3.458.130-3.459.224		189	3.459.221-3.459.409
	5	BTB_9p	+	1080	3.453-4.532		147	4.529-4.675
	6	BTB_502p	+	1095	251.328-252.422		150	252.422-252.571
	7	BTB_78p	-	1113	11.208-12.320		135	11.077-11.211
	8	BTB_6p	-	1095	3.566-4.660	*	141	3.430-3.570

Strains	*rap-phr*	replicon	DNA strand	*rap* genes			*phr* genes	
				size (bp)	Location (bp)		size (bp)	Location (bp)
***Bt kurstaki* HD-73**	1	chromosome	+	1089	1.159.766-1.160.854		192	1.160.854-1.161.045
	2		+	1074	2.283.515-2.284.588		126	2.284.585-2.284.710
	3		-	1095	3.591.157-3.592.251		132	3.590.971-3.591.102
	4		+	1116	3.626.757-3.627.872		162	3.627.872-3.628.033
	5		-	1116	5.238.153-5.239.268		219	5.237.935-5.238.153
	6	pAW63	+	1074	52.289-53.362		135	53.362-53.496
	7	pHT77	+	1101	55.858-56.958		159	56.958-57.116
	8	pHT8_1	+	1080	1.566-2.645		147	2.642-2.788
***Bt thuringiensis* IS 5056**	1	chromosome	+	1110	1.005.250-1.006.359		138	1.006.356-1.006.493
	2		+	1095	1.045.887-1.046.981		201	1.046.981-1.047.181
	3		-	1095	1.702.539-1.703.633		138	1.702.402-1.702.539
	4		+	1095	3.449.075-3.450.169		189	3.450.166-3.450.354
	5	pIS56-39	-	1116	15.764-16.879		246	15.519-15.764
	6	pIS56-107	+	1101	81.226-82.326		159	82.326-82.484
	7	pIS56-233	+	1101	11.065-12.165		159	12.165-12.323
	8		+	1095	112.091-113.185		129	113.182-113.310
	9	pIS56-16	-	1149	3.106-4.254		141	2.964-3.104
	10	pIS56-285	+	1095	30.707-31.801		138	31.798-31.935
	11		+	1074	72.279-73.352		135	73.352-73.486
	12		+	1092	210.255-211.346		135	211.343-211.477
	13	pIS56-328	+	1092	18.946-20.037		135	20.034-20.168
	14		-	1116	64.081-65.196		162	63.920-64.081
	15	pIS56-6	+	1095	3.502-4.596		141	4.593-4.733
	16	pIS56-68	-	1113	16.944-18.056		135	16.813-16.947
***Bt* YBT-1518**	1	chromosome	+	1110	1.228.754-1.229.863		138	1.229.860-1.229.997
	2		+	1095	1.266.722-1.267.816		246	1.267.816-1.268.061
	3		-	1095	1.966.382-1.967.476		138	1.966.245-1.966.382
	4		+	1095	3.752.095-3.753.189		189	3.753.186-3.753.374
***Bt kurstaki* YBT-1520**	1	chromosome	+	1089	1.160.683-1.161.771		192	1.161.771-1.161.962
	2		+	1074	2.244.009-2.245.082		126	2.245.079-2.245.204
	3		-	1095	3.548.026-3.549.120		132	3.547.840-3.547.971
	4		+	1116	3.583.627-3.584.742		162	3.584.742-3.584.903
	5	pBMB293	+	1074	33.358-34.431		135	34.431-34.565
	6		+	1092	171.195-172.286		135	172.283-172.417
	7		+	1095	284.928-286.022		138	286.019-286.156
	8	pBMB422	-	1095	125.155-126.249		132	125.027-125.158
	9		+	1092	224.207-225.298	*	132	225.296-225.427
	10		-	1074	242.914-243.987		126	242.792-242.917
	11		-	1092	260.849-261.940		159	260.691-260.849
	12		+	1095	331.871-332.965		129	332.962-333.090
	13	pBMB67	+	1113	10.900-12.012		135	12.009-12.143
	14	pBMB8513	+	1080	7.058-8.137		147	8.134-8.280
	15	pBMB94	+	1101	59.096-60.196		159	60.196-60.354
***Bt kurstaki* HD-1**	1	chromosome	+	1089	1.171.581-1.172.669		192	1.172.669-1.172.860
	2		-	1095	3.578.614-3.579.708		132	3.578.428-3.578.559
	3		+	1116	3.608.764-3.609.879		162	3.609.879-3.610.040
	4		-	1116	5.232.494-5.233.609		219	5.232.276-5.232.494
	5	pBMB299	+	1074	32.488-33.561		135	33.561-33.695
	6		+	1092	173.756-174.847		135	174.844-174.978
	7		+	1095	290.350-291.444		138	291.441-291.578
	8	pBMB431	-	1095	105.363-106.457		132	105.235-105.366
	9		+	1092	204.410-205.501	*	132	205.499-205.630
	10		-	1092	241.056-242.147		159	240.898-241.056
	11		+	1095	322.926-324.020		129	324.017-324.145
	12	pBMB64	+	1113	46.384-47.496		135	47.493-47.627
	13	pBMB74	+	1116	64.696-65.811		129	65.811-65.939
	14	pBMB8513	+	1080	5.407-6.486		147	6.483-6.629
	15	pBMB95	+	1101	47.062-48.162		159	48.162-48.320
***Bt galleriae* HD-29**	1	chromosome	+	1089	1.246.958-1.248.046		192	1.248.046-1.248.237
	2		+	1074	2.251.547-2.252.620		126	2.252.617-2.252.742

Strains	*rap-phr*	replicon	DNA strand	*rap* genes			*phr* genes	
				size (bp)	Location (bp)		size (bp)	Location (bp)
	3		-	1095	3.538.921-3.540.015		132	3.538.735-3.538.866
	4		+	1116	3.570.883-3.571.998		162	3.571.998-3.572.159
	5		-	1116	5.206.194-5.207.309		219	5.205.976-5.206.194
	6	pBMB267	+	1074	27.631-28.704	*	135	28.705-28.839
	7		+	1092	184.074-185.165	*	135	185.163-185.297
	8		-	1101	77.440-78.540		159	77.282-77.440
	9	pBMB426	-	1089	122.752-123.840	*	132	122.620-122.751
	10		+	1095	326.101-327.195		129	327.192-327.320
	11	pBMB126	+	1101	11.663-12.763	*	159	12.764-12.922
	12	pBMB71	+	1113	41.451-42.563		135	42.560-42.694
***Bt* HD1011**	1		+	1089	2.180.383-2.181.471		204	2.181.471-2.181.674
	2	chromosome	-	1101	3.566.815-3.567.915		159	3.566.657-3.566.815
	3		+	1116	4.280.640-4.281.755		162	4.281.755-4.281.916
	4		+	1098	4.516.228-4.517.325		189	4.517.328-4.517.516
***Bt* HD571**	1		-	1101	171.412-172.512		159	171.254-171.412
	2		+	1116	844.716-845.831		162	845.831-845.992
	3	chromosome	+	1095	1.079.992-1.081.086		138	1.081.086-1.081.223
	4		+	1110	4.000.508-4.001.617		138	4.001.614-4.001.751
	5		+	1095	4.038.985-4.040.079		246	4.040.079-4.040.324
***Bt* HD682**	1		-	1095	1.466.465-1.467.559		189	1.466.280-1.466.468
	2	chromosome	-	1116	1.705.845-1.706.960		162	1.705.684-1.705.845
	3		+	1101	2.422.702-2.423.802		159	2.423.802-2.423.960
***Bt* HD1002**	1		-	1116	488.251-489.366		162	488.090-488.251
	2		+	1089	531.234-532.331		132	532.386-532.517
	3		+	1101	1.489.738-1.490.838		159	1.490.838-1.490.996
	4	chromosome	-	1074	1.813.773-1.814.846		126	1.813.651-1.813.776
	5		+	1095	2.175.723-2.176.817		138	2.176.817-2.176.954
	6		-	1095	2.836.570-2.837.664		201	2.836.370-2.836.570
	7	plasmid 3	+	1098	60.748-61.845		150	61.845-61.994
***Bt morrisoni* BGSC 4AA1**	1		+	1074	2.142.635-2.143.708		126	2.143.705-2.143.830
	2		-	1101	2.497.875-2.498.975		159	2.497.717-2.497.875
	3	chromosome	-	1095	3.488.313-3.489.407		132	3.488.127-3.488.258
	4		+	1116	3.531.255-3.532.370		162	3.532.370-3.532.531
	5	pBMB232	-	1095	183.115-184.209	*	141	182.979-183.119
	6		+	1074	229.038-230.111	*	135	230.112-230.246
	7	pBMB68	+	1107	57.098-58.204		135	58.206-58.340
***Ba* 'Ames Ancestor'**	1		+	1113	1.009.329-1.010.441		132	1.010.438-1.010.569
	2		-	1101	2.345.742-2.346.842		159	2.345.584-2.345.742
	3	chromosome	+	1116	3.021.381-3.022.496		162	3.022.496-3.022.657
	4		-	1080	3.457.189-3.458.268		150	3.457.043-3.457.192
	5	pXO1	+	1095	172.308-173.402	*	141	173.400-173.540
***B. weihenstephanensis* KBAB4**	1	chromosome	+	1095	1.015.638-1.016.732		246	1.016.732-1.016.977
	2		-	1113	3.270.543-3.271.655		192	3.270.352-3.270.543
	3		-	1095	70.268-71.362	*	129	70.144-70.272
	4	pBWB401	-	1101	95.773-96.873		159	95.615-95.773
	5		-	1101	342.867-343.967		159	342.709-342.867
***B. mycoides* ATCC 6462**	1		-	1104	2.694.691-2.695.794		288	2.694.407-2.694.694
	2	chromosome	-	1095	4.694.615-4.695.709		237	4.694.379-4.694.615
	3		+	1095	5.217.111-5.218.205		267	5.218.205-5.218.471
	4	pBMX_1	-	1095	320.291-321.385		327	319.965-320.291
***B. pseudomycoides* DSM 12442**	1		+	1095	929.301-930.395		198	930.395-930.592
	2		+	1095	1.176.425-1.177.519		126	1.177.525-1.177.650
	3		+	1083	2.343.042-2.344.124		129	2.344.130-2.344.258
	4		+	1089	2.949.185-2.950.273		261	2.950.273-2.950.533
	5	chromosome	-	1032	3.117.170-3.118.201		207	3.116.964-3.117.170
	6		-	1098	3.540.792-3.541.889		294	3.540.499-3.540.792
	7		-	1095	4.775.718-4.776.812	*	123	4.775.600-4.775.722
	8		-	1095	5.593.711-5.594.805	*	150	5.593.566-5.593.715
***B. cytotoxicus* NVH 391-98**	1	chromosome	-	1095	524.265-525.359	*	135	524.135-524.269
	2		+	1107	855.631-856.737	*	135	856.735-856.869

Strains	*rap-phr*	replicon	DNA strand	*rap* genes			*phr* genes	
				size (bp)	Location (bp)		size (bp)	Location (bp)
	3		+	1113	1.095.291-1.096.403	*	150	1.096.401-1.096.550
	4		-	1095	1.108.182-1.109.276	*	129	1.108.058-1.108.186
	5		-	1086	2.034.056-2.035.141		150	2.033.910-2.034.059
	6		+	1056	2.376.098-2.377.153		141	2.377.150-2.377.290
	7		+	1137	3.794.252-3.795.388		246	3.795.385-3.795.630
	8		+	1095	4.047.541-4.048.635		225	4.048.632-4.048.856

Phr genes with an asterisk were not annotated as ORFs in NCBI. Bc: *B. cereus*; Bt: *B. thuringiensis*; Ba: *B. anthracis*.

Table 5. Consensus of the key residues involved in the sporulation activity of the Rap proteins already described to act as a phosphatase over Spo0F. Profile of chromosomal and plasmid Rap proteins of the *B. cereus* group. Orange patterns are predicted as Spo+ and green patterns as Spo-; residues divergent from the consensus are typed in white. Residues typed in green do not differ from the consensus but are not found in this exact order in Rap proteins validated as Spo+, as consequence, Rap proteins with these residues were predicted as Spo-. The number of proteins is the sum of Rap corresponding to each profile in the group.

Rap proteins		Amino acid residues									Nb of proteins	% in the group	Tested Rap
Sequence position		45	46	47	50	58	96	134	137	175			
Rap H		**E**	**D**	**Q**	**L**	**F**	**L**	**D**	**E**	**Y**			
RapA/RapQ		E	D	Q	L	F	L	D	E	Y			
RapE		E	D	Q	L	F	M	D	E	Y			
RapBXA0205		E	D	Q	L	F	F	D	E	Y			
Rap8		E	D	Q	L	L	F	D	E	Y			
RapB		E	D	Q	L	Y	L	D	E	Y			
Rap60		E	N	Q	L	F	M	D	E	Y			
RapJ		E	N	Q	L	F	L	D	E	Y			
RapBA3790		Q	D	Q	L	F	F	D	E	Y			
Consensus Spo+		**E, Q**	**D, N**	**Q**	**L**	**F, L, Y**	**L, F, M**	**D**	**E**	**Y**			
Bc group - Chromosome Rap	Group 1	V	D	Q	L	F	V	D	E	Y	10	100%	
		V	D	Q	L	F	F	D	E	Y	8		
		V	D	Q	L	F	L	N	E	Y	1		
		V	D	Q	S	F	F	H	E	C	4		
		L	D	E	L	F	Y	N	E	C	2		
		E	D	Q	A	F	L	S	E	Y	4		
		E	E	K	S	F	V	N	E	Y	1		
	Group 2	T	D	Q	S	F	F	D	E	Y	13	100%	
		T	D	Q	L	F	F	D	E	Y	1		
		A	D	H	S	F	F	D	E	Y	5		
		T	N	Q	S	F	F	D	E	Y	1		
		Q	D	Q	L	F	Y	D	E	Y	3	16%	5-BtHD73
	Group 3	E	D	K	L	F	F	N	E	Y	3	84%	
		E	D	K	L	F	F	A	E	Y	1		
		E	D	T	Q	F	L	D	E	C	1		
		E	D	E	L	F	F	T	E	Y	2		
		E	D	E	S	F	F	N	E	Y	2		
		K	D	T	L	F	C	E	E	Y	7		
	Group 4	S	D	V	S	F	S	D	E	Y	7	100%	
		S	D	I	S	F	S	D	E	Y	5		
		G	D	I	L	F	L	D	E	H	1		
	Group 5	E	D	E	A	F	P	D	E	Y	5	100%	
		E	D	E	A	F	P	D	E	F	2		
		E	D	E	A	F	Q	D	E	Y	9		
		E	D	G	W	F	R	D	E	Q	3		
		E	D	G	W	F	R	E	E	Q	1		
		K	D	Q	L	F	T	D	E	H	1		
		K	D	R	L	F	T	D	E	H	2		
		E	D	P	L	F	S	D	E	Y	1		
		L	N	S	H	F	Q	S	E	C	2		

