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RESEARCH ARTICLE

Rap Protein Paralogs of *Bacillus thuringiensis*: a Multifunctional and Redundant Regulatory Repertoire for the Control of Collective Functions

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ABSTRACT Quorum sensing (QS) is a mechanism of synthesis and detection of signaling molecules to regulate gene expression and coordinate behaviors in bacterial populations. In *Bacillus subtilis*, multiple paralog Rap-Phr QS systems (receptor-signaling peptides) are highly redundant and multifunctional, interconnecting the regulation of differentiation processes such as sporulation and competence. However, their functions in the *Bacillus cereus* group are largely unknown. We evaluated the functions of Rap proteins in *Bacillus thuringiensis* Bt8741, which codes for eight Rap-Phr systems; these were individually overexpressed to study their participation in sporulation, biofilm formation, spreading, and extracellular proteolytic activity. Our results show that four Rap-Phr systems (RapC, RapK, RapF, and RapLike) inhibit sporulation, two of which (RapK and RapF) probably dephosphorylate Spo0F from the Spo0A phosphorelay; these two Rap proteins also inhibit biofilm formation. Four systems (RapC, RacF1, RacF2, and RapLike) participate in spreading inhibition; finally, six systems (RapC, -F, -F2, -I, and -I1 and RapLike) decrease extracellular proteolytic activity. We foresee that functions performed by Rap proteins of Bt8741 could also be carried out by Rap homologs in other strains within the *B. cereus* group. These results indicate that Rap-Phr systems constitute a highly multifunctional and redundant regulatory repertoire that enables *B. thuringiensis* and other species from the *B. cereus* group to efficiently regulate collective functions during their 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 about the ecology of these bacteria is hindered by our limited understanding 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, spreading, extracellular proteolytic activity, and probably other functions in species from the *B. cereus* group.

KEYWORDS *Bacillus cereus*, *Bacillus thuringiensis*, biofilm formation, collective functions, quorum sensing, Rap-Phr, sporulation

Bacteria perform many functions that depend on multicellular-organism-like behaviors, such as cell differentiation and specialization. These 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

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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) and 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*. In *B. subtilis* cultures, several mutually exclusive cell types (motile, competent, sporulating, cannibal, biofilm matrix producing, surfactant producing, and mining [9, 10]) mediate the emergence of ecological interactions such as cooperation, cheating, and cross-feeding (5, 6, 11). These phenomena, which ultimately affect the manifestation of collective traits such as sporulation efficiency, surface colonization, biofilm architecture complexity, etc. (2, 9, 12) depend on global modifications of transcriptional regulation; they are triggered by environmental cues, stress conditions, and 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 protein family (Rgg, Rap, NprR, PlcR, and 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). To carry out signaling, peptides need to be secreted, matured by proteolysis and reinternalized at high cell density or quorum state (17, 20). Rgg, NprR, PlcR, and PrgX proteins are transcriptional activators that bind directly to DNA. Rap proteins, however, lack a DNA-binding domain, and they function by binding to and inhibiting response regulators or transcriptional activators (21, 23, 24). In high cell density, Phr signaling peptides bind to specific Rap proteins and release their inhibitory functions (25). Eleven Rap paralogs from *B. subtilis* strain 168 (RapA, -B, -C, -D, -E, -F, -G, -H, -I, -J, and -K), RapP from the *B. subtilis* 168 parental strain NCIB3610 (26), and Rap60 in strains carrying the plasmid pTA1060 (27) control diverse functions. The RapG-PhrG pair regulates the activation of DegU, a transcriptional regulator that controls *aprE* and *comK* genes encoding extracellular proteases and a transcription factor for competence in *B. subtilis*, respectively (15, 28); activity of ComA—the master regulator of competence genes—is inhibited by RapC, -D, -F, -G, -H, -K, and -60 (14, 27, 29–33); Spo0A—the transcriptional activator of many differentiation genes—is indirectly regulated by RapA, -B, -E, -H, -J, -P, and -60 (24, 27, 34–38). Hence, Rap protein paralogs from *B. subtilis* 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 autophosphorylate 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 (39). 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 (34, 40).

While the regulation of collective traits in *B. subtilis* is well known, these phenomena remain largely understudied in the *Bacillus cereus* group, which includes bacteria with clinical and biotechnological relevance (41). Although *B. subtilis* and the *B. cereus* group species share similar characteristics, such as the sporulation process and the Spo0A phosphorelay components, and have many protein families in common, they also present several genetic differences (42). In *Bacillus thuringiensis* (the most widely used biopesticide), the bifunctional QS receptor NprR, which is not present in *B. subtilis* (43–45), modulates the Spo0A phosphorelay through binding to Spo0F (similar to the activity of Rap proteins) and functions as a transcriptional activator through DNA

binding. On the other hand, ComA and DegU response regulators are not encoded in *B. thuringiensis*. Additionally, Rap-Phr QS systems also differ in both groups. These QS systems have evolved by duplication and divergence mechanisms; even though multiple Rap protein paralogs are also found in the *B. cereus* group species, they have evolved independently, and no Rap homologs are shared between the two groups (46, 47). 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 *B. subtilis*.

Some Rap-Phr systems from species of the *B. cereus* group have been studied. First, Rap BXA0205 and BA3790 from *Bacillus anthracis* strain A2012 were demonstrated to regulate sporulation initiation and to dephosphorylate Spo0F (48). Later, it was shown that Rap8 from *B. thuringiensis* HD73 regulates the sporulation process *in vitro* and in the insect cadaver as well as biofilm formation *in vitro* (49). A more recent study showed the participation of Rap6, -7, and -8—also known as RapC, RapK, and RapF, respectively (50)—in the modulation of the sporulation process in *B. thuringiensis* Bt407 (51). However, other Rap paralogs with unknown functions have been identified in the genomes of *B. cereus* group bacteria (47, 50), and these may be relevant to their ecology.

In this study, we evaluated the functions of Rap proteins using *B. thuringiensis* Bt8741 as a model. We generated eight Rap overexpression strains from Bt8741 to evaluate the role of each Rap paralog in sporulation efficiency, biofilm formation, spreading, and extracellular proteolytic activity. Once we assigned functions to Bt8741 Rap proteins, we identified homolog Rap proteins in the genomes of other strains and species from the *B. cereus* group; this allows the prediction of their functions based on Rap proteins from Bt8741 studied here.

(This article was submitted to an online preprint archive [52].)

