Rap-protein paralogs of *B. thuringiensis*: a multifunctional and redundant regulatory repertoire for the control of collective functions.

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Running Head: Control of collective functions by Rap proteins in Bt

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Data Deposition Statement: Analysis scripts and input files associated with reconstruction of the phylogenetic tree are available at https://github.com/gabyga16/rap_phylogenetics

A file with supplemental material is available.

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 five Rap-Phr systems (RapC, K, F, I and RapLike) inhibit sporulation, two of which (RapK and RapF) probably dephosphorylate of Spo0F from the Spo0A phosphorelay; these two Rap proteins 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 enables 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 understanding about 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, 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

71 by environmental cues, stress conditions, cell-cell signaling, and are tightly modulated by
72 complex, overlapping regulatory circuits (13–15).
73
74 Bacteria detect cell density through quorum sensing (QS), which depends on self-produced
75 signaling molecules that accumulate in the extracellular space as the population grows.
76 Specific receptors in the cell membrane or in the cytoplasm recognize these signaling
77 molecules and regulate downstream cellular processes (16–18). Collective traits such as
78 virulence, competence, sporulation and bioluminescence are regulated by QS. Gram-
79 positive bacteria use small peptides as signaling molecules for QS (17).
80
81 The RRNPP family (Rgg, Rap, NprR, PlcR, PrgX) are intracellular QS receptors that
82 regulate several functions across gram-positive bacteria (19–21). Genes coding for receptor
83 proteins and their associated signaling peptides are encoded in transcriptional cassettes (22).
84 Rgg, NprR, PlcR and PrgX proteins are transcriptional activators that bind directly to DNA
85 in quorum state. Rap proteins, however, lack a DNA binding domain and they function by
86 binding and inhibiting proteins, specifically response regulators and transcriptional
87 activators (21, 23, 24). Twelve Rap paralogs (RapA, B, C, D, E, F, G, H, I, J, K, 60) control
88 diverse functions in *B. subtilis* 168 (Bs168). The RapG-PhrG pair regulates the activation
89 of DegU, a transcriptional regulator that controls *aprE* and *comK* genes encoding for
90 extracellular proteases and a transcription factor for competence in Bs, respectively (15, 25);
91 ComA – the master regulator of competence genes – is repressed by RapC, D, F, G, H, K
92 and Rap60 (14, 26–31); Spo0A – the transcriptional activator of many differentiation genes
93 – 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 present 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 encoded 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

117 functions of Rap proteins in the *B. cereus* group based on what is known of Rap proteins
118 from Bs.
119

120 Some Rap-Phr systems from species of the *B. cereus* group have been studied. First, Rap
121 BXA0205 and BA3790 from *B. anthracis* str. A2012, were demonstrated to regulate
122 sporulation initiation and to dephosphorylate Spo0F (45). Later, it was shown that Rap8
123 from Bt-HD73, regulates the sporulation and biofilm formation processes *in vitro* (46). A
124 more recent study showed the participation of Rap6, 7 and 8 – also known as RapC, K and
125 RapF, respectively (47) – in the modulation of the sporulation process in Bt407 (48).
126 However, other Rap paralogs with unknown functions have been identified in the genomes
127 of *B. cereus* group bacteria (44, 47) that may be relevant to their ecology.
128

129 In this study we aimed at evaluating the diversification of the Rap-Phr systems in the *B.*
130 *cereus* group as well as their functions, using *Bacillus thuringiensis* Bt8741 as model. We
131 generated eight Rap-overexpression strains of Bt8741 to evaluate the role of each Rap
132 paralog in sporulation efficiency, biofilm formation, extracellular proteolytic activity and
133 spreading. We also studied the evolution of Rap-Phr paralogs in the *B. cereus* group, by
134 identifying Rap homologs from other species and analyzing its phylogeny. This allowed the
135 prediction of their functions, based on those of Rap proteins from Bt8741.
136

137 **Results**

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

139 In order to predict the capacity of Rap proteins from Bt407 (a strain closely related to
140 Bt8741) to bind to Spo0F, we analyzed the conservation of the amino acids previously
141 reported to be involved in Spo0F-binding by RapH from *B. subtilis*. In this analysis, we
142 included reported sequences of Bs-Rap proteins that bind to Spo0F (RapA, B, E, H, J) as
143 well as the sequence of RapD from Bs, which does not bind to Spo0F (34) (Fig. 1). We
144 found more conservation of the functional amino acids of RapH, in the sequences of both
145 Bs168 and Bt407, compared to the corresponding full sequences (Fig. 1B). In Bs168, the
146 full sequence conservation of the Rap proteins known to bind to Spo0F (RapA, B, E, J)
147 compared to RapH, ranged from 59% to 66%, and the functional amino acids conservation
148 percentage, from 82.3% to 100%. In RapD, which does not bind to Spo0F, the full-length
149 sequence is conserved at 50% and the functional residues are only 64.7% conserved (Fig.
150 1B). In the case of Rap proteins from Bt407, the full sequence conservation in comparison
151 to RapH of Bs168 ranged from 45% to 48%. On the other hand, conservation of the
152 functional residues ranged from 64.7% to 88.2% (Fig 1B). Since more conservation occurs
153 in the Spo0F-binding functional residues, these residues could be important for the function
154 of Bt Rap proteins.
155
156 RapK exhibited the highest conservation percentage of Spo0F-binding residues (88.2%),
157 followed by RapF, I and RapI1 (82.3%), RapF1, F2 and RapLike (70.5%) and finally RapC,
158 with 64.6%. Although RapF1 and RapF2 had a high conservation of functional residues,
159 neither these Rap paralogs, nor RapC, conserve the residue Q47 found in the catalytic site
160 and previously shown to be essential for the phosphatase activity of RapH (34). This
161 analysis enables the prediction that some Rap protein paralogs from Bt8741, with a high
162 conservation percentage of putative Spo0F-binding amino acids, could dephosphorylate

163 Spo0F, while other paralogs could have evolved to participate in other regulatory processes.
164 Indeed, RapK, RapF and unexpectedly RapC from Bt407, Rap8 from Bt-HD73 (ortholog to
165 RapI from Bt407) and Rap BXA0205 and BA3790 from *B. anthracis*, (homologs of RapK
166 and RapF2, respectively) have been shown to participate in the modulation of sporulation
167 (45, 46, 48). Previous to this work, RapF1, I1, and RapLike from Bt407 (or its homologs in
168 other species), had not been tested for their role in sporulation.
169
170 **RapC, K, F and RapLike control sporulation in Bt8741.**
171 We constructed nine Rap-overexpression strains in the Bt8741 background (Table S1), one
172 for each endogenous Rap protein identified in Bt407 (RapC, K, F, F1, F2, I, I1, Like) and
173 one more for RapA from Bs168 ($RapA_{Bs}$). We also generated a control strain of Bt8741
174 carrying the empty plasmid pHT315-P_{xylA} (Table S1). DNA sequencing showed correct, in-
175 frame insertion of P_{xylA} and *rap* genes in the pHT315 plasmid (not shown). We followed a
176 growth time-course experiment of all strains in shaking flasks for 24 hours and confirmed
177 that neither xylose addition, nor Rap overexpression, affected bacterial growth (Fig. S1).
178
179 In order to identify the Rap proteins involved in the regulation of sporulation initiation, we
180 studied the effect of Rap overexpression in the sporulation efficiency of each strain. In this
181 experiment, we observed that both addition of xylose to the culture medium and the
182 presence of *rap* genes in the plasmid had minor effects on total and thermoresistant CFU
183 counts of Bt8741 at 72 h. In the control strain, addition of xylose caused a decrease of ≈1
184 log10 in total and thermoresistant CFU (Fig. S2A and S2B). Similarly, when *rapF1* and
185 *rapF2* genes were carried in the plasmid – but not overexpressed – total CFU decreased by
186 up to one logarithm of CFU in comparison to the control strain (Fig. S2A). Additionally,

187 sporulation decreased one logarithm in strains carrying *rapF1*, *rapF2* and *rapI1* in
188 comparison to the control strain when overexpression was not induced (Fig. S2C). These
189 unspecific effects were probably related to basal expression from the P_{xylA} promoter, even
190 when xylose is not added, since pHT315 replicates at 15 copies per cell (49). However, the
191 most dramatic effect was found in thermoresistant CFU of strains overexpressing Rap
192 proteins (Fig. S2D).
193
194 In spite of the unspecific effect of xylose addition on growth and sporulation, sporulation
195 efficiency of the control strain remained unchanged by the addition of inducer (Fig. 2). In
196 contrast, overexpression of $RapA_{Bs}$ caused a decrease in sporulation efficiency from 7.9%
197 to 0.0005%. In fact, thermoresistant CFU were undetectable when $RapA_{Bs}$ was
198 overexpressed (Fig. S2D). We also found undetectable levels of spores in strains
199 overexpressing RapK and RapF (Fig. S2D). Sporulation efficiency decreased from 32.93%
200 to 0.0002% in the strain overexpressing RapK and from 9.24% to 0.0026% in the strain
201 overexpressing RapF (Fig. 2). In Bs, RapA dephosphorylates Spo0F in the Spo0A
202 phosphorelay (32) and this result indicates that it performs the same function in B8741;
203 furthermore, it suggests that RapK and RapF carry out the same mechanism for regulation
204 of sporulation initiation.
205
206 Strains carrying P_{xylA}*'rapC* and P_{xylA}*'rapLike* also exhibited reduced sporulation efficiency
207 when xylose was added to the medium. Sporulation efficiency decreased from 5.43% to
208 0.0357% and from 12.34% to 0.1352% when RapC and RapLike were overexpressed,
209 respectively (Fig 2). Additionally, RapI overexpression slightly decreased sporulation