Rap proteins		Amino acid residues									Nb of proteins	% in the group	Tested Rap
		T	I	P	Q	F	H	S	E	C	2		
		T	F	P	Y	F	H	S	E	C	1		
		Q	D	Q	L	F	F	D	E	Y	25	68%	
		E	N	Q	L	L	L	D	E	Y	1		1-Bc14579
	Group 6	E	D	Q	L	F	L	N	E	Y	2		
		A	D	Q	S	F	D	G	E	Y	8	32%	
		E	N	Q	L	F	F	N	E	C	1		
		Q	N	Q	L	F	L	D	E	Y	1		
		E	D	Q	L	F	L	D	E	Y	5		
		E	D	Q	L	L	L	D	E	Y	1	32%	
		E	N	Q	L	F	L	D	E	Y	1		
		Q	D	Q	L	F	L	D	E	Y	2		2-Bc14579
		E	D	Q	L	F	L	D	E	C	1		
		E	D	Q	F	F	L	D	E	Y	1		
		D	N	S	L	F	L	D	E	N	2		
	Group 7	A	D	S	L	F	L	D	E	Y	5		
		E	D	K	L	F	L	D	E	Y	1	68%	
		D	D	K	L	F	L	D	E	Y	3		
		N	N	P	Q	F	L	D	E	F	3		
		Q	K	Q	L	F	L	D	E	Y	1		
		P	D	Q	L	F	L	D	E	Y	1		
		Q	N	Q	L	F	L	D	E	Y	1		
		Q	N	Q	L	Y	Y	N	E	Y	1		
	Group 8	Q	E	Q	L	F	F	E	E	Y	1	100%	
		Q	D	Q	L	Y	Y	N	E	Y	3		
	Group 9	E	D	S	S	F	F	D	E	Y	2	100%	
		E	D	Q	L	A	F	D	E	Y	10		
	Group 10	E	C	D	L	W	R	D	E	F	3	100%	
	Group 11	E	N	T	F	F	L	D	E	Y	1	100%	
		K	H	T	F	F	F	N	E	Y	1		
	Group 12	E	N	Q	T	F	S	D	E	Y	8	100%	
		E	D	R	T	F	S	D	E	Y	4		
Bc group - Plasmid Rap		E	D	Q	L	F	F	D	E	Y	11		
	Group 1	E	D	Q	L	F	L	D	E	Y	4	95%	8-Bt407
		E	N	Q	L	F	L	D	E	Y	6		
		N	D	Q	L	F	M	D	E	Y	1	5%	
		E	D	Q	L	F	F	D	E	Y	9	65%	8-BtHD1
		Q	D	Q	L	F	F	D	E	Y	8		7-Bt407
	Group 2	E	D	Q	L	F	T	D	E	Y	2		
		K	D	Q	L	F	T	D	E	Y	5	35%	6-BtHD1
		S	D	Q	H	F	I	D	E	H	2		
		Q	D	Q	L	F	F	D	E	Y	2	40%	
	Group 3	Q	N	Q	L	F	L	D	E	Y	1		
		Q	H	Q	L	F	L	D	E	Y	1	60%	
		D	D	K	L	F	L	D	E	C	1		
	Group 4	L	S	E	Q	F	V	D	E	N	1	100%	6-Bt407
	Group 5	K	D	S	L	F	T	D	E	Y	1	100%	
	Group 6	Q	D	Q	L	F	M	D	E	Y	2	100%	
		E	N	Q	L	F	F	D	E	Y	3	18%	10-BtHD1
	Group 7	Q	D	Q	L	F	Y	D	E	Y	2		
		K	D	E	S	F	F	N	E	Y	1	82%	
		T	D	P	S	F	F	D	E	Y	1		

Rap proteins	Amino acid residues									Nb of proteins	% in the group	Tested Rap
	T	D	P	S	F	F	N	E	Y	1		
	T	D	Q	S	F	F	D	E	Y	9		7-BtHD3
Group 8	Q	D	Q	L	Y	Y	N	E	Y	2	100%	
Group 9	E	D	Q	L	F	F	D	E	Y	8		
	E	D	Q	L	L	F	D	E	Y	5	93%	
	E	N	Q	L	F	L	D	E	Y	1		
	E	D	Q	L	A	F	D	E	Y	1	7%	

Rap proteins used to construct the consensus: RapA, RapB, RapE, RapH, RapJ, and Rap60 from *B. subtilis* (Koetje et al. 2003; Parashar et al. 2011); RapQ from *B. amyloliquefaciens* (Yang et al. 2015); RapBXA0205 and RapBA3790 from *B. anthracis* (Bongiorni et al. 2006); and Rap8 from *B. thuringiensis* (Fazion et al. 2018).

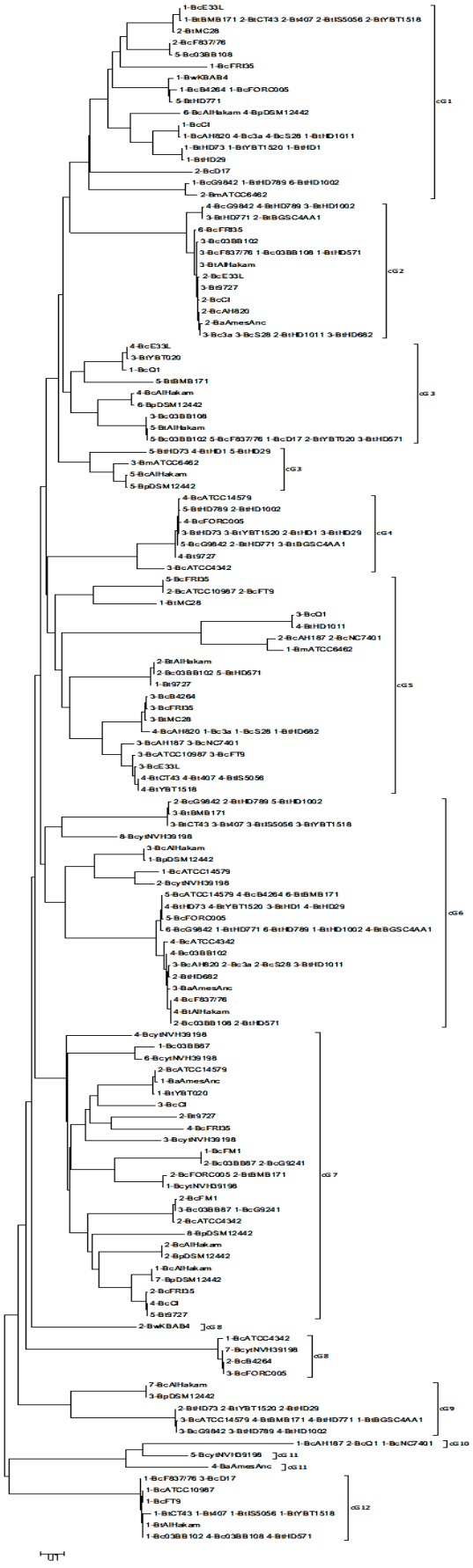
Table 6. Efficiency of sporulation of strains expressing *rap* genes.

Strains	Viable cells	Heat-resistant spores	% spores	Prediction
315xyl	1.21E+08 (±7.58E+07)	1.21E+08 (±5.23E+07)	95 (±10)	-
Rap6-HD1	1.21E+08 (±4.54E+07)	1.30E+08 (±5.01E+07)	93 (±10)	Spo-
Rap8-HD1	1.41E+08 (±6.55E+07)	1.57E+08 (±4.62E+07)	99 (±2)	Spo+
Rap10-HD1	7.13E+07 (±1.20E+07)	1.33E+05 (±3.51E+04)	0.2 (±0.04)	Spo+
Rap6-Bt407	1.45E+08 (±7.35E+07)	1.47E+08 (±4.58E+07)	91 (±9)	Spo-
Rap7-Bt407	1.01E+08 (±1.04E+07)	1.59E+05 (±1.26E+04)	0.2 (±0.02)	Spo+
Rap8-Bt407	6.13E+07 (±2.10E+07)	7.15E+06 (±1.56E+06)	13 (±8)	Spo+
Rap5-Bt HD73	4.03E+07 (±6.48E+06)	2.07E+05 (±1.26E+05)	0.51 (±0.3)	Spo+
Rap7-Bt HD73	3.08E+08 (±2.93E+07)	2.35E+08 (±2.25E+07)	77 (±3.7)	Spo-
Rap1-BcATCC14579	3.92E+08 (±4.73E+07)	2.80E+08 (±3.28E+07)	71.5 (±2)	Spo+
Rap2-BcATCC14579	3.51E+08 (±3.89E+07)	2.47E+08 (±9.29E+06)	70.8 (±6)	Spo+

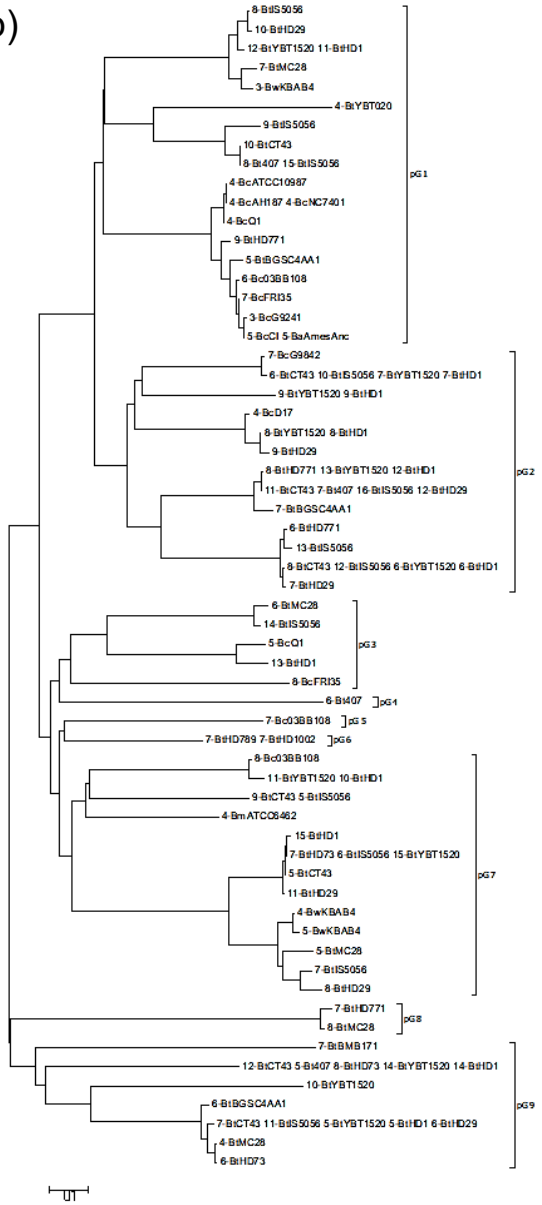
The viable cells and heat-resistant spores were counted after 48 h in HCT medium at 30°C. Results are given as mean ± standard error of the mean (SEM). The percentages of spores were calculated as 100 × the ratio between heat-resistant spores per milliliter and total viable cells per milliliter. The data were analyzed by using One-way Analysis of Variance (ANOVA) followed by Tukey-Kramer Multiple Comparisons Test - * ($P < 0.01$) and ** ($P < 0.001$): % of spores is statistically different from control (315xyl).

Fig. 1 Rap protein sequence groups (refer to Fig. 2): a) Chromosomal Rap sequences distributed within 12 groups; b) Plasmid Rap sequences distributed within nine groups. Phylogenetic trees were inferred by Neighbor-Joining method and the evolutionary distances were computed using the JTT matrix-based method and are in the units of the number of amino acid substitutions per site. Evolutionary analyses were conducted in MEGA6.

a)



b)



4 ARTICLE 2

Rap-Phr systems from pAW63 and pHT8-1 plasmids act synergistically to regulate sporulation in the *Bacillus thuringiensis kurstaki* HD73 strain

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Abstract

Rap-Phr quorum sensing systems regulate different bacterial processes, notably the commitment to sporulation in *Bacillus* species. Rap proteins act as phosphatases on Spo0F, intermediate of the sporulation phosphorelay, and are inhibited by Phr peptides that function as quorum sensors. *Bacillus thuringiensis* is a spore-forming Gram positive bacterium that is pathogenic to larvae of diverse insect species. *B. thuringiensis* contains a remarkable amount of extra-chromosome DNA molecules and a great number of plasmid *rap-phr* genes. In this study, we describe the Rap63-Phr63 system from the *B. thuringiensis* var. *kurstaki* HD73 strain, encoded on the pAW63 plasmid. Rap63 have a moderate activity on sporulation that is inhibited by the Phr63 whose the mature form is included in the C-terminal end of its precursor. The two components of this signaling cassette are co-transcribed and the *phr63* gene is also transcribed by an additional promoter depending partially of the σH factor. Interestingly, we show that the ΔPhr8ΔPhr63 mutant strain strongly prevent sporulation in the insect larvae. Despite the Phr8 and Phr63 similarities, there is no cross-talk between the Rap63-Phr63 and Rap8-Phr8 systems. Our results suggest a synergistic activity of these two Rap-Phr systems in the regulation of the sporulation process ensuring the survival and the dissemination of the *B. thuringiensis* at the end of the infectious cycle. Our results also pointed out the relevance of the plasmid network to the fitness of *B. thuringiensis* bacteria.

Introduction

Bacillus thuringiensis belongs to the *Bacillus cereus* group of Gram positive rod-shaped spore-forming bacteria and distinguishes from the other seven closely related species of this group by the production of a crystal inclusion that is toxic to larvae of various insects (Vilas-Bôas et al., 2007). Due to its entomopathogenic properties, *B. thuringiensis* is widely used as biopesticide to control agricultural pests or human disease vectors (Schnepf et al., 1998). Crystal inclusions are encoded by *cry* (and *cyt*) genes mainly harbored on plasmids and the production of Cry toxins are generally regulated by sporulation sigma factors (Deng et al., 2014). *B. thuringiensis* strains were shown to carry a complex pattern of plasmids, up to 17 different extrachromosomal elements with different size, from a cryptic plasmid of 2Kb to

megaplasmids of 600Kb (Cardoso et al., in press; Lereclus et al., 1982; Reyes-Ramirez & Ibarra, 2008; Zhong et al., 2011). As result of its biotechnological relevance, plasmids encoding *cry* genes are the most studied. Some of the other plasmids have also been investigated because of their conjugation properties, such as pAW63 (Van der Auwera & Mahillon, 2008; Wilcks et al., 1998) and pXO16 (Jensen et al., 1995; Makart et al., 2015) or for the role of plasmid-borne Rap-Phr system, as pHT8_1 (Fazion et al., 2018).

B. thuringiensis plasmids carry a great amount of Rap-Phr systems with 38% of its plasmids harboring at least one Rap-Phr system (Cardoso et al., in press). Rap-Phr quorum sensing systems were first described and extensively studied in *B. subtilis* (Perego, 2013; Perego et al., 1994). In this species, they regulate various processes such as sporulation, competence, transfer of mobile genetic elements, production of proteases and biofilm formation (Perego, 2013). Firstly identified on the chromosome, *rap-phr* genes were also described to be carried on plasmids of *Bacillus* species (Bongiorni et al., 2006; Fazion et al., 2018; Koetje et al., 2003; Parashar et al., 2013; Qiao et al., 2011; Singh et al., 2013).

Rap-Phr systems belong to the RNPP family of quorum sensing systems from Gram positive bacteria, which consist in a response regulator with tetratricopeptide repeat (TPR) domains and a signaling oligopeptide that modulates the regulator activities (Declerck et al., 2007). The infectious cycle of *B. thuringiensis* in the insect is regulated by the sequential activation of these RNPP systems (Slamti et al., 2014). Firstly, PlcR-PapR regulates the virulence stage (Agaisse et al., 1999); next, NprR-NprX controls the necrotrophic stage and the transition to sporulation (Dubois et al., 2012; Perchat et al., 2016); finally, Rap-Phr also controls the initiation of the sporulation process (Fazion et al., 2018).

Commitment to sporulation is regulated by the phosphorylation state of the major response regulator Spo0A (Molle et al., 2003; Sonenshein, 2000). Different signals, such as nutritional deprivation, are recognized by sporulation kinases which are able to autophosphorylate (Jiang et al., 2000b). Next, kinases phosphorylate Spo0F, which is used as substrate by the phosphotransferase Spo0B to phosphorylate Spo0A (Burbulys et al., 1991). Response regulator aspartate phosphatases (Rap) are able to hamper this signal transduction pathway by dephosphorylating the Spo0F response regulator (Perego et al., 1994). Rap protein

activity is counteract by its cognate Phr peptide, which is translated in a premature form that needs to be secreted, processed and reimported by oligopeptide permeases to be active (Perego & Hoch, 1996; Pottathil & Lazazzera, 2003).

Rap-Phr systems are largely found in bacteria of the *B. cereus* group, with a relevant amount in *B. thuringiensis* plasmids, whose systems were rather predicted to have an effect on sporulation (Cardoso et al., in press; Even-Tov et al., 2016). The pXO1 pathogenicity-plasmid from *B. anthracis* (Bongiorni et al., 2006) and the pHT8_1 cryptic plasmid of *B. thuringiensis* HD73 (Fazion et al., 2018) carry a Rap-Phr system that regulate the sporulation process. Moreover, Fazion et al. (2018) demonstrated that the Rap8-Phr8 system control the sporulation of *B. thuringiensis* in its ecological niche, the insect larvae. Sequencing of the plasmid pAW63 (Van der Auwera et al., 2005), also revealed the presence of *rap-phr* alleles. pAW63 and pHT8_1 are found in the *B. thuringiensis* serovar *kurstaki* HD73 strain with another five plasmids (Liu et al., 2013), among them the pHT77 also harbors a Rap-Phr system which does not have a role in sporulation (Cardoso et al., in press).

pAW63 is a theta-replicating conjugative plasmid of 72Kb (Wilcks et al., 1998, 1999) that efficiently conjugates and mobilizes non-conjugative plasmids even in food matrices (Modrie et al., 2010; Van der Auwera et al., 2007) and in adverse conditions (Beuls et al., 2009, 2012). pAW63 also harbors a toxin-antitoxin system that promotes plasmid stabilization during vegetative growth or sporulation (Short et al., 2015), and two Group II introns: B.th.I1 and B.th.I2 (Van der Auwera et al., 2005). These RNA based mobile elements were found to interrupt genes of the transfer module. Furthermore, pAW63 shares a common backbone with the second pathogenic plasmid of *B. anthracis* pXO2 and with pBT9727 from the pathogenic *B. thuringiensis* serovar *konkukian* strain 97-27, including replication and transfer modules (Van der Auwera et al., 2005).

In this study, we characterized the Rap63-Phr63 quorum sensing system from the pAW63 plasmid. Although this system presents a moderate effect on sporulation, we show a synergistic sporulation effect of this system together with the Rap8-Phr8 from the pHT8_1 *in vitro* and *in vivo*. However, no cross-talk between these close related Rap-Phr systems was detected in this study, revealing a high specificity of the Phr peptides for their cognate Rap proteins. These results reinforce the relevance of plasmid-borne Rap-Phr systems to bacteria development in its naturalistic conditions.

Materials and Methods

Bacterial strains and growth conditions

All strains used in this study derived from the *B. thuringiensis* var. *kurstaki* HD73 acrystalliferous (Cry-, cured of the pHT73 plasmid) strain (Wilcks et al., 1998). *Escherichia coli* K-12 strains TG1 was used as host strain for plasmid construction. *E. coli* strain ET12567 (Dam- Dcm-) was used to prepare plasmids to transform *B. thuringiensis* strains by electroporation (Lereclus et al., 1989). *E. coli* strains were transformed by thermal shock and were cultivated in Luria Bertani (LB) medium at 37°C. *B. thuringiensis* strains were grown in LB or in the sporulation-specific medium HCT (Lecadet et al., 1980; Lereclus et al., 1982) at 30°C or 37°C. Liquid cultures were maintained with shaking at 175 r.p.m. For bacterial selection, the antibiotics were used at the following concentrations: ampicillin 100 µg/ml and spectinomycin 50 µg/ml for *E. coli* and erythromycin 10 µg/ml, spectinomycin 200 µg/ml and kanamycin 200 µg/ml for *B. thuringiensis*. LB plates with 100 µg/ml of X-gal were used for colorimetric screening during pMAD mutagenesis. When required, xylose was used at a concentration of 20 mM.

DNA manipulations

Genomic DNA from *B. thuringiensis* strains was extracted using the Puregene Yeast/Bact. Kit B (Qiagen, France) and plasmid DNA from *E. coli* was extracted with the QIAprep Spin Miniprep Kit (Qiagen, France). Phusion High-Fidelity DNA polymerase, Standard Taq DNA Polymerase, restriction enzymes and T4 DNA ligase were used following the manufacturer's recommendations (New England Biolabs, USA). PCRs were performed in an Applied Biosystems 2720 Thermal cycler using oligonucleotides listed in Table S1, synthesized by Eurofins Genomics (Germany). The amplified DNA fragments were purified using QIAquick PCR Purification Kit (Qiagen, France) and the QIAquick Gel Extraction Kit (Qiagen, France) was used to purify digested DNA fragments separated on 1% agarose gels. All constructs were verified by DNA sequencing (GATC Biotech, Germany).

Plasmid and strains constructions

The plasmid pHT315-P*xylA* (Grandvalet et al. 2001), a multi-copy vector with xylose-inducible promoter, was used to express *rap63* and/or *phr63*. Promoter regions of *rap63* or *phr63* genes were inserted in the plasmid pHT304.18'*yfp* (Fagerlund et al., 2014) for evaluation of their expression. The plasmid pMAD (Arnaud et al., 2004) was used for homologous recombination.

The *rap63-phr63* or *phr63* genes were disrupted in the wild-type HD73- strain and on its derivative HD73 Δ*rap8-phr8* or Δ*phr8* (Fazion et al., 2018) by using pMADΩ*rap63-phr63*::*specR* and pMADΩ*phr63*::*specR*. These genes were replaced by the spectinomycin resistance gene with its own promoter by homologous recombination (Lereclus & Arantes, 1992). The recombinant strains were Lac⁻, erythromycin sensible and spectinomycin resistant. All the constructed plasmids used in this study are described in Table S2.

RT-PCR experiment

This experiment was performed as described by Fazion et al. (2018), concerning growth conditions, RNA extraction and reverse transcriptase reaction. Three different fragments were amplified by PCR reactions with oligonucleotides PromRapF7557 and RT7557-2 for the *rap63* gene and its upstream region, RT7557-3 and RT7557-4 for the *rap63* and *phr63* genes and RT7557-5 and RT7557-7 for the *phr63* gene and its downstream region. Sequences of the oligonucleotides are given in Table S1.

Synthetic oligopeptides

Several Phr peptides, corresponding to the C-terminal end of the *phr63* gene product, were synthesized, purified and identified by mass spectrophotometry by GenScript (USA). To determine the active oligopeptide, the synthetic peptides were tested in sporulation assays (see below) at final concentrations of 50 µM.

In vitro sporulation assays

In vitro sporulation efficiency tests were carried in the sporulation-specific medium HCT. *B. thuringiensis* strains were grown at 30°C for 48h and serial dilutions were plated before and after heat treatment (12 min at 80°C). When required, xylose and

synthetic peptides were added to the culture at the beginning of stationary growth phase (t0). The sporulation percentage was calculated as 100 × the ratio between heat-resistant spores per milliliter and total viable cells per milliliter. Experiments and plating were done at least in triplicate and mean values were calculated. Results were analyzed by statistical test ANOVA followed by Tukey ($p<0.05$).

In vivo sporulation assays

Experiments with insect larvae were performed as described in Fazion et al. (2018). Essentially, larvae of *Galleria mellonella* were infected with intrahemocoelic injection of vegetative bacteria and were maintained at 30°C. Dead larvae were crushed after 96h of treatment and diluted in 0.9% NaCl solution. Sporulation efficiencies of the harvested *B. thuringiensis* cells were calculated as described for the *in vitro* assays. Results were statistically analyzed by unpaired t test with Welch correction ($p<0.05$).

Fluorescence analysis

Yfp fluorescence produced from different promoters regions was measured from *B. thuringiensis* strains cultures grown in HCT medium at 37°C. Cells were harvest at determined time points and were fixed as described by Fagerlund et al. (2014). At the first sample time, cultures were supplemented with xylose, if necessary. Fixed cells were kept at 4°C until analysis. Samples were distributed into a 96 wells black micro plate of polystyrene (Greiner) and measured at an Infinite 200 Pro micro plate reader device (TECAN, Switzerland), applying an excitation wavelength of 485 nm and recovered at an emission wavelength of 535 nm. Data were recovered by Tecan i-control software (TECAN, Switzerland), and each time point were expressed as the arbitrary unit per DO_{600}. Strain harboring plasmid pHT304.18'*yfp* was used as auto-fluorescence control. Promoter analysis were done in triplicate and mean values were calculated.

Aggregation kinetics

The aggregation curve was determined in HCT medium with xylose at 30°C. Cultures were sampled each hour from the onset of stationary phase. The optical densities at 600nm (OD_{600}) of each sample were measured once they were collected and after 15

minutes. The percentage of aggregation was calculated as the formula: % aggregation = [1-(DO_{600} t15min / DO_{600} t0)]*100.

Microscopy

The cultures used for analysis of aggregation kinetics were also sampled for microscopy at t4, t8, t14 and t32h after the onset of the stationary phase. The cells were observed with a Zeiss AxioObserver.Z1 inverted microscope in phase contrast. Pictures were taken with a Zeiss AxioCam MRm CCD camera connected to the microscope.

Results

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

rap and *phr* genes were shown to form transcriptional units with a promoter located upstream the *rap* gene (Perego & Hoch, 1996). As generally described for these genes, *phr63* gene slightly overlaps the *rap63* gene (by just one bp). To verify if *rap63* and *phr63* genes are co-transcribed, a reverse transcription PCR reaction (RT-PCR) was performed from RNA extracted 3 hours after the onset of the stationary phase. The result demonstrates that *rap63* and *phr63* genes are co-transcribed (Figure1A and 1B) and that the transcription unit is only formed by these two genes.

Transcription of most *phr* genes are also regulated by an additional specific promoter situated upstream the *phr* gene and inside the *rap* gene, generally controlled by the alternate sigma factor sigmaH (σ^H or *sigH*) (McQuade et al., 2001). To measure the expression of the *rap63-phr63* signaling cassette, DNA regions upstream from the *rap63* and the *phr63* genes were coupled to the *yfp* fluorescent reporter gene on the plasmid pHT304.18'yfp. Transcription of P*rap63* starts one hour after the entry on stationary phase (t1) and extended at least until t5 (Figure 1C). The expression of P*phr63* begins at t2 and lasts until t5 (Figure 1D) demonstrating that the *phr63* transcription is also controlled by its own promoter. Interestingly, the expression from P*phr63* is one hour delayed in a *sigH*-deficient strain (Δ*sigH*) suggesting a role of SigH in *phr* expression activation (Figure 1D). However, as the P*phr63* transcription is not abolished in the Δ*sigH* mutant strain, this result shows that another transcription factor is involved on the regulation of this promoter.

Promoter prediction using different tools were not capable to figure out a concise result about the prediction of transcription factors binding sites. Considering the timing of the P*phr63* expression in the *sigH*-deficient strain which starts at t3 and the role of SigE in the expression of the P*phr8* in the same strain (Fazion et al., 2018), transcription from P*phr63* was assessed in the *sigE*-deficient strain (Δ*sigE*). As shown in Figure 1D, the expression of P*phr63* on the Δ*sigE* strain is similar to the wild-type strain suggesting that SigE is not required for P*phr63* transcription.

Rap63 inhibits sporulation

Our previous Rap-Phr systems analyses in the *B. cereus* group predicted that the Rap63 could affect the sporulation (Cardoso et al., in press). To verify this activity, *rap63*, *rap63-phr63* and *phr63* genes were introduced into the pHT315-P*xylA*, a multicopy plasmid in which the expression of these genes is under regulation of a xylose inducible promoter. These constructions were transformed into the *B. thuringiensis* HD73- wild-type strain. The control strain, bearing the empty pHT315-P*xylA* plasmid, sporulates efficiently (82% of spores) upon 48h culture at 30°C in a HCT medium supplemented with xylose (Figure 2; Table S3). In sharp contrast, the strain expressing *rap63* gene presented less sporulation efficiency (38% of spores) with a 5-fold reduction of heat-resistant spore production in the same conditions (5.80E+07 versus 3.25E+08 in the control strain). The sporulation efficiencies of strains expressing *rap63-phr63* or *phr63* genes present a similar sporulation profile that the control strain (84% and 85%, respectively). Therefore, these results confirm that Rap63 inhibits sporulation, in a moderate manner, and that the Rap63 activity is counteracted by its cognate Phr63.

Rap63 delays expression of Spo0A-regulated genes

Rap proteins affect sporulation by acting as phosphates on Spo0F~P resulting in phosphorylation inhibition of the major response regulator Spo0A (Perego et al., 1994). The phosphorylated form of Spo0A regulates the expression of several genes related to sporulation. The *spoIIE* gene is regulated by Spo0A-P in *B. subtilis* (Molle et al., 2003) and in *B. thuringiensis* (Perchat et al., 2016). The promoter region of *spoIIE* gene (*PspoIIE*) was coupled to the *yfp* fluorescent reporter gene and inserted in the pHT315-P*xylA*-*rap63* or *rap63-phr63* plasmids (xylΩr*ap63*-P*spoIIE*'*yfp* and

xylΩ*rap63-phr63*-P*spoIIE*'*yfp*, respectively). In the control strain (harboring plasmid xyl-P*spoIIE*'yfp) the transcription of *spoIIE* starts two hours after the onset of the stationary phase (Figure 3). When the transcriptional fusion is carried together with the *rap63* gene, the expression from P*spoIIE* is delayed, beginning around T5. When both Rap63 and Phr63 are produced, expression from the P*spoIIE* is restored to the same kinetics and level found with the control strain. Therefore, these results demonstrate that the Rap63 delays the expression of Spo0A-regulated genes and that Phr63 inhibits Rap63 activity. This is in accordance with the sporulation test, confirming the moderate role of the Rap63 on the control of the sporulation process.

Auto-aggregation phenotype linked to sporulation

At the end of the sporulation assays, after 48h of culture, the HD73 strain expressing Rap63 displayed a remarkable aggregative phenotype. To a better understanding of this phenomenon, we realized an aggregation kinetic in HCT medium with shaking at 30°C with the three HD73-derivated strains tested (315xyl, xylΩ*rap63* and xylΩ*rap-phr63*). The results showed that all the strains presented a transitory aggregation phenotype which starts at t4 with a maximum at t8 and the disaggregation beginning between t10 and t12 (Figure 4A). Interestingly, the decrease in the aggregation is coincident to spore liberation, as revealed by the microscopic observations (Figure 4B). In the strain expressing *rap63*, the disaggregation is postponed and does not reach a null aggregation as observed with the control strain and with the strain expressing *rap63-phr63*. This result shows that this aggregation phenotype is not specifically dependent on Rap63. Moreover, the defect in the disaggregation phenotype could be correlated to the regulation profile of Rap63 delaying the Spo0A-regulated genes expression and thus the sporulation process.

The ΔPhr8ΔPhr63 mutant strain negatively affects the commitment to sporulation

The complete infectious cycle – pathogenesis, necrotrophism and sporulation – of *B. thuringiensis* in insects larvae was shown to be controlled by quorum sensing systems of the RNPP family (Slamti et al., 2014). Hence, we analyzed the role of Rap63-Phr63 in a naturalistic condition, on death larvae of *G. mellonella*. For this purpose, HD73- mutant strains with *rap63-phr63* or *phr63* deletions were constructed. Since the ΔPhr8 strain was already shown to present a reduction on

sporulation efficiency in this environment (Fazion et al., 2018), a double Phr mutant (ΔPhr8ΔPhr63) was also built. These strains were evaluated for their sporulation efficiencies in insect 96h after intra hemocelic injection (Figure 5A, Table S3). The wild type strain (HD73) and its derivatives mutant strains ΔRap63-Phr63 and ΔPhr63 presented a similar sporulation efficiency (22%, 24% and 16% of spores, respectively). However, sporulation efficiency was strongly reduced in the ΔPhr8ΔPhr63 mutant strain (0.23%), in a greater extent than the ΔPhr8 strain (Fazion et al., 2018). The results obtained *in vitro*, in HCT medium, showed that the ΔRap-Phr63 and ΔPhr63 strains sporulate as the wild type strain whereas the sporulation was slightly but significantly affected in the ΔPhr8ΔPhr63 strain. These results suggest a synergic effect of the Rap8-Phr8 and Rap63-Phr63 systems to regulate the sporulation process.

Determination of the Phr63 active form

Rap proteins are inhibited by Phr oligopeptides, which active form commonly correspond to the C-terminal end of the *phr* gene product and were already described to contain five, six or seven amino acids (Fazion et al., 2018; Mirouze et al., 2011; Perego, 1997; Pottathil & Lazazzera, 2003). To determine whether the mature form of Phr63 correspond to the C-terminal end of the pro-peptide, we constructed a plasmid expressing the *rap63* gene with a truncated *phr* gene at the 3' end (xyl_*rap63-phr63*_R3'), missing codons related to the six C-terminal amino-acids of the *phr* gene product (Figure 6A). The sporulation efficiency of the strain harboring this plasmid (32%) is similar to the strain expressing the Rap63 (Figure 6B, Table S3). This result suggests that the active form of the Phr63 is included in the C-terminal end of the premature Phr.

To define the Phr63 active form, various Phr63 peptides corresponding to the C-terminal end were synthetized: GETI (Phr63-4); HGETI (Phr63-5); AHGETI (Phr63-6); YAHGETI (Phr63-7) and QYAHGETI (Phr63-8) (Figure 6A). To evaluate the capacity of the synthetic peptides to inhibit Rap63 activity, they were separately added in the culture medium of the strain expressing *rap63* (xyl_*rap63*). Phr63-4 was not able to inhibit Rap63 activity (30% of spores), while Phr63-5 (77%), Phr63-6 (84%), Phr63-7 (87%), and Phr63-8 (87%) efficiently counteracted Rap63 effect on sporulation (Figure 6B, Table S3). These results demonstrated that the active form is

well in the C-terminal end of the Phr63 peptide but did not allow precise identification the mature sequence.

A crosstalk between Rap-Phr63 and Rap8-Phr8 systems?

B. thuringiensis HD73 strain possess eight Rap-Phr systems, five on the chromosome and three on plasmids (pHT8_1, pAW63, and pHT77). Two of them were more deeply studied: Rap8-Phr8 from pHT8_1 (Fazion et al., 2018) and Rap63-Phr63 from pAW63 (this study). The C-terminal region of both Phr peptides including the mature form shows a high similarity (Figure 7A), especially considering the eight last amino acids where just two residues are divergent. Due to these sequence similarities and the results of the ΔPhr8ΔPhr63 mutant strain, we investigated the possibility of cross talk between Rap8-Phr8 and Rap63-Phr63 systems. Hence, we constructed the ΔRap8-Phr8ΔRap63-Phr63 mutant strain to avoid the effect of intrinsic systems. This mutant strain was transformed with pHT315-P*xylA*, pHT315-P*xylA-rap8* and pHT315-P*xylA-rap63* plasmids and the sporulation of the resultant strains was assessed (Figure 7B, Table S3). The control strain (ΔΔ 315xyl) sporulates efficiently (84% of spores). Likewise the sporulation test with the wild-type strain, the strain expressing *rap63* presented reduced sporulation efficiency (32%). In addition, in the presence of the Phr63-7 peptides this strain showed sporulation efficiency similar to the control (82%). However, when the strain expressing *rap63* was cultured in the presence of Phr8-6, Phr8-7 or Phr8-8 no effect on sporulation was recorded (31%, 35% and 29%, respectively). Then, none of the Phr8 peptides used was able to counteract the Rap63 activity. Furthermore, the ΔΔ 315xyl strain expressing *rap8* is strongly affected in sporulation (1.3%) and the Phr8-7 peptides inhibited Rap8 activity (80%) as already described in the wild-type strain by Fazion et al. (2018) (Figure 7B, Table S3). Unfortunately, none of the Phr63 peptides tested was able to prevent the Rap8 sporulation effect (Figure 7B). Altogether, these results showed that there is no crosstalk between Rap8-Phr8 and Rap63-Phr63 systems.

Discussion

In agreement with the rich plasmid profile of the *B. thuringiensis* species (Reyes-Ramirez & Ibarra, 2008), the complete genome sequence of the HD73 strain (Liu et

al., 2013) includes seven plasmids (pHT7, pHT8_1, pHT8_2, pHT11, pAW63, pHT73, pHT77). Among them, three harbor a Rap-Phr system. The Rap8-Phr8 from the pHT8_1 is involved in biofilm formation and regulation of the sporulation (Fazion et al., 2018) and the Rap-Phr from the pHT77 did not have a sporulation activity (Cardoso et al., in press). In this study, we described the Rap63-Phr63 system harbored in the pAW63. Plasmid pAW63 is a broad range conjugative plasmid able to mobilize other plasmids (Wilcks et al., 1998). It shows high conjugation rates in various conditions such as in food matrices (Modrie et al., 2010; Van der Auwera et al., 2007), simulated microgravity (Beuls et al., 2009) and salt stress (Beuls et al., 2012). This plasmid also carries a Type III toxin-antitoxin system, based on a RNA antitoxin and an endoribonuclease toxin, that were shown to play a role in plasmid maintenance in vegetative growth, sporulation and germination (Short et al., 2015). The pAW63 possesses B.th.I1 and B.th.I2 introns that interrupt genes whose products are related to conjugation function. Interestingly, the B.th.I2 was demonstrated to excise during transcription allowing expression of host gene (Van der Auwera et al., 2005; Van der Auwera & Mahillon, 2008). The pAW63 shares a common backbone with pXO2 (the second toxin-plasmid of *B. anthracis*) and pBT9727 from the pathogenic *B. thuringiensis* strain *konkukian* 97-27 (Van der Auwera et al., 2005). Even if these similar plasmids share 42 CDSs, more than half of pAW63 CDSs, pAW63 is the only one harboring a regulatory Rap-Phr system (Cardoso et al., in press).

Our data show that the *rap63-phr63* genes are co-transcribed from a promoter activated in the beginning of the stationary phase in sporulation medium. The *phr63* gene is also independently transcribed from a promoter located in the *rap63* gene. Its transcription starts 2h after the onset of the stationary phase and is partially activated by the σ^H factor. In *B. subtilis*, most of the *phr* genes are controlled by σ^H ensuring Phr accumulation to trigger sporulation (McQuade 2001). Transcriptional analyses of pAW63 coding-sequences detected the expression of *rap63* (CDS 57) and *phr63* (CDS 57b) at mid exponential growth but in different growth conditions (Van der Auwera & Mahillon, 2008). They compared the expression pattern of a wild type HD73 strain and the same strain cured of the pAW63 plasmid by a microarray analysis. Their results showed that the *rap63* gene is expressed at a moderate level while the *phr63* gene was found in high level (Van der Auwera & Mahillon, 2008).

However, the Phr signal also presented a high level in the cured strain suggesting that the probe used to detect *phr63* gene is not specific and reveals the presence of another *phr* gene in the *B. thuringiensis* HD73 genome. The transcription of *rap63-phr63* genes happen concomitantly with this of *rap8-phr8* genes from pHT8_1 plasmid (Fazion et al., 2018). Interestingly, both Rap proteins are able to inhibit the sporulation process but with a different extent. While Rap8 strongly inhibits sporulation by preventing expression of Spo0A-regulated genes (Fazion et al., 2018), Rap63 has a moderate inhibition of sporulation and delays the expression of the *spoIIE* gene controlled by Spo0A. Moreover, the Rap63 presents the same profile concerning the key residues involved in sporulation activity than the Rap-BXA0205 from the pXO1 plasmid that strongly inhibits sporulation (Bongiorni et al., 2006; Cardoso et al., in press). This suggests that other residues play an important role in Rap-Spo0F interaction resulting in a modulation in sporulation inhibition.

Rap proteins are inhibited by their cognate Phr oligopeptides (Perego & Hoch, 1996). In accordance, we showed that the expression of the *phr63* gene prevent Rap63 activity on sporulation. The ΔPhr63 mutant strain did not show significant effect on sporulation but the deletion of both *phr* genes in the ΔPhr8ΔPhr63 mutant strain strongly prevents the commitment to sporulation *in vivo*. In insect larvae, the ecological niche of *B. thuringiensis*, sporulation of the ΔPhr8 strain showed a repression about 4-fold (Fazion et al., 2018) while in the double mutant strain the inhibition was of almost 100-fold, suggesting a synergistic activity of both quorum sensing systems. Generally, the mature peptide corresponds to the C-terminal end of the Phr peptide sequence and contains a positively charged residue (Pottathil & Lazazzera, 2003). We demonstrated that the active form of Phr63 is part of the C-terminal end. However, it was not possible to determine the exact size of the mature Phr63 by using synthetic peptides. Indeed, synthetic oligopeptides Phr63-5, Phr63-6, Phr63-7, and Phr63-8 effectively inhibit Rap63 activity on sporulation. This result suggests that the Phr-binding pocket in the Rap63 could be more versatile to Phr63 binding than Rap8 that was inhibited only by Phr8-7(Fazion et al., 2018); or, yet, that the longer peptides might undergo further processing to achieve its active form. In *B. subtilis*, the mature Phr are penta or hexapeptides (Mirouze et al., 2011; Pottathil & Lazazzera, 2003) while mature peptides of the RNPP regulators in *B. cereus* group

bacteria are commonly heptapeptides, such as NprX (Perchat et al., 2011), PapR (Bouillaut et al., 2008) and Phr8 (Fazion et al., 2018).

The C-terminal ends of the Phr8 and Phr63 present a high sequence similarity with six out eight amino acids identical and a histidine residue (positively charged) at the -5 position from the C-terminal end of the precursor peptides. Consequently, we hypothesize a possible crosstalk between the Rap63-Phr63 and Rap8-Phr8 plasmid systems. Unfortunately, ours results show that neither Phr8 is able to inhibit Rap63 nor Phr63 prevents Rap8 activity. In *B. subtilis,* the absence of crosstalk between RapA-PhrA, RapC-PhrC and RapE-PhrE has been also demonstrated (Jiang et al., 2000a; Perego, 1997). Therefore, this absence of crosstalk demonstrates the high specificity of the Rap and Phr interactions supporting the hypothesis of the co-evolution of Rap-Phr system components (Perego, 2013). In the other *B. thuringiensis* RNPP systems, a cross-activation has been described among different pherotypes but not within one given strain (Perchat et al., 2011; Slamti & Lereclus, 2005).

In this study, we described an auto-aggregation phenotype that seems to be linked to sporulation. This aggregation phenotype is a transitory phenomenon extending from 4 to 14 hours after the onset of the stationary phase. During this time, bacteria develop their sporulation process and the disaggregation is concomitant with the spore release. In the strain expressing *rap63*, we observed a prolonged aggregation phenotype that could be due to the delay that the Rap63 causes on Spo0A-regulated genes. For example, the expression of CwlB and CwlC autolysins described to play a role in spore release are regulated by the late sporulation sigma factor σ^K (Chen et al., 2018; Yang et al., 2013), thus, indirectly regulated by Spo0A. The aggregation observed during the sporulation process differs from the known aggregation involved in conjugation induced by pXO16 plasmid in *B. thuringiensis israelensis* (Andrup et al., 1993, 1996; Jensen et al., 1995). Indeed, these two phenomena occur at different growth phases. The aggregation system related to pXO16 conjugation arises during exponential growth, beginning about 15-20 minutes after the combination of the strains and disappearing at yet a low optic density (OD_{600} > 1) (Andrup et al., 1996). Moreover, while the conjugation aggregation is formed between donor and recipient strains in a mixed culture (Andrup et al., 1993), here we observe an auto-aggregation phenotype. Recently, a cell-cell aggregation related to

the expression of c-di-GMP second messenger was described in the *B. thuringiensis* BMB171 strain (Fu et al., 2018; Tang et al., 2016). In this strain, a c-di-GMP-binding riboswitch controls the expression of a collagen binding protein that was shown to modulate different bacterial physiological process, such as motility, biofilm formation, aggregation and virulence (Tang et al., 2016). Moreover, an aggregation phenotype related to sporulation was described for the Gram negative bacteria *Myxococcus xanthus*. In this species, the aggregation is the first step for fruiting body formation, within which the bacteria sporulate (Kruse et al., 2001). However, this process occurs in a solid surface while the aggregation observed on this work arises in liquid medium and with vigorous shaking. Several questions remain unsolved to better understand this phenotype. It would be interesting to characterize the occurrence of this phenotype within the *B. cereus* group and its importance in the developmental pathway of bacteria, particularly in the sporulation process. This auto-aggregation might play a key role in quorum sensing, for example by ensuring a high local concentration of Phr peptides within the aggregates in order to optimize the sporulation process. In *Vibrio harveyi*, cell aggregates were demonstrated to display stronger cell-cell communication than a dispersed population with the same number of cells (Gao et al., 2016).

Horizontal gene transfer can increase the variability of the genetic set of a bacterial species allowing the bacteria to better adapt to environmental changes. The *B. thuringiensis* plasmids had been widely studied, mainly due to the *cry* and *cyt* genes coding for the insecticidal toxin genes and preferably located on large conjugative plasmids. However, *B. thuringiensis* strains harbor a wide and diverse amount of plasmids, many of which contain Rap-Phr systems predicted to regulate sporulation. For the first time, the integrative activity of two plasmid-borne Rap-Phr systems in a given strain was tested. The Rap8-Phr8 and the Rap63-Phr63 systems from *B. thuringiensis* HD73 strain act synergistically to control the commitment to sporulation in insect larvae. Since *B. thuringiensis* is an invertebrate specialized pathogen, it is expected that this species presented a rich repertoire of mechanism that facilitate its development and survival in its environmental niche. Our results reinforce the relevance of plasmid Rap-Phr quorum sensing systems and highlight that plasmids other than the *cry*-harboring plasmids play an important role in the *B. thuringiensis* infectious cycle regulation and bacterial survival.

Acknowledgements

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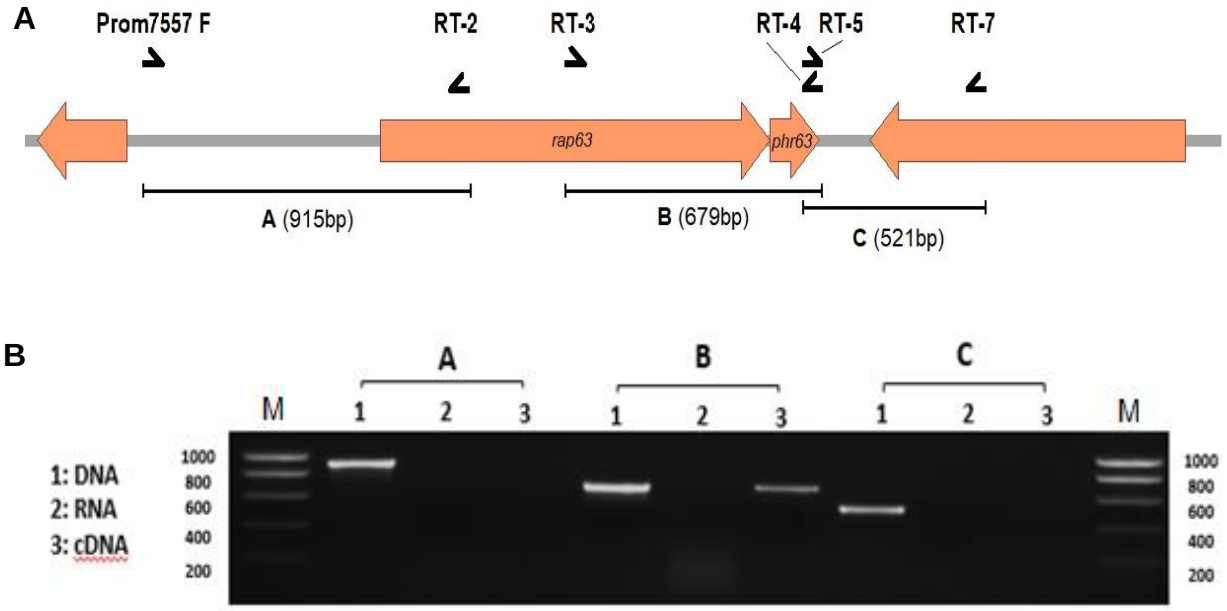
Figures



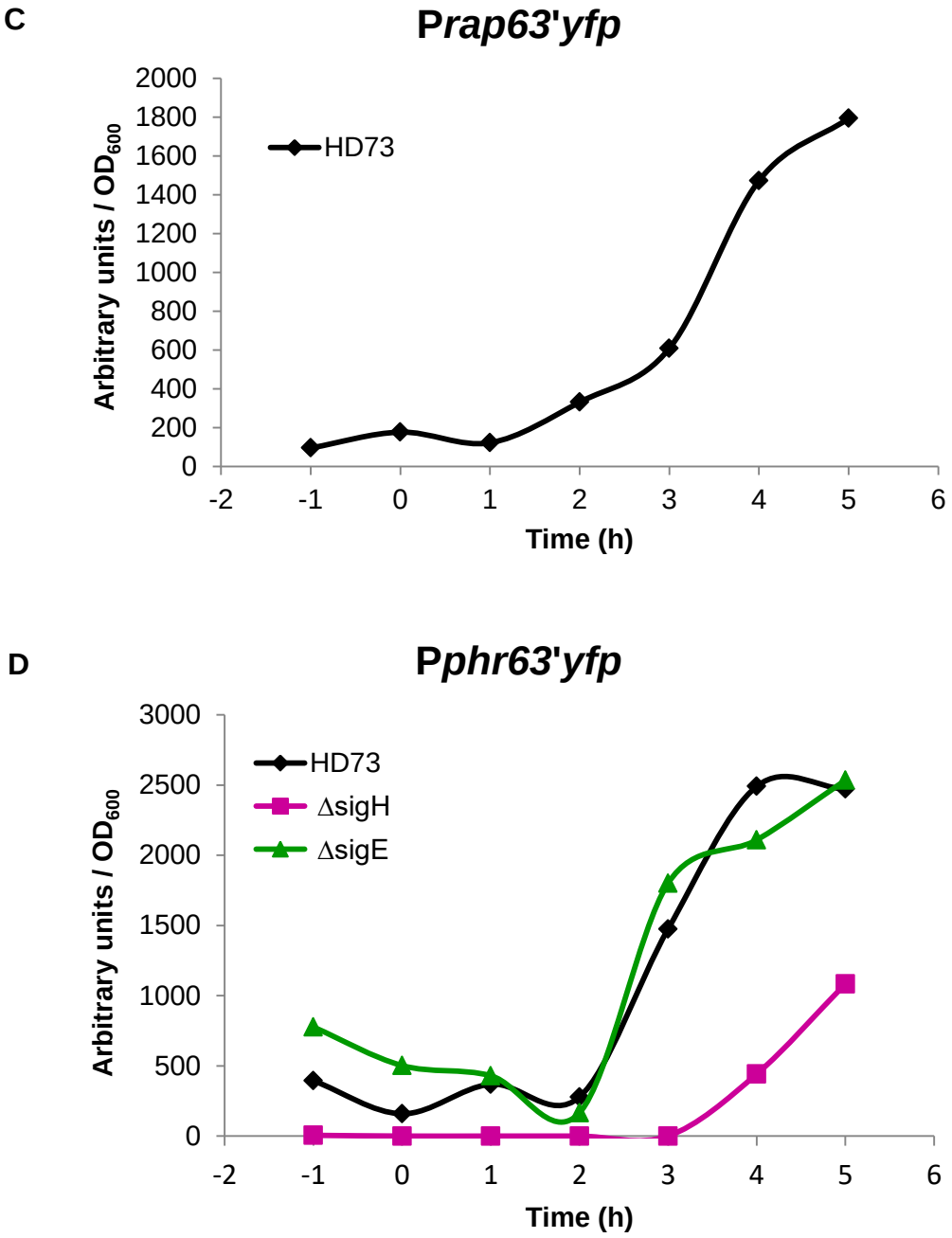


Figure 1. The *rap63-phr63* transcription unit. A) Schematic representation of the *rap-phr63* locus in the pAW63 plasmid. Small arrows correspond to primers used to amplify the three RT-PCR fragments. B) RT-PCR experiment. Total RNA was extracted from a t3 culture in HCT medium at 37°C and 175 r.p.m. Genomic DNA (1), RNA (2) and cDNA (3) were used as template to PCR amplification, verified by 1% agarose gel and compared to molecular weight markers (M) (Smartladder Small fragments – Eurogentec). C) Kinetics of the *rap63-phr63* expression. YFP fluorescence production of the HD73 wild-type strain carrying the P*rap63'yfp*. D) Kinetics of the *phr63* expression. YFP fluorescence production of the HD73 wild-type, HD73 Δ*sigH*, and HD73 Δ*sigE* mutant strains carrying the P*phr63'yfp*. Time zero corresponds to the entry into stationary phase. The results are expressed in arbitrary units per OD_{600} unit.

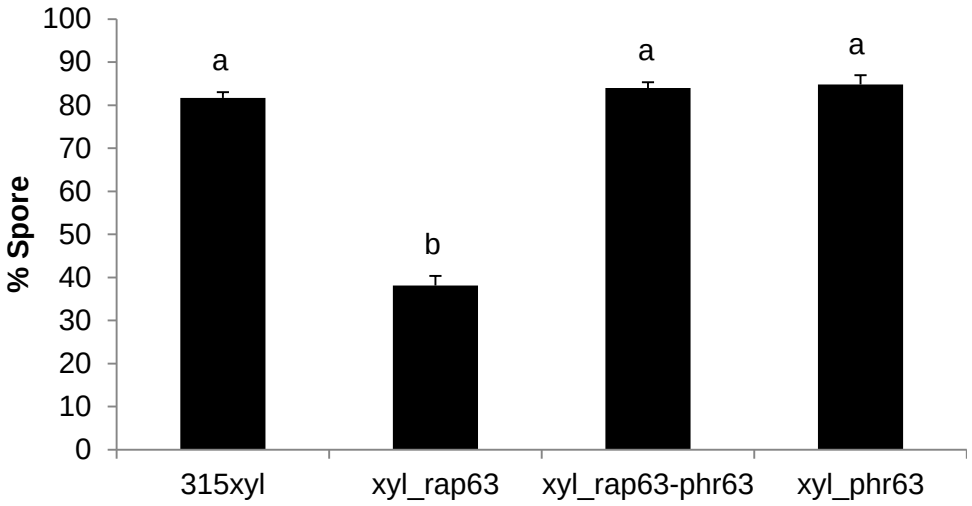


Figure 2. Rap63 inhibits moderately the sporulation. Sporulation efficiency measured in the HD73 control strain (315xyl) and HD73 strain expressing *rap63* (xyl_*rap63*), *rap63-phr63* (xyl_*rap63-phr63*) or *phr63* (xyl_*phr63*). The percentages of spores were calculated as 100 × the ratio between heat-resistant spores per ml and total viable cells per ml. Error bars represent the Standard Error of the Mean (SEM). Experimental values are detailed in Table S3. Different letters correspond to significant differences in the main values ($p<0.001$).

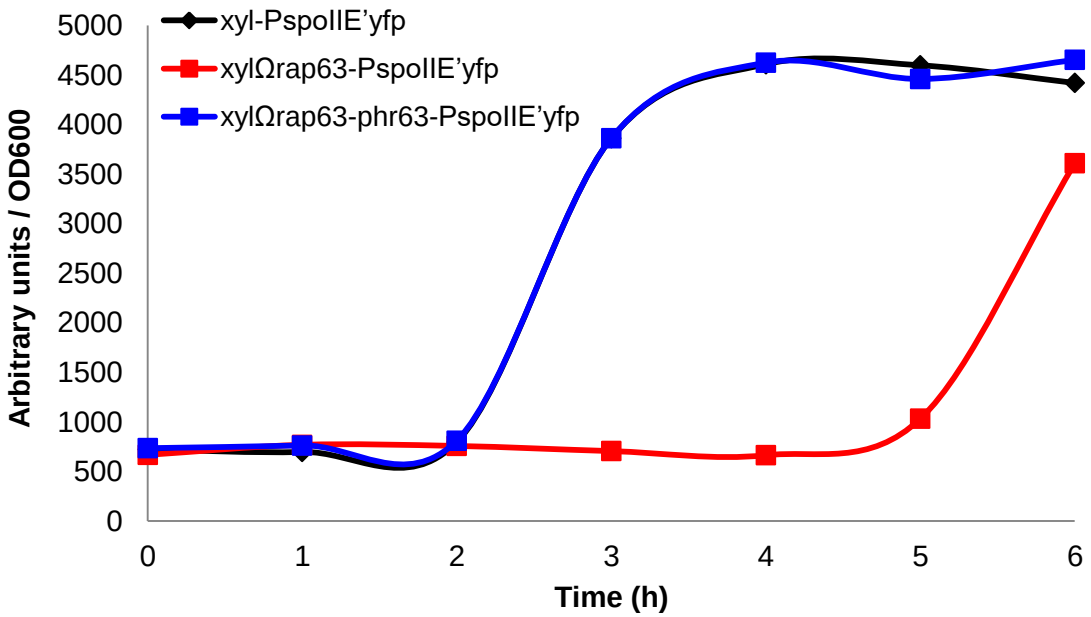


Figure 3. Rap63 delays expression of Spo0A-regulated genes. Kinetics of *spoIIE* expression in the HD73 wild-type strain carrying the pHT315xyl-P*spoIIE*'*yfp*, the pHT315xylΩ*rap63*_P*spoIIE*'*yfp,* or the pHT315xylΩ*rap63-phr63*_P*spoIIE*'*yfp* plasmids. The YFP fluorescence was measured during growth in HCT medium at 30°C in the presence of xylose 20 mM added at t0 (entry into stationary phase). The results are expressed in arbitrary units per OD_{600} unit.

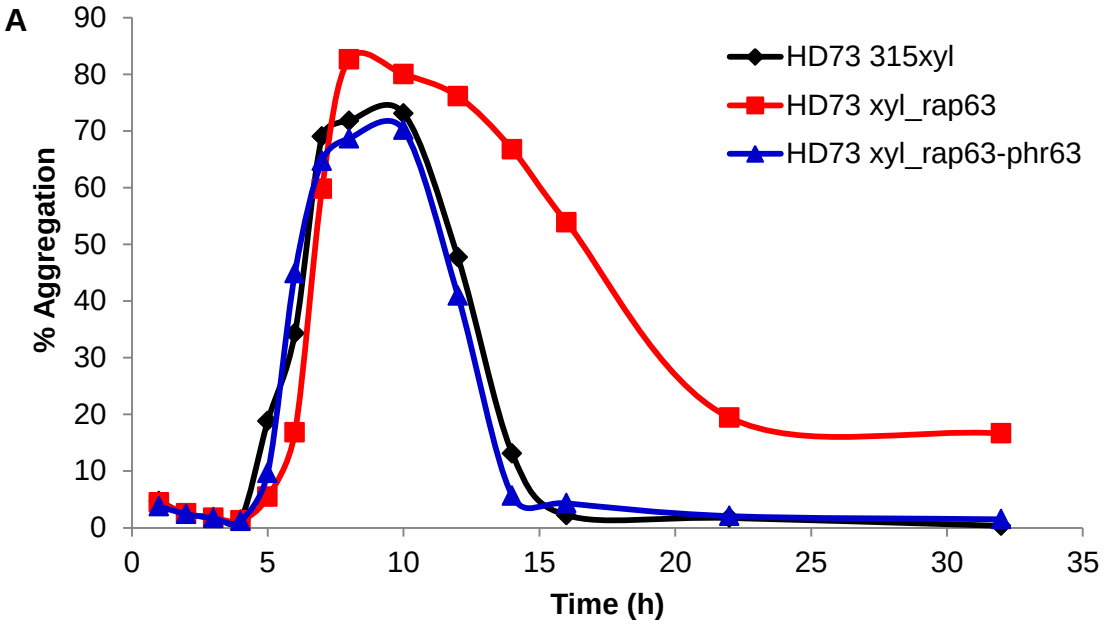
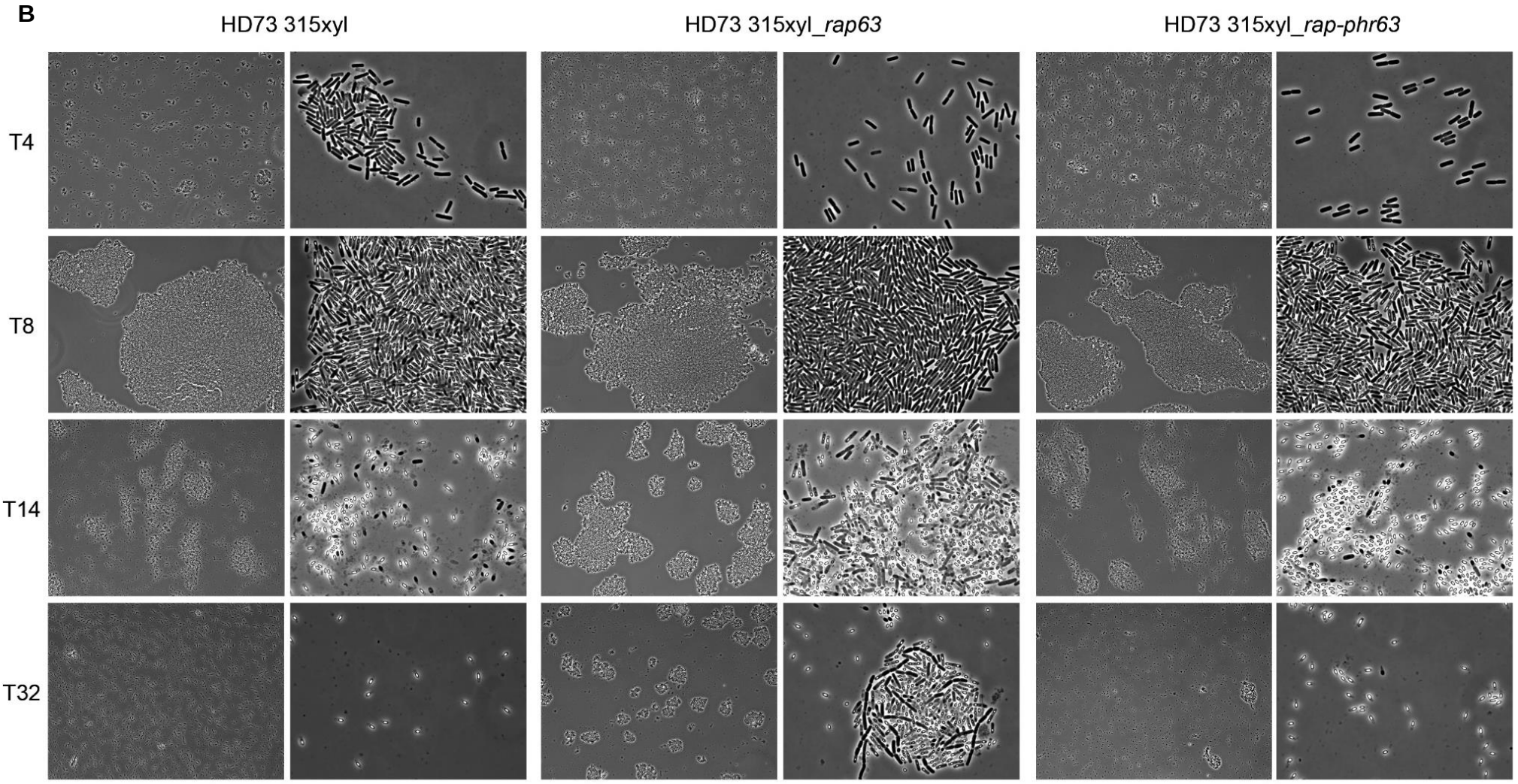


Figure 4. Kinetics of the auto-aggregation phenotype. Comparison of the aggregation in the HD73 control strain (HD73 315xyl) and the HD73 strains expressing *rap63* (HD73 xyl_*rap63*) or *rap63-phr63* (HD73 xyl_*rap63-phr63*) during growth in HCT medium at 30°C with xylose 20 mM. Time 0 corresponds to the onset of stationary phase. A) Percentage of aggregation calculated after 15 minutes at room temperature. B) Phase contrast microscopy. Pictures were taken at four different times during the kinetics assay with a 10x-objective (left pictures) and a 100x-objective (right pictures).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HD73 315xyl_rap63
HD73 315xyl_rap-phr63
T4
T8
T14
T32

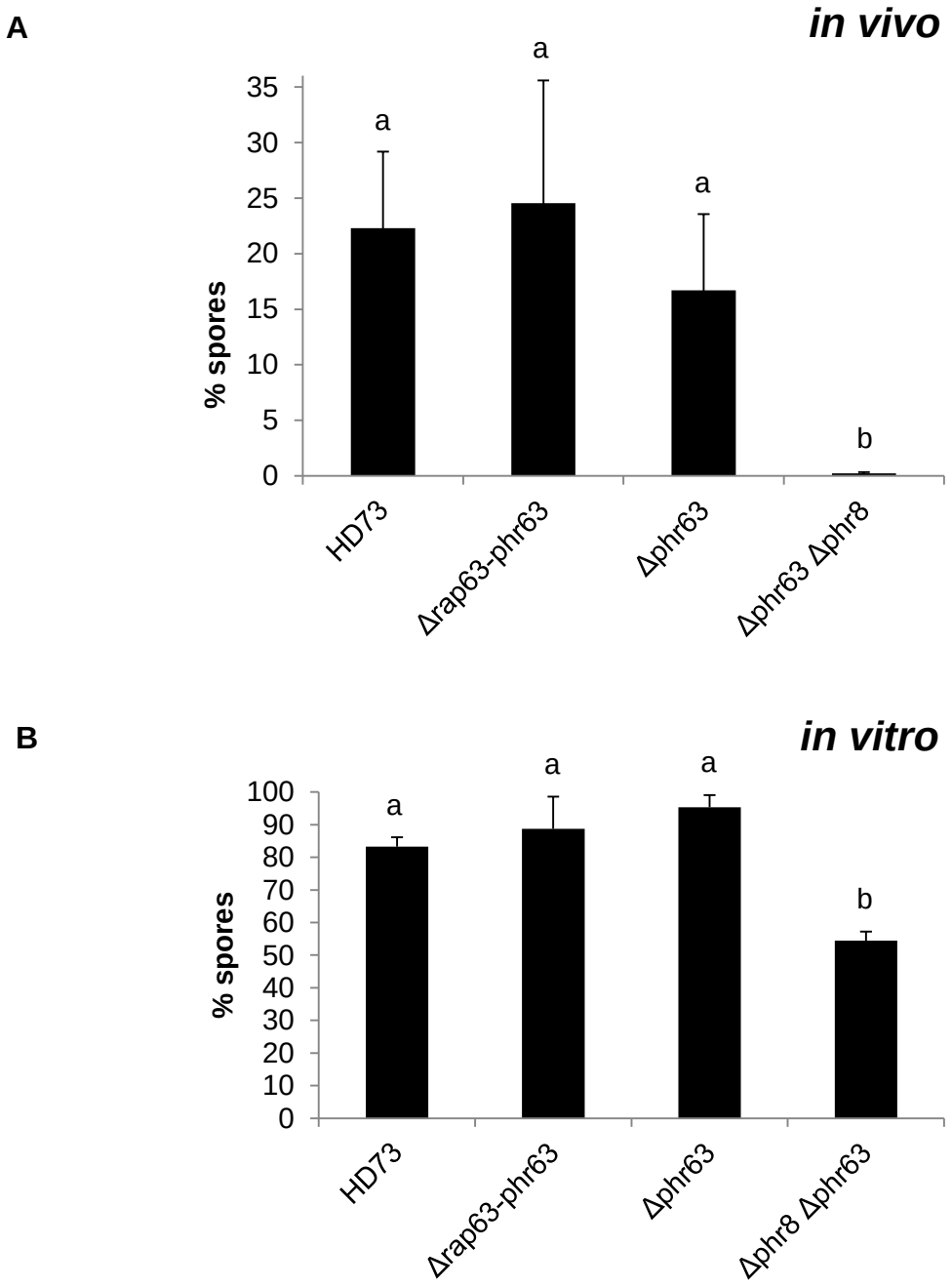

Figure 5. Synergistic action of the Rap63-Phr63 and the Rap8-Phr8 systems on sporulation. Sporulation efficiency of HD73 wild-type, Δ*rap63-phr63*, Δ*phr63* and Δ*phr8*Δ*phr63* mutant strains. A) In death larvae of *Galleria mellonella* (*in vivo*). The viable cells and spores were counted in death larvae 4 days after treatment maintained at 30°C. B) In HCT medium culture (*in vitro*) the viable cells and spores were counted after 48h of culture at 30°C. The percentages of spores were calculated as 100 × the ratio between heat-resistant spores per ml and total viable cells per ml. Error bars represent the Standard Error of the Mean (SEM). Experimental values are detailed in Table S3. Different letters correspond to significant differences in the main values ($p<0.01$).

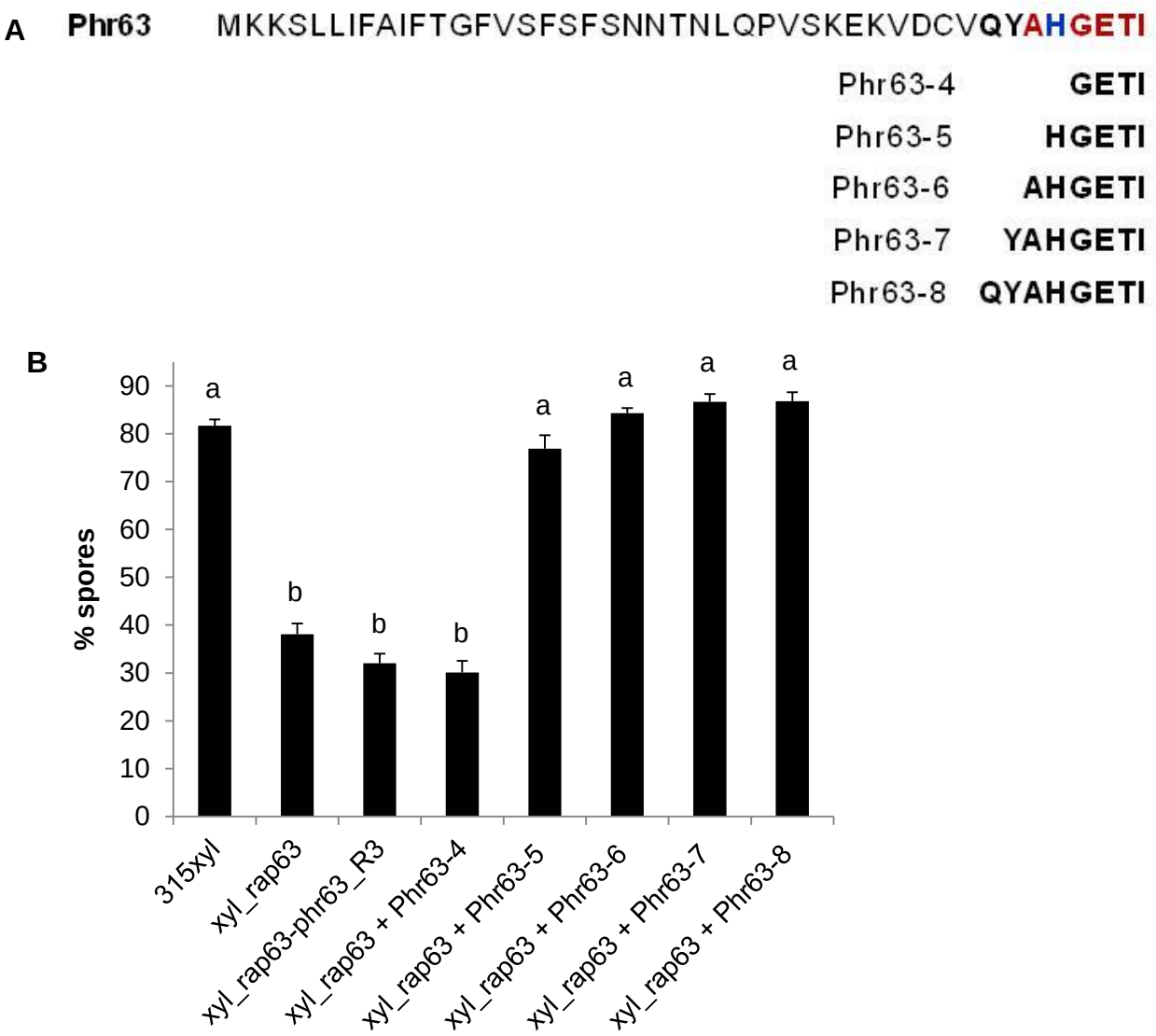

Figure 6. Characterization of Phr63 active form. A) Amino acid sequence of the pre-Phr63 peptide and of the five different synthetic peptides used in complementation experiments. C-terminal end sequence truncated in the xyl_*rap63-phr63*_R3 is colored in red, and the positively charged residue in blue. B) Sporulation efficiencies of the HD73 control strain (315xyl), HD73 expressing *rap63* (xyl_*rap63*) and expressing *rap63-phr63*_R3 producing the C-terminal truncated Phr63. The strain expressing *rap63* was complemented with the synthetic peptides Phr63-4, Phr63-5, Phr63-6, Phr63-7 and Phr63-8 added independently 1 h after the onset of the stationary phase at 50 mM final concentrations. The percentages of spores were calculated as 100 × the ratio between heat-resistant spores per ml and total viable cells per ml after 48h in HCT medium at 30°C in the presence of xylose 20 mM. Error bars represent the Standard Error of the Mean (SEM). Experimental values are detailed in Table S3. Different letters correspond to significant differences in the main values ($p<0.001$).

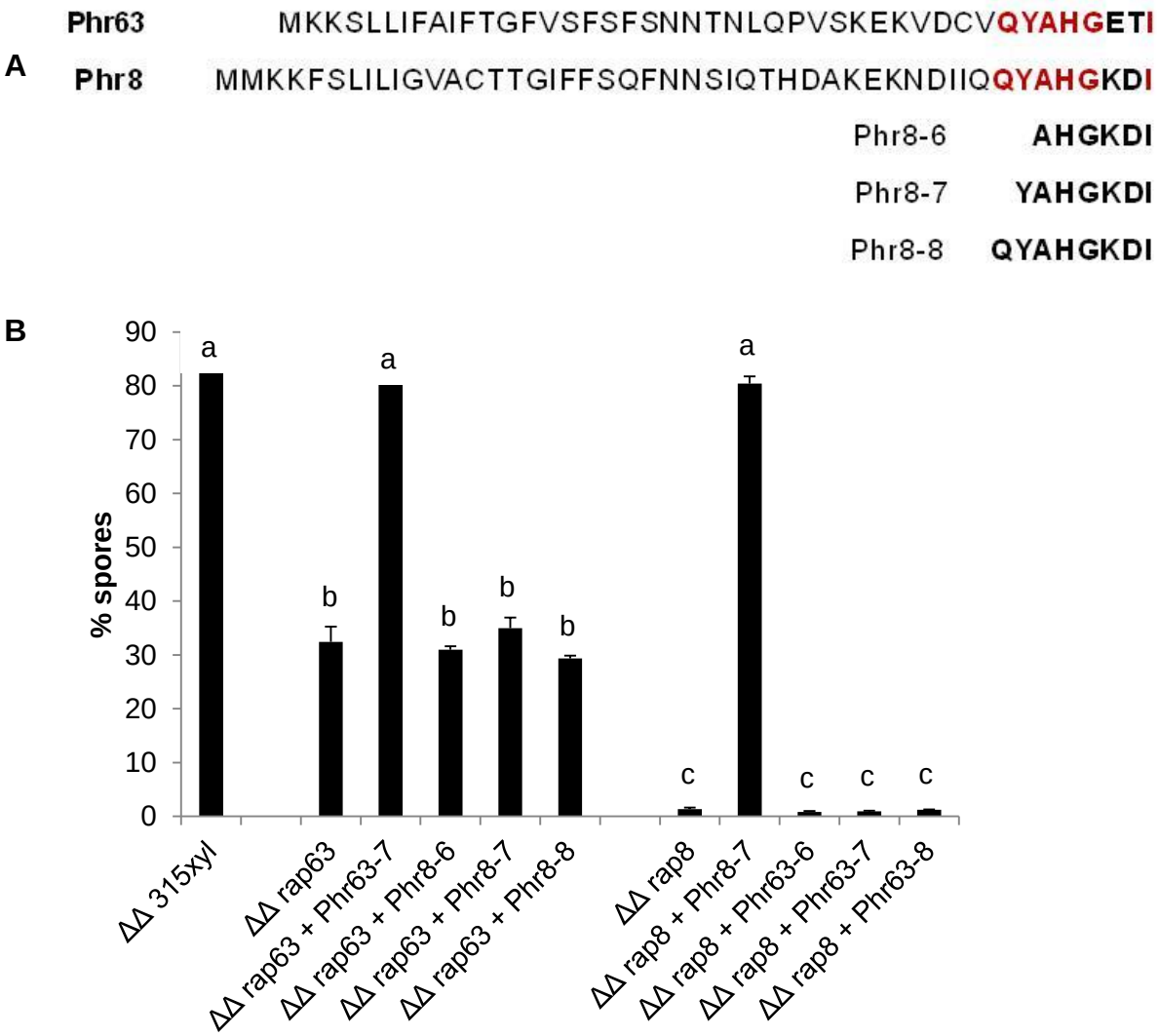

Figure 7. Evaluation of cross-talk between the Rap8-Phr8 and Rap63-Phr63 systems. A) Amino acid sequences of Phr63 and Phr8 peptides. C-terminal ends similarities are highlighted in red and the two divergent residues are in black bold. Phr8-6, Phr8-7 and Phr8-8 correspond to the Phr8 synthetic peptides described by Fazion et al. (2018). B) Sporulation efficiency of the HD73 Δ*rap8-phr8*Δ*rap63-phr63* control strain (ΔΔ 315xyl), and expressing *rap63* (ΔΔ *rap63*) or *rap8* (ΔΔ *rap8*) strains in HCT medium at 30°C in the presence of xylose 20 mM. The strain expressing *rap63* was complemented with Phr8-6, Phr8-7 and Phr8-8 synthetic peptides and the strain expressing *rap8* with Phr63-6, Phr63-7 and Phr63-8 synthetic peptides. Peptides were added independently 1h after the onset of the stationary phase at 50 mM final concentrations. The percentages of spores were calculated as 100 × the ratio between heat-resistant spores per ml and total viable cells per ml. Error bars represent the Standard Error of the Mean (SEM). Experimental values are detailed in Table S3. Different letters correspond to significant differences in the main values ($p<0.001$).

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Supporting Information

Table S1. Primers used in this study

Primer name	Sequence	Restriction Site*
Rap7557-F	CGC**GGATCC**GAATGAGGGGATTAAATATGAATGTG	*Bam*HI
Rap7557-R	CCC**AAGCTT**TCATTATTTTAAAGCTCCTTTCTCGG	*Hind*III
Phr7557-F	CGC**GGATCC**TGATAAAAAGGCTTCCGAGAAAG	*Bam*HI
Phr7557-R	CCC**AAGCTT**GGTGTTAAATAGTTTCACCATGTGC	*Hind*III
7557Amont1-F	CATG**CCATGG**CGCCTTTATTGTCAAGATACATCTACTC	*Nco*I
7557Amont1-R	CGG**GGTACC**ACATTCATATTTAATCCCCTCATTC	*Kpn*I
7557Amont2-F	CATG**CCATGG**TATCAATCCATCATTTCACAACATG	*Nco*I
7557Amont2-R	CGG**GGTACC**ATTATTTTAAAGCTCCTTTCTCGG	*Kpn*I
7557Aval-F	CG**TCTAGA**CACCATAAAGTACTAAAAAGTTATGTCATTAC	*Xba*I
7557Aval-R	CCG**GAATTC**CAATTTTGACCAAAGTCAATCCAC	*Eco*RI
Prom7557-F	CCC**AAGCTT**CGTTACTTATAAGAAACAAACAAGAGCC	*Hind*III
Prom7557-R	CGC**GGATCC**ACATTCATATTTAATCCCCTCATTC	*Bam*HI
Prom7557Phr-F	CCC**AAGCTT**GCTGCTTGTAATAACACACTAGG	*Hind*III
Prom7557Phr-R	CGC**GGATCC**ATTATTTTAAAGCTCCTTTCTCGG	*Bam*HI
Phr7557R3	CCC**AAGCTT**AATATTGAACACAGTCTACTTTTTCTTTTG	*Hind*III
RT7557-2	GAAGGCATCTGCTTGATCAGGTATAC	/
RT7557-3	GCTTGTAATAACACACTAGGTCTTGC	/
RT7557-4	CCATGTGCATATTGAACACAGTCTAC	/
RT7557-5	GTAGACTGTGTTCAATATGCACATGG	/
RT7557-7	CTTCAAGACATAGAAGACCAACATGTG	/
PU-EcoRI	CG**GAATTC**GCCAGGGTTTTCCCAGTCACGAC	*Eco*RI
YFP-R	CG**GAATTC**TTATTTGTATAGTTCATCCATGC	*Eco*RI
PspoIIE-F	AACTGCAGCTGGCTAGAGCGTACGG	/
xylRout3'	GGAATGTCCTCCATTGTGATTGATC	/

* Restriction sites are highlighted in bold in primer sequences

Table S2. Plasmid constructions used in this study

Plasmids	Description
pHT315xylΩ*rap63* (xyl_*rap63*)	*rap63* gene was amplified using primers Rap7557-F/Rap7557-R and the *B. thuringiensis* HD73 genomic DNA as template. The fragment was inserted between the *Bam*HI and *Hind*III sites of pHT315xyl plasmid.
pHT315xylΩ*phr63* (xyl_*phr63*)	*phr63* gene was amplified using primers Phr7557-F/Phr7557-R and the *B. thuringiensis* HD73 genomic DNA as template. The fragment was inserted between the *Bam*HI and *Hind*III sites of pHT315xyl plasmid.
pHT315xylΩ*rap63-phr63* (xyl_*rap63-phr63*)	*rap-phr63* genes was amplified using primers Rap7557-F/Phr7557-R and the *B. thuringiensis* HD73 genomic DNA as template. The fragment was inserted between the *Bam*HI and *Hind*III sites of pHT315xyl plasmid.
pHT315xylΩ*rap63-phr63*_R3 (xyl_*rap63-phr63*_R3)	*rap63* gene together with *phr63* truncated gene was amplified using primers Rap7557-F/Phr7557R3 and the *B. thuringiensis* HD73 genomic DNA as template. The fragment was inserted between the *Bam*HI and *Hind*III sites of pHT315xyl plasmid.
pHT304.18-P*rap63*'*yfp*	Primers Prom7557-F and Prom7557-R were used to amplify the promoter region of *rap63* gene, using the *B. thuringiensis* HD73 genomic DNA as template. The fragment was inserted between the *Hind*III and *Bam*HI sites of pHT304-18YFP.
pHT304.18-P*phr63*'*yfp*	Primers Prom7557Phr-F and Prom7557Phr-R were used to amplify the promoter region of *phr63* gene, using the *B. thuringiensis* HD73 genome as template. The fragment was inserted between the *Hind*III and *Bam*HI sites of pHT304-18YFP.
pHT315xyl-P*spoIIE*'*yfp*	Plasmid described by Fazion et al. (2018).
pHT315xylΩ*rap63*_P*spoIIE*'*yfp*	The P*spoIIE*'*yfp* fragment was amplified using primers PU-*Eco*RI /YFP-R and pHT315xyl-P*spoIIE*'*yfp* plasmid as template; and the fragment was inserted into the *Eco*RI site of pHT315xylΩ*rap63* plasmid. To avoid influence of P*xylA* promoter, the orientation of the inserted fragment was verified by PCR using primers PspoIIE-F/xylRout3'.
pHT315xylΩ*rap63-phr63*_ P*spoIIE*'*yfp*	The P*spoIIE*'*yfp* fragment was amplified using primers PU-*Eco*RI /YFP-R and pHT315xyl-P*spoIIE*'*yfp* plasmid as template; and the fragment was inserted into the *Eco*RI site of pHT315xylΩ*rap63-phr63* plasmid. To avoid influence of P*xylA* promoter, the orientation of the inserted fragment was verified by PCR using primers PspoIIE-F/xylRout3'.
pMADΩ*rap-phr63*::*spec*	5' and 3' regions of *rap63-phr63* genes were amplified using primers 7557Amont1-F/7557Amont1-R and 7557Aval-F/Aval7557-R, respectively, and *B. thuringiensis* HD73 genomic DNA as template. The 5' end was purified as an *Nco*I/*Kpn*I fragment and the 3' end as an *Xba*I/*Eco*RI fragment. The spectinomycin resistance gene was purified as a *Kpn*I/*Xba*I fragment from the pUC18Ω*spec* and inserted together with the 5' and 3' regions of *rap63-phr63* between the *Nco*I and *Eco*RI sites of pMAD.
pMADΩ*phr63*::*spec*	5' and 3' regions of *phr63* genes were amplified using primers 7557Amont2-F/7557Amont2-R and 7557Aval-F/Aval7557-R, respectively, and *B. thuringiensis* HD73 genomic DNA as template. The 5' end was purified as an *Nco*I/*Kpn*I fragment and the 3' end as an *Xba*I/*Eco*RI fragment. The spectinomycin resistance cassette was purified as a *Kpn*I/*Xba*I fragment from the pUC18Ω*spec* and inserted together with the 5' and 3' regions of *phr63* 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 pHT315xyl (xyl)	HCT	3.97E+08 (±1.53E+07)	3.25E+08 (±1.46E+07)	81.69 (±1.33)
xyl_*rap63*	HCT	1.60E+08 (±2.31E+07)	5.80E+07 (±6.89E+06)	38.11 (±2.26)
xyl_*rap63-phr63*	HCT	4.10E+08 (±1.68E+07)	3.44E+08 (±1.47E+07)	83.99 (±1.37)
xyl_*phr63*	HCT	3.37E+08 (±1.25E+07)	2.86E+08 (±1.53E+07)	84.79 (±2.17)
xyl_*rap-phr63*_R3'	HCT	2.31E+08 (±4.37E+07)	7.38E+07 (±1.44E+07)	32.06 (±1.96)
xyl_*rap63* + Phr63-4	HCT	2.57E+08 (±3.76E+06)	7.73E+07 (±6.38E+06)	30.12 (±2.38)
xyl_*rap63* + Phr63-5	HCT	4.00E+08 (±2.19E+07)	3.07E+08 (±7.13E+06)	76.90 (±2.77)
xyl_*rap63* + Phr63-6	HCT	2.86E+08 (±4.46E+07)	2.42E+08 (±4.11E+07)	84.33 (±1.04)
xyl_*rap63* + Phr63-7	HCT	3.57E+08 (±1.92E+07)	3.09E+08 (±1.72E+07)	86.69 (±1.63)
xyl_*rap63* + Phr63-8	HCT	3.20E+08 (±2.94E+07)	2.79E+08 (±2.99E+07)	86.84 (±1.87)
HD73	HCT	5.46E+08 (±4.52E+07)	4.54E+08 (±3.89E+07)	83.31 (±2.82)
HD73 ΔRap-Phr63	HCT	6.20E+08 (±9.51E+07)	5.50E+08 (±9.76E+07)	88.68 (±9.86)
HD73 ΔPhr63	HCT	5.70E+08 (±6.19E+07)	5.45E+08 (±7.03E+07)	95.29 (±3.78)
HD73 ΔPhr63 ΔPhr8	HCT	4.62E+08 (±2.73E+06)	2.51E+08 (±1.44E+07)	54.41 (±2.82)
HD73 Δ*rap63-phr63* Δ*rap8-phr8* (ΔΔ) xyl	HCT	2.56E+08 (±2.42E+07)	2.16E+08 (±2.48E+07)	83.83 (±3.11)
ΔΔ xyl_*rap63*	HCT	1.25E+08 (±7.88E+06)	4.07E+07 (±5.70E+06)	32.40 (±2.87)
ΔΔ xyl_*rap63-phr63*	HCT	2.78E+08 (±2.10E+07)	2.44E+08 (±2.09E+07)	87.68 (±2.24)
ΔΔ xyl_*rap63* + Phr63-6	HCT	2.57E+08 (±2.08E+07)	2.03E+08 (±1.66E+07)	79.15 (±2.35)
ΔΔ xyl_*rap63* + Phr63-7	HCT	2.79E+08 (±1.47E+07)	2.30E+08 (±1.40E+07)	82.41 (±2.31)
ΔΔ xyl_*rap63* + Phr63-8	HCT	2.78E+08 (±2.58E+07)	2.39E+08 (±2.14E+07)	86.03 (±0.46)
ΔΔ xyl_*rap63* + Phr8-6	HCT	1.46E+08 (±2.08E+06)	4.52E+07 (±4.41E+05)	30.95 (±0.64)
ΔΔ xyl_*rap63* + Phr8-7	HCT	1.78E+08 (±1.05E+07)	6.18E+07 (±4.41E+05)	34.97 (±1.94)
ΔΔ xyl_*rap63*+ Phr8-8	HCT	2.25E+08 (±1.15E+06)	6.60E+07 (±1.56E+06)	29.33 (±0.54)
ΔΔ xyl_*rap8*	HCT	4.45E+07 (±6.55E+06)	6.08E+05 (±1.59E+05)	1.34 (±0.29)
ΔΔ xyl_*rap8-phr8*	HCT	3.21E+08 (±2.01E+07)	2.66E+08 (±9.40E+06)	83.10 (±3.27)
ΔΔ xyl_*rap8* + Phr8-6	HCT	6.17E+07 (±7.78E+06)	6.99E+06 (±4.66E+05)	11.48 (±0.64)
ΔΔ xyl_*rap8* + Phr8-7	HCT	2.53E+08 (±1.39E+07)	2.03E+08 (±1.28E+07)	80.42 (±1.32)
ΔΔ xyl_*rap8* + Phr8-8	HCT	2.32E+08 (±1.27E+07)	3.04E+07 (±3.26E+06)	13.09 (±1.30)
ΔΔ xyl_*rap8* + Phr63-6	HCT	9.83E+07 (±2.10E+07)	7.61E+05 (±1.40E+04)	0.84 (±0.16)
ΔΔ xyl_*rap8* + Phr63-7	HCT	1.42E+08 (±6.15E+07)	1.27E+06 (±4.40E+05)	0.96 (±0.08)
ΔΔ xyl_*rap8* + Phr63-8	HCT	1.18E+08 (±3.61E+07)	1.49E+06 (±5.12E+05)	1.23 (±0.07)
HD73	Insect larvae	1.84E+08 (±1.08E+08)	3.58E+07 (±1.41E+07)	22.30 (±6.89)
HD73 ΔRap63-Phr63	Insect larvae	1.52E+08 (±8.91E+07)	2.16E+07 (±6.11E+06)	24.53 (±11.06)
HD73 ΔPhr63	Insect larvae	1.04E+08 (±4.55E+07)	2.25E+07 (±1.13E+07)	16.69 (±6.88)
HD73 ΔPhr8 ΔPhr63	Insect larvae	1.12E+07 (±) 2.68E+06	2.95E+04 (±1.90E+04)	0.23 (±0.11)

For the *in vitro* sporulation assays, the viable cells and heat-resistant spores were counted after 48 hours of growth in HCT medium at 30°C. For *in vivo* sporulation assays, the viable cells and heat-resistant spores were counted in dead larvae of *Galleria mellonella* 96 hours after injection at 30°C. Results are given as mean ± standard error of the mean (SEM). The percentages of spores were calculated as 100x the ratio between heat-resistant spores per ml and total viable cells per ml. Phr63-4: GETI. Phr63-5: HGETI. Phr63-6: AHGETI. Phr63-7: YAHGETI. Phr63-8: QYAHGETI. Phr8-6: AHGKDI. Phr8-7: YAHGKDI. Phr8-8: QYAHGKDI.

5 DISCUSSION

Rap-Phr systems were shown to regulate relevant pathways on *Bacillus* species, such as sporulation, competence, genetic mobile element transfer and production of proteases. The residues involved in their phosphatase activity on Spo0F are generally conserved even in Rap proteins with another function suggesting that the ancestor Rap protein regulated the sporulation phosphorelay (Even-Tov et al., 2016). The phosphorelay is a cascade of phosphoryl group transfer that modulates the phosphorylate state of Spo0A, the master regulator of commitment to sporulation (Burbulys et al., 1991). The spore formation is an important developmental process of bacteria from the Firmicutes phylum, namely *Bacillus* and *Clostridium* genera (Fimlaid & Shen, 2015). Spore is a metabolic dormant cell-type that is highly resistant to adverse conditions, which enable survival and dispersion of the bacteria in unfavorable environments.

Rap-Phr systems belong to the RNPP family of quorum sensing systems from Gram positive bacteria. RNPP cell-cell signaling is characterized by a regulator containing TPR domain that is regulated by its signaling peptide intracellularly (Declerck et al., 2007). The infectious cycle of *B. thuringiensis* in insect larvae is regulated by the sequential activation of RNPP systems: PlcR-PapR controls the virulence stage, NprR-NprX regulates the saprophytic stage and sporulation, and Rap-Phr also plays a role in the initiation of sporulation (Slamti et al., 2014). Among these three systems, the Rap-Phr quorum sensing system was the less studied in the *B. cereus* group.

5.1 Overview of Rap-Phr systems in *B. cereus* group

In *B. subtilis* type strain 168, 11 chromosomal Rap-Phr systems have been described (Kunst et al., 1997), a number corresponding to the average of Rap-Phr systems by strain in this species (Even-Tov et al., 2016). Moreover, some plasmid-borne *rap-phr* signaling cassettes were further studied in *B. subtilis* strains and related bacteria (Koetje et al., 2003; Parashar et al., 2013b; Qiao et al., 2011; Singh et al., 2013). Sequencing projects have revealed the presence of *rap-phr* alleles on chromosome and plasmids of *B. cereus* group genomes. The construction of a Rap-

Phr database from *B. cereus* group allowed to a more complete description of the distribution and diversity of these systems among bacteria of this group. *rap* genes were found in all studied strains and a *phr* gene was detected downstream from each *rap* gene, diverging from *B. subtilis* in which there are nearly three orphans *rap* genes per strain (Even-Tov et al., 2016).

Compare to *B. subtilis*, strains from the *B. cereus* group possess less *rap-phr* alleles, six in average. Furthermore, Rap proteins from the *B. cereus* and *B. subtilis* groups form two distinct clusters suggesting that these proteins have differentiated after the evolutionary separation of the two species (Even-Tov et al., 2016). This variation in the mechanism for sensing and responding to environmental changes enable these groups to occupy different niches (Alcaraz et al., 2010). Considering that the Rap protein is described as the most ancestor regulator from the RNPP family (Declerck et al., 2007), it could have undergone modifications that give rise to the other regulators of the family, such as NprR and then PlcR. The fact that, in *B. cereus* group, NprR and PlcR are described to control pathways, namely protease production (Dubois et al., 2012; Gohar et al., 2008) and sporulation (Perchat et al., 2016a), that are normally associated to Rap proteins in *B. subtilis* reinforces this hypothesis.

Plasmids were shown to play an important role in the adaptation of the bacteria from the *B. cereus* group to their ecological niche (Fazion et al., 2018; Vilas-Bôas et al., 2007). Interestingly, 30% of the identified Rap-Phr systems are encoded on plasmids. This amount appears to be more relevant when *B. cereus* (*sensu lato*) is compared to *B. thuringiensis*. While 13% of the *rap-phr* genes of *B. cereus* are harbored on plasmid, in *B. thuringiensis* this amount reaches 45%. Furthermore, the average of plasmid-borne systems in *B. thuringiensis* strains is six-fold higher than in *B. cereus* strains (3.6 and 0.6, respectively). Interestingly, the greater number of plasmids in *B. thuringiensis* does not only explain this difference.

As described for the Rap-Phr systems in *B. subtilis* (Even-Tov et al., 2016), Rap proteins from the *B. cereus* group show a great diversity which allowed to cluster them onto 12 chromosomal groups and nine plasmid groups. However, none of these groups occurs in all strains but close related strains present a similar Rap profile suggesting a similar evolutionary history with the housekeeping genes.

5.2 Sporulation activity prediction

Rap proteins from each group were aligned with RapH of *B. subtilis*, in which the residues involved in the phosphatase function on Spo0F were described (Parashar et al., 2011). Only 32% of the Rap regulators from the *B. cereus* group were predicted to inhibit the phosphorelay (Spo+). The predicted sporulation function was not related to the groups established by phylogenetic analysis, as three from 12 chromosome groups and five from nine plasmid groups are composed by proteins with different predicted effect on sporulation. Moreover, the Spo+ Rap proteins are mainly located on plasmids and thus in *B. thuringiensis* strains. The higher amount of Rap-Phr systems with effect on sporulation enables *B. thuringiensis* to better regulate its development, preventing the commitment to sporulation when enough nutrients are available.

To validate the sporulation activity prediction, ten Rap proteins were tested in sporulation assays. All Rap predicted as Spo- did not affect sporulation efficiency whereas among the seven Rap proteins predicted as Spo+, three did not inhibit spore formation. This absence of the expected activity could be due to the conditions of culture used, since the HCT medium is extremely favorable to sporulation, hindering the effect of Rap proteins that may act in a slight extent. Or yet, variation in other residues than the depicted ones could lead to conformational changes that impair the interaction with Spo0F. Hence, the residues assigned as implicated in the phosphatase activity of Rap proteins seems to be required to the sporulation activity but not sufficient to an accurate prediction. Consequently, the estimation of Spo+ Rap in the *B. cereus* group must be overestimated.

Although 14% of the selected strains did not carry any Spo+ Rap, this absence may be supplanted by the Rap-like phosphatase activity of NprR, which was recently demonstrated to also regulate sporulation (Perchat et al., 2016a). The high ratio of Rap proteins predicted as Spo- raise the question of the role of these proteins. Unlike *B. subtilis* bacteria, in which the ComA competence response regulator is the second main target pathway of Rap proteins, *B. cereus* group strains do not bear ComA homologs (Even-Tov et al., 2016). Considering the great amount of plasmid-borne systems and the role of Rap_{LS20}-Phr_{LS20} in the conjugation of the pLS20 plasmid (Singh et al., 2013), the effect of these systems on their encoding plasmid transfer could be an attractive subject to future analyses.

5.3 Rap63-Phr63 regulates sporulation

Since sporulation is the major process known to be regulated by Rap-Phr systems in *B. cereus* group bacteria and considering the interesting amount of plasmid-borne systems, the Rap63-Phr63 system from the well-studied pAW63 plasmid was further assessed with regard to sporulation activity. *rap63* gene is co-transcribed with the *phr63* gene, as largely described for the *rap-phr* signaling cassette (Perego & Hoch, 1996; Pottathil & Lazazzera, 2003). The expression from the *rap63* promoter begins one hour after the onset of the stationary phase. Furthermore, the *phr63* gene is transcribed by its own promoter, partially controlled by the sporulation specific sigma factor δ^H, likewise *phr* genes from *B. subtilis* (McQuade et al., 2001).

Contrasting to the strong effect on sporulation already described for the plasmid-borne Rap8 (Fazion et al., 2018) and Rap-BXA0205 (Bongiorni et al., 2006), the Rap63 moderately inhibits sporulation. Rap63 and Rap-BXA0205 (Rap5 – *B. anthracis* Ames Ancestor) display the same profile of residues implicate in sporulation activity but they are positioned in opposite extremity groups of the plasmid Rap phylogenetic tree (Group 9 and Group 1, respectively). These data reinforce the idea that other residues could affect the interaction between Rap and Spo0F. Moreover, while the Rap8 completely inhibits the expression of Spo0A-dependent genes (Fazion et al., 2018), Rap63 delays the expression of these genes. In sharp contrast, no effect was observed in the Δ*phr63* mutant strain, as already reported for other Rap-Phr systems (Auchtung et al., 2006; Fazion et al., 2018; Omer Bendori et al., 2015).

The strain expressing Rap63 present an auto-aggregation phenotype at the end of sporulation assays. Aggregation kinetics and microscope observation revealed a transient phenomenon that initiates four hours after the entry into stationary growth phase and dissipates with spore release. The peptidoglycan hydrolases that allow spores to release are under indirect control of Spo0A transcription regulator, as the autolysins CwlB and CwlC which expression are regulated by the sporulation sigma factor σ^K (Chen et al., 2018; Yang et al., 2013). Since Spo0A-regulated genes expression is delayed by Rap63 protein, the autolysins are also impacted leading to prevention of the disaggregation. One hypothesis for the occurrence of the aggregation phenotype is that the bunch of cells favors cell-cell

communication and then ensures an amount of Phr enough to achieve the sporulation process. Several questions remain unsolved about the aggregation phenotype, especially its regulation and the aggregation factor. Preliminary analysis has shown that most of strains of the *B. cereus* group present this phenotype, except for *B. cereus* ATCC 10987.

5.4 Synergistic activity of Rap63-Phr63 and Rap8-Phr8 on regulation of the commitment to sporulation

As already demonstrated for multiple sporulation (Bischofs et al., 2009) and ComA-related Rap regulation (Auchtung et al., 2006; Bongiorni et al., 2005) on *B. subtilis*, Rap proteins cohabitating in a given strain may act synergistically on the target process. The *B. thuringiensis* var. *kurstaki* HD73 strain contains eight *rap-phr* signaling cassettes, five on the chromosome and three on plasmids (pAW63; pHT77; pHT8_1) with half of these systems predicted to be Spo+, among them Rap8-Phr8 system was already described (Fazion et al., 2018).

Interestingly, the Δ*phr8*Δ*phr63* mutant strain exhibits an inhibition of sporulation efficiency both *in vitro* and *in vivo*. Indeed, in death larvae of *G. mellonella*, the sporulation was strongly affected with reduction of almost 100-fold compared to the wild-type strain. This effect is also more important than in the Δ*phr8* mutant strain that reduced four-times the sporulation efficiency (Fazion et al., 2018). These results suggest a synergistic action of Rap8-Phr8 and Rap63-Phr63 systems on the commitment to sporulation in the *B. thuringiensis* HD73 strain. Consequently, this regulation reinforces the relevance of the rich plasmid-pattern of *B. thuringiensis* strains for adaptation and survival in its ecological niche.

5.5. The Phr63 peptide and the specificity of Rap-Phr systems

The mature form of the Phr oligopeptides are generally found in the C-terminal end of the premature peptide (Pottathil & Lazazzera, 2003). The Phr63 active form is part of the C-terminal end of the pro-Phr63 peptide. However, the exact size of the mature peptide was not unveiled, since synthetic peptides corresponding to five, six, seven or eight last amino acids of the C-terminal end of Phr63 were able to inhibit the Rap63 activity. Similarly, the active form of Phr-BA3791 encoded on *B. anthracis*

chromosome is included in the C-terminal region of the peptide sequence but the exact mature peptide was not known (Bongiorni et al., 2006).

The C-terminal part of the Phr63 sequence is highly similar to that of Phr8. In addition to the synergic effect of these two systems, this similarity suggests a cross-talk between Rap63-Phr63 and Rap8-Phr8. Nevertheless, the Phr from one system was not able to inhibit the Rap regulator of the other. Similarly, the absence of cross-reactivity between RapA-PhrA, RapC-PhrC and RapE-PhrE was demonstrated in *B. subtilis* (Jiang et al., 2000a; Perego, 1997). Thus, the absence of cross-talk between the Rap63-Phr63 and Rap8-Phr8 systems strengthen the hypothesis of the specificity of the Rap-Phr signaling pair.

6 CONCLUSION

Cell-cell communication is now regarded as a general trait of bacteria species. Indeed, the quorum sensing enables bacteria to coordinate their metabolic processes regarding to the population density. Gram positive bacteria utilize peptides as signaling molecules and the quorum sensing systems from the RNPP family play a key role in the infectious cycle of *B. thuringiensis* in its naturalistic environment. Rap-Phr systems are crucial for the coordination of bacterial process in *B. cereus* group species, specifically sporulation, as wide described for *B. subtilis* systems. The precise control of sporulation allows the bacteria to survive and to better adapt in their ecological niche, taking into account the modifications of the environment. The great number of Rap-Phr systems encoded by plasmid reinforces the importance of the wide plasmid pattern of *B. cereus* group, especially in *B. thuringiensis*. The fact that the main virulence gene and regulator of bacterial development (such as Rap-Phr systems) are encoded on plasmids emphasize that these replicative extrachromosomal elements play an important role in adaptation of their host bacteria.

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Titre : Diversité et analyse fonctionnelle des systèmes Rap-Phr du groupe *Bacillus cereus*

Mots clés : *Bacillus thuringiensis*, sporulation, quorum sensing, famille RNPP, plasmide

Résumé : Le groupe *Bacillus cereus* est composé de huit espèces de bactéries à Gram positif sporulantes qui peuvent coloniser plusieurs niches écologiques. Les espèces les plus importantes sont *B. cereus*, une bactérie ubiquitaire du sol et un pathogène opportuniste; *B. thuringiensis*, un entomopathogène très utilisé comme biopesticide; et *B. anthracis* l'agent de la maladie du charbon. Bien que ces espèces présentent différents phénotypes, elles sont étroitement liées génétiquement et leurs facteurs de virulences principaux sont portés par des plasmides. Le cycle infectieux de *B. thuringiensis* dans la larve d'insecte est régulé par l'activation séquentielle de systèmes de quorum sensing de la famille RNPP. Parmi eux, les systèmes Rap-Phr, caractérisés chez *B. subtilis*, ont très peu été étudiés dans le groupe *B. cereus*. Ces systèmes régulent divers processus bactériens importants dont la sporulation. L'objectif de cette étude est d'analyser les systèmes Rap-Phr dans le groupe *B. cereus*, pour connaitre leur distribution, leur localisation et leur diversité afin d'obtenir une vue globale de ces systèmes chez ces bactéries. De plus, leur possible implication dans la régulation du processus de sporulation a été prédite sur la base de données structurales décrites chez RapH de *B. subtilis*. Les gènes *rap*, toujours associés à un gène *phr*, sont présents dans toutes les souches étudiées avec une moyenne de six gènes *rap-phr* par souche et avec 30% de ces systèmes qui sont portés par des plasmides. Les souches de *B. thuringiensis* portent six fois plus de systèmes Rap-Phr plasmidiques que les souches de *B. cereus*. Par ailleurs, les souches phylogénétiquement proches possèdent un profil de gènes *rap-phr* similaire. Un tiers des protéines Rap sont prédites pour inhiber la sporulation et ces protéines sont préférentiellement localisées sur les plasmides et donc plus fréquemment présentes chez *B. thuringiensis* que chez *B. cereus*. Cette prédiction a été partiellement validée par des tests de sporulation suggérant que les résidus impliqués dans cette activité chez *B. subtilis* sont conservés mais insuffisants pour prédire cette fonction. Le système Rap63-Phr63 porté par le plasmide pAW63 de la souche *B. thuringiensis* HD73 a ensuite été caractérisé. La protéine Rap63 a un effet modéré sur la sporulation et retarde l'expression des gènes régulés par Spo0A. La Rap63 est inhibée par son peptide Phr63, dont la forme mature correspond à l'extrémité C-terminale du pro-peptide. Les résultats de sporulation dans l'insecte suggèrent une activité synergique des systèmes Rap63-Phr63 et Rap8-Phr8 (porté par le pHT8_1) dans la régulation de la sporulation. Malgré la similarité entre les Phr63 et Phr8 aucun cross-talk n'a pu être mis en évidence, ce qui confirme la spécificité de ces systèmes de communication cellulaire. L'ensemble de ces résultats démontre la grande diversité des systèmes Rap-Phr dans le groupe *B. cereus* et souligne l'impact des systèmes plasmidiques dans le développement de ces bactéries. Par conséquent, les plasmides sont des éléments importants pour l'adaptation et la survie de ces bactéries et particulièrement pour *B. thuringiensis*.

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Title: Diversity and functional analysis of Rap-Phr systems from *Bacillus cereus* group

Keywords: *Bacillus thuringiensis*, sporulation, quorum sensing, RNPP family, plasmid

Abstract: The *Bacillus cereus* group of Gram positive spore forming bacteria is comprised by eight species that are able to colonize several ecological niches. The most important species are *B. cereus*, a ubiquitous soil bacterium and an opportunistic pathogen; *B. thuringiensis*, an entomopathogen widely used as biopesticide; and *B. anthracis*, the causative agent of anthrax. Even if they present different phenotypes, they are genetic closely related and their main virulence factors are encoded on plasmids. The infectious cycle of *B. thuringiensis* in the insect larvae is regulated by the sequential activation of quorum sensing systems from the RNPP family. Among them, the Rap-Phr was extensively studied in *B. subtilis* but just punctually in *B. cereus* group species. The Rap-Phr systems were shown to regulate various bacterial processes, including the sporulation. The objective of this study was to analyze the Rap-Phr systems in the *B. cereus* group, regarding their distribution, location and diversity to achieve an overview of these systems in these bacteria. Moreover, their possible involvement in the control of the sporulation process was predicted based on structural data described for RapH in *B. subtilis*. The *rap* genes, always associated with a *phr* gene, were present in all 49 studied strains with an average of six *rap-phr* genes per strain and 30% were located on plasmids. Comparison among *B. cereus* and *B. thuringiensis* strains revealed that the last one harbors six-fold more plasmid *rap-phr* system then the former. Moreover, phylogenetic closer strains possess a similar profile of *rap-phr* genes. Interestingly, 32% of the Rap proteins were predicted to inhibit sporulation and these proteins were preferentially located on plasmids and therefore in *B. thuringiensis* strains. This prediction was partially validated by sporulation efficiency assays suggesting that residues identified in *B. subtilis* as involved in the phosphatase activity are conserved but not sufficient to predict the sporulation function. Then, the plasmid-borne Rap63-Phr63 system from pAW63 plasmid of *B. thuringiensis* HD73 strain was further studied. The Rap63 protein moderately inhibits the sporulation and delays the expression of Spo0A-regulated genes. Rap63 is counteracted by its cognate Phr63 peptide, which mature form corresponds to the C-terminal end of the pro-peptide. Sporulation assays in insect larvae suggest a synergistic activity of Rap63-Phr63 and Rap8-Phr8 (from pHT8_1 of *B. thuringiensis* HD73 strain) systems on sporulation efficiency. Despite the similarities of Phr63 and Phr8 no cross-talk was found between these two systems, confirming their specificity. Altogether, these results reveal the high diversity of the Rap-Phr systems in the *B. cereus* group and highlight the relevance of the plasmid-borne systems to cell development. Therefore, the results demonstrated the importance of the plasmids in the adaptation and the survival of these bacteria, especially for *B. thuringiensis*.

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Título: Diversidade e análise funcional de sistemas Rap-Phr no grupo *Bacillus cereus*

Palavras-chave: *Bacillus thuringiensis*, esporulação, *quorum sensing*, família RNPP, plasmídeo

Resumo: O grupo *Bacillus cereus* é formado por oito espécies de bactérias Gram positivas esporulantes que podem colonizar diversos nichos ecológicos. As espécies mais importantes do grupo são *B. cereus*, bactéria ubíqua do solo e patógeno oportunista; *B. thuringiensis*, entomopatógeno amplamente utilizado como biopesticida; e *B. anthracis*, agente etiológico do antraz. Embora apresentem fenótipos diferentes, essas espécies são próximas geneticamente e seus principais fatores de virulência são codificados por plasmídeos. O ciclo infeccioso de *B. thuringiensis* na larva de inseto é regulado pela ativação consecutiva de sistemas de *quorum sensing* da família RNPP. Dentre eles, o sistema Rap-Phr foi amplamente estudado em *B. subtilis*, porém apenas pontualmente explorado nas espécies do grupo *B. cereus*. Os sistemas Rap-Phr regulam vários processos fisiológicos bacterianos, inclusive a esporulação. O objetivo deste estudo foi analisar os sistemas Rap-Phr no grupo *B. cereus*, com intuito de conhecer sua distribuição, localização e diversidade a fim de obter um panorama desses sistemas neste grupo. Além disso, o possível envolvimento desses sistemas no controle do processo de esporulação foi predito com base nos dados estruturais descritos para RapH de *B. subtilis*. Genes *rap*, sempre associados a um gene *phr*, estão presentes em todas as 49 linhagens estudadas com uma média de seis alelos *rap-phr* por linhagem e 30% dos sistemas estão localizados em plasmídeos. As linhagens de *B. thuringiensis* possuem seis vezes mais sistemas Rap-Phr plasmidiais do que as linhagens de *B. cereus*. Ademais, linhagens filogeneticamente próximas apresentam um perfil similar de genes *rap-phr*. Um terço das proteínas Rap foram preditas como inibidoras da esporulação e estas proteínas estão preferencialmente localizadas em plasmídeos e, portanto, em linhagens de *B. thuringiensis*. A predição foi parcialmente validada por ensaios de esporulação sugerindo que os resíduos identificados pelo envolvimento na atividade de fosfatase em *B. subtilis* são conservados no grupo *B. cereus*, porém não são suficientes para predizer a função sobre a esporulação. Em seguida, o sistema Rap63-Phr63 codificado pelo plasmídeo pAW63 da linhagem *B. thuringiensis* HD73 foi caracterizado. A proteína Rap inibe moderadamente a esporulação e retarda a expressão de genes regulados por Spo0A. Rap63 é inibida por seu peptídeo cognato Phr63, cuja forma madura corresponde à extremidade carboxi-terminal do pro-peptídeo. Ensaios de esporulação em larvas de inseto sugerem uma atividade sinérgica dos sistemas Rap63-Phr63 e Rap8-Phr8 (do plasmídeo pHT8_1 da linhagem *B. thuringiensis* HD73) sobre a esporulação. Apesar da similaridade entre Phr63 e Phr8 não foi observado *cross-talk* entre os dois sistemas, confirmando sua especificidade. Desta forma, o conjunto dos resultados demonstra a grande diversidade dos sistemas Rap-Phr no grupo *B. cereus* e destaca o impacto de sistemas plasmidiais no desenvolvimento destas bactérias. Consequentemente, reforça a importância dos plasmídeos na adaptação e sobrevivência dessas espécies, particularmente em *B. thuringiensis*.