RESULTS

Spo0F-binding residues from $RapH_{Bs}$ 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 amino acids involved in Spo0F-binding by RapH from *B. subtilis* ($RapH_{Bs}$) (Fig. 1) (36). Rap proteins with high conservation of Spo0F-binding residues should retain the activity of inhibiting sporulation initiation, while Rap proteins with lower conservation could be specialized for other functions. We found more conservation of the functional amino acids of $RapH_{Bs}$ in the sequences of both *B. subtilis* 168 and Bt407 compared to the corresponding full sequences (Fig. 1B). In *B. subtilis* 168, the full sequence conservation of the Rap proteins known to bind to Spo0F (RapA, -B, -E, and -J) compared to $RapH_{Bs}$ ranged from 59% to 66%, and the functional amino acid conservation percentage, from 82% to 100%. In $RapD_{Bs}$, which does not bind to Spo0F, the full-length sequence is conserved at 50%, and the functional residues are only 64% conserved (Fig. 1B). In the case of Rap proteins from Bt407, the full sequence conservation in comparison to $RapH_{Bs}$ ranged from 45% to 48%. On the other hand, conservation of the functional residues ranged from 64% to 88% (Fig. 1B). Since more conservation occurs in the Spo0F-binding functional residues, these amino acids could be important for the function of *B. thuringiensis* Rap proteins.

RapK exhibited the highest conservation percentage of Spo0F-binding residues (88%), followed by RapF, RapI, and RapI1 (82%), RapF1, RapF2, and RapLike (70%), and, finally, RapC (64%). We found that RapF1, RapF2, and RapC do not conserve the residue Q47, which is essential for the phosphatase activity of $RapH_{Bs}$ (36). This analysis enables the prediction that some Rap protein paralogs from Bt8741 with a high conservation percentage of putative Spo0F-binding amino acids could dephosphorylate Spo0F. Indeed, RapK and RapF from Bt407, Rap8 from *B. thuringiensis* HD73 (ortholog to RapI from Bt407), and Rap BXA0205 and BA3790 from *B. anthracis* (homologs of RapK and RapF2, respectively) have been shown to participate in the modulation of sporulation (48, 49, 51). Previous to this work, RapF1, RapI1, and RapLike from Bt407 (or its homologs in other strains) had not been tested for their role in sporulation.

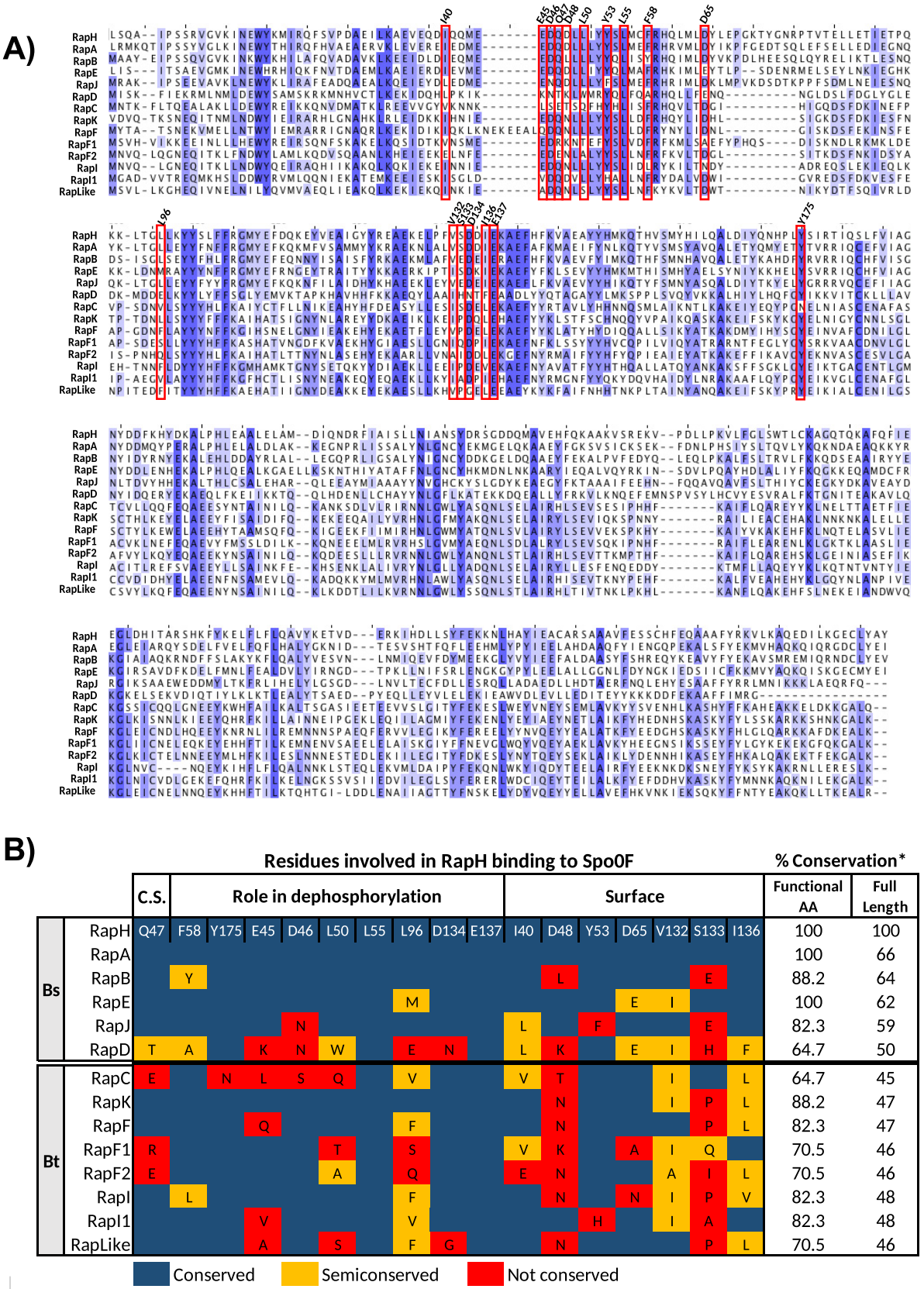

FIG 1 Prediction of the capacity of Rap proteins from Bt8741 to bind and dephosphorylate Spo0F. (A) Multiple sequence alignment of the complete amino acid sequences of RapH, -A, -B, -E, -J, and -D from *B. subtilis* 168 and eight Rap proteins from Bt8741. Blue highlighting

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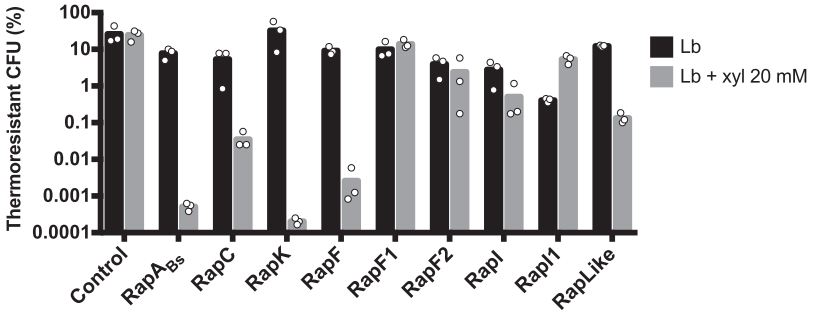

FIG 2 Sporulation efficiency of Bt8741 carrying overexpression plasmids for Rap proteins with and without addition of inducer. In the cases where thermoresistant CFU were undetectable, we considered a value of 166 spores/ml, which is the detection limit for this assay. Columns represent the average of three individual measurements, shown as dots.

