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 two Rap-Phr systems (RapK and RapF) inhibit sporulation, probably through dephosphorylation of Spo0F; these two Raps also inhibit biofilm formation. Five systems (RapC, F, F2, I1 and RapLike) decrease extracellular proteolytic activity; finally, four systems (RapC, F1, F2 and RapLike) participate in spreading inhibition. Our bioinformatic analyses showed that Rap proteins from the *B. cereus* group diversified into five pherogroups, and we foresee that functions performed by Rap proteins of Bt8741 could also be carried out by Rap homologs in other species within the group. These results indicate that Rap-Phr systems constitute a highly multifunctional and redundant regulatory repertoire that enable bacteria from the *B. cereus* group to efficiently regulate collective functions during the bacterial life cycle, in the face of changing environments.

Importance

The *Bacillus cereus* group of bacteria includes species of high economic, clinical, biological warfare and biotechnological interest, e.g. *B. anthracis* in bioterrorism, *B. cereus* in food intoxications and *B. thuringiensis* in biocontrol. Knowledge on the ecology of these bacteria is hindered due to our limited knowledge of the regulatory circuits that control differentiation and specialization processes. Here, we uncover the participation of eight Rap quorum-sensing receptors in collective functions of *B. thuringiensis*. These

48 proteins are highly multifunctional and redundant in their functions, linking ecologically
49 relevant processes such as sporulation, biofilm formation, extracellular proteolytic activity
50 and spreading, and probably other additional functions in species from the *B. cereus* group.
51

52 **Introduction**

53 Bacteria perform many functions that depend on multicellular-like behaviors, such as cell
54 differentiation and specialization. These behaviors, also known as collective functions,
55 allow the emergence of complex ecological interactions, including cooperation and division
56 of labor in biofilms (1, 2). Collective functions are only evident and effective when
57 performed by large groups in bacterial populations or communities (3–6). Some of the most
58 studied examples include bioluminescence by the squid symbiont *Vibrio fischeri* (7), or
59 fruiting body formation during sporulation of *Myxococcus xanthus* (8).
60

61 In gram positive bacteria, collective functions and the molecular mechanisms for their
62 control have been widely studied in *Bacillus subtilis* (Bs). In Bs cultures, several mutually-
63 exclusive cell-types have been identified (motile, competent, sporulating, cannibal, biofilm
64 matrix producers, surfactant producers and miners (9, 10)), where emerging ecological
65 interactions such as cooperation, cheating and cross-feeding, have been described (5, 6, 11).
66 The presence of these cell differentiation phenomena and the resulting ecological
67 interactions, ultimately affect the manifestation of collective traits such as sporulation
68 efficiency, surface colonization, biofilm architecture complexity, etc. (2, 9, 12). These
69 phenomena depend on global modifications of transcriptional regulation; they are triggered
70 by environmental cues, stress conditions, cell-cell signaling, and are tightly modulated by
71 complex, overlapping regulatory circuits (13–15).

72

73 Bacteria detect cell density through quorum sensing (QS), which depends on self-produced
74 signaling molecules that accumulate in the extracellular space as the population grows.
75 Specific receptors in the cell membrane or in the cytoplasm recognize these signaling
76 molecules and regulate downstream cellular processes (16–18). Collective traits such as
77 virulence, competence, sporulation and bioluminescence are regulated by QS. Gram-
78 positive bacteria use small peptides as signaling molecules for QS (17).

79

80 The RRNPP family (Rgg, Rap, NprR, PlcR, PrgX) are intracellular QS receptors that
81 regulate several functions across gram-positive bacteria (19–21). Genes coding for receptor
82 proteins and their associated signaling peptides are encoded in transcriptional cassettes (22).
83 Rgg, NprR, PlcR and PrgX proteins are transcriptional activators that bind directly to DNA
84 in quorum state. Rap proteins, however, lack a DNA binding domain and they function by
85 binding and inhibiting proteins, specifically response regulators and transcriptional
86 activators (21, 23, 24). Twelve Rap paralogs (RapA, B, C, D, E, F, G, H, I, J, K, 60) control
87 diverse functions in *B. subtilis* 168 (Bs168). The RapG-PhrG pair regulates the activation
88 of DegU, a transcriptional regulator that controls *aprE* and *comK* genes encoding for
89 extracellular proteases and a transcription factor for competence in Bs, respectively (15, 25);
90 ComA – the master regulator of competence genes – is repressed by RapC, D, F, G, H, K
91 and Rap60 (14, 26–31); Spo0A – the transcriptional activator of many differentiation genes
92 – is indirectly regulated by RapA, B, E, H, J, and Rap60 (24, 31–35). Hence, Rap protein
93 paralogs from Bs are highly multifunctional and redundant and they connect several
94 differentiation processes and coordinate collective traits.