210 efficiency, from 2.82% to 0.51%. Sporulation efficiency was not decreased by the
211 overexpression of either RapF1, F2, I or RapI1.
212
213 Samples of the Rap-overexpressing strain cultures at 72 h were observed in a microscope.
214 We detected free spores and bacterial debris in all cultures, when Rap proteins were not
215 overexpressed (Fig. S3). Figure 3 shows representative fields of view with cells from each
216 induced culture. Strains overexpressing Rap proteins that did not affect sporulation
217 efficiency (RapF1, F2 I1) showed cell morphology similar to that of the control strain, i.e. a
218 sporulated bacilli with defined endospores. In samples from strains overexpressing $RapA_{Bs}$,
219 K and RapF, that had acutely decreased sporulation efficiency, we observed chained,
220 wrinkled cells with no spores (Fig. 3). On the other hand, cells from strains overexpressing
221 RapC and RapLike, were observed as rod-shaped and no spores were evident (Fig. 3).
222 Finally, in cells from the strain overexpressing RapI, which had a slight effect on
223 sporulation efficiency, cell morphology was similar to strains overexpressing RapF1, F2, I1
224 and the control strain (Fig. 3), showing a defined endospore.
225
226 **Overexpression of RapF and RapK prevents biofilm formation of Bt8741.**
227 In nature, over 80% of bacteria live in biofilms (49), therefore, biofilm formation is likely a
228 relevant trait – albeit an understudied one – during the life cycle of Bt and other bacteria
229 belonging to the *B. cereus* group. To determine which Rap proteins were involved in the
230 regulation of biofilm development in Bt8741, we quantified biofilm formation of the Rap-
231 overexpression strains in the air-liquid interphase at 48 h. For this, we suspended the cells
232 form the biofilm and measured optical density (OD_{600}). Since 20 mM of xylose in the
233 media caused a complete inhibition of biofilm formation in the Bt8741 control strain (not

234 shown), we first tested the effect of xylose concentration on this phenotype. We found that
235 biofilm formation was not affected at 2 mM, but was decreased at higher concentrations of
236 5, 10 and 15 mM (Fig. S4); therefore, overexpression of Rap proteins was performed with 2
237 mM of xylose (50).
238
239 Overexpression of RapK and RapF caused an inhibition of biofilm formation of Bt8741
240 (Fig. 4A), evident by the significant decrease ($p<0.0001$) in the OD_{600} measured from a
241 sample obtained from the surface of the culture (Fig. 4B). The OD_{600} of the biofilms
242 decreased from 0.7115 to 0.0977 and from 0.6577 to 0.0961 in strains overexpressing
243 RapK and RapF, respectively (Fig. 4B). On the other hand, biofilms were normally formed
244 by strains overexpressing $RapA_{Bs}$, C, F2, I, I1 and RapLike (Fig. 4). Interestingly, the strain
245 overexpressing RapF1 was unable to form biofilms even when P_{xylA}*'rapF1* was not induced
246 (Fig. 4A and B).
247
248 In order to discard possible global growth defects in this assay when RapK and RapF were
249 overexpressed, we measured planktonic growth through OD_{600} of the liquid culture media
250 from the same experiments where biofilm formation was assessed. We found that
251 planktonic growth was higher in conditions where a biofilm was not formed (Fig. S5). This
252 suggests that RapK and RapF specifically inhibit biofilm formation (e.g. secretion of
253 extracellular matrix components).
254
255 **Extracellular proteolytic activity is downregulated by RapC, F, F2, I1 and RapLike in**
256 **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 in the media 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 the 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, F1, F2 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 protein overexpression on radial spreading of colonies of Bt8741 growing on agar media.

We observed that addition of xylose in the media did not affect the 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

281 5.05 mm to 0.78 mm (decrease of 84.5%); and RapLike from 3.64 mm to 0.65 mm
282 (decrease of 82.1%) (Fig. 6B). Spreading inhibition is evident in the colony morphology of
283 these strains (Fig. 6C). We observed that the overexpression of RapC, F2 and RapLike,
284 completely eliminated this phenotype, while overexpression of RapF1 only decreased
285 spreading ($p<0.05$) (Fig. 6B and C).
286
287 The overexpression of $RapA_{Bs}$, K, F, I and RapI1 did not affect the spreading of Bt8741
288 ($p>0.05$) (Fig. 6B). Spreading of the strains carrying overexpression plasmids for these Rap
289 proteins ranged from 4.68 mm to 7.29 mm without induction and from 3.37 mm to 6.84
290 mm when induced (Fig. 6B). In some cases, Rap overexpression affected colony
291 morphology, i.e. colonies of strains overexpressing RapABs, RapK and RapI showed an
292 increased dendritic phenotype; however, the spreading phenotype measured as colony
293 radius, was still present (Fig. 6C).
294
295 **Rap-Phr systems diversified into five pherogroups in the *B. cereus* group.**
296 In order to predict the functions of Rap paralogs in *B. cereus* (Bc), *B. anthracis* (Ba), *B.*
297 *mycoides* (Bm), *B. pseudomycoides* (Bps) and *B. cytotoxicus* (Bcyt), we analyzed their
298 sequences to deduce the evolution of Rap proteins in these species and identify their
299 putative signaling peptide sequences (mature Phr). Additional to the 8 *rap* genes in Bt407
300 (of which 4 are located in the chromosome, and 4 in plasmids) we found 32 *rap-phr*
301 systems in the *B. cereus* group (Table S2), 30 of which are located in the chromosome and
302 2 in plasmids (Table 1).
303

304 The phylogeny of Rap proteins from the *B. cereus* group shows that clades are composed of
305 Rap proteins from different species, i.e., phylogenetically close Rap homologs can be found
306 in different species. This suggests that Rap-Phr divergence occurred before speciation in
307 this group (Fig. 7). Hence, it is possible that Rap functions uncovered in this work could be
308 extrapolated to the rest of the *B. cereus* group, e.g. Rap proteins found in the same clade as
309 BtRapK and BtRapF (Bps28285, Bps05775, Bps24285, Bcyt05320, Bc1026, Ba05875,
310 Ba29315, Bcyt11595, Bcyt05405 and Bcyt02700) may modulate sporulation initiation and
311 biofilm formation. Since we found that several Rap paralogs are coded in every species of
312 the *B. cereus* group, we suggest that they could regulate a variety of collective functions in
313 all these species, as we describe here for Bt8741.
314
315 We identified 5 pherogroups, each with a putative mature Phr peptide consensus sequence.
316 All five pherogroups include Rap proteins from different species (Fig. S7). These five
317 pherogroups are identified with colors in the branches of the phylogeny in figure 7. We
318 found that the mature Phr corresponding to pherogroups 1 and 2 are located at the C-
319 terminal domain of the pro-peptides (exported Phr sequence). RapI, F, F1 and RapK from
320 Bt are found at these pherogroups. On the other hand, for pherogroup 3 – where RapF2
321 from Bt is found – consensus sequences are located at the N-terminal domain of the pro-
322 peptide. Finally, putative mature Phr peptides from pherogroups 4 and 5 – which include
323 RapLike, C and RapI1 from Bt – are located in the middle of the exported sequence (Table
324 S3). We observed that only Bt and Bps encode Rap proteins from all five pherogroups; Rap
325 proteins from Ba and Bcyt are found in pherogroups 1, 2, 3 and 4; Rap proteins in Bm
326 correspond to pherogroups 1, 3 and 5; Rap proteins from Bc are found only in pherogroups
327 1 and 4 (Fig. 7). Pherogroup 1 is the only one present in all evaluated species.

328

329 **Discussion**

330 Few studies have addressed multicellular behaviors such as differentiation, cell-
331 specialization, collective functions, and the resulting ecological interactions in species from
332 the *B.* cereus group (11, 54). Similarly, molecular mechanisms for the control of
333 differentiation processes in the *B. cereus* group bacteria remain understudied (45, 46, 48, 54,
334 55). Here we show that Rap-Phr systems in Bt8741 regulate collective functions such as
335 sporulation, biofilm formation, production of extracellular proteases and spreading motility.
336 In fact, Rap-Phr systems in this strain are highly multifunctional and redundant, since five
337 out of eight Rap paralogs modulate more than one collective trait, and all four collective
338 traits studied were inhibited by more than one Rap protein. Hence, Rap paralogs appear to
339 constitute a regulatory repertoire that allows Bt populations to respond efficiently to
340 environmental changes, which contributes to fitness of the population.

341

342 Although it is well known how the Rap-Phr systems participate in differentiation processes
343 of the gram-positive model bacteria Bs, speciation resulted in divergent Rap proteins in the
344 *B. cereus* and *B. subtilis* groups (43, 44). Therefore, no homologs are shared between the
345 groups; however, in both cases, speciation resulted in the presence of multiple Rap paralogs
346 per genome. We propose that Rap proteins that are phylogenetically close to Rap proteins
347 from Bt8741, could have the same functions in other bacteria of the *B. cereus* group.

348

349 It is not yet clear how bacteria benefit from keeping multiple receptor-signaling peptide
350 gene pairs comprising this complex signaling network of Rap-Phr systems; however, it has
351 been shown that redundancy in Rap-Phr systems in Bs has been selected for because it

352 provides social advantages (56). Because Rap proteins have a repressive function upon its
353 target, the gain of a novel Rap-Phr system for the regulation of extracellular public good
354 production enables a facultative cheating mechanism in which variants with an extra system
355 exploit their ancestral strain. Here we showed that extracellular public goods such as
356 biofilm matrix components, extracellular proteases or surfactants, are likely controlled by
357 Rap proteins in Bt8741; therefore, the same facultative cheating mechanism could be
358 expected during duplication of *rap-phr* genes in the *B. cereus* group. This represents a
359 selective advantage by a fitness increase of the novel population. Multifunctionality seems
360 to have evolutionary advantages as well. Perhaps, because Rap-Phr systems are known to
361 be parallel signaling pathways (44) they are not all activated simultaneously; instead, some
362 of them may be active only under specific conditions, achieving the regulation of various
363 differentiation processes and collective functions while optimizing energetic costs. Overall,
364 keeping multiple redundant and multifunctional Rap paralogs that control important
365 collective functions results in a better adaptation and population survival in nature.
366
367 Sporulation in the *Bacillus* genus is essential for bacterial survival and dissemination in
368 their habitats; it is also important for the biotechnological uses of *Bacillus* species. Six Rap-
369 Phr systems from Bs, including RapA, negatively regulate Spo0A phosphorelay by
370 dephosphorylating Spo0F, and therefore prevent the activation of Spo0A (32). We found
371 that $RapA_{Bs}$, retained this function when it was overexpressed in Bt8741. Furthermore, five
372 Rap-Phr systems from Bt8741 (RapK, F, C, Like and RapI) also regulate sporulation in this
373 species. We propose that RapK and RapF may function by dephosphorylating Spo0F,
374 similar to the mechanism carried out by RapA in Bs. This suggestion is supported by three
375 findings: 1) both RapK and RapF retain the highest conservation of Spo0F binding residues

376 from RapH, including the catalytic residue Q47; 2) their overexpression resulted in
377 undetectable number of spores, similar to $RapA_{Bs}$ overexpression; 3) the overexpression of
378 $RapA_{Bs}$, K and RapF caused an identical cell morphology in the three overexpressing
379 strains. Additionally, RapK and RapF are closely related and both belong to pherogroup 2,
380 which may indicate that they resulted from a gene duplication event of a Rap ancestor that
381 dephosphorylated Spo0F. Other Rap proteins that decreased sporulation efficiency are
382 RapC, Like and RapI; of these, RapC does not contain the catalytic site residue Q47.
383 Further studies are needed in order to elucidate the mechanisms by which all these
384 receptors regulate sporulation in Bt and other species from the *B. cereus* group.
385
386 RapK and RapF are the only Rap proteins from Bt8741 that prevented biofilm formation.
387 Because Spo0A-P levels regulate both sporulation and biofilm formation in Bs, we
388 speculate that bifunctionality of RapK and RapF in Bt8741 results from their activity on
389 Spo0F. We noted, however, that the overexpression of $RapA_{Bs}$, which completely
390 prevented sporulation, did not affected biofilm formation in Bt. Overexpression of $RapA_{Bs}$
391 may allow low levels of Spo0A-P in Bt8741, which in Bs are sufficient for the activation of
392 genes related to production of extracellular matrix components, but not for the activation of
393 early sporulation genes (13). This picture is probably more complex, as different feedback
394 loops modulate sporulation and biofilm formation (57). It is noteworthy that overexpression
395 of $RapA_{Bs}$, in Bt8741 did not inhibit any phenotype, other than sporulation; this reflects the
396 fact that Rap proteins co-evolve with specific protein targets in each bacterial species; it
397 also indicates that Rap target regulators involved in the control of extracellular proteases
398 and spreading may not be conserved betweem Bs and the *B. cereus* group.
399

400 We suggest that Rap proteins have diversified according to the ecological needs of each
401 species. For example, Bs is a soil dwelling bacteria found associated to rhizosphere forming
402 biofilms (58). In Bs, six Rap proteins modulate Spo0A-P levels (21, 59), affecting
403 sporulation and biofilm formation. Here we demonstrate that five Rap proteins modulated
404 sporulation (RapC, K, F, I and RapLike) while only two of these (RapK and RapF) affected
405 biofilm formation, perhaps through the Spo0A phosphorelay. This highlights the
406 importance of sporulation regulation in both species and that probably, biofilm formation is
407 not as essencial in the lifecylce of Bt, as it is in Bs. In contrast, Bt is a soil inhabitant, insect
408 patogenic and necrotrophic bacteria (60). In this species, extracellular protease production
409 is essential for nutrient scavenging, which is normally associated to the necrotrophic stage
410 of bacterial development in the insect cadaver (40). Additionally, it could be relevant
411 during the transition from exponential growth to stationary phase in controlled
412 fermentations or for adaptation against fluctuations in nutrient availability in the
413 environment. While only one of the twelve Rap proteins from Bs modulates its extracellular
414 proteolytic activity (RapG) (25), Bt has extended the modulation of extracellular protease
415 production to five Rap-Phr systems (RapC, F, F2, I1 and RapLike).
416
417 We found that the Spo0A phosphorelay and production of extracellular proteases are highly
418 interconnected in Bt8741 through the functions of RapC, F and RapLike. Additionally,
419 extracellular proteolytic activity (specifically the NprA protease) is regulated by the QS
420 system NprR-NprRB (61), which is also involved in the modulation of the Spo0A
421 phosphorelay (41, 42). Likewise, NprR also participates in the spreading phenotype of
422 Bt8741 (A. Verdugo *et. al*, unpublished data), as well as RapC and RapLike. Because
423 sporulation, extracellular protease production and spreading of Bt have evolved to be

424 regulated and coordinated by multiple QS systems, these collective traits may be important
425 in the life cycle of Bt and represent essential mechanisms for its ecology.
426
427 Mature Phr signaling peptides from Bs correspond to at least five residues located in the C-
428 terminal end of the pro-Phr or in the middle of the sequence. Sequence analyses of mature
429 Phrs in Bs have shown that a basic amino acid is found in the second position from the N-
430 terminal end, and an alanine residue is necessary in the position before the cleavage site for
431 Phr maturation (22, 62, 63). Our analysis of consensus putative mature Phr sequences
432 showed that these characteristics are not maintained in mature Phr peptides of the *B cereus*
433 group. This suggests that signaling peptides are processed differently in these bacteria, i.e.,
434 using different sets of extracellular proteases and peptidases that recognize distinct
435 sequences. In Bt, the identity of a mature Phr has only been shown for Rap8-Phr8 from Bt-
436 HD73. In this case, the active heptapeptide YAHGKDI is located in the C-terminal end
437 from its exported sequence (46). RapI from Bt8741, ortholog protein to Rap8, is found in
438 pherogroup 1, in which the consensus sequence HGKDI corresponds to the five residues in
439 the C-terminal end from the exported sequence. This indicates that the consensus sequences
440 determined in this study may not exactly predict the signaling peptide sequence, but they
441 can direct their search in future studies.
442
443 We found that Rap-Phr systems in the *B. cereus* group have evolved into five pherogroups,
444 each including Rap homologs from different species. This means that signaling peptides
445 shared by more than one species, could mediate crosstalk or eavesdropping phenomena in
446 nature, allowing the regulation of collective functions in response to interspecific signals as
447 described for other gram-positive species (64, 65).

448

449 The *B. cereus* group comprise bacteria with clinical and biotechnological relevance such as
450 Ba, Bc, Bt, and other environmental and facultative species (38). We show that Rap-Phr QS
451 systems in Bt are involved in the regulation of ecologically important collective traits, and
452 our findings are highly relevant for further studies about the *B. cereus* group and contribute
453 to the knowledge about its ecology. Understanding the regulatory processes for cell
454 differentiation and specialization in these bacteria may enhance the use of
455 biotechnologically-relevant species, or the strategies to control human pathogens, through
456 the intervention of their collective functions at the molecular level. For instance, Ba and Bc
457 are known for their pathogenic nature against mammals; therefore, elucidating the role of
458 Rap-Phr systems in the production of virulence factors of these species such as anthrax
459 toxin and capsule of Ba, or enterotoxins of Bc, could be of high relevance. Additionally, it
460 is known that QS systems can be synthetically engineered (66, 67). As a result, Rap-Phr
461 systems could be manipulated in order to enhance Bt survival, insect pathogenesis or cry
462 protein production. This work serves as a starting point for the study of cell specialization
463 of the *B. cereus* group bacteria.

464

465 **Materials and Methods**

466 **Bacterial strains, media and culture conditions**

467 *Bacillus thuringiensis* strain 8741 (Bt8741) (43), derived from Bt407 (Acc. No.
468 NC_018877.1, 51), was used as host for the overexpression of Rap proteins. *Bacillus*
469 *subtilis* strain 168 (Bs168) was used for the amplification of *rapA*. *Escherichia coli* strain
470 TOP10 (69) was used for construction and cloning of overexpression plasmids before
471 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 ºC for *Bacillus* cultures and at 37 ºC 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 the BlastP tool (71). Then, all sequences were aligned together using 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 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_{xylA}, 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 *Hin*dIII and *Pst*I 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*, 47) and

519 *rapA* from Bs168 ($RapA_{Bs}$, 32) were amplified using the corresponding primers pairs listed
520 in Table S4, and inserted in-frame between the *PstI* and *SalI* sites of pHT315-P_{xylA}. Nine
521 overexpression plasmids, one for each Rap protein, were transformed into *E. coli* Top10
522 competent cells. All plasmids were then transformed into Bt8741 electrocompetent cells,
523 using the protocol described in previous studies (41), generating nine Bt8741 strains for the
524 overexpression each Rap protein. Additionally, we transformed Bt8741 with the pHT315-
525 P_{xylA} (without a *rap* gene), and the resulting strain was used as control strain throughout the
526 Rap induction experiments. The complete sequence of pHT315-P_{xylA}'*rapI* was verified by
527 Illumina sequencing (MGH DNA Core, Cambridge, MA, USA), and the rest of the
528 P_{xylA}'*rap* constructions were verified by Sanger sequencing (Unidad de Servicios
529 Genómicos, LANGEBIO-CINVESTAV, Irapuato, Mexico) using primers GG26 and DS17
530 (Table S4).
531
532 **Sporulation efficiency**
533 We assessed the effect of the overexpression of Rap proteins on sporulation efficiency in
534 Bt8741. Preinoculums were prepared by picking a single colony of each strain into 5 mL of
535 liquid media and grown overnight. Then, 1 mL of preinoculum was centrifuged, washed
536 and suspended in 1 mL of sterile PBS. Glass culture tubes (25 mm diameter) with 5 mL of
537 LB with erythromycin were inoculated with 50 µL (1% v/v) of preinoculum containing
538 $\approx 10^7$ cfu ml^{-1} and incubated for 72 h. All strains were cultured in triplicate, in LB with and
539 without the addition of xylose. To determine growth and sporulation, total and
540 thermoresistant CFU were calculated by plating 10-fold serial dilutions in nutrient agar. For
541 thermoresistant CFU, samples of 100 µL were incubated at 80 ºC for 20 min prior to

542 diluting and plating. Sporulation efficiency was calculated as the percentage of
543 thermoresistant CFU in total CFU.
544
545 **Biofilm formation assay**
546 We evaluated the effect of the overexpression of Rap proteins on the capacity of Bt874 to
547 form biofilms. For this assay, we used 13 x 100 mm glass tubes with 3 mL Nutrient Broth +
548 erythromycin, with and without the addition of xylose to a final concentration of 2 mM.
549 Three µL of preinoculum was added in triplicates, and the inoculated tubes were incubated
550 without agitation at 31 ºC ± 1 ºC for 48 hours. The culture media was then removed with a
551 syringe with needle. The biofilm and ring attached to the wall of the tube, composed of
552 cells from the biofilm, were suspended in 1.5 mL of sterile PBS and the optical density
553 (OD_{600}) was measured. The OD_{600} was also measured from the removed liquid media to
554 address planktonic growth. At least 5 replicates of each treatment were performed.
555
556 **Extracellular proteolytic activity assay**
557 To evaluate the effect of Rap overexpression in extracellular proteolytic activity of Bt8741,
558 2 µL of preinoculums of each Rap-overexpression strain, prepared as described above, were
559 spotted in triplicate on milk agar with and without the addition of xylose. The hydrolysis
560 halo area was measured after 24 h of incubation using the Image Lab™ Software
561 (BIORAD). To correct for differences in colony growth, we subtracted the colony area.
562
563 **Spreading phenotype assay**
564 The spreading phenotype of Rap-overexpression Bt8741 variants was followed in colonies
565 spotted on agar. For this assay, we used diluted nutrient agar (NA) (0.8 g L^{-1} Nutrient broth,

566 1.5 g L^{-1} agar) with erythromycin and with or without the addition of xylose. Plates were
567 air-dried inside a biological hood for 60 minutes prior to inoculation. Then, 5 µL of
568 preinoculum cultures were spotted in the center of the plate, dried for 5 minutes and
569 incubated at 30 ºC for 14 days. The inoculated agar plates were photographed at days 1, 3,
570 5, 7 and 14, using a gel documentation system (Gel Doc™ XR+, BIORAD). Colony area
571 was measured using the Image Lab™ Software (BIORAD) and radial growth was
572 calculated. For normalization of radial dispersion, we subtracted from all observations the
573 colony radius at day 1, which corresponds to the inoculated droplet area. Three replicates of
574 each treatment were performed.
575
576 **Phylogenetic Analysis**
577 To reconstruct the phylogeny of Rap proteins in the *B. cereus* group, we first selected one
578 representative strain of each species from NCBI GenBank, including *Bacillus cereus*
579 ATCC14579 (Accession NC_004722.1), *Bacillus anthracis* A0248 (NC_012659.1),
580 *Bacillus thuringiensis* 407 (NC_018877.1), *Bacillus mycoides* ATCC6442
581 (NZ_CP009692.1), *Bacillus pseudomycoides* DMS12442 (NZ_CM000745.1) and *Bacillus*
582 *cytotoxicus* NVH391-98 (NC_009674.1). Strains were selected based on the availability of
583 a complete genome (as of July of 2018) and thus, *Bacillus weihenstephanensis* was
584 excluded. We searched for Rap protein homologs in the selected genomes by querying the
585 amino acid sequence of *B. subtilis* RapA (NP_389125.1) and each of the eight Rap
586 sequences of *B. thuringiensis* 407: RapC (AFV21721.1), RapK (AFV22194.1), RapF
587 (AFV22088.1), RapF1 (AFV16731.1), RapF2 (AFV19251.1), RapI (AFV22208.1), RapI1
588 (AFV16776.1) and RapLike (AFV17466.1). Homologs were searched using BLAST tool
589 (71), the tBlastn tool and a local script designed for performing the blast search in an

590 assembled database of the selected genomes. To ensure the identity of the Rap protein
591 homologs, Blast hits were submitted manually to the Conserved Domain Search-NCBI tool
592 (75) in order to determine if they presented the characteristic TPR-containing domain. Rap
593 protein amino acid sequences were aligned in MAFFT version 7 (72) using the G-INS-i
594 iterative refinement method which incorporates pairwise alignment algorithms (73). RapA
595 and RapH from Bs168 were also included as outgroups for the phylogenetic reconstruction.
596 The selection of the best substitution evolutionary model (JTT+G+I+F) was made using the
597 Smart Model Selection with the Akaike Information Criterion in PhyML 3.0 (76, 77), as
598 well as the phylogeny reconstruction by the Maximum Likelihood method using 1000
599 bootstraps to support the phylogenetic prediction.
600
601 **Phr pro-peptide identification and pherogroup prediction**
602 Additional to the identification of Rap homologs in the *B. cereus* group, we also analyzed
603 the putative *phr* genes, which code for pro-Phr, the precursor of the quorum sensing signal
604 peptide. For this, we performed a manual search targeting open reading frames (ORFs)
605 between 30 and 100 amino acids of length, downstream from the *rap* gene sequences.
606 When present, each Phr amino acid sequence was analyzed for the presence of a signal
607 peptide for secretion and a cleavage site using SignalP4.1 (78). The putative mature
608 signaling peptide (mature Phr) and pherogroup prediction were performed from the
609 exported Phr amino acid sequences (pro-Phr). For this, Phrs corresponding to Rap proteins
610 from different clades of the phylogenetic reconstruction were analyzed separately. The
611 amino acid sequences of the pro-Phr from each clade were aligned using ClustalW (79).
612 Pherogroups were identified by manually, by modifying the groups of aligned Phrs and
613 looking for consensus sequences in the alignments. For better identification of consensus

614 sequences, sequence Logos were created for each pherogroup using the Seq2Logo 2.0
615 online service (80).
616
617 **Statistics**
618 All the statistical analyses were performed using GraphPad Prism version 7.0a. Data
619 obtained from the extracellular proteolytic activity assay, spreading (at day 7) and biofilm
620 formation were analyzed with multiple *t*-tests to search for differences between not induced
621 and induced Rap protein overexpression conditions of each strain. Significance of 0.05 was
622 used in all statistical tests.
623
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632
633 **References**
634 1. Crespi BJ. 2001. The evolution of social behavior in microorganisms. Trends Ecol
635 Evol 16:178–183.
636 2. Vlamakis H, Aguilar C, Losick R, Kolter R. 2008. Control of cell fate by the
637 formation of an architecturally complex bacterial community. Genes Dev 22:945–

953.

3. Shapiro JA. 1998. Thinking about bacterial populations as multicellular organisms. Annu Rev Microbiol 52:81–104.

4. Jeckel H, Matthey N, Drescher K. 2019. Biophysics: Common concepts for bacterial collectives. Elife 8:e47019.

5. van Vliet S, Ackermann M. 2015. Bacterial Ventures into Multicellularity: Collectivism through Individuality. PLoS Biol 13:1–5.

6. Claessen D, Rozen DE, Kuipers OP, Søgaard-Andersen L, Van Wezel GP. 2014. Bacterial solutions to multicellularity: A tale of biofilms, filaments and fruiting bodies. Nat Rev Microbiol 12:115–124.

7. Ruby EG. 1996. LESSONS FROM A COOPERATIVE, BACTERIAL-ANIMAL ASSOCIATION: The *Vibrio fischeri–Euprymna scolopes* Light Organ Symbiosis. Annu Rev Microbiol 50:591–624.

8. Muñoz-Dorado J, Marcos-Torres FJ, García-Bravo E, Moraleda-Muñoz A, Pérez J. 2016. *Myxobacteria*: Moving, killing, feeding, and surviving together. Front Microbiol 7:781.

9. Lopez D, Vlamakis H, Kolter R. 2008. Generation of multiple cell types in *Bacillus subtilis*. FEMS Microbiol Rev 33:152–163.

10. López D, Kolter R. 2010. Extracellular signals that define distinct and coexisting cell fates in *Bacillus subtilis*. FEMS Microbiol Rev 34:134–149.

11. Kovács ÁT, Dragoš A. 2019. Evolved Biofilm: Review on the Experimental Evolution Studies of *Bacillus subtilis* Pellicles. J Mol Biol in press.

12. Kearns DB, Chu F, Rudner R, Losick R. 2004. Genes governing swarming in *Bacillus subtilis* and evidence for a phase variation mechanism controlling surface

662 motility. Mol Microbiol 52:357–369.

663 13. Fujita M, González-Pastor JE, Losick R. 2005. High- and low-threshold genes in the
664 Spo0A regulon of *Bacillus subtilis*. J Bacteriol 187:1357–1368.

665 14. Auchtung JM, Lee CA, Grossman AD. 2006. Modulation of the ComA-Dependent
666 Quorum Response in *Bacillus subtilis* by Multiple Rap Proteins and Phr Peptides. J
667 Bacteriol 188:5273–5285.

668 15. Mukai K, Kawata M, Tanaka T. 1990. Isolation and phosphorylation of the *Bacillus*
669 *subtilis degS* and *degU* gene products. J Biol Chem 265:20000–20006.

670 16. Bassler BL, Losick R. 2006. Bacterially speaking. Cell 125:237–246.

671 17. Waters CM, Bassler BL. 2005. Quorum sensing: cell-to-cell communication in
672 bacteria. Annu Rev Cell Dev Biol 21:319–346.

673 18. Williams P. 2007. Quorum sensing, communication and cross-kingdom signalling in
674 the bacterial world. Microbiology 153:3923–3938.

675 19. Declerck N, Bouillaut L, Chaix D, Rugani N, Slamti L, Hoh F, Lereclus D, Arold ST.
676 2007. Structure of PlcR: Insights into virulence regulation and evolution of quorum
677 sensing in Gram-positive bacteria. Proc Natl Acad Sci 104:18490–18495.

678 20. Rocha-Estrada J, Aceves-Diez AE, Guarneros G, De La Torre M. 2010. The RNPP
679 family of quorum-sensing proteins in Gram-positive bacteria. Appl Microbiol
680 Biotechnol 87:913–923.

681 21. Neiditch MB, Capodagli GC, Prehna G, Federle MJ. 2017. Genetic and structural
682 analyses of RRNPP intercellular peptide signaling of Gram-positive bacteria. Annu
683 Rev Genet 51:311–333.

684 22. Pottathil M, Lazazzera BA. 2003. The extracellular Phr peptide–Rap phosphatase
685 signaling circuit of *Bacillus subtilis*. Front Biosci 8:32–45.

686 23. Core L, Perego M. 2003. TPR-mediated interaction of RapC with ComA inhibits
687 response regulator-DNA binding for competence development in *Bacillus subtilis*.
688 Mol Microbiol 49:1509–1522.
689 24. Perego M. 1997. A peptide export-import control circuit modulating bacterial
690 development regulates protein phosphatases of the phosphorelay. Proc Natl Acad Sci
691 U S A 94:8612–8617.
692 25. Ogura M, Shimane K, Asai K, Ogasawara N, Tanaka T. 2003. Binding of response
693 regulator DegU to the *aprE* promoter is inhibited by RapG, which is counteracted by
694 extracellular PhrG in *Bacillus subtilis*. Mol Microbiol 49:1685–1697.
695 26. Solomon JM, Lazazzera BA, Grossman AD. 1996. Purification and characterization
696 of an extracellular peptide factor that affects two different developmental pathways
697 in *Bacillus subtilis*. Genes Dev 10:2014–2024.
698 27. Ogura M, Fujita Y. 2007. *Bacillus subtilis rapD*, a direct target of transcription
699 repression by RghR, negatively regulates *srfA* expression. FEMS Microbiol Lett
700 268:73–80.
701 28. Bongiorni C, Ishikawa S, Stephenson S, Ogasawara N, Perego M. 2005. Synergistic
702 regulation of competence development in *Bacillus subtilis* by two Rap-Phr systems.
703 J Bacteriol 187:4353–4361.
704 29. Hayashi K, Kensuke T, Kobayashi K, Ogasawara N, Ogura M. 2006. *Bacillus*
705 *subtilis* RghR (YvaN) represses *rapG* and *rapH*, which encode inhibitors of
706 expression of the *srfA* operon. Mol Microbiol 59:1714–1729.
707 30. Smits WK, Bongiorni C, Veening J-W, Hamoen LW, Kuipers OP, Perego M. 2007.
708 Temporal separation of distinct differentiation pathways by a dual specificity Rap-
709 Phr system in *Bacillus subtilis*. Mol Microbiol 65:103–120.

710 31. Boguslawski KM, Hill PA, Griffith KL. 2015. Novel mechanisms of controlling the
711 activities of the transcription factors Spo0A and ComA by the plasmid-encoded
712 quorum sensing regulators Rap60-Phr60 in *Bacillus subtilis*. Mol Microbiol 96:325–
713 348.
714 32. Perego M, Hanstein C, Welsh KM, Djavakhishvili T, Glaser P, Hoch JA. 1994.
715 Multiple protein-aspartate phosphatases provide a mechanism for the integration of
716 diverse signals in the control of development in *B. subtilis*. Cell 79:1047–1055.
717 33. Jiang M, Grau R, Perego M. 2000. Differential processing of propeptide inhibitors of
718 Rap phosphatases in *Bacillus subtilis*. J Bacteriol 182:303–310.
719 34. Parashar V, Mirouze N, Dubnau DA, Neiditch MB. 2011. Structural basis of
720 response regulator dephosphorylation by Rap phosphatases. PLoS Biol 9:e1000589.
721 35. Parashar V, Jeffrey PD, Neiditch MB. 2013. Conformational Change-Induced
722 Repeat Domain Expansion Regulates Rap Phosphatase Quorum-Sensing Signal
723 Receptors. PLoS Biol 11:e1001512.
724 36. Burbulys D, Trach KA, Hoch JA. 1991. Initiation of Sporulation in *B. subtilis* is
725 Controlled by a Multicomponent Phosphorelay. Cell 64:545–552.
726 37. Perego M. 2001. A new family of aspartyl phosphate phosphatases targeting the
727 sporulation transcription factor Spo0A of *Bacillus subtilis*. Mol Microbiol 42:133–
728 143.
729 38. Ehling-Schulz M, Koehler TM, Lereclus D. 2019. The *Bacillus cereus* Group:
730 *Bacillus* species with Pathogenic Potential. Microbiol Spectr 6 GPP3-0032–2018.
731 39. Anderson I, Sorokin A, Kapatral V, Reznik G, Bhattacharya A, Mikhailova N, Burd
732 H, Joukov V, Kaznadzey D, Walunas T, Souza MÕ, Larsen N, Pusch G, Liolios K,
733 Grechkin Y, Lapidus A, Goltsman E, Chu L, Fonstein M, Ehrlich SD. 2005.

734 Comparative genome analysis of *Bacillus cereus* group genomes with *Bacillus*
735 *subtilis*. FEMS Microbiol Lett 250:175–184.
736 40. Dubois T, Lemy C, Buisson C, Faegri K, Nielsen-leroux C, Gohar M, Jacques P,
737 Ramarao N, Kolstø A. 2012. Necrotrophism is a quorum-sensing-regulated lifestyle
738 in *Bacillus thuringiensis*. PLoS Pathog 8:e1002629.
739 41. Cabrera R, Rocha J, Flores V, Vázquez-Moreno L, Guarneros G, Olmedo G,
740 Rodríguez-Romero A, de la Torre M. 2014. Regulation of sporulation initiation by
741 NprR and its signaling peptide NprRB: molecular recognition and conformational
742 changes. Appl Microbiol Biotechnol 98:9399–9412.
743 42. Cabrera R, Rodríguez-Romero A, Guarneros G, de la Torre M. 2016. New insights
744 into the interaction between the quorum-sensing receptor NprR and its DNA target,
745 or the response regulator Spo0F. FEBS Lett 590:3243–3253.
746 43. Rocha J, Flores V, Cabrera R, Soto-Guzmán A, Granados G, Juaristi E, Guarneros G,
747 De La Torre M. 2012. Evolution and some functions of the NprR-NprRB quorum-
748 sensing system in the *Bacillus cereus* group. Appl Microbiol Biotechnol 94:1069–
749 1078.
750 44. Even-Tov E, Omer Bendori S, Pollak S, Eldar A. 2016. Transient Duplication-
751 Dependent Divergence and Horizontal Transfer Underlie the Evolutionary Dynamics
752 of Bacterial Cell-Cell Signaling. PLoS Biol 14:1–23.
753 45. Bongiorni C, Stoessel R, Shoemaker D, Perego M. 2006. Rap Phosphatase of
754 Virulence Plasmid pXO1 Inhibits *Bacillus anthracis* Sporulation. J Bacteriol
755 188:487–498.
756 46. Fazion F, Perchat S, Buisson C, Vilas-Bôas G, Lereclus D. 2018. A plasmid-borne
757 Rap-Phr system regulates sporulation of *Bacillus thuringiensis* in insect larvae.

758 Environ Microbiol 20:145–155.

759 47. Slamti L, Perchat S, Huillet E, Lereclus D. 2014. Quorum sensing in *Bacillus*
760 *thuringiensis* is required for completion of a full infectious cycle in the insect.
761 Toxins (Basel) 6:2239–2255.

762 48. Cardoso P de F, Perchat S, Vilas-Boas LA, Lereclus D, Vilas-Bôas GT. 2019.
763 Diversity of the Rap–Phr quorum-sensing systems in the *Bacillus cereus* group. Curr
764 Genet 1–15.

765 49. Flemming HC, Wuertz S. 2019. Bacteria and archaea on Earth and their abundance
766 in biofilms. Nat Rev Microbiol 17:247–260.

767 50. Bhavsar AP, Zhao X, Brown ED. 2001. Development and characterization of a
768 xylose-dependent system for expression of cloned genes in *Bacillus subtilis*:
769 Conditional complementation of a teichoic acid mutant. Appl Environ Microbiol
770 67:403–410.

771 51. Kinsinger RF, Shirk MC, Fall R. 2003. Rapid surface motility in *Bacillus subtilis* is
772 dependent on extracellular surfactin and potassium ion. J Bacteriol 185:5627–5631.

773 52. Kearns DB. 2010. A field guide to bacterial swarming motility. Nat Rev Microbiol
774 8:634–644.

775 53. Hölscher T, Kovács ÁT. 2017. Sliding on the surface: bacterial spreading without an
776 active motor. Environ Microbiol 19:2537–2545.

777 54. Fagerlund A, Dubois T, Økstad OA, Verplaetse E, Gilois N, Bennaceur I, Perchat S,
778 Gominet M, Aymerich S, Kolstø AB, Lereclus D, Gohar M. 2014. SinR controls
779 enterotoxin expression in *Bacillus thuringiensis* biofilms. PLoS One 9:e87532.

780 55. Raynor MJ, Roh JH, Widen SG, Wood TG, Koehler TM. 2018. Regulons and
781 protein–protein interactions of PRD-containing *Bacillus anthracis* virulence

782 regulators reveal overlapping but distinct functions. Mol Microbiol 109:1–22.

783 56. Even-Tov E, Omer Bendori S, Valastyan J, Ke X, Pollak S, Bareia T, Ben-Zion I,
784 Bassler BL, Eldar A. 2016. Social Evolution Selects for Redundancy in Bacterial
785 Quorum Sensing. PLoS Biol 14:e1002386.

786 57. Dubnau EJ, Carabetta VJ, Tanner AW, Miras M, Diethmaier C, Dubnau D. 2016. A
787 protein complex supports the production of Spo0A-P and plays additional roles for
788 biofilms and the K-state in *Bacillus subtilis*. Mol Microbiol 101:606–624.

789 58. Earl AM, Losick R, Kolter R. 2008. Ecology and genomics of *Bacillus subtilis*.
790 Trends Microbiol 16:269–275.

791 59. Mirouze N, Dubnau D. 2013. Chance and Necessity in *Bacillus subtilis*
792 Development. Microbiol Spectr 1:TBS-0004-2012.

793 60. Argôlo-Filho RC, Loguercio LL. 2014. *Bacillus thuringiensis* is an environmental
794 pathogen and host-specificity has developed as an adaptation to human-generated
795 ecological niches. Insects 5:62–91.

796 61. Perchat S, Dubois T, Zouhir S, Gominet M, Poncet S, Lemy C, Aumont-Nicaise M,
797 Deutscher J, Gohar M, Nessler S, Lereclus D. 2011. A cell-cell communication
798 system regulates protease production during sporulation in bacteria of the *Bacillus*
799 *cereus* group. Mol Microbiol 82:619–633.

800 62. Perego M, Brannigan JA. 2001. Pentapeptide regulation of aspartyl-phosphate
801 phosphatases. Peptides 22:1541–1547.

802 63. Stephenson S, Mueller C, Jiang M, Perego M. 2003. Molecular analysis of Phr
803 peptide processing in *Bacillus subtilis*. J Bacteriol 185:4861–4871.

804 64. Miller EL, Kjos M, Abrudan MI, Roberts IS, Veening JW, Rozen DE. 2018.
805 Eavesdropping and crosstalk between secreted quorum sensing peptide signals that

regulate bacteriocin production in *Streptococcus pneumoniae*. ISME J 12:2363–2375.

65. Fleuchot B, Guillot A, Mézange C, Besset C, Chambellon E, Monnet V, Gardan R. 2013. Rgg-Associated SHP Signaling Peptides Mediate Cross-Talk in *Streptococci*. PLoS One 8:e66042.

66. Zhang F, Kwan A, Xu A, Süel GM. 2015. A synthetic quorum sensing system reveals a potential private benefit for public good production in a biofilm. PLoS One 10:e0132948.

67. Geddes BA, Paramasivan P, Joffrin A, Thompson AL, Christensen K, Jorrin B, Brett P, Conway SJ, Oldroyd GED, Poole PS. 2019. Engineering transkingdom signalling in plants to control gene expression in rhizosphere bacteria. Nat Commun 10:3430.

68. Sheppard AE, Poehlein A, Rosenstiel P, Liesegang H, Schulenburg H. 2013. Complete genome sequence of *Bacillus thuringiensis* strain 407 Cry-. Genome Announc 1:e00158-12.

69. Maniatis T, Fritsch EF, Sambrook J. 1988. Molecular Cloning : a laboratory manual. 2nd edition. Cold Spring Harb, New York.

70. Grandvalet C, Gominet M, Lereclus D. 2001. Identification of genes involved in the activation of the *Bacillus thuringiensis inhA* metalloprotease gene at the onset of sporulation. Microbiology 147:1805–1813.

71. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. J Mol Biol 215:403–410.

72. Katoh K, Rozewicki J, Yamada KD. 2017. MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. Brief Bioinform in press.

73. Katoh K, Kuma KI, Toh H, Miyata T. 2005. MAFFT version 5: Improvement in

accuracy of multiple sequence alignment. Nucleic Acids Res 33:511–518.

74. Arantes O, Lereclus D. 1991. Construction of Cloning Vectors for *Bacillus thuringiensis*. Gene 108:115–119.

75. Marchler-Bauer A, Bo Y, Han L, He J, Lanczycki CJ, Lu S, Chitsaz F, Derbyshire MK, Geer RC, Gonzales NR, Gwadz M, Hurwitz DI, Lu F, Marchler GH, Song JS, Thanki N, Wang Z, Yamashita RA, Zhang D, Zheng C, Geer LY, Bryant SH. 2016. CDD/SPARCLE: functional classification of proteins via subfamily domain architectures. Nucleic Acids Res 45:D200–D203.

76. Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O. 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: Assessing the performance of PhyML 3.0. Syst Biol 59:307-321.

77. Lefort V, Longueville JE, Gascuel O. 2017. SMS: Smart Model Selection in PhyML. Mol Biol Evol 34:2422-2424.

78. Nielsen H. 2017. Predicting secretory proteins with SignalP Protein Function Prediction. Humana Press, New York, NY.

79. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG. 2007. Clustal W and Clustal X version 2.0. Bioinformatics 23:2947–2948.

80. Thomsen MCF, Nielsen M. 2012. Seq2Logo: a method for construction and visualization of amino acid binding motifs and sequence profiles including sequence weighting, pseudo counts and two-sided representation of amino acid enrichment and depletion. Nucleic Acids Res 40:W281–W287.

854 **Tables**

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

856 group.

Specie	Number of Rap-Phr systems	Location	
		Chromosome	Plasmid
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

857

858

Figures

Figure 1

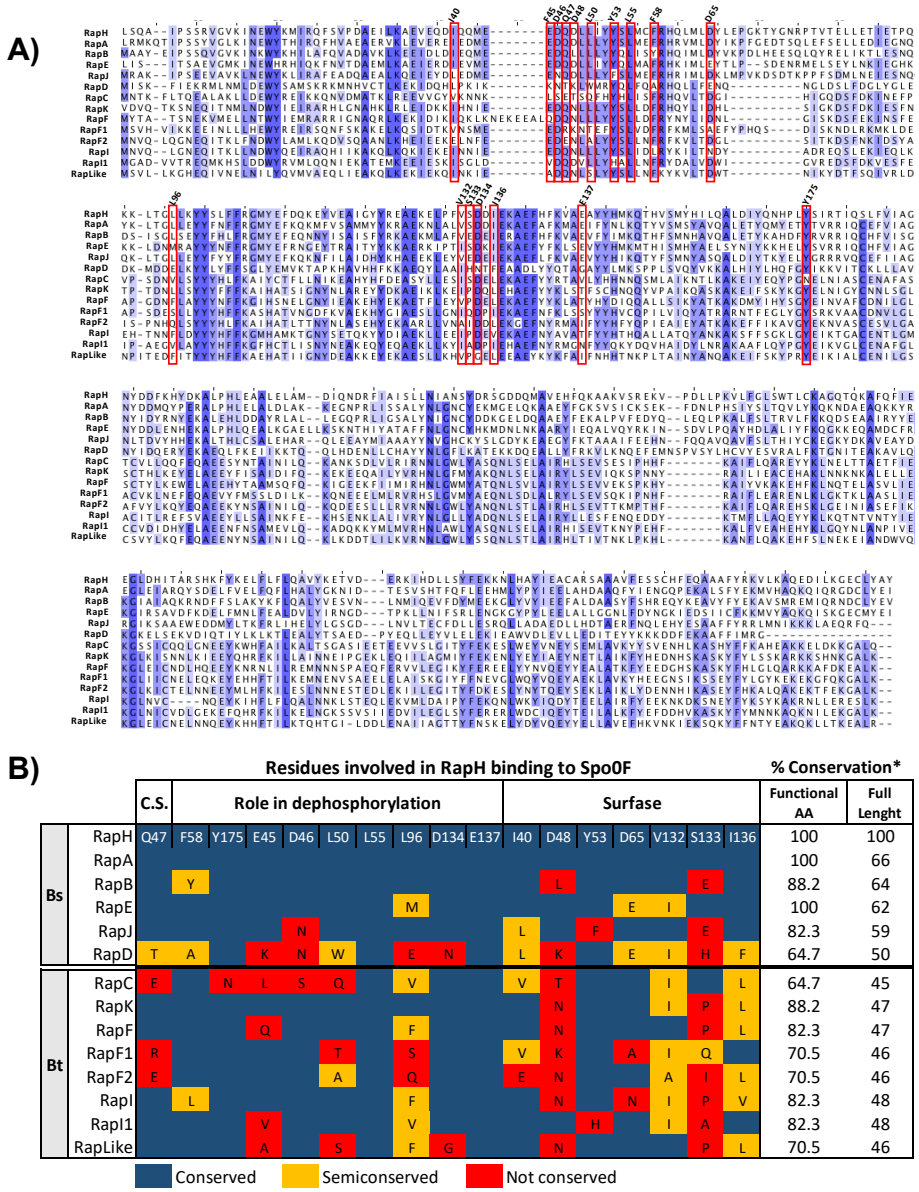
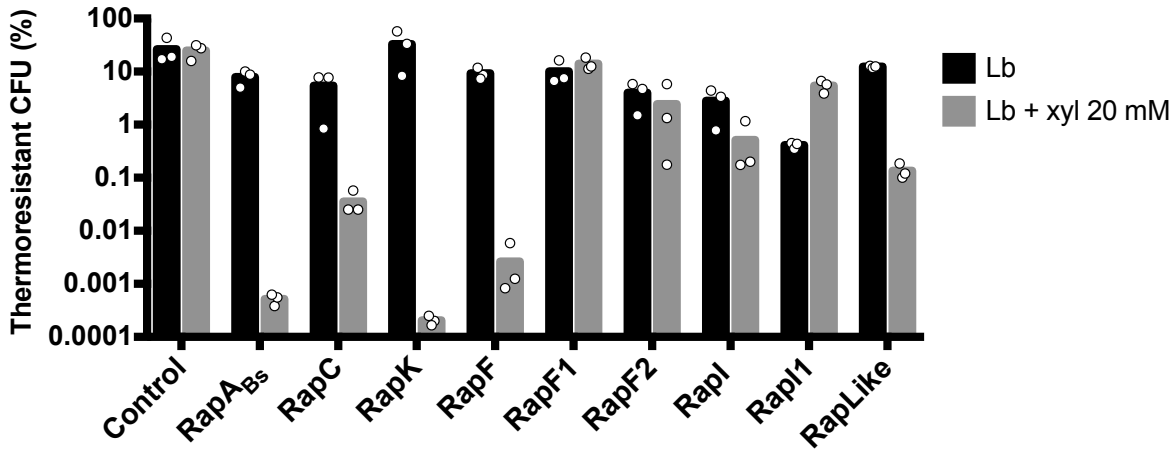


Figure 1. Prediction of the capacity of Rap proteins from Bt8741 to bind and dephosphorylate Spo0F. A) Multiple sequence alignment of the complete amino acid sequences of RapH, A, B, E, J and RapD from *B. subtilis* 168, and eight Rap proteins from

866 Bt8741. Blue highlights indicate highly conserved amino acids. Residues involved in the
867 RapH-Spo0F binding are indicated in red rectangles and its position in RapH is shown on
868 top of the alignment. B) Conservation of residues involved in RapH binding to Spo0F.
869 Residues were considered as semiconserved when a functional amino acid of RapH was
870 substituted with another amino acid with similar characteristics. Bs, *Bacillus subtilis* 168;
871 Bt, *Bacillus thuringiensis* 407; C.S., Catalytic Site; *Percentage of conserved and
872 semiconserved amino acids in pairwise alignment to RapH.
873
874

875 **Figure 2**

876



877

878 **Figure 2.** Sporulation efficiency of Bt8741 carrying overexpression plasmids for Rap

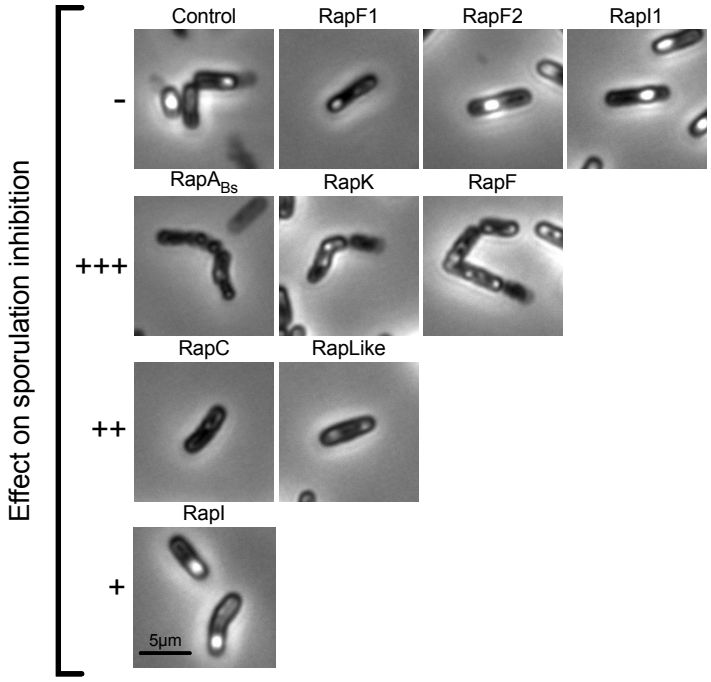
879 proteins, with and without addition of inducer. In the cases where thermoresistant CFU

880 were undetectable, we considered a value of 166 spores/ml, which is the detection limit for

881 this assay. Columns represent average of three individual measurements, shown as dots.

882

883 **Figure 3**



884

885 **Figure 3.** Cell morphology of strains with induced Rap protein overexpression at 72 h.

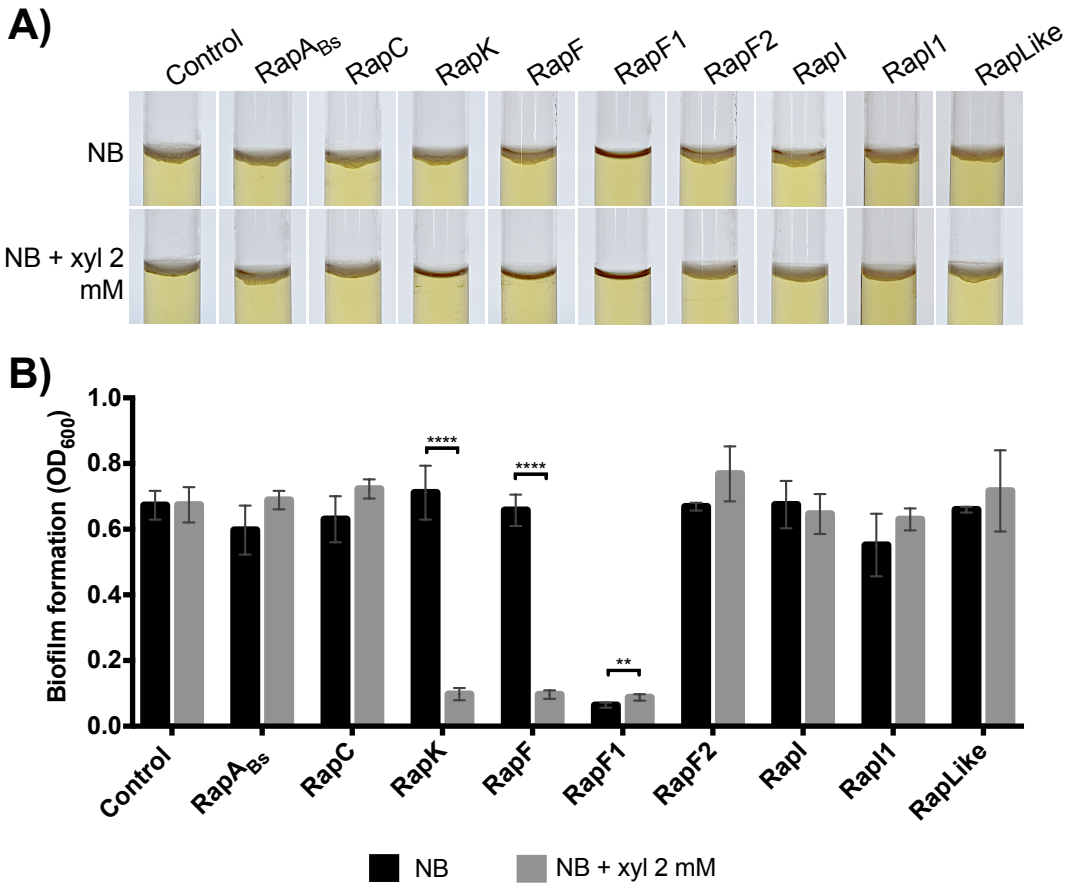
886 Phase contrast microscopy 63X and 1.8X magnification. -, no effect; +, decrease under 10-

887 fold; decrease between 90 and 160-fold; +++, decrease >1,000-fold.

888

889

890 **Figure 4**

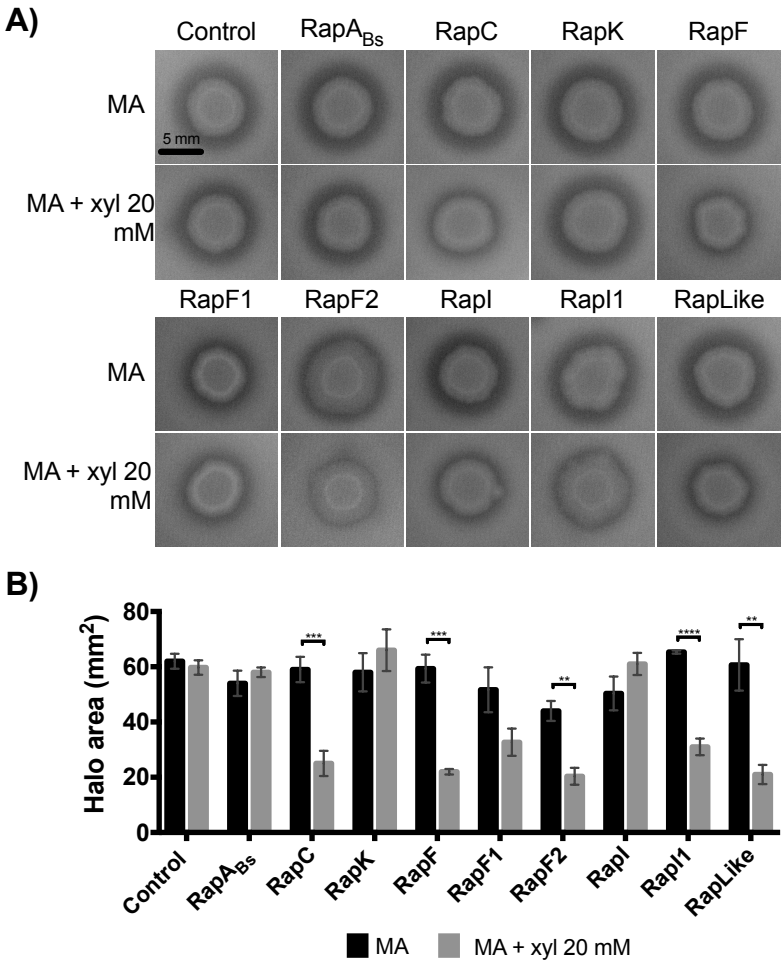


891

892 **Figure 4.** Biofilm formation of Rap-overexpression strains at 48 h. A) Biofilms formed in
893 the liquid-air interphase in 13 x 100 mm glass tubes at 48 h. Biofilms are identified as a
894 white layer on the surface. B) Biofilm formation quantification of Rap-overexpression
895 strains in induced and not induced media after 48 h. Columns represent average of 5
896 replicates ± SD. NB, Nutrient Broth; **, $p<0.005$; ****, $p<0.0001$.

897

898 **Figure 5**



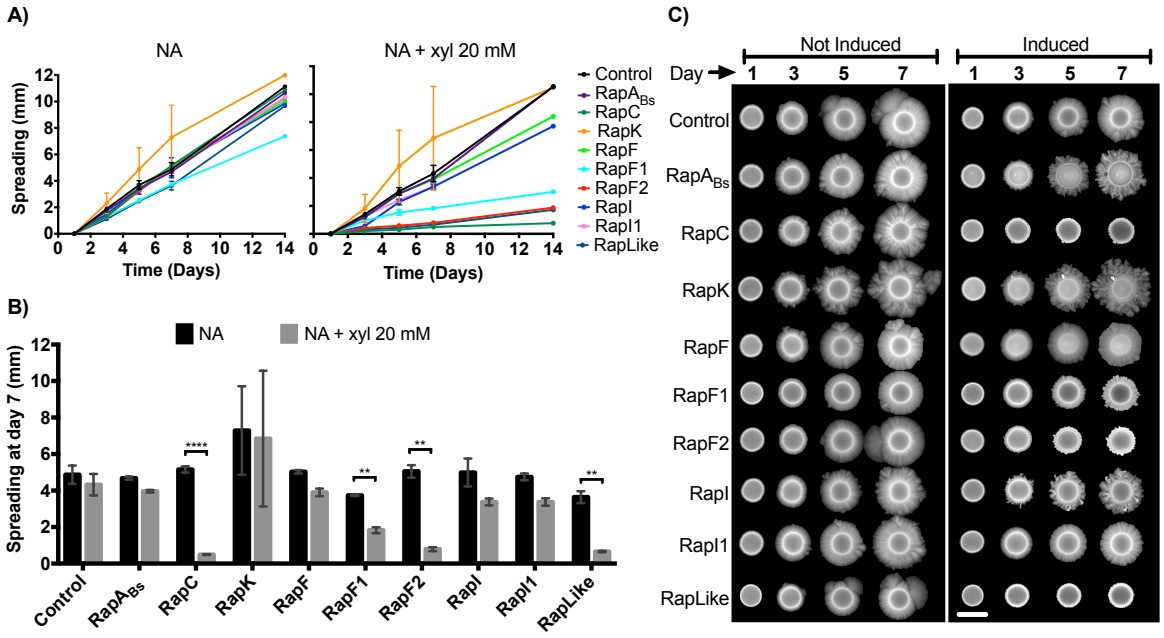
899

900 **Figure 5.** Extracellular proteolytic activity of Rap-overexpression strains. A) Effect of Rap
901 protein overexpression in the hydrolysis halo of Rap-overexpression strains colonies. B)
902 Hydrolysis halo area with and without Rap-overexpression induction. Columns represent
903 average of 3 replicates ± SD. MA, milk agar; **, $p<0.005$; ***, $p< 0.0005$; ****, $p<0.0001$.

904

905

906 **Figure 6**



907

908 **Figure 6.** Spreading phenotype of Rap-overexpression strains. A) Spreading kinetics of

909 Rap-overexpression colonies on agar. Each point represents the media of triplicates ± SD;

910 only one data point is shown at day 14. B) Spreading quantification of Rap-overexpression

911 colonies at day 7. Columns represent average of triplicates ± SD. C) Pictures of

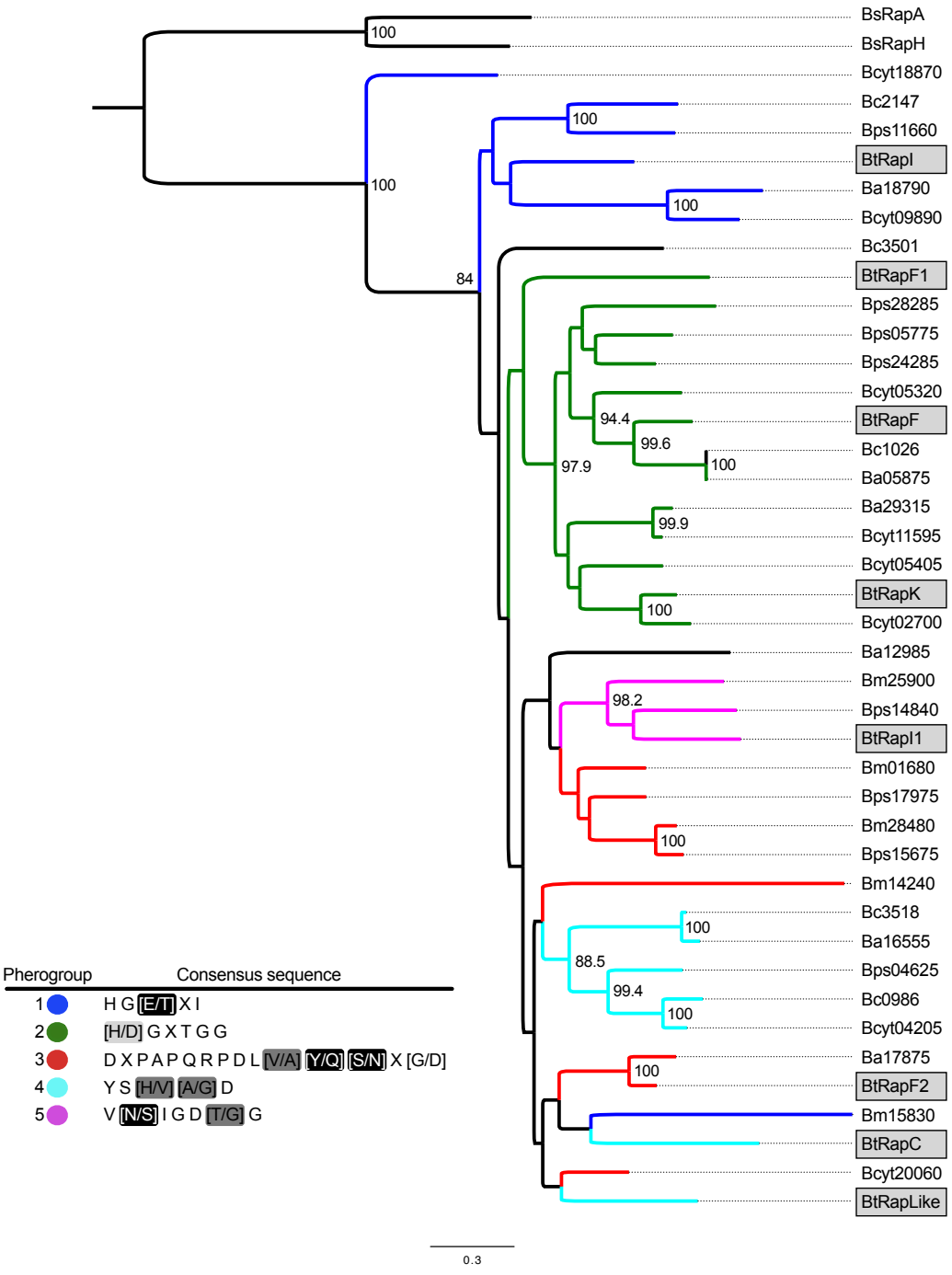
912 representative Rap-overexpression strains spreading during 7 days. Scale bar indicates 5

913 mm. NA, Nutrient Agar; **, p<0.005; ****, p<0.0001.

914

915

916 **Figure 7**



917

918 **Figure 7.** Maximum likelihood phylogeny of Rap proteins from the *B. cereus* group. Rap

919 proteins from Bt8741 are highlighted in gray boxes. Branches from each pherogroup are

920 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.