RapC, RapK, RapF, and RapLike control sporulation in Bt8741. We constructed nine Rap overexpression strains in the Bt8741 wild-type (WT) background (see Table S1 in the supplemental material), one for each endogenous Rap protein identified in Bt407 (RapC, -K, -F, -F1, -F2, -I, and -I1 and RapLike) (50) and one for RapA from *B. subtilis* 168 (RapA$_{Bs}$). We also generated a control strain of Bt8741 carrying the empty plasmid pHT315-P$_{xylA}$ (Table S1). Because some Rap proteins of *B. subtilis* are known to negatively regulate sporulation (24, 27, 34–37), the overexpression of Rap proteins involved in the regulation of sporulation in *B. thuringiensis* should result in the decrease of thermoresistant CFU (spores). A growth time course experiment confirmed that neither xylose addition nor Rap overexpression affected bacterial growth (Fig. S1).

Sporulation efficiency of the control strain remained unchanged by the addition of xylose (Fig. 2). In contrast, the strain carrying P$_{xylA}$′*rapA$_{Bs}$* had decreased sporulation efficiency (from 7% to 0.0005%, ≈14,000-fold) caused by the induction with xylose. In fact, thermoresistant CFU were undetectable when RapA$_{Bs}$ was overexpressed (Fig. S2; CFU data of Fig. 2). We also found undetectable levels of spores in strains overexpressing RapK and RapF (Fig. S2); sporulation efficiency decreased ≈160,000-fold in the strain overexpressing RapK and ≈3,400-fold in the strain overexpressing RapF (Fig. 2). Strains carrying P$_{xylA}$′*rapC* and P$_{xylA}$′*rapLike* also exhibited reduced sporulation efficiency of ≈140-fold and ≈88-fold, respectively (Fig. 2). Finally, we found a slight ≈5-fold decrease in sporulation efficiency when RapI was overexpressed, and sporulation efficiency was not affected by the overexpression of RapF1, RapF2, or RapI1.

In this assay, we found that the addition of xylose to the medium and the presence of *rap* genes in plasmids had unspecific effects on growth and sporulation; e.g., in the control strain, the addition of xylose caused a decrease of ≈1 $\log_{10}$ in total and thermoresistant CFU (Fig. S2). For this reason, we used sporulation efficiency instead of CFU to identify Rap proteins that decrease sporulation. Additionally, our analysis was based on comparisons within each strain in induced versus not induced conditions, instead of comparing each overexpression strain against the control strain in induced conditions; this could be the source of discrepancies between our results and those of previous work (e.g., RapI versus the homolog Rap8 in *B. thuringiensis* HD73 [49]).

Samples of the Rap-overexpressing strain cultures at 72 h were observed with a microscope. We detected free spores and bacterial debris in all cultures when Rap proteins were not overexpressed (Fig. S3). Strains overexpressing Rap proteins that did not affect sporulation efficiency (RapF1, -F2, -I, and -I1) showed cell morphology similar

FIG 1 Legend (Continued)
indicates highly conserved amino acids. Residues involved in the RapH$_{BS}$-Spo0F binding are shown in red rectangles, and their position in RapH$_{Bs}$ is shown on top of the alignment. (B) Conservation of residues involved in RapH$_{Bs}$ binding to Spo0F. Residues were considered semiconserved when a functional amino acid of RapH$_{Bs}$ was substituted with another amino acid with similar characteristics. Bs, *Bacillus subtilis* 168; Bt, *Bacillus thuringiensis* Bt407; C.S., catalytic site; *, percentage of conserved and semiconserved amino acids in pairwise alignment to RapH$_{Bs}$.

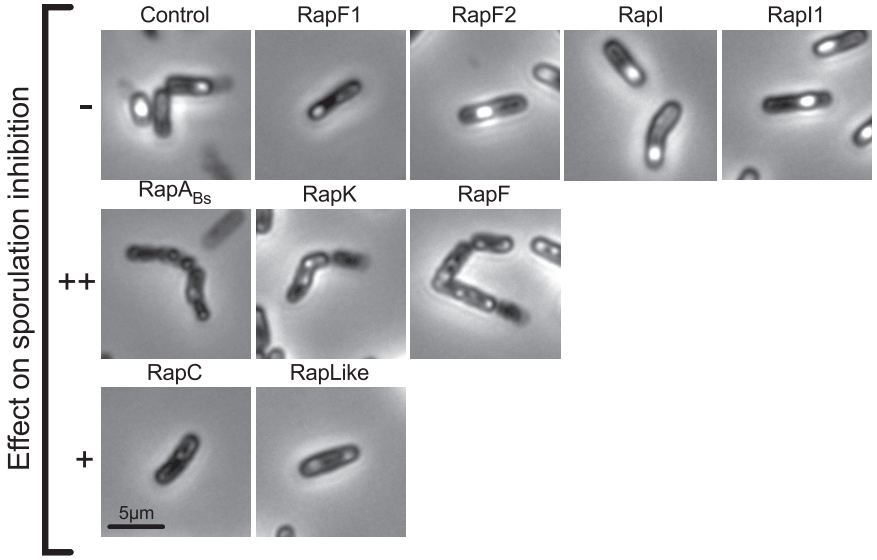

FIG 3 Cell morphology of strains with induced Rap protein overexpression at 72 h. Phase-contrast microscopy of 63× and 1.8× magnification. –, no effect; ++, undetectable thermoresistant CFU; +, decreased sporulation efficiency, detectable thermoresistant CFU.

to that of the control strain, i.e., bacilli with defined endospores. In samples from strains overexpressing RapA$_{Bs}$, RapK, and RapF that acutely decreased sporulation efficiency, we observed chained, wrinkled cells with no spores (Fig. 3). Cells from strains overexpressing RapC and RapLike, were observed as rod shaped, and no spores were evident (Fig. 3).

The analysis of Spo0F-interacting residues (Fig. 1) was partially accurate at predicting the participation of Rap proteins in sporulation. RapK and RapF that highly conserve the Spo0F-binding residues had the strongest effect on sporulation inhibition (Table 1); however, RapI and RapI1 also had a high conservation of Spo0F-binding residues, and their overexpression had no effect on sporulation. Additionally, RapC and RapLike, which had lower conservation of Spo0F-binding residues, decreased sporulation efficiency.

Overexpression of RapF and RapK prevents biofilm formation of Bt8741. In nature, over 80% of bacteria live in biofilms (53); therefore, biofilm formation may be a relevant trait—albeit an understudied one—during the life cycle of *B. thuringiensis*. We quantified biofilm formation of the Rap overexpression strains in the air-liquid interphase at 48 h. Since 20 mM xylose in the medium caused a complete inhibition of biofilm formation in the Bt8741 control strain (not shown), we tested the effect of

TABLE 1 Summarized results of the participation of Rap proteins in collective functions of Bt8741

Name[a]	Name[b]	Spo0F-binding prediction (%)	Phenotype for:[c]				Multifunctional Rap?
			Sporulation	Biofilm formation	Spreading	Extracellular proteases	
RapA$_{Bs}$		100	++				
RapC	Rap6	64.7	+		++	+	Yes
RapK	Rap8	88.2	++	++			Yes
RapF	Rap7	82.3	++	++		+	Yes
RapF1	Rap1	70.5			+		
RapF2	Rap4	70.5			++	+	Yes
RapI	Rap5	82.3				+	
RapI1	Rap2	82.3				+	
RapLike	Rap3	70.5	+		++	+	Yes

[a]Nomenclature of *B. thuringiensis* Rap proteins used in this study (50).
[b]Nomenclature of *B. thuringiensis* Rap proteins in reference 51.
[c]+, phenotype decreased; ++ phenotype eliminated.

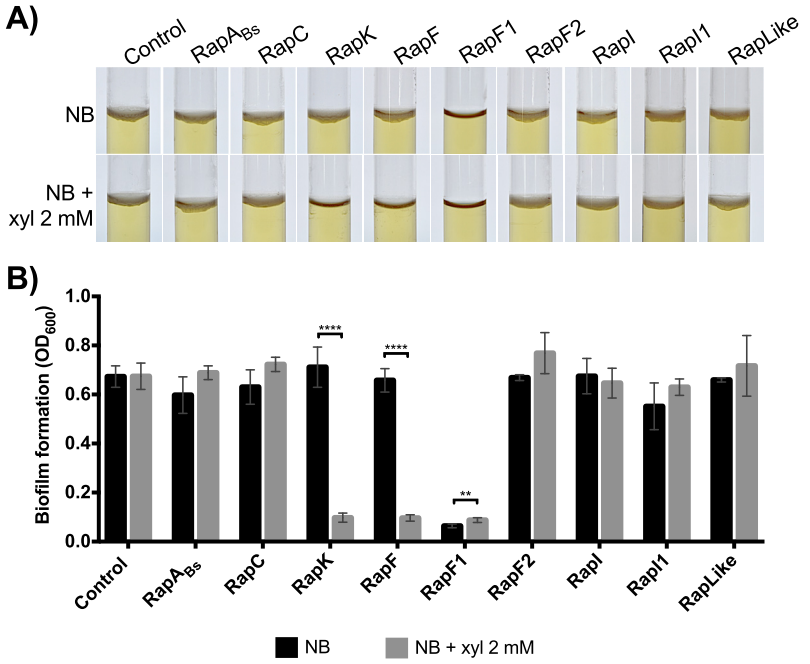

FIG 4 Biofilm formation of Rap overexpression strains at 48 h. (A) Biofilms formed in the air-liquid interphase in glass tubes at 48 h. Biofilms are identified as a white layer on the surface of the medium. (B) Biofilm formation quantification of Rap overexpression strains in induced and not induced media after 48 h. Columns represent the average from 5 replicates ± standard deviation (SD). NB, nutrient broth; **, $P < 0.005$; ****, $P < 0.0001$.

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 xylose (54).

Overexpression of RapK and RapF caused the inhibition of biofilm formation of Bt8741 (Fig. 4A), evident by the significant decrease ($P < 0.0001$) in the optical density at 600 nm (OD_{600}) measured in samples obtained from the surface of the cultures (Fig. 4B). On the other hand, biofilms were normally formed by strains overexpressing $RapA_{Bs}$, -C, -F2, -I, and -I1 and RapLike (Fig. 4). The strain overexpressing RapF1 was unable to form biofilms even when overexpression was not induced (Fig. 4).

In order to discard possible global growth defects in this assay when RapK and RapF were overexpressed, we measured planktonic growth through OD_{600} of the liquid media where biofilm formation was assessed. We found that planktonic growth was higher in conditions where a biofilm was not formed (Fig. S5). This suggests that RapK and RapF specifically inhibit biofilm formation (e.g., expression of genes related to synthesis of extracellular matrix components).

RapC, RapF1, RapF2, and RapLike regulate spreading of Bt8741 colonies. Colonies of Bt8741 present a spreading phenotype that could be associated with its capacity to colonize hosts and habitats. Similar passive motility phenotypes have been described in other species of *Bacillus*, associated with the production of extracellular surfactant molecules (55–57). We observed that the overexpression of RapC, RapF1, RapF2, and RapLike caused a decrease in spreading ($P < 0.05$) of Bt8741 colonies at day 7 (Fig. 5A and B). The overexpression of RapC reduced 90% of the colony dispersion; RapF1 reduced 50%, RapF2 reduced 84%, and RapLike reduced 82% (Fig. 5B). We observed that the overexpression of RapC, RapF2, and RapLike completely eliminated this phenotype, while overexpression of RapF1 only decreased spreading ($P < 0.05$) (Fig. 5B and C).

The overexpression of $RapA_{Bs}$, RapK, RapF, RapI, and RapI1 did not affect the spread of Bt8741 ($P > 0.05$) (Fig. 5B). In some cases, Rap overexpression affected colony morphology; i.e., colonies of strains overexpressing $RapA_{Bs}$, RapK, and RapI showed an

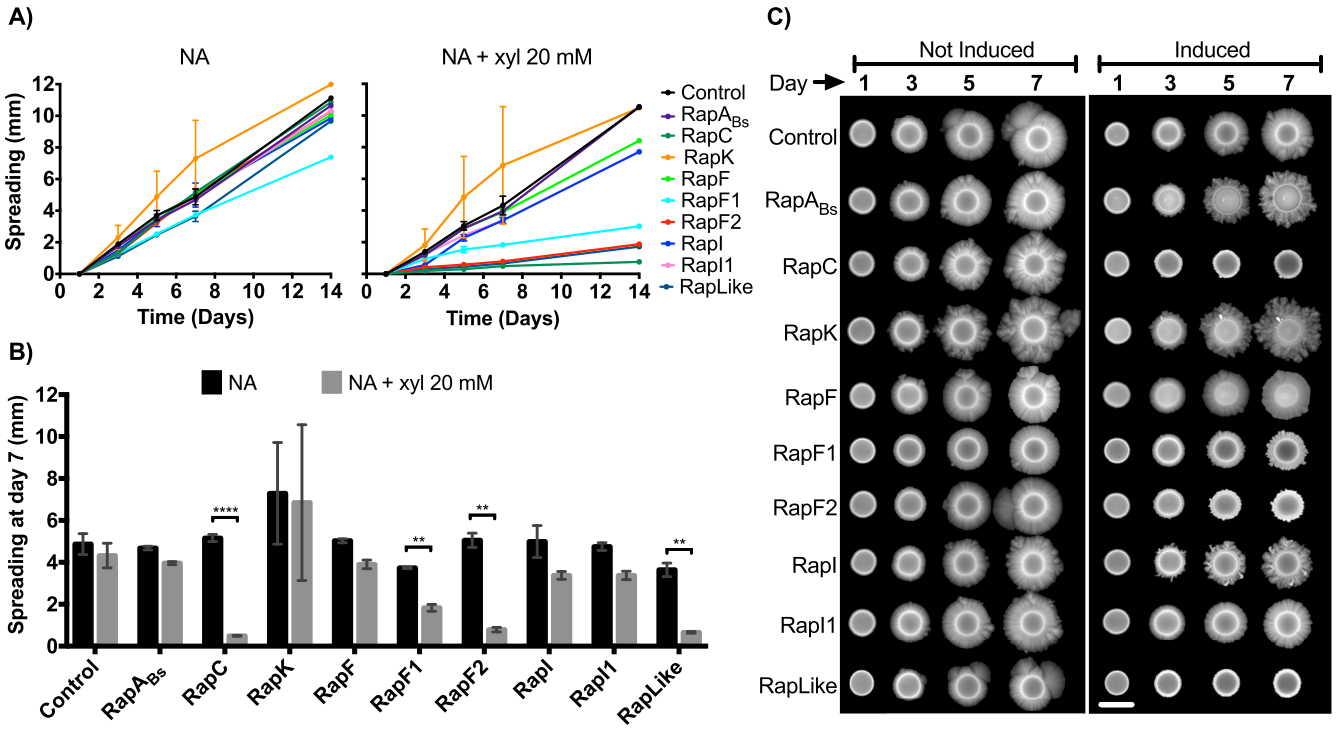

FIG 5 Spreading phenotype of Rap overexpression strains. (A) Spreading kinetics of colonies on agar. Each point represents the average from triplicates ± SD; only one data point is shown at day 14. (B) Spreading quantification of Rap overexpression colonies at day 7. Columns represent the average from triplicates ± SD. (C) Pictures of representative Rap overexpression strains spreading during 7 days. The scale bar indicates 5 mm. NA, nutrient agar; **, $P < 0.005$; ****, $P < 0.0001$.

increased dendritic phenotype. However, the spreading phenotype, measured as colony radius, was still present (Fig. 5C).

Extracellular proteolytic activity is downregulated by RapC, -F, -F2, -I, and -I1 and RapLike in Bt8741. In *B. thuringiensis*, the production of extracellular proteases is crucial during its necrotrophic phase, i.e., development in insect cadavers (43, 46). 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. Overexpression of RapC, -F, -F2, -I, and -I1 and RapLike decreased the halo area ($P < 0.05$; Fig. 6B). In contrast, the proteolytic activity of the control strain and strains overexpressing RapA$_{Bs}$, RapK, and RapF1 was not affected by the induction ($P > 0.05$; Fig. 6).

We noted a coincidence between Rap proteins that participate in inhibiting extracellular proteolytic activity and spreading (RapC, RapF2, and RapLike). However, we determined that the effects on spreading could not affect our measurements of extracellular proteases, because the spreading phenotype is not yet relevant at day 1 (Fig. 5), when proteolytic activity was assessed. Indeed, colony area was not affected by the overexpression of Rap proteins (except for RapF2), and the colony size of Rap-overexpressing strains was not reduced compared to that of the control strain (Fig. S6).

Are Rap paralogs a multifunctional and redundant regulatory repertoire across the *B. cereus* group? Our phenotypic analyses showed that all Rap proteins participate in the regulation of at least one of the collective functions studied here (Table 1). We found that five out of eight Rap proteins from Bt8741 (RapC, RapK, RapF, RapF2, and RapLike) participate in more than one collective function, and all four collective functions were inhibited by more than one Rap protein.

In order to predict the functions of Rap paralogs across the *B. cereus* group, we analyzed Rap amino acid sequences from representative strains of *B. thuringiensis*, *B. cereus*, *B. anthracis*, *B. weihenstephanensis*, *B. mycoides*, *B. pseudomycoides*, and *B. cytotoxicus* (51) (Data Set S1). Since several Rap paralogs can be found in all species (47,

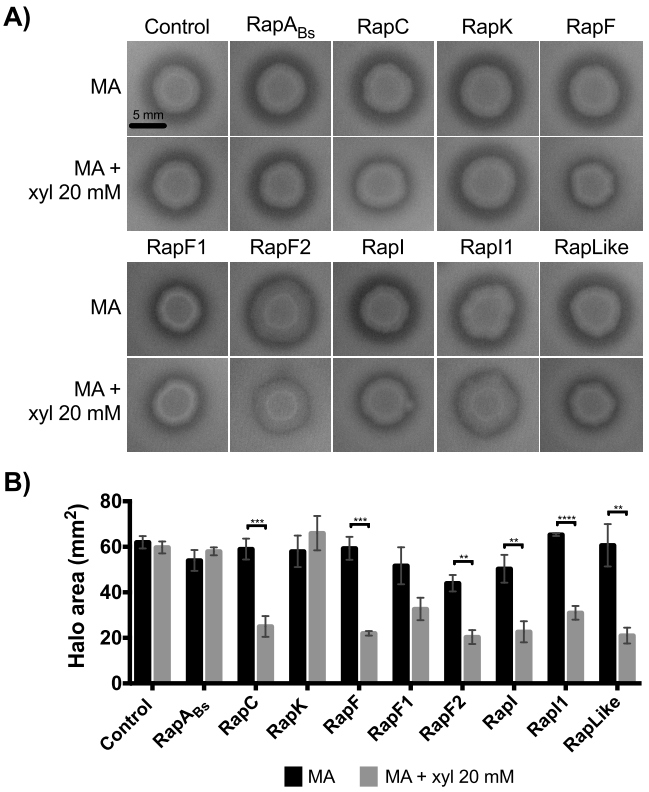

FIG 6 Extracellular proteolytic activity of Rap overexpression strains. (A) Effect of Rap protein overexpression in the hydrolysis halo of Rap overexpression strains colonies. (B) Hydrolysis halo area with and without Rap overexpression induction. Columns represent the average of 3 replicates ± SD. MA, milk agar; **, $P < 0.005$; ***, $P < 0.0005$; ****, $P < 0.0001$.

51), we hypothesized that the detection of Rap homologs to Bt8741 Raps could be useful in order to predict their functions. Seven out of eight Rap proteins from Bt8741 share homologs (identity, <90%) in other strains from the *B. cereus* group (Fig. 7). Homologs of RapK, RapF, and RapI were only found in other *B. thuringiensis* strains, while homologs of RapF1, RapF2, RapI1, and RapLike were found in strains from both *B. thuringiensis* and *B. cereus*. Notably, we did not detect RapC homologs in any strain (other than Bt407) or homologs to any Bt8741 Rap proteins in *B. anthracis*, *B. weihenstephanensis*, *B. mycoides*, *B. pseudomycoides*, or *B. cytotoxicus*; this was verified by querying amino acid sequences of Bt8741 Rap proteins against the NCBI GenBank database (as of November 2019) (Data Set S1). These results provide useful hypotheses for experimental testing of Rap protein functions in future studies.

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, 58). Similarly, molecular mechanisms for the control of differentiation processes in the *B. cereus* group bacteria remain understudied (48, 49, 51, 58, 59). In this work, we uncovered the functions of Rap protein paralogs from Bt8741. Rap proteins are highly redundant in *B. subtilis*, which could result in the compensation of phenotypes of *rap* deletion mutants. For this reason, we chose to generate overexpression strains of Bt8741. Through this clean, reductionist approach, we found that Rap-Phr paralogs in this strain regulate collective functions such as sporulation, biofilm formation, spreading motility, and production of extracellular proteases. Our results show that Rap paralogs constitute a regulatory repertoire that may allow *B. thuringiensis* populations to respond efficiently to environmental changes, contributing to the fitness of the population. Moreover, the functions uncovered here

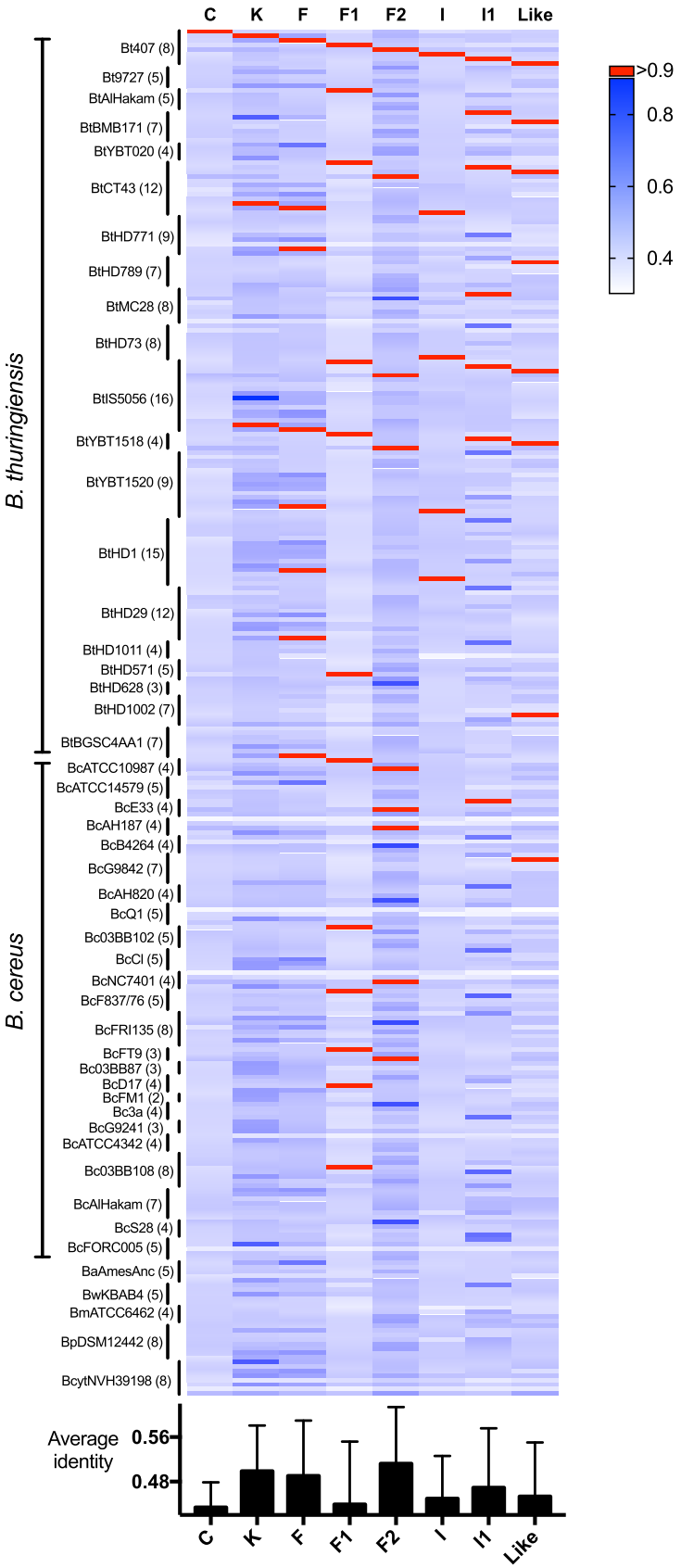

FIG 7 Homologs of Rap proteins from Bt8741 in strains from the *B. cereus* group. (Top) Heat map representing the identity of each Rap protein from Bt8741 against all Raps identified in strains from the

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can be extrapolated to other *B. thuringiensis* and *B. cereus* strains that encode in their genomes Rap proteins highly conserved to Bt8741 Raps, but experimental confirmation is needed.

Recent studies have shown that bacteria benefit from keeping multiple Rap-Phr systems, as redundancy has been selected for due to the social advantages it provides (60). Because Rap proteins have a repressive function upon their target, the gain of a novel Rap-Phr system for the regulation of extracellular public good production enables a facultative cheating mechanism in which variants with an extra system exploit their ancestral strain. Here, we show that the production of extracellular public goods, such as biofilm matrix components (58, 61), extracellular proteases (62), and surfactants (such as kurstakins [43, 63, 64]), 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, resulting in the presence of multiple Rap paralogs generated in an evolutionary process independent from that of *B. subtilis* (47). Multifunctionality may also provide evolutionary advantages. Because Rap-Phr systems are known to be parallel signaling pathways (47), 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. Our phenotypic studies show that Raps from *B. thuringiensis* have specialized for a variety of functions, and diversification was probably facilitated by those mechanisms. The phylogeny of Raps from the *B. cereus* group (47, 51) indicates that this is the case for all species within the group, due to the presence of several Rap paralogs encoded in their genomes.

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. In *B. subtilis*, five Rap-Phr systems negatively regulate Spo0A phosphorelay by dephosphorylating Spo0F and therefore prevent the activation of Spo0A (34). We found that $RapA_{Bs}$ retained this function when it was overexpressed in Bt8741. Furthermore, four Rap-Phr systems from Bt8741 (RapK, RapF, RapC, and RapLike) also regulate sporulation in this species. We propose that RapK and RapF may function by dephosphorylating Spo0F; this suggestion is supported by the following findings: (i) both RapK and RapF retain a high conservation of Spo0F-binding residues from RapH, including the catalytic residue Q47; (ii) their overexpression resulted in an undetectable number of spores, similar to $RapA_{Bs}$ overexpression; (iii) the overexpression of $RapA_{Bs}$, RapK, and RapF caused an identical cell morphology in the three overexpressing strains; and (iv) RapK and RapF also regulated biofilm formation, probably through the same activity on Spo0F.

Our results show that the participation of Rap proteins in sporulation cannot be fully predicted from the conservation of Spo0F-binding residues or the presence of the catalytic site residue Q47. This suggests that some Rap proteins could inhibit sporulation through other, unknown mechanisms. For this reason, experimental validation (e.g., direct measurement of Spo0F binding and bona fide phosphatase activity [21, 34, 44]) is essential for showing the participation of Rap proteins in the Spo0A phosphorelay. Further studies are needed in order to directly test the mechanisms by which RapC, RapK, RapF, and RapLike regulate sporulation in *B. thuringiensis* and other species from the *B. cereus* group.

We noted that the overexpression of $RapA_{Bs}$, which completely prevented sporulation, did not affect biofilm formation in *B. thuringiensis* or any other phenotype studied here. This reflects the fact that Rap proteins are not regulators that establish promiscuous protein-protein interactions; instead, they coevolve with specific protein targets

FIG 7 Legend (Continued)
B. cereus group (51). (Bottom) Average identity of each Rap protein from Bt8741 against all Raps from the *B. cereus* group. Bt, *B. thuringiensis*; Bc, *B. cereus*; Ba, *B. anthracis*; Bw, *B. weihenstephanensis*; Bm, *B. mycoides*; Bp, *B. pseudomycoides*; Bcyt, *B. cytotoxicus*. Numbers in parentheses indicate the number of Rap paralogs found in each strain. Values of identity for individual comparisons are available in Data Set S1.

in each bacterial species. *B. subtilis* Rap proteins target DegU, ComA, and Spo0F (21), but Rap targets in the *B. cereus* group (besides Spo0F, which is highly conserved in *B. subtilis* and *B. thuringiensis*) have not been studied; however, the coincidence between Rap proteins that inhibit extracellular proteolytic activity and spreading suggests that one target regulator mediates both functions.

We propose that Rap proteins have diversified according to the ecological needs of each species. For example, *B. subtilis* is a soil-dwelling bacterium that can be found in root-associated biofilms (65); in *B. subtilis*, five Rap proteins modulate Spo0A-P levels (21, 66), affecting sporulation and biofilm formation. Here, we demonstrate that four Rap proteins modulated sporulation (RapC, RapK, RapF, and RapLike), while only two of these (RapK and RapF) affected biofilm formation. This highlights the importance of sporulation regulation in both species and shows that, probably, biofilm formation is not as essential in the life cycle of *B. thuringiensis*. In contrast, *B. thuringiensis* is a soil-inhabiting, insect-pathogenic, and necrotrophic bacterium (67). In this species, extracellular protease production is essential for nutrient scavenging, which is normally associated with the necrotrophic stage of bacterial development in the insect cadaver (43); it could also be relevant for adaptation against fluctuations in nutrient availability in the environment. While only 1 out of 11 Rap proteins from *B. subtilis* 168 modulates extracellular proteolytic activity (RapG) (28), *B. thuringiensis* has extended the modulation of extracellular protease production to 6 Rap-Phr systems (RapC, -F, -F2, -I, and -I1 and RapLike). The divergence of Raps within the *B. cereus* group suggests that Raps from *B. anthracis*, *B. weihenstephanensis*, *B. mycoides*, *B. pseudomycoides*, and *B. cytotoxicus*, which do not include any homologs to Bt8741 Raps, have specialized for functions that may contrast to those found here for Bt8741 Raps.

The *B. cereus* group comprises bacteria with clinical and biotechnological relevance, such as *B. anthracis*, *B. cereus*, and *B. thuringiensis*, as well as other environmental and facultative species (41). Understanding the regulatory processes of 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, *B. anthracis* and *B. cereus* are known for their pathogenic nature against mammals. Therefore, elucidating the role of Rap-Phr systems in the production of virulence factors in these species, such as anthrax toxin and capsule of *B. anthracis* or enterotoxins of *B. cereus*, could be of high relevance. Additionally, it is known that QS systems can be synthetically engineered (68, 69), and Rap-Phr systems could be manipulated in order to enhance *B. thuringiensis* survival, insect pathogenesis, or Cry protein production.

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. *Bacillus thuringiensis* strain Bt8741 (46), a laboratory strain closely related to Bt407 (GenBank accession no. NC_018877.1), was used as the host for the overexpression of Rap proteins. *Bacillus subtilis* strain 168 was used for the amplification of *rapA*. *Escherichia coli* strain TOP10 was used for the construction and cloning of overexpression plasmids before transferring into Bt8741. Luria-Bertani (LB) broth (10 g liter^{-1} tryptone, 5 g liter^{-1} yeast extract, and 5 g liter^{-1} NaCl) and nutrient agar (8 g liter^{-1} nutrient broth and 15 g liter^{-1} agar) were used at 30°C for *Bacillus* cultures and at 37°C for *E. coli* and at 200 rpm for liquid cultures. Milk agar was prepared using nutrient agar supplemented with 5% skim milk (44). 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 *B. subtilis* 168 (36), 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 *B. subtilis* 168 and Bt8741 in comparison to RapH from *B. subtilis* 168 ($RapH_{Bs}$). For this, we performed pairwise alignments of the $RapH_{Bs}$ amino acid sequence (GenBank accession no. NP_388565.2) with $RapA_{Bs}$ (GenBank accession no. (NP_389125.1), $RapB_{Bs}$ (GenBank accession no. NP_391550.1), $RapE_{Bs}$ (GenBank accession no. NP_390460.2), $RapJ_{Bs}$ (GenBank accession no. NP_388164.1), $RapD_{Bs}$ (GenBank accession no. NP_391519.1) from *B. subtilis* 168, and each of the eight Raps from Bt407 (GenBank accession no. AFV21721.1, AFV22194.1, AFV22088.1, AFV16731.1, AFV19251.1, AFV22208.1, AFV16776.1, AFV17466.1), using the BLASTP tool (71). Then, all sequences were aligned together using the MAFFT version 7 online service (72) with the G-INS-i iterative refinement method (73). Finally, we identified in the alignment the amino acids of Rap protein sequences that correspond to the residues of $RapH_{Bs}$ that participate in binding and dephosphorylation of Spo0F.

DNA manipulation. All primers used in this study are listed in Table S2 in the supplemental material. DNA was isolated from *B. subtilis* 168 and Bt8741 using the PureLink genomic DNA minikit (Invitrogen, Carlsbad, CA). The QIAprep Spin miniprep kit (Qiagen, Germantown, MD) was used routinely for plasmid extraction and purification. Oligonucleotides were designed for amplifying each Rap gene from the Bt407 chromosome or plasmids (GenBank accession no. NC_018877.1, NC_018883.1, NC_018886.1, NC_018879.1, NC_018878.1) and the *B. subtilis* 168 genome (GenBank accession no. NC_000964.3) and synthesized by 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). The enzymes DreamTaq master mix, HindIII, SalI (Thermo Scientific, Waltham, MA), PstI, and T4 DNA ligase (New England Biolabs, Inc., Ipswich, MA) were used as recommended by the manufacturers.

Construction of Rap overexpression Bt8741 strains. All strains and plasmids used in this study are listed in Table S1. *rap* genes from Bt407 were previously identified (50). We performed an independent search using the amino acid sequence of *B. subtilis* RapA (GenBank accession no. NP_389125.1) as a query in BLAST (71) and identified the same proteins. Additionally, to ensure the identity of the Rap proteins, the sequences were submitted to the NCBI conserved domain search tool (74) in order to determine the presence of a tetratricopeptide repeat-containing domain. For the construction of the overexpression plasmid pHT315-P_{xylA}, the regulatory region of the xylose operon, including the *xylA* promoter (P_{xylA}) and the repressor gene *xylR*, was amplified using PCR from the *B. subtilis* 168 genome using the primers GG1 and GG2 (Table S2). This PCR product was inserted into the HindIII and PstI sites of the pHT315 plasmid (75), and colonies were PCR checked using primers DS16 and DS17 (Table S2). The resulting plasmid, pHT315-P_{xylA}, was transferred 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* [50]) and *rapA* from *B. subtilis* 168 ($RapA_{Bs}$ [34]) were amplified using the corresponding primer pairs listed in Table S2 and inserted in-frame between the PstI and SalI sites of pHT315-P_{xylA}. Nine overexpression plasmids, one for each Rap protein, were transferred into *E. coli* TOP10 competent cells. All plasmids were then transferred into Bt8741 electrocompetent cells using a protocol described in previous studies (44), generating nine Bt8741 strains for the overexpression of each Rap protein. Additionally, we transformed Bt8741 with pHT315-P_{xylA} (without a *rap* gene), and the resulting strain was used as a control strain throughout the Rap induction experiments. We used a wild-type strain of Bt8741 for overexpression of Rap proteins; therefore, expression is expected from all Phrs present in the genome, which could antagonize Rap overexpression effects on the phenotypes studied. The complete sequence of pHT315-P_{xylA}′*rapI* was verified using Illumina sequencing (MGH DNA Core, Cambridge, MA), and the rest of the P_{xylA}′*rap* constructions were verified using Sanger sequencing (Unidad de Servicios Genómicos, Langebio-Cinvestav, Irapuato, Mexico) using primers GG26 and DS17 (Table S2).

Sporulation efficiency. We assessed the effect of the overexpression of Rap proteins on sporulation efficiency in Bt8741. Preinoculums were prepared by picking a single colony of each strain into 5 ml of liquid medium and were grown overnight. Then, 1 ml of preinoculum was centrifuged, washed, and suspended in 1 ml of sterile phosphate-buffered saline (PBS). Glass culture tubes (25 mm diameter) with 5 ml of LB with erythromycin were inoculated with 50 μl (1% vol/vol) 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°C 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 glass tubes (13 by 100 mm) with 3 ml nutrient broth plus erythromycin, with and without the addition of xylose, to a final concentration of 2 mM. Then, 3 μl of preinoculum was added in triplicate, and the inoculated tubes were incubated without agitation at 31°C $\pm$ 1°C for 48 hours. The culture medium was then removed with a syringe with a needle. The biofilm and ring attached to the wall of the tube, composed of cells from the biofilm, were suspended 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 medium to address planktonic growth. At least 5 replicates of each treatment were performed.

Spreading 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 $liter^{-1}$ nutrient broth and 1.5 g $liter^{-1}$ agar) with erythromycin and with or without the addition of xylose. Plates were air dried inside a biological hood for 60 min prior to inoculation. Then, 5 μl of preinoculum cultures was spotted in the center of the plate, dried for 5 min, and incubated at 30°C for 14 days. The inoculated agar plates were photographed at days 1, 3, 5, and 7 using a gel documentation system (Gel Doc XR+; Bio-Rad). The colony area was measured using Image Lab software (Bio-Rad), and radial growth was calculated. 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.

Extracellular proteolytic activity assay. To evaluate the effect of Rap overexpression in extracellular proteolytic activity of Bt8741, 2 μl of preinoculum of each Rap overexpression strain was 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 Image Lab software (Bio-Rad). To correct for differences in colony growth, we subtracted the colony area.

Identification of Bt8741 Rap homologs in the *B. cereus* group. We used a data set from previous work (51) consisting of Rap protein sequences from representative strains from the *B. cereus* group. The amino acid sequence of each Rap protein from Bt8741 was queried against each sequence from the *B. cereus* Rap protein data set using the tBlastn Blast2 online tool (71). We considered homologs the cases where identity was >90% between a Rap from Bt8741 and another Rap from the *B. cereus* group.

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 multiple *t* tests to search for differences between not induced and induced Rap protein overexpression conditions of each strain. Colony size data from the extracellular proteolytic activity assay were analyzed with a two-way analysis of variance (ANOVA), and the Tukey-Kramer test was used for multiple comparisons. A significance of $P = 0.05$ was used in all statistical tests.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.2 MB.

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J.R. and M.D.L.T. conceived the study. J.R. and G.G. designed the experimental work. G.G. conducted molecular protocols and lab experiments. G.G. conducted the computational analyses. G.G., M.D.L.T., and J.R. interpreted experimental data. G.G. wrote the first draft of the manuscript, and G.G., M.D.L.T., and J.R. added critical revisions.

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