95

96 Spo0A is activated by phosphorylation through a multicomponent phosphorelay system. Up
97 to five kinases auto-phosphorylate in response to intracellular and environmental stress
98 signals and transfer the phosphate group to Spo0F, which is then transferred to Spo0B and
99 finally to Spo0A (36). Spo0A-P activates the transcription of multiple genes, including
100 biofilm formation (at low concentrations) and early sporulation genes (at high
101 concentrations (13)). Rap QS proteins prevent the phosphate transfer in the phosphorelay
102 by binding to Spo0F (32, 37).
103
104 While the regulation of collective traits in Bs is well known, these phenomena remain
105 largely understudied in the *B. cereus* group, which includes bacteria with clinical and
106 biotechnological relevance (38). Although Bs and *B. cereus* group species share similar
107 characteristics such as the sporulation process, the Spo0A phosphorelay components, and
108 have many protein families in common, they also have notorious genetic differences (39).
109 In *B. thuringiensis* (Bt, the most widely used biopesticide), the Spo0A phosphorelay is
110 modulated by the bifunctional QS receptor NprR, which is not present in Bs (40–42). On
111 the other hand, ComA and DegU response regulators are not present in Bt. Additionally,
112 Rap-Phr QS systems also differ in both groups. These QS systems have evolved by
113 duplication and divergence mechanisms; even though multiple Rap proteins paralogs are
114 also found *B. cereus* group species, they have evolved independently and no Rap homologs
115 are shared between the two groups (43, 44). Therefore, it is not possible to predict the
116 functions of Rap proteins in the *B. cereus* group based on what is known of Rap proteins
117 from Bs.
118

119 Some Rap-Phr systems from species of the *B. cereus* group have been studied. First, Rap
120 BXA0205 and BA3790 from *B. anthracis* str. A2012, were demonstrated to regulate
121 sporulation initiation and to dephosphorylate Spo0F (45). Later, it was shown that Rap8
122 from Bt-HD73, regulates the sporulation and biofilm formation processes *in vitro* (46). A
123 more recent study showed the participation of Rap6, 7 and 8 – also known as RapC, K and
124 RapF, respectively (47) – in the modulation of the sporulation process in Bt407 (48).
125 However, other Rap paralogs with unknown functions have been identified in the genomes
126 of *B. cereus* group bacteria (44, 47) and they may be relevant to their ecology.
127
128 In this study we aimed at evaluating the diversification of the Rap-Phr systems in the *B.*
129 *cereus* group as well as their functions, using *Bacillus thuringiensis* Bt8741 as a model. We
130 generated eight Rap-overexpression strains of Bt8741 to evaluate the role of each Rap
131 paralog in sporulation efficiency, biofilm formation, extracellular proteolytic activity and
132 spreading. We also studied the evolution of Rap-Phr paralogs in the *B. cereus* group, by
133 identifying Rap homologs from other species and analyzing its phylogeny. This allowed the
134 prediction of their functions, based on those of Rap proteins from Bt.
135
136 **Results**
137 **Spo0F-binding residues from Bs RapH are conserved in Rap proteins from Bt8741.**
138 In order to predict the capacity of Rap proteins from Bt407 (a strain closely related to
139 Bt8741) to bind to Spo0F, we analyzed the conservation of the amino acids previously
140 reported to be involved in Spo0F-binding by RapH from *B. subtilis*. In this analysis, we
141 included reported sequences of Bs-Rap proteins that bind to Spo0F (RapA, B, E, H, J) as
142 well as the sequence of RapD from Bs, which does not bind to Spo0F (34) (Fig. 1). We

143 found higher percentage of conservation of the functional amino acids of RapH, in the
144 sequences of both Bs168 and Bt407, compared to the corresponding complete sequences
145 (Fig. 1B). In Bs168 the full sequence conservation of the Spo0F binding Raps (RapA, B, E,
146 J) compared to RapH, ranges from 59% to 66% and the functional amino acids
147 conservation percentage from 82.3% to 100%. In RapD, the full-length sequence is
148 conserved at 50% and functional residues are only 64.7% conserved (Fig. 1B). In the case
149 of Rap proteins from Bt407, the full sequence conservation in comparison to RapH of
150 Bs168 ranged from 45% to 48%. On the other hand, conservation of functional residues
151 ranged from 64.7% to 88.2% (Fig 1B). This indicates that the analyzed residues are
152 important for the function of Bt Rap proteins.
153
154 RapK presented the highest conservation percentage of functional residues (88.2%),
155 followed by RapF, I and RapI1 (82.3%), RapF1, F2 and RapLike (70.5%) and finally RapC
156 with 64.6%. Although RapF1 and RapF2 presented a high conservation of functional
157 residues, these Rap paralogs, as well as RapC, do not conserve the residue Q47 found in the
158 catalytic site and previously shown to be essential for the phosphatase activity of RapH (34).
159 This analysis enables the prediction that some Rap protein paralogs from Bt8741, with a
160 high conservation percentage of functional amino acids, could dephosphorylate Spo0F,
161 while other paralogs could have evolved to participate in other regulatory processes. Indeed,
162 RapK, RapF, and unexpectedly RapC from Bt407, Rap8 from Bt-HD73 (ortholog to RapI
163 from Bt407) and Rap BXA0205 and BA3790 from *B. anthracis*, (homologs of RapK and
164 RapF2, respectively) have been shown to participate in the modulation of sporulation (45,
165 46, 48). Previous to this work, RapF1, I1, and RapLike from Bt407 (or its homologs in
166 other species), had not been tested for their role in sporulation.

167

168 **RapC, K, F and RapLike control sporulation in Bt8741.**

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

176

177 In order to identify the Rap proteins involved in the regulation of the sporulation initiation,
178 we studied the effect of the overexpression in the sporulation efficiency of each strain. In
179 this experiment, we observed that both addition of xylose to the culture medium and the
180 presence of *rap* genes in the plasmid had minor effects on total and thermoresistant CFU
181 counts of Bt8741 at 72 h. In the control strain, addition of xylose caused a decrease of ≈1
182 log10 in total and thermoresistant CFU (Fig. S2A and S2B). Similarly, when *rapF1* and
183 *rapF2* genes were carried in the plasmid – but not overexpressed – total CFU decreased by
184 up to one logarithm of CFU in comparison to the control strain (Fig. S2A). Additionally,
185 sporulation decreased one logarithm in strains carrying *rapF1*, *rapF2* and *rapI1* in
186 comparison to the control strain when Rap was not overexpressed (Fig. S2C). These
187 unspecific effects were probably related to basal expression from the P_{xylA} promoter, even
188 when xylose is not added, since pHT315 replicates at 15 copies per cell (49). Finally, the
189 most dramatic effect was found in thermoresistant CFU of strains that overexpressed Rap
190 proteins (Fig. S2D).

191

192 In spite of unspecific effect of xylose addition on growth and sporulation, sporulation
193 efficiency of the control strain remained unchanged by the addition of inducer (Fig. 2). In
194 contrast, overexpression of $RapA_{Bs}$ caused a decrease in sporulation efficiency from 7.9%
195 to 0.0005%. In fact, thermoresistant CFU were undetectable when $RapA_{Bs}$ was
196 overexpressed (Fig. S2D). We also found undetectable levels of spores in strains
197 overexpressing RapK and RapF (Fig. S2D). Sporulation efficiency decreased from 32.93%
198 to 0.0002% in the strain overexpressing RapK and from 9.24% to 0.0026% in the strain
199 overexpressing RapF (Fig. 2). In Bs, RapA dephosphorylates Spo0F in the Spo0A
200 phosphorelay (32) and this result indicates that it performs the same function in B8741;
201 furthermore, it suggests that RapK and RapF carry out the same mechanism for regulation
202 of sporulation initiation.

203

204 Strains carrying P_{xylA}*'rapC* and P_{xylA}*'rapLike* also had reduced sporulation efficiency in
205 induced media. Sporulation efficiency decreased from 5.43% to 0.0357% and from 12.34%
206 to 0.1352% when RapC and RapLike were overexpressed, respectively (Fig 2).
207 Additionally, RapI overexpression slightly decreased sporulation efficiency, from 2.82% to
208 0.51%. Sporulation efficiency was not decreased by the overexpression of RapF1, F2, I or
209 RapI1.

210

211 Samples of the Rap-overexpressing strain cultures at 72 h were observed in a microscope.
212 We detected free spores and bacterial debris in all strains, when Rap proteins were not
213 overexpressed (Fig. S3). Figure 3 shows representative cells from each induced culture.
214 Strains overexpressing Rap proteins that did not affect sporulation efficiency (RapF1, F2

215 and RapI1) showed cell morphology similar to that of the control strain, i.e. a sporulated
216 bacilli with defined endospores. In strains overexpressing $RapA_{Bs}$, K and RapF, that had
217 acutely decreased sporulation efficiency, we observed chained, wrinkled cells with no
218 spores (Fig. 3). On the other hand, cells from strains overexpressing RapC and RapLike,
219 were rod-shaped with no evident spore (Fig. 3). Finally, cells from the strain
220 overexpressing RapI, which had a slight effect on sporulation efficiency, cell morphology
221 was similar to strains overexpressing RapF1, F2, I1 and the control strain (Fig. 3), showing
222 a defined endospore.
223
224 **Overexpression of RapF and RapK prevents biofilm formation of Bt8741.**
225 In nature, over 80% of bacteria live in biofilms (49), therefore, biofilm formation is likely a
226 relevant trait – albeit an understudied one – during the life cycle of Bt and other *B. cereus*
227 group bacteria. To determine which Rap proteins are involved in the regulation of biofilm
228 development in Bt8741, we quantified biofilm formation in the air-liquid interphase at 48 h
229 using Rap-overexpression strains. For this, we suspended cells form the biofilm and
230 measured optical density (OD_{600}). Since 20 mM xylose caused a complete inhibition of
231 biofilm formation in the Bt8741 control strain (not shown), we first tested the effect of
232 xylose concentration on this phenotype. We found that biofilm formation was not affected
233 at 2 mM, but was decreased at higher concentrations of 5, 10 and 15 mM (Fig. S4);
234 therefore, overexpression of Rap proteins was performed with 2 mM of xylose (50).
235
236 Overexpression of RapK and RapF caused a complete inhibition of biofilm formation of
237 Bt8741 (Fig. 4A), evident by the significant decrease ($p<0.0001$) in the OD_{600} of the
238 resuspended biofilm (Fig. 4B). The OD_{600} of the biofilms decreased from 0.7115 to 0.0977

239 and from 0.6577 to 0.0961 in strains overexpressing RapK and RapF, respectively (Fig. 4B).
240 On the other hand, biofilms were normally formed by strains overexpressing $RapA_{Bs}$, C, F2,
241 I, I1 and RapLike (Fig. 4). Interestingly, the strain overexpressing RapF1 was unable to
242 form biofilms even when the overexpression was not induced (Fig. 4A and B).
243
244 In order to discard global growth defects in this assay when RapK and RapF are
245 overexpressed, we measured planktonic growth through OD_{600} of the liquid culture media
246 from the same experiments where biofilm formation was assessed. We found that
247 planktonic growth was higher in conditions where a biofilm is not formed (Fig. S5). This
248 indicates that RapK and RapF specifically inhibit biofilm formation (e.g. secretion of
249 extracellular matrix components).
250
251 **Extracellular proteolytic activity is downregulated by RapC, F, F2, I1 and RapLike in**
252 **Bt8741.**
253 In Bt, the production of extracellular proteases is crucial during its necrotrophic phase, i.e.
254 development in insect cadavers. We tested the role of Rap proteins in extracellular
255 proteolytic activity by measuring the effect of Rap overexpression on hydrolysis halos of
256 colonies on milk agar (MA) plates. Addition of xylose had no effect ($p>0.05$) on the
257 hydrolysis halo of the control strain (Fig. 5). In contrast, overexpression of RapC, F, F2, I1
258 and RapLike decreased the halo area ($p<0.05$; Fig. 5B). In these strains, the halo area
259 decreased to 41.98%, 37.81%, 46.65%, 47.51% and 34.93%, respectively (Fig. 5B, Fig. S6)
260 compared to halos in plates where overexpression was not induced (100%). Proteolytic
261 activity of strains overexpressing $RapA_{Bs}$, K, I and RapF1 was not affected by the induction
262 ($p>0.05$; Fig. 5A and B).

263

264 **RapC, RapF1, RapF2 and RapLike regulate spreading of Bt8741 colonies.**

265 Colonies of Bt8741 present a spreading phenotype that could be associated to its capacity to
266 colonize hosts and habitats. Similar passive motility phenotypes have been described in
267 other species of *Bacillus*, associated to the production of extracellular surfactant molecules
268 (51–53). To gain insights on this understudied collective trait, we determined the effect of
269 Rap proteins on radial spreading of colonies of Bt8741 growing on agar media.

270

271 We observed that addition of xylose in the media did not affect spreading of the control
272 strain (Fig. 6). In contrast, the overexpression of RapC, F1, F2 and RapLike caused a
273 decrease in spreading ($p<0.05$) of Bt8741 colonies at day 7 (Fig. 6A and B). The
274 overexpression of RapC reduced the colony dispersion from 5.15 mm to 0.49 mm
275 (reduction of 90.4%); RapF1, from 3.73 mm to 1.83 mm (decrease of 50.9%); RapF2 from
276 5.05 mm to 0.78 mm (decrease of 84.5%); and RapLike from 3.64 mm to 0.65 mm
277 (decrease of 82.1%) (Fig. 6B). Spreading inhibition is evident in the colony morphology of
278 these strains (Fig. 6C). We observed that the overexpression of RapC, F2 and RapLike,
279 completely eliminated this phenotype, while overexpression of RapF1 only decreased
280 spreading ($p<0.05$) (Fig. 6B and C).

281

282 The overexpression of $RapA_{Bs}$, K, F, I and RapI1 did not affect the spreading of Bt8741
283 ($p>0.05$) (Fig. 6B). Spreading of the strains carrying overexpression plasmids for these Rap
284 proteins ranged from 4.68 mm to 7.29 mm without induction and from 3.37 mm to 6.84
285 mm when induced (Fig. 6B). In some cases, Rap overexpression affected colony
286 morphology, i.e. colonies of strains overexpressing RapK and RapF show an increased

287 dendritic phenotype; however, the spreading phenotype measured as colony radius, is still
288 present (Fig. 6C).
289
290 **Rap-Phr systems diversified into five pherogroups in the *B. cereus* group.**
291 In order to predict functions of Rap paralogs in *B. cereus* (Bc), *B. anthracis* (Ba), *B.*
292 *mycoides* (Bm), *B. pseudomycoides* (Bps) and *B. cytotoxicus* (Bcyt), we studied the
293 evolution of Rap proteins in these species and identified their putative signaling peptide
294 sequences (mature Phr). Additional to the 8 Raps in Bt407 (of which 4 are coded in the
295 chromosome, and 4 in plasmids) we found 32 *rap-phr* systems in the *B. cereus* group
296 (Table S2), 30 of which are encoded in chromosome and 2 in plasmids (Table 1).
297
298 The phylogeny of Rap proteins from the *B. cereus* group shows that clades are composed of
299 Rap proteins from different species, i.e., phylogenetically close Rap homologs can be found
300 in different species. This indicates that Rap-Phr divergence occurred before speciation in
301 this group (Fig. 7). Hence, Rap functions discovered in this work could be extrapolated to
302 the rest of the *B. cereus* group, e.g. Rap proteins found in the same clade as BtRapK and
303 BtRapF (Bps28285, Bps05775, Bps24285, Bcyt05320, Bc1026, Ba05875, Ba29315,
304 Bcyt11595, Bcyt05405 and Bcyt02700) may modulate sporulation initiation and biofilm
305 formation. Since we found that several Rap paralogs are coded in every species of the *B.*
306 *cereus* group, we suggest that they could regulate a variety of collective functions in all
307 species, as we describe here for Bt8741.
308
309 We identified 5 pherogroups, each with a putative mature Phr peptide sequence. All five
310 pherogroup comprise Rap proteins from different species, which share Phr consensus

311 sequences corresponding to the putative mature Phr (Fig. S7). These five pherogroups are
312 identified with colors in the branches of the phylogeny in figure 7. We found that mature
313 Phr corresponding to pherogroups 1 and 2 are located in the C-terminal domain of the pro-
314 peptides (exported Phr sequence). RapI, F, F1 and RapK from Bt are found in these
315 pherogroups. On the other hand, pherogroup 3 – were RapF2 from Bt is found – consensus
316 sequences are located in the N-terminal domain of the pro-peptide. Finally, putative mature
317 Phr peptides from pherogroups 4 and 5 – which include RapLike, C and RapI1 from Bt –
318 are located in the middle of the exported sequence (Table S3). We observed that only Bt
319 and Bps encode Rap proteins from all five pherogroups; Rap proteins from Ba and Bcyt are
320 found in pherogroups 1, 2, 3 and 4; Rap proteins in Bm correspond to pherogroups 1, 3 and
321 5; finally Rap proteins from Bc are found only in pherogroups 1 and 4 (Fig. 7).
322

323 **Discussion**

324 Few studies have addressed multicellular behaviors such as differentiation, cell-
325 specialization, collective functions, and the resulting ecological interactions in species from
326 the *B.* cereus group (11, 56). Similarly, molecular mechanisms for the control
327 differentiation processes in the *B. cereus* group bacteria remain understudied (45, 46, 48, 56,
328 57). Here we demonstrate that Rap-Phr systems in Bt8741 regulate collective functions
329 such as sporulation, biofilm formation, production of extracellular proteases and spreading
330 motility. In fact, Rap-Phr paralogs in this strain are highly multifunctional and redundant,
331 since five out of eight Rap paralogs modulate more than one collective trait, and all four
332 collective traits studied were inhibited by more than one Rap protein. Hence, Rap paralogs
333 appear to constitute a regulatory repertoire that allow Bt populations to respond efficiently
334 to environmental changes, which aid for survival of the population.

335

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

342

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

358 keeping multiple redundant and multifunctional Rap paralogs that control important
359 collective functions results in a better adaptation and population survival in nature.
360
361 Sporulation in the *Bacillus* genus is essential for bacterial survival and dissemination in
362 their habitats; it is also important for the biotechnological uses of *Bacillus* species. Six Rap-
363 Phr systems from Bs, including RapA, negatively regulate Spo0A phosphorelay by
364 dephosphorylating Spo0F, and therefore prevent the activation of Spo0A (32). We found
365 that $RapA_{Bs}$, retained this function when it was overexpressed in Bt8741. Furthermore, five
366 Rap-Phr systems from Bt8741 (RapK, F, C, Like and RapI) also regulate sporulation in this
367 species. We propose that RapK and RapF may function by dephosphorylating Spo0F,
368 similar to the mechanism carried out by RapA in Bs. This suggestion is supported by three
369 findings: 1) both RapK and RapF retain the highest conservation of Spo0F binding residues
370 from RapH, including the catalytic residue Q47; 2) their overexpression resulted in
371 undetectable number of spores, similar to $RapA_{Bs}$ overexpression; 3) the overexpression of
372 $RapA_{Bs}$, K and RapF caused an identical cell morphology in the three overexpressing
373 strains. Additionally, RapK and RapF are closely related and both belong to pherogroup 2,
374 which may indicate that they resulted from a gene duplication event of a Rap ancestor that
375 dephosphorylated Spo0F. Other Rap proteins that decreased sporulation efficiency are
376 RapC, Like and RapI; of these, RapC do not contain the catalytic site residue Q47. Further
377 studies are needed in order to elucidate the mechanisms by which all these receptors
378 regulate sporulation in Bt and other species from the *B. cereus* group.
379 RapK and RapF are the only Rap proteins from Bt8741 that prevented biofilm formation.
380 Because Spo0A-P levels regulate both sporulation and biofilm formation in Bs, we
381 speculate that bifunctionality of RapK and RapF in Bt8741 results from their activity on

382 Spo0F. We noted that the overexpression of $RapA_{Bs}$, which completely prevented
383 sporulation, did not affected biofilm formation of Bt. Overexpression of $RapA_{Bs}$ may allow
384 low levels of Spo0A-P in Bt8741, which in Bs are sufficient for the activation of genes
385 related to production of extracellular matrix components, but not for the activation of early
386 sporulation genes (13).
387
388 We suggest that Rap proteins have diversified according to the ecological needs of each
389 species. For example, Bs is a soil dwelling bacteria found associated to rhizosphere forming
390 biofilms (59). In Bs, six Rap proteins modulate Spo0A-P levels (21, 60), affecting
391 sporulation and biofilm formation. Here we demonstrate that five Rap proteins modulated
392 sporulation (RapC, K, F, I and RapLike) while only two of these (RapK and RapF) affected
393 biofilm formation, perhaps througout the Spo0A phosphorelay. This highlights the
394 importance of sporulation regulation in both species and that probably, biofilm formation is
395 not as essencial in the lifecylce of Bt, as it is in Bs. In contrast, Bt is a soil inhabitant, insect
396 patogenic and necrotrophic bacteria (61). In this species, extracellular protease production
397 is essential for nutrient scavenging, which is normally associated to the necrotrophic stage
398 of bacterial development in the insect cadaver (40). Additionally, it could be relevant
399 during the transition from exponential growth to stationary phase in controlled
400 fermentations or for adaptation against fluctuations in nutrient availability in the
401 environment. While only one of the twelve Rap proteins from Bs modulates its extracellular
402 proteolytic activity (RapG) (25), Bt has extended the modulation of extracellular protease
403 production to five Rap-Phr systems (RapC, F, F2, I1 and RapLike).
404

405 We found that the Spo0A phosphorelay and production of extracellular proteases are highly
406 interconnected in Bt8741 through the functions of RapC, F and RapLike. Additionally,
407 extracellular proteolytic activity (specifically the NprA protease) is regulated by the QS
408 system NprR-NprRB (62), which is also involved in the modulation of the Spo0A
409 phosphorelay (41, 42). Likewise, NprR also participates in the spreading phenotype of
410 Bt8741 (A. Verdugo *et. al*, unpublished data), as well RapC and RapLike. Because
411 sporulation, extracellular protease production and spreading of Bt have evolved to be
412 regulated and coordinated by multiple QS systems, these collective traits may be important
413 in the life cycle of Bt and represent essential mechanisms for its ecology.
414
415 Mature Phr signaling peptides from Bs correspond to at least five residues in the C-terminal
416 end of the pro-Phr or in the middle of the sequence. Sequence analyses of matuyre Phrs in
417 Bs has showed that a basic amino acid is found in the second position from the N-terminal
418 end, and an alanine residue is necessary in the position before the cleavage site for Phr
419 maturation (22, 63, 64). Our analysis of consensus putative mature Phr sequences showed
420 that these characteristics are not maintained in mature Phr peptides of the *B cereus* group.
421 This suggests that signaling peptides are processed differently in these bacteria, this is,
422 using different sets of extracellular proteases and peptidases that recognize distinct
423 sequences. In Bt, the identity of a mature Phr has only been shown for Rap8-Phr8 from Bt-
424 HD73. In that case, the active heptapeptide YAHGKDI is located in the C-terminal end
425 from its exported sequence (46). RapI from Bt8741, ortholog protein to Rap8, is found in
426 pherogroup 1, in which the consensus sequence HGKDI corresponds to the five residues in
427 the C-terminal end from the exported sequence. This indicates that the consensus sequences

428 determined in this study may not exactly predict the signaling peptide sequence, but they
429 can direct their search in future studies.
430
431 We found that Rap-Phr systems in the *B. cereus* group have evolved into five pherogroups,
432 each including Rap homologs from different species. This means that signaling peptides
433 shared by more than one species, could mediate crosstalk or eavesdropping phenomena in
434 nature, allowing the regulation of collective functions in response to interspecific signals as
435 described for other gram-positive species (65, 66).
436
437 The *B. cereus* group comprise bacteria with clinical and biotechnological relevance such as
438 Ba, Bc, Bt, and other environmental and facultative species (38). We show that Rap-Phr QS
439 systems in Bt are involved in the regulation of ecologically important collective traits, and
440 our findings are highly relevant for further studies about the *B. cereus* group and contribute
441 to the knowledge about its ecology. Understanding the regulatory processes for cell
442 differentiation and specialization in these bacteria may enhance the use of
443 biotechnologically-relevant species, or the strategies to control human pathogens, through
444 the intervention of their collective functions at the molecular level. For instance, Ba and Bc
445 are known for their pathogenic nature against mammals; therefore, elucidating the role of
446 Rap-Phr systems in the production of virulence factors of these species such as anthrax
447 toxin and capsule of Ba, or enterotoxins of Bc, could be of high relevance. Additionally, it
448 is known that QS systems can be synthetically engineered (67, 68). As a result, Rap-Phr
449 systems could be manipulated in order to enhance Bt survival, insect pathogenesis or cry
450 protein production. This work serves as a starting point for the study of cell specialization
451 of the *B. cereus* group bacteria.

452

453 **Materials and Methods**

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

455 *Bacillus thuringiensis* strain 8741 (Bt8741) (43), derived from Bt407 (Acc. No.
456 NC_018877.1, 51), was used as host for the overexpression of Rap proteins. *Bacillus*
457 *subtilis* strain 168 (Bs168) was used for the amplification of *rapA*. *Escherichia coli* strain
458 TOP10 was used for construction and cloning of overexpression plasmids before
459 transforming into Bt8741. Luria-Bertani (LB) broth (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract
460 and 5 g L^{-1} NaCl) and Nutrient Agar (8 g L^{-1} nutrient broth, 15 g L^{-1} agar) were used at 30
461 ºC for *Bacillus* cultures and at 37 ºC for *E. coli* and 200 rpm for liquid cultures. Milk Agar
462 was prepared using Nutrient Agar, supplemented with 5% skim milk (41). When needed,
463 ampicillin (100 µg mL^{-1}) or erythromycin (5 µg mL^{-1}) was added to media. To induce
464 expression from the *xylA* promoter in Bt8741, xylose was used to a final concentration of
465 20 mM (70), unless otherwise specified.

466

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

468 Based on the RapH residues involved in Spo0F binding in Bs168 (34) we determined the
469 conservation of the corresponding residues in Raps from Bt407, in order to predict their
470 capacity to bind to Spo0F. First, we analyzed the conservation of full-length Rap proteins
471 from Bs168 and Bt8741 in comparison to RapH from Bs168. For this, we performed
472 pairwise alignments of RapH amino acid sequence (NP_388565.2) with RapA
473 (NP_389125.1), RapB (NP_391550.1), RapE (NP_390460.2), RapJ (NP_388164.1), RapD
474 (NP_391519.1) from Bs168, and each of the eight Raps from Bt407 (AFV21721.1,
475 AFV22194.1, AFV22088.1, AFV16731.1, AFV19251.1, AFV22208.1, AFV16776.1,

476 AFV17466.1), using BlastP tool (71). Then, all sequences were aligned together using
477 MAFFT version 7 online service (72) with the G-INS-i interative refinement method (73).
478 Finally, we identified in the alignment the amino acids of Rap protein sequences that
479 correspond to the residues of RapH that participate in binding and dephosphorylation of
480 Spo0F.
481
482 **DNA manipulation**
483 All primers used in this study are listed in Table S4. DNA was isolated from Bs168 and
484 Bt8741 using the PureLink Genomic DNA Mini Kit (Invitrogen, Carlsbad CA, USA).
485 QIAprep Spin Miniprep Kit (Qiagen, Germantown, MD, USA) was used routinely for
486 plasmid extraction and purification. Oligonucleotides were designed for amplifying each
487 Rap gene from Bt8741 genome or plasmids (Acc. No. NC_018877.1, NC_018883.1,
488 NC_018886.1, NC_018879.1, NC_018878.1) and Bs168 genome (Acc. No. NC_000964.3),
489 and synthesized as a commercial service (T4 Oligo, Irapuato, Mexico). PCR products and
490 restriction reactions were purified using the PureLink Quick PCR Purification Kit
491 (Invitrogen). When needed, PCR products were isolated from 0.8% agarose gels using the
492 Zymoclean™ Gel DNA Recovery Kit (ZYMO Research, Irvine, CA, USA). Enzymes
493 Dream Taq Master Mix, *HindIII*, *SalI* (Thermo Scientific, Waltham, MA, USA), *PstI* and
494 T4 DNA Ligase (New England Biolabs Inc., Ipswich, MA, USA) were used as
495 recommended by the manufacturer.
496
497 **Construction of Rap-overexpression Bt8741 strains**
498 All strains and plasmids used in this study are listed in Table S1. For the construction of the
499 overexpression plasmid pHT315-P_{xylA}, the regulatory region of the xylose operon, including

500 the *xylA* promoter (P_{xylA}) and the repressor gene *xylR,* were amplified by PCR from Bs168
501 genome using primers GG1 and GG2 (Table S4). This PCR product was inserted into the
502 *Hin*dIII and *Pst*I sites of pHT315 plasmid (74), and colonies were PCR checked using
503 primers DS16 and DS17 (Table S4). The resulting plasmid pHT315-P_{xylA} was transformed
504 into *E. coli* Top10 competent cells. Then, this plasmid was used for the inducible
505 overexpression of Rap proteins with xylose in Bt8741. For this, *rap* genes encoded in the
506 genome of Bt8741 (*rapC*, *rapK*, *rapF*, *rapF1*, *rapF2*, *rapI*, *rapI1* and *rapLike*, Slamti *et al.*,
507 2014) and *rapA* from Bs168 ($RapA_{Bs}$, Perego *et al.*, 1994) were amplified using the
508 corresponding primers pairs listed in Table S4, and inserted in-frame between the *PstI* and
509 *SalI* sites of pHT315-P_{xylA}. Nine overexpression plasmids, one for each Rap protein, were
510 transformed into *E. coli* Top10 competent cells. All plasmids were then transformed into
511 Bt8741 electrocompetent cells, using the protocol described in previous studies (41),
512 generating nine Bt8741 strains for the overexpression each Rap protein. Additionally, we
513 transformed Bt8741 with the pHT315-P_{xylA} (without a *rap* gene), and the resulting strain
514 was used as control strain throughout the Rap induction experiments. The complete
515 sequence of pHT315-P_{xylA}'*rapI* was verified by Illumina sequencing (MGH DNA Core,
516 Cambridge, MA, USA), and the rest of the P_{xylA}'*rap* constructions were verified by Sanger
517 sequencing (Unidad de Servicios Genómicos, LANGEBIO-CINVESTAV, Irapuato,
518 Mexico) