Abstract

Quorum Sensing (QS) are mechanisms of synthesis and detection of signaling molecules to regulate gene expression and coordinate behaviors in bacterial populations. In *Bacillus subtilis* (Bs), multiple paralog Rap-Phr QS systems (receptor-signaling peptide) are highly
redundant and multifunctional, interconnecting the regulation of differentiation processes such as sporulation and competence. However, their functions in the B. cereus group are largely unknown. We evaluated the diversification of Rap-Phr systems in the B. cereus group as well as their functions, using Bacillus thuringiensis Bt8741 as model. Bt8741 codes for eight Rap-Phr systems; these were overexpressed to study their participation in sporulation, biofilm formation, extracellular proteolytic activity and spreading. Our results show that two Rap-Phr systems (RapK and RapF) inhibit sporulation, probably through dephosphorylation of Spo0F; these two Raps also inhibit biofilm formation. Five systems (RapC, F, F2, I1 and RapLike) decrease extracellular proteolytic activity; finally, four systems (RapC, F1, F2 and RapLike) participate in spreading inhibition. Our bioinformatic analyses showed that Rap proteins from the B. cereus group diversified into five pherogroups, and we foresee that functions performed by Rap proteins of Bt8741 could also be carried out by Rap homologs in other species within the group. These results indicate that Rap-Phr systems constitute a highly multifunctional and redundant regulatory repertoire that enable bacteria from the B. cereus group to efficiently regulate collective functions during the bacterial life cycle, in the face of changing environments.

Importance

The Bacillus cereus group of bacteria includes species of high economic, clinical, biological warfare and biotechnological interest, e.g. B. anthracis in bioterrorism, B. cereus in food intoxications and B. thuringiensis in biocontrol. Knowledge on the ecology of these bacteria is hindered due to our limited knowledge of the regulatory circuits that control differentiation and specialization processes. Here, we uncover the participation of eight Rap quorum-sensing receptors in collective functions of B. thuringiensis. These
proteins are highly multifunctional and redundant in their functions, linking ecologically relevant processes such as sporulation, biofilm formation, extracellular proteolytic activity and spreading, and probably other additional functions in species from the B. cereus group.

**Introduction**

Bacteria perform many functions that depend on multicellular-like behaviors, such as cell differentiation and specialization. These behaviors, also known as collective functions, allow the emergence of complex ecological interactions, including cooperation and division of labor in biofilms (1, 2). Collective functions are only evident and effective when performed by large groups in bacterial populations or communities (3–6). Some of the most studied examples include bioluminescence by the squid symbiont *Vibrio fischeri* (7), or fruiting body formation during sporulation of *Myxococcus xanthus* (8).

In gram positive bacteria, collective functions and the molecular mechanisms for their control have been widely studied in *Bacillus subtilis* (Bs). In Bs cultures, several mutually-exclusive cell-types have been identified (motile, competent, sporulating, cannibal, biofilm matrix producers, surfactant producers and miners (9, 10)), where emerging ecological interactions such as cooperation, cheating and cross-feeding, have been described (5, 6, 11). The presence of these cell differentiation phenomena and the resulting ecological interactions, ultimately affect the manifestation of collective traits such as sporulation efficiency, surface colonization, biofilm architecture complexity, etc. (2, 9, 12). These phenomena depend on global modifications of transcriptional regulation; they are triggered by environmental cues, stress conditions, cell-cell signaling, and are tightly modulated by complex, overlapping regulatory circuits (13–15).
Bacteria detect cell density through quorum sensing (QS), which depends on self-produced signaling molecules that accumulate in the extracellular space as the population grows. Specific receptors in the cell membrane or in the cytoplasm recognize these signaling molecules and regulate downstream cellular processes (16–18). Collective traits such as virulence, competence, sporulation and bioluminescence are regulated by QS. Gram-positive bacteria use small peptides as signaling molecules for QS (17).

The RRNPP family (Rgg, Rap, NprR, PlcR, PrgX) are intracellular QS receptors that regulate several functions across gram-positive bacteria (19–21). Genes coding for receptor proteins and their associated signaling peptides are encoded in transcriptional cassettes (22). Rgg, NprR, PlcR and PrgX proteins are transcriptional activators that bind directly to DNA in quorum state. Rap proteins, however, lack a DNA binding domain and they function by binding and inhibiting proteins, specifically response regulators and transcriptional activators (21, 23, 24). Twelve Rap paralogs (RapA, B, C, D, E, F, G, H, I, J, K, 60) control diverse functions in \textit{B. subtilis} 168 (Bs168). The RapG-PhrG pair regulates the activation of DegU, a transcriptional regulator that controls \textit{aprE} and \textit{comK} genes encoding for extracellular proteases and a transcription factor for competence in Bs, respectively (15, 25); ComA – the master regulator of competence genes – is repressed by RapC, D, F, G, H, K and Rap60 (14, 26–31); Spo0A – the transcriptional activator of many differentiation genes – is indirectly regulated by RapA, B, E, H, J, and Rap60 (24, 31–35). Hence, Rap protein paralogs from Bs are highly multifunctional and redundant and they connect several differentiation processes and coordinate collective traits.
Spo0A is activated by phosphorylation through a multicomponent phosphorelay system. Up to five kinases auto-phosphorylate in response to intracellular and environmental stress signals and transfer the phosphate group to Spo0F, which is then transferred to Spo0B and finally to Spo0A (36). Spo0A-P activates the transcription of multiple genes, including biofilm formation (at low concentrations) and early sporulation genes (at high concentrations (13)). Rap QS proteins prevent the phosphate transfer in the phosphorelay by binding to Spo0F (32, 37).

While the regulation of collective traits in Bs is well known, these phenomena remain largely understudied in the B. cereus group, which includes bacteria with clinical and biotechnological relevance (38). Although Bs and B. cereus group species share similar characteristics such as the sporulation process, the Spo0A phosphorelay components, and have many protein families in common, they also have notorious genetic differences (39). In B. thuringiensis (Bt, the most widely used biopesticide), the Spo0A phosphorelay is modulated by the bifunctional QS receptor NprR, which is not present in Bs (40–42). On the other hand, ComA and DegU response regulators are not present in Bt. Additionally, Rap-Phr QS systems also differ in both groups. These QS systems have evolved by duplication and divergence mechanisms; even though multiple Rap proteins paralogs are also found B. cereus group species, they have evolved independently and no Rap homologs are shared between the two groups (43, 44). Therefore, it is not possible to predict the functions of Rap proteins in the B. cereus group based on what is known of Rap proteins from Bs.
Some Rap-Phr systems from species of the *B. cereus* group have been studied. First, Rap BXAO205 and BA3790 from *B. anthracis* str. A2012, were demonstrated to regulate sporulation initiation and to dephosphorylate Spo0F (45). Later, it was shown that Rap8 from Bt-HD73, regulates the sporulation and biofilm formation processes *in vitro* (46). A more recent study showed the participation of Rap6, 7 and 8 – also known as RapC, K and RapF, respectively (47) – in the modulation of the sporulation process in Bt407 (48).

However, other Rap paralogs with unknown functions have been identified in the genomes of *B. cereus* group bacteria (44, 47) and they may be relevant to their ecology.

In this study we aimed at evaluating the diversification of the Rap-Phr systems in the *B. cereus* group as well as their functions, using *Bacillus thuringiensis* Bt8741 as a model. We generated eight Rap-overexpression strains of Bt8741 to evaluate the role of each Rap paralog in sporulation efficiency, biofilm formation, extracellular proteolytic activity and spreading. We also studied the evolution of Rap-Phr paralogs in the *B. cereus* group, by identifying Rap homologs from other species and analyzing its phylogeny. This allowed the prediction of their functions, based on those of Rap proteins from Bt.

**Results**

Spo0F-binding residues from Bs RapH are conserved in Rap proteins from Bt8741.

In order to predict the capacity of Rap proteins from Bt407 (a strain closely related to Bt8741) to bind to Spo0F, we analyzed the conservation of the amino acids previously reported to be involved in Spo0F-binding by RapH from *B. subtilis*. In this analysis, we included reported sequences of Bs-Rap proteins that bind to Spo0F (RapA, B, E, H, J) as well as the sequence of RapD from Bs, which does not bind to Spo0F (34) (Fig. 1). We...
found higher percentage of conservation of the functional amino acids of RapH, in the sequences of both Bs168 and Bt407, compared to the corresponding complete sequences (Fig. 1B). In Bs168 the full sequence conservation of the Spo0F binding Raps (RapA, B, E, J) compared to RapH, ranges from 59% to 66% and the functional amino acids conservation percentage from 82.3% to 100%. In RapD, the full-length sequence is conserved at 50% and functional residues are only 64.7% conserved (Fig. 1B). In the case of Rap proteins from Bt407, the full sequence conservation in comparison to RapH of Bs168 ranged from 45% to 48%. On the other hand, conservation of functional residues ranged from 64.7% to 88.2% (Fig 1B). This indicates that the analyzed residues are important for the function of Bt Rap proteins.

RapK presented the highest conservation percentage of functional residues (88.2%), followed by RapF, I and RapI1 (82.3%), RapF1, F2 and RapLike (70.5%) and finally RapC with 64.6%. Although RapF1 and RapF2 presented a high conservation of functional residues, these Rap paralogs, as well as RapC, do not conserve the residue Q47 found in the catalytic site and previously shown to be essential for the phosphatase activity of RapH (34). This analysis enables the prediction that some Rap protein paralogs from Bt8741, with a high conservation percentage of functional amino acids, could dephosphorylate Spo0F, while other paralogs could have evolved to participate in other regulatory processes. Indeed, RapK, RapF, and unexpectedly RapC from Bt407, Rap8 from Bt-HD73 (ortholog to RapI from Bt407) and Rap BX0205 and BA3790 from B. anthracis, (homologs of RapK and RapF2, respectively) have been shown to participate in the modulation of sporulation (45, 46, 48). Previous to this work, RapF1, I1, and RapLike from Bt407 (or its homologs in other species), had not been tested for their role in sporulation.
RapC, K, F and RapLike control sporulation in Bt8741.

We constructed nine Rap-overexpression strains in the Bt8741 background (Table S1), one for each endogenous Rap protein identified in Bt407 (RapC, K, F, F1, F2, I, I1, Like) and one more for RapA from Bs168 (RapABs). We also generated a control strain of Bt8741 carrying the empty plasmid pHT315-P\(_{xylA}\) (Table S1). DNA sequencing showed correct, in-frame insertion of P\(_{xylA}\) and rap genes in the pHT315 plasmid (not shown). We followed a growth time-course experiment of all strains in shaking flasks for 24 hours and confirmed that neither xylose addition, nor Rap overexpression, affected bacterial growth (Fig. S1).

In order to identify the Rap proteins involved in the regulation of the sporulation initiation, we studied the effect of the overexpression in the sporulation efficiency of each strain. In this experiment, we observed that both addition of xylose to the culture medium and the presence of rap genes in the plasmid had minor effects on total and thermoresistant CFU counts of Bt8741 at 72 h. In the control strain, addition of xylose caused a decrease of \(\approx 1\) log10 in total and thermoresistant CFU (Fig. S2A and S2B). Similarly, when rapF1 and rapF2 genes were carried in the plasmid – but not overexpressed – total CFU decreased by up to one logarithm of CFU in comparison to the control strain (Fig. S2A). Additionally, sporulation decreased one logarithm in strains carrying rapF1, rapF2 and rapI1 in comparison to the control strain when Rap was not overexpressed (Fig. S2C). These unspecific effects were probably related to basal expression from the P\(_{xylA}\) promoter, even when xylose is not added, since pHT315 replicates at 15 copies per cell (49). Finally, the most dramatic effect was found in thermoresistant CFU of strains that overexpressed Rap proteins (Fig. S2D).
In spite of unspecific effect of xylose addition on growth and sporulation, sporulation efficiency of the control strain remained unchanged by the addition of inducer (Fig. 2). In contrast, overexpression of RapA_{Bs} caused a decrease in sporulation efficiency from 7.9\% to 0.0005\%. In fact, thermoresistant CFU were undetectable when RapA_{Bs} was overexpressed (Fig. S2D). We also found undetectable levels of spores in strains overexpressing RapK and RapF (Fig. S2D). Sporulation efficiency decreased from 32.93\% to 0.0002\% in the strain overexpressing RapK and from 9.24\% to 0.0026\% in the strain overexpressing RapF (Fig. 2). In Bs, RapA dephosphorylates Spo0F in the Spo0A phosphorelay (32) and this result indicates that it performs the same function in B8741; furthermore, it suggests that RapK and RapF carry out the same mechanism for regulation of sporulation initiation.

Strains carrying P_{xylA}‘rapC and P_{xylA}‘rapLike also had reduced sporulation efficiency in induced media. Sporulation efficiency decreased from 5.43\% to 0.0357\% and from 12.34\% to 0.1352\% when RapC and RapLike were overexpressed, respectively (Fig 2). Additionally, RapI overexpression slightly decreased sporulation efficiency, from 2.82\% to 0.51\%. Sporulation efficiency was not decreased by the overexpression of RapF1, F2, I or RapI1.

Samples of the Rap-overexpressing strain cultures at 72 h were observed in a microscope. We detected free spores and bacterial debris in all strains, when Rap proteins were not overexpressed (Fig. S3). Figure 3 shows representative cells from each induced culture. Strains overexpressing Rap proteins that did not affect sporulation efficiency (RapF1, F2
and RapI1) showed cell morphology similar to that of the control strain, i.e. a sporulated bacilli with defined endospores. In strains overexpressing RapA, K and RapF, that had acutely decreased sporulation efficiency, we observed chained, wrinkled cells with no spores (Fig. 3). On the other hand, cells from strains overexpressing RapC and RapLike, were rod-shaped with no evident spore (Fig. 3). Finally, cells from the strain overexpressing RapI, which had a slight effect on sporulation efficiency, cell morphology was similar to strains overexpressing RapF1, F2, I1 and the control strain (Fig. 3), showing a defined endospore.

Overexpression of RapF and RapK prevents biofilm formation of Bt8741.

In nature, over 80% of bacteria live in biofilms (49), therefore, biofilm formation is likely a relevant trait – albeit an understudied one – during the life cycle of Bt and other B. cereus group bacteria. To determine which Rap proteins are involved in the regulation of biofilm development in Bt8741, we quantified biofilm formation in the air-liquid interphase at 48 h using Rap-overexpression strains. For this, we suspended cells form the biofilm and measured optical density ($OD_{600}$). Since 20 mM xylose caused a complete inhibition of biofilm formation in the Bt8741 control strain (not shown), we first tested the effect of xylose concentration on this phenotype. We found that biofilm formation was not affected at 2 mM, but was decreased at higher concentrations of 5, 10 and 15 mM (Fig. S4); therefore, overexpression of Rap proteins was performed with 2 mM of xylose (50).

Overexpression of RapK and RapF caused a complete inhibition of biofilm formation of Bt8741 (Fig. 4A), evident by the significant decrease ($p<0.0001$) in the $OD_{600}$ of the resuspended biofilm (Fig. 4B). The $OD_{600}$ of the biofilms decreased from 0.7115 to 0.0977
and from 0.6577 to 0.0961 in strains overexpressing RapK and RapF, respectively (Fig. 4B).

On the other hand, biofilms were normally formed by strains overexpressing RapA Bs, C, F2, I, I1 and RapLike (Fig. 4). Interestingly, the strain overexpressing RapF1 was unable to form biofilms even when the overexpression was not induced (Fig. 4A and B).

In order to discard global growth defects in this assay when RapK and RapF are overexpressed, we measured planktonic growth through OD$_{600}$ of the liquid culture media from the same experiments where biofilm formation was assessed. We found that planktonic growth was higher in conditions where a biofilm is not formed (Fig. S5). This indicates that RapK and RapF specifically inhibit biofilm formation (e.g. secretion of extracellular matrix components).

Extracellular proteolytic activity is downregulated by RapC, F, F2, I1 and RapLike in Bt8741.

In Bt, the production of extracellular proteases is crucial during its necrotrophic phase, i.e. development in insect cadavers. We tested the role of Rap proteins in extracellular proteolytic activity by measuring the effect of Rap overexpression on hydrolysis halos of colonies on milk agar (MA) plates. Addition of xylose had no effect (p>0.05) on the hydrolysis halo of the control strain (Fig. 5). In contrast, overexpression of RapC, F, F2, I1 and RapLike decreased the halo area (p<0.05; Fig. 5B). In these strains, the halo area decreased to 41.98%, 37.81%, 46.65%, 47.51% and 34.93%, respectively (Fig. 5B, Fig. S6) compared to halos in plates where overexpression was not induced (100%). Proteolytic activity of strains overexpressing RapA Bs, K, I and RapF1 was not affected by the induction (p>0.05; Fig. 5A and B).
RapC, RapF1, RapF2 and RapLike regulate spreading of Bt8741 colonies.

Colonies of Bt8741 present a spreading phenotype that could be associated to its capacity to colonize hosts and habitats. Similar passive motility phenotypes have been described in other species of Bacillus, associated to the production of extracellular surfactant molecules (51–53). To gain insights on this understudied collective trait, we determined the effect of Rap proteins on radial spreading of colonies of Bt8741 growing on agar media.

We observed that addition of xylose in the media did not affect spreading of the control strain (Fig. 6). In contrast, the overexpression of RapC, F1, F2 and RapLike caused a decrease in spreading (p<0.05) of Bt8741 colonies at day 7 (Fig. 6A and B). The overexpression of RapC reduced the colony dispersion from 5.15 mm to 0.49 mm (reduction of 90.4%); RapF1, from 3.73 mm to 1.83 mm (decrease of 50.9%); RapF2 from 5.05 mm to 0.78 mm (decrease of 84.5%); and RapLike from 3.64 mm to 0.65 mm (decrease of 82.1%) (Fig. 6B). Spreading inhibition is evident in the colony morphology of these strains (Fig. 6C). We observed that the overexpression of RapC, F2 and RapLike, completely eliminated this phenotype, while overexpression of RapF1 only decreased spreading (p<0.05) (Fig. 6B and C).

The overexpression of RapA Bs, K, F, I and RapI1 did not affect the spreading of Bt8741 (p>0.05) (Fig. 6B). Spreading of the strains carrying overexpression plasmids for these Rap proteins ranged from 4.68 mm to 7.29 mm without induction and from 3.37 mm to 6.84 mm when induced (Fig. 6B). In some cases, Rap overexpression affected colony morphology, i.e. colonies of strains overexpressing RapK and RapF show an increased...
dendritic phenotype; however, the spreading phenotype measured as colony radius, is still present (Fig. 6C).

**Rap-Phr systems diversified into five pherogroups in the *B. cereus* group.**

In order to predict functions of Rap paralogs in *B. cereus* (Bc), *B. anthracis* (Ba), *B. mycoides* (Bm), *B. pseudomycoides* (Bps) and *B. cytotoxicus* (Bcyt), we studied the evolution of Rap proteins in these species and identified their putative signaling peptide sequences (mature Phr). Additional to the 8 Raps in Bt407 (of which 4 are coded in the chromosome, and 4 in plasmids) we found 32 *rap-phr* systems in the *B. cereus* group (Table S2), 30 of which are encoded in chromosome and 2 in plasmids (Table 1).

The phylogeny of Rap proteins from the *B. cereus* group shows that clades are composed of Rap proteins from different species, i.e., phylogenetically close Rap homologs can be found in different species. This indicates that Rap-Phr divergence occurred before speciation in this group (Fig. 7). Hence, Rap functions discovered in this work could be extrapolated to the rest of the *B. cereus* group, e.g. Rap proteins found in the same clade as BtRapK and BtRapF (Bps28285, Bps05775, Bps24285, Bcyt05320, Bc1026, Ba05875, Ba29315, Bcyt11595, Bcyt05405 and Bcyt02700) may modulate sporulation initiation and biofilm formation. Since we found that several Rap paralogs are coded in every species of the *B. cereus* group, we suggest that they could regulate a variety of collective functions in all species, as we describe here for Bt8741.

We identified 5 pherogroups, each with a putative mature Phr peptide sequence. All five pherogroup comprise Rap proteins from different species, which share Phr consensus...
sequences corresponding to the putative mature Phr (Fig. S7). These five pherogroups are identified with colors in the branches of the phylogeny in figure 7. We found that mature Phr corresponding to pherogroups 1 and 2 are located in the C-terminal domain of the pro-peptides (exported Phr sequence). RapI, F, F1 and RapK from Bt are found in these pherogroups. On the other hand, pherogroup 3 – were RapF2 from Bt is found – consensus sequences are located in the N-terminal domain of the pro-peptide. Finally, putative mature Phr peptides from pherogroups 4 and 5 – which include RapLike, C and RapI1 from Bt – are located in the middle of the exported sequence (Table S3). We observed that only Bt and Bps encode Rap proteins from all five pherogroups; Rap proteins from Ba and Bcyt are found in pherogroups 1, 2, 3 and 4; Rap proteins in Bm correspond to pherogroups 1, 3 and 5; finally Rap proteins from Bc are found only in pherogroups 1 and 4 (Fig. 7).

Discussion

Few studies have addressed multicellular behaviors such as differentiation, cell-specialization, collective functions, and the resulting ecological interactions in species from the B. cereus group (11, 56). Similarly, molecular mechanisms for the control differentiation processes in the B. cereus group bacteria remain understudied (45, 46, 48, 56, 57). Here we demonstrate that Rap-Phr systems in Bt8741 regulate collective functions such as sporulation, biofilm formation, production of extracellular proteases and spreading motility. In fact, Rap-Phr paralogs in this strain are highly multifunctional and redundant, since five out of eight Rap paralogs modulate more than one collective trait, and all four collective traits studied were inhibited by more than one Rap protein. Hence, Rap paralogs appear to constitute a regulatory repertoire that allow Bt populations to respond efficiently to environmental changes, which aid for survival of the population.
Although it is well known how Rap-Phr systems participate in differentiation processes of
the gram-positive model bacteria Bs, speciation resulted in divergent Rap proteins in the B. 
cereus and B. subtilis groups (43, 44). Therefore, no homologs are shared between the
groups; however, in both cases, speciation resulted in the presence of multiple Rap paralogs
per genome. We propose that Rap proteins that are phylogenetically close to Rap proteins
from Bt8741, could have the same functions in other bacteria of the B. cereus group.

It is not yet clear how bacteria benefit from keeping multiple receptor-signaling peptide
gene pairs comprising this complex signaling network of Rap-Phr systems; however, it has
been shown that redundancy in Rap-Phr systems on Bs is selected by social advantages
(58). Because Rap proteins have a repressive function upon its target, the gain of a novel
Rap-Phr system for the regulation of extracellular public good production enable a
facultative cheating mechanism in which variants with an extra system exploit their
ancestral strain. Here we showed that extracellular public goods such as biofilm matrix
components, extracellular proteases or surfactants, are likely controlled by Rap proteins in
Bt8741; therefore, the same facultative cheating mechanism could be expected during
duplication of rap-phr genes in the B. cereus group. This represents a selective advantage
by a fitness increasement of the novel population. Multifunctionality seems to have
evolutionary advantages as well. Perhaps, because Rap-Phr systems are known to be
parallel signaling pathways (44) they are not all activated simultaneously; instead, some of
them may be active only under specific conditions, achieving the regulation of various
differentiation processes and collective functions while optimizing energetic costs. Overall,
keeping multiple redundant and multifunctional Rap paralogs that control important collective functions results in a better adaptation and population survival in nature.

Sporulation in the *Bacillus* genus is essential for bacterial survival and dissemination in their habitats; it is also important for the biotechnological uses of *Bacillus* species. Six Rap-Phr systems from Bt, including RapA, negatively regulate Spo0A phosphorelay by dephosphorylating Spo0F, and therefore prevent the activation of Spo0A (32). We found that RapA<sub>Bs</sub> retained this function when it was overexpressed in Bt8741. Furthermore, five Rap-Phr systems from Bt8741 (RapK, F, C, Like and RapI) also regulate sporulation in this species. We propose that RapK and RapF may function by dephosphorylating Spo0F, similar to the mechanism carried out by RapA in Bs. This suggestion is supported by three findings: 1) both RapK and RapF retain the highest conservation of Spo0F binding residues from RapH, including the catalytic residue Q47; 2) their overexpression resulted in undetectable number of spores, similar to RapA<sub>Bs</sub> overexpression; 3) the overexpression of RapA<sub>Bs</sub>, K and RapF caused an identical cell morphology in the three overexpressing strains. Additionally, RapK and RapF are closely related and both belong to pherogroup 2, which may indicate that they resulted from a gene duplication event of a Rap ancestor that dephosphorylated Spo0F. Other Rap proteins that decreased sporulation efficiency are RapC, Like and RapI; of these, RapC do not contain the catalytic site residue Q47. Further studies are needed in order to elucidate the mechanisms by which all these receptors regulate sporulation in Bt and other species from the *B. cereus* group.

RapK and RapF are the only Rap proteins from Bt8741 that prevented biofilm formation. Because Spo0A-P levels regulate both sporulation and biofilm formation in Bs, we speculate that bifunctionality of RapK and RapF in Bt8741 results from their activity on
Spo0F. We noted that the overexpression of RapA<sub> Bs </sub>, which completely prevented sporulation, did not affect biofilm formation of Bt. Overexpression of RapA<sub> Bs </sub> may allow low levels of Spo0A-P in Bt8741, which in Bs are sufficient for the activation of genes related to production of extracellular matrix components, but not for the activation of early sporulation genes (13).

We suggest that Rap proteins have diversified according to the ecological needs of each species. For example, Bs is a soil dwelling bacteria found associated to rhizosphere forming biofilms (59). In Bs, six Rap proteins modulate Spo0A-P levels (21, 60), affecting sporulation and biofilm formation. Here we demonstrate that five Rap proteins modulated sporulation (RapC, K, F, I and RapLike) while only two of these (RapK and RapF) affected biofilm formation, perhaps throughout the Spo0A phosphorelay. This highlights the importance of sporulation regulation in both species and that probably, biofilm formation is not as essential in the lifecycle of Bt, as it is in Bs. In contrast, Bt is a soil inhabitant, insect patogenic and necrotrophic bacteria (61). In this species, extracellular protease production is essential for nutrient scavenging, which is normally associated to the necrotrophic stage of bacterial development in the insect cadaver (40). Additionally, it could be relevant during the transition from exponential growth to stationary phase in controlled fermentations or for adaptation against fluctuations in nutrient availability in the environment. While only one of the twelve Rap proteins from Bs modulates its extracellular proteolytic activity (RapG) (25), Bt has extended the modulation of extracellular protease production to five Rap-Phr systems (RapC, F, F2, I1 and RapLike).
We found that the Spo0A phosphorelay and production of extracellular proteases are highly interconnected in Bt8741 through the functions of RapC, F and RapLike. Additionally, extracellular proteolytic activity (specifically the NprA protease) is regulated by the QS system NprR-NprRB (62), which is also involved in the modulation of the Spo0A phosphorelay (41, 42). Likewise, NprR also participates in the spreading phenotype of Bt8741 (A. Verdugo et al, unpublished data), as well RapC and RapLike. Because sporulation, extracellular protease production and spreading of Bt have evolved to be regulated and coordinated by multiple QS systems, these collective traits may be important in the life cycle of Bt and represent essential mechanisms for its ecology.

Mature Phr signaling peptides from Bs correspond to at least five residues in the C-terminal end of the pro-Phr or in the middle of the sequence. Sequence analyses of matuyre Phrs in Bs has showed that a basic amino acid is found in the second position from the N-terminal end, and an alanine residue is necessary in the position before the cleavage site for Phr maturation (22, 63, 64). Our analysis of consensus putative mature Phr sequences showed that these characteristics are not maintained in mature Phr peptides of the B. cereus group. This suggests that signaling peptides are processed differently in these bacteria, this is, using different sets of extracellular proteases and peptidases that recognize distinct sequences. In Bt, the identity of a mature Phr has only been shown for Rap8-Phr8 from Bt-HD73. In that case, the active heptapeptide YAHGKDI is located in the C-terminal end from its exported sequence (46). RapI from Bt8741, ortholog protein to Rap8, is found in pherogroup 1, in which the consensus sequence HGKDI corresponds to the five residues in the C-terminal end from the exported sequence. This indicates that the consensus sequences
determined in this study may not exactly predict the signaling peptide sequence, but they can direct their search in future studies.

We found that Rap-Phr systems in the *B. cereus* group have evolved into five pherogroups, each including Rap homologs from different species. This means that signaling peptides shared by more than one species, could mediate crosstalk or eavesdropping phenomena in nature, allowing the regulation of collective functions in response to interspecific signals as described for other gram-positive species (65, 66).

The *B. cereus* group comprise bacteria with clinical and biotechnological relevance such as Ba, Bc, Bt, and other environmental and facultative species (38). We show that Rap-Phr QS systems in Bt are involved in the regulation of ecologically important collective traits, and our findings are highly relevant for further studies about the *B. cereus* group and contribute to the knowledge about its ecology. Understanding the regulatory processes for cell differentiation and specialization in these bacteria may enhance the use of biotechnologically-relevant species, or the strategies to control human pathogens, through the intervention of their collective functions at the molecular level. For instance, Ba and Bc are known for their pathogenic nature against mammals; therefore, elucidating the role of Rap-Phr systems in the production of virulence factors of these species such as anthrax toxin and capsule of Ba, or enterotoxins of Bc, could be of high relevance. Additionally, it is known that QS systems can be synthetically engineered (67, 68). As a result, Rap-Phr systems could be manipulated in order to enhance Bt survival, insect pathogenesis or cry protein production. This work serves as a starting point for the study of cell specialization of the *B. cereus* group bacteria.
Materials and Methods

Bacterial strains, media and culture conditions

*Bacillus thuringiensis* strain 8741 (Bt8741) (43), derived from Bt407 (Acc. No. NC_018877.1, 51), was used as host for the overexpression of Rap proteins. *Bacillus subtilis* strain 168 (Bs168) was used for the amplification of *rapA*. *Escherichia coli* strain TOP10 was used for construction and cloning of overexpression plasmids before transforming into Bt8741. Luria-Bertani (LB) broth (10 g L\(^{-1}\) tryptone, 5 g L\(^{-1}\) yeast extract and 5 g L\(^{-1}\) NaCl) and Nutrient Agar (8 g L\(^{-1}\) nutrient broth, 15 g L\(^{-1}\) agar) were used at 30 °C for *Bacillus* cultures and at 37 °C for *E. coli* and 200 rpm for liquid cultures. Milk Agar was prepared using Nutrient Agar, supplemented with 5% skim milk (41). When needed, ampicillin (100 µg mL\(^{-1}\)) or erythromycin (5 µg mL\(^{-1}\)) was added to media. To induce expression from the *xylA* promoter in Bt8741, xylose was used to a final concentration of 20 mM (70), unless otherwise specified.

Analysis of putative Spo0F-binding amino acids in Raps from Bt407

Based on the RapH residues involved in Spo0F binding in Bs168 (34) we determined the conservation of the corresponding residues in Raps from Bt407, in order to predict their capacity to bind to Spo0F. First, we analyzed the conservation of full-length Rap proteins from Bs168 and Bt8741 in comparison to RapH from Bs168. For this, we performed pairwise alignments of RapH amino acid sequence (NP_388565.2) with RapA (NP_389125.1), RapB (NP_391550.1), RapE (NP_390460.2), RapJ (NP_388164.1), RapD (NP_391519.1) from Bs168, and each of the eight Raps from Bt407 (AFV21721.1, AFV22194.1, AFV22088.1, AFV16731.1, AFV19251.1, AFV22208.1, AFV16776.1,
AFV17466.1), using BlastP tool (71). Then, all sequences were aligned together using MAFFT version 7 online service (72) with the G-INS-i interactive refinement method (73). Finally, we identified in the alignment the amino acids of Rap protein sequences that correspond to the residues of RapH that participate in binding and dephosphorylation of Spo0F.

**DNA manipulation**

All primers used in this study are listed in Table S4. DNA was isolated from Bs168 and Bt8741 using the PureLink Genomic DNA Mini Kit (Invitrogen, Carlsbad CA, USA). QIAprep Spin Miniprep Kit (Qiagen, Germantown, MD, USA) was used routinely for plasmid extraction and purification. Oligonucleotides were designed for amplifying each Rap gene from Bt8741 genome or plasmids (Acc. No. NC_018877.1, NC_018883.1, NC_018886.1, NC_018879.1, NC_018878.1) and Bs168 genome (Acc. No. NC_000964.3), and synthesized as a commercial service (T4 Oligo, Irapuato, Mexico). PCR products and restriction reactions were purified using the PureLink Quick PCR Purification Kit (Invitrogen). When needed, PCR products were isolated from 0.8% agarose gels using the Zymoclean™ Gel DNA Recovery Kit (ZYMO Research, Irvine, CA, USA). Enzymes Dream Taq Master Mix, HindIII, SalI (Thermo Scientific, Waltham, MA, USA), PstI and T4 DNA Ligase (New England Biolabs Inc., Ipswich, MA, USA) were used as recommended by the manufacturer.

**Construction of Rap-overexpression Bt8741 strains**

All strains and plasmids used in this study are listed in Table S1. For the construction of the overexpression plasmid pHT315-P$_{xyd}$, the regulatory region of the xylose operon, including
the xylA promoter (P_xylA) and the repressor gene xylR, were amplified by PCR from Bs168 genome using primers GG1 and GG2 (Table S4). This PCR product was inserted into the HindIII and PstI sites of pHT315 plasmid (74), and colonies were PCR checked using primers DS16 and DS17 (Table S4). The resulting plasmid pHT315-P_xylA was transformed into E. coli Top10 competent cells. Then, this plasmid was used for the inducible overexpression of Rap proteins with xylose in Bt8741. For this, rap genes encoded in the genome of Bt8741 (rapC, rapK, rapF, rapF1, rapF2, rapI, rapI1 and rapLike, Slamti et al., 2014) and rapA from Bs168 (RapA_Bs, Perego et al., 1994) were amplified using the corresponding primers pairs listed in Table S4, and inserted in-frame between the PstI and Sall sites of pHT315-P_xylA. Nine overexpression plasmids, one for each Rap protein, were transformed into E. coli Top10 competent cells. All plasmids were then transformed into Bt8741 electrocompetent cells, using the protocol described in previous studies (41), generating nine Bt8741 strains for the overexpression each Rap protein. Additionally, we transformed Bt8741 with the pHT315-P_xylA (without a rap gene), and the resulting strain was used as control strain throughout the Rap induction experiments. The complete sequence of pHT315-P_xylA’rapI was verified by Illumina sequencing (MGH DNA Core, Cambridge, MA, USA), and the rest of the P_xylA’rap constructions were verified by Sanger sequencing (Unidad de Servicios Genómicos, LANGEBIO-CINVESTAV, Irapuato, Mexico) using primers GG26 and DS17 (Table S4).

Sporulation efficiency

We assessed the effect of the overexpression of Rap proteins on sporulation efficiency in Bt8741. Preinoculum were prepared by picking a single colony of each strain into 5 mL of liquid media and grown overnight. Then, 1 mL of preinoculum was centrifuged, washed
and suspended in 1 mL of sterile PBS. Glass culture tubes (25 mm diameter) with 5 mL of LB with erythromycin were inoculated with 50 µL (1% v/v) of preinoculum containing \( \approx 10^7 \) cfu ml\(^{-1} \) and incubated for 72 h. All strains were cultured in triplicate, in LB with and without the addition of xylose. To determine growth and sporulation, total and thermoresistant CFU were calculated by plating 10-fold serial dilutions in nutrient agar. For thermoresistant CFU, samples of 100 µL were incubated at 80 ºC for 20 min prior to diluting and plating. Sporulation efficiency was calculated as the percentage of thermoresistant CFU in total CFU.

**Biofilm formation assay**

We evaluated the effect of the overexpression of Rap proteins on the capacity of Bt874 to form biofilms. For this assay, we used 13 x 100 mm glass tubes with 3 mL Nutrient Broth + erythromycin, with and without the addition of xylose to a final concentration of 2 mM. Three µL of preinoculum was added in triplicates, and the inoculated tubes were incubated without agitation at 31 ºC ± 1 ºC for 48 hours. The culture media was then removed with a syringe with needle. The biofilm and ring attached to the wall of the tube, composed of cells from the biofilm, were resuspended in 1.5 mL of sterile PBS and the optical density (OD\(_{600}\)) was measured. The OD\(_{600}\) was also measured from the removed liquid media to address planktonic growth. At least 5 replicates of each treatment were performed.

**Extracellular proteolytic activity assay**

To evaluate the effect of Rap overexpression in extracellular proteolytic activity of Bt8741, 2 µL preinoculums of each Rap-overexpression strain, prepared as described above, were spotted in triplicate on milk agar with and without the addition of xylose. The hydrolysis
halo area was measured after 24 h of incubation using the Image Lab™ Software (BIORAD). To correct for differences in colony growth, we subtracted the colony area.

**Spreading phenotype assay**

The spreading phenotype of Rap-overexpression Bt8741 variants was followed in colonies spotted on agar. For this assay, we used diluted nutrient agar (NA) (0.8 g L\(^{-1}\) Nutrient broth, 1.5 g L\(^{-1}\) agar) with erythromycin and with or without the addition of xylose. Plates were air-dried inside a biological hood for 60 minutes prior to inoculation. Then, 5 µL of preinoculum cultures were spotted in the center of the plate, dried for 5 minutes and incubated at 30 ºC for 14 days. The inoculated agar plates were photographed at day 1, 3, 5, 7 and 14, using a gel documentation system (Gel Doc™ XR+, BIORAD). Colony area was measured using the Image Lab™ Software (BIORAD) and radial growth was calculated. For normalization of radial dispersion, we subtracted from all observations the colony radius at day 1, which corresponds to the inoculated droplet area. Three replicates of each treatment were performed.

**Phylogenetic Analysis**

To reconstruct the phylogeny of Rap proteins in the *B. cereus* group, we first selected one representative strain of each species from NCBI GenBank, including *Bacillus cereus* ATCC14579 (Accession NC_004722.1), *Bacillus anthracis* A0248 (NC_012659.1), *Bacillus thuringiensis* 407 (NC_018877.1), *Bacillus mycoides* ATCC6442 (NZ_CP009692.1), *Bacillus pseudomyoides* DMS12442 (NZ_CM000745.1) and *Bacillus cytotoxicus* NVH391-98 (NC_009674.1). Strains were selected based on the availability of a complete genome (as of July of 2018) and thus, *Bacillus weihenstephanensis* was
excluded. We searched for Rap protein homologs in the selected genomes by querying the amino acid sequence of B. subtilis RapA (NP_389125.1) and each of the eight Rap sequences of B. thuringiensis 407: RapC (AFV21721.1), RapK (AFV22194.1), RapF (AFV22088.1), RapF1 (AFV16731.1), RapF2 (AFV19251.1), RapI (AFV22208.1), RapI1 (AFV16776.1) and RapLike (AFV17466.1). Homologs were searched using BLAST tool (71), the tBlastn tool and a local script designed for performing the blast search in an assembled database of the selected genomes. To ensure the identity of the Rap protein homologs, Blast hits were submitted manually to the Conserved Domain Search-NCBI tool (75) in order to determine if they presented the characteristic TPR-containing domain. Rap protein amino acid sequences were aligned in MAFFT version 7 (72) using the G-INS-i iterative refinement method which incorporates pairwise alignment algorithms (73). RapA and RapH from Bs168 were also included as outgroups for the phylogenetic reconstruction. The selection of the best substitution evolutionary model (JTT+G+I+F) was made using the Smart Model Selection with the Akaike Information Criterion in PhyML 3.0 (76, 77), as well as the phylogeny reconstruction by the Maximum Likelihood method using 1000 bootstraps to support the phylogenetic prediction.

**Phr pro-peptide identification and pherogroup prediction**

Additional to the identification of Rap homologs in the B. cereus group, we also analyzed the putative phr genes, which code for pro-Phr, the precursor of the quorum sensing signal peptide. For this, we performed a manual search targeting open reading frames (ORFs) between 30 and 100 amino acids of length, downstream from the rap gene sequences. When present, each Phr amino acid sequence was analyzed for the presence of a signal peptide for secretion and a cleavage site using SignalP4.1 (78). The putative mature
signaling peptide (mature Phr) and pherogroup prediction were performed from the exported Phr amino acid sequences (pro-Phr). For this, Phrs corresponding to Rap proteins from different clades of the phylogenetic reconstruction were analyzed separately. The amino acid sequences of the pro-Phr from each clade were aligned using ClustalW (79). Pherogroups were identified by manually, by modifying the groups of aligned Phrs and looking for consensus sequences in the alignments. For better identification of consensus sequences, sequence Logos were created for each pherogroup using the Seq2Logo 2.0 online service (80).

**Statistics**

All the statistical analyses were performed using GraphPad Prism version 7.0a. Data obtained from the extracellular proteolytic activity assay, spreading (at day 7) and biofilm formation were analyzed with a multiple t-tests to search for differences between not induced and induced Rap protein overexpression conditions of each strain. Significance of 0.05 was used in all statistical tests.

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Table 1. Rap-Phr systems encoded in *B. subtilis* 168 and in species from the *B. cereus* group.

| Specie                        | Number of Rap-Phr systems | Location |         |         |
|-------------------------------|---------------------------|----------|---------|---------|
| *Bacillus subtilis* 168       | 12                        | 11       | 1       |
| *Bacillus cereus* ATCC 14579  | 5                         | 5        | 0       |
| *Bacillus anthracis* str. A0248 | 6                        | 5        | 1       |
| *Bacillus thuringiensis* 407  | 8                         | 4        | 4       |
| *Bacillus mycoides* ATCC 6442 | 5                         | 4        | 1       |
| *Bacillus pseudomycoides* DMS 12442 | 8           | 8        | 0       |
| *Bacillus cytotoxicus* NVH 391-98 | 8                       | 8        | 0       |
Prediction of the capacity of Rap proteins from Bt8741 to bind and dephosphorylate Spo0F. A) Multiple sequence alignment of the complete amino acid sequences of RapH, A, B, E, J and D from *B. subtilis* 168, and eight Rap proteins from *B. thuringiensis*.
Bt8741. Blue highlights indicate highly conserved amino acids. Residues involved in the RapH-Spo0F binding are indicated in red rectangles and its position in RapH is shown on top of the alignment. B) Conservation of residues involved in RapH binding to Spo0F. Residues were considered as semiconserved when a functional amino acid of RapH was substituted with another amino acid with similar characteristics. Bs, *Bacillus subtilis* 168; Bt, *Bacillus thuringiensis* 407; C.S., Catalytic Site; *Percentage of conserved and semiconserved amino acids in pairwise alignment to RapH.*
Figure 2

Figure 2. Sporulation efficiency of Bt8741 carrying overexpression plasmids for Rap proteins, with and without addition of inducer. Columns represent average of three individual measurements, shown as dots.
**Figure 3.** Cell morphology in strains with induced Rap protein overexpression at 72 h.

Phase contrast microscopy 63X and 1.8X magnification. -, no effect; +, decrease under 10-fold; decrease between 90 and 160-fold; ++, decrease >1,000-fold.
Figure 4. Biofilm formation of Rap-overexpression strains at 48 h. A) Biofilms formed in the liquid-air interphase in 13 x 100 mm glass tubes at 48 h. Biofilms are identified as a white layer on the surface. B) Biofilm formation quantification of Rap-overexpression strains in induced and not induced media after 48 h. Columns represent average of 5 replicates, ± SD. NB, Nutrient Broth; **, p<0.005; ****, p<0.0001.
**Figure 5.** Extracellular proteolytic activity of Rap-overexpression strains. A) Effect of Rap protein overexpression in the hydrolysis halo of Rap-overexpression strains colonies. B) Hydrolysis halo area with and without Rap-overexpression induction. Columns represent average of 3 replicates ± SD. MA, milk agar; **, p<0.005; ***, p<0.0005; ****, p<0.0001.
Figure 6

Figure 6. Spreading phenotype of Rap-overexpression strains. A) Spreading kinetics of Rap-overexpression colonies on agar. Each point represents the media of triplicates ± SD; only one data point is shown at day 14. B) Spreading quantification of Rap-overexpression colonies at day 7. Columns represent average of triplicates ± SD. C) Pictures of representative Rap-overexpression strains spreading during 7 days. Scale bar indicates 5 mm. NA, Nutrient Agar; **, p<0.005; ****, p<0.0001.
Figure 7. Maximum likelihood phylogeny of Rap proteins from the *B. cereus* group. Rap proteins from Bt8741 are highlighted in gray boxes. Branches from each pherogroup are identified in colors. Bootstraps higher than 80% are shown in each node. Insert table:
pherogroups and consensus sequences of mature Phr. Semiconservations in the consensus sequences are highlighted: black, polar residues; gray, hydrophobic residues; silver, polar and charged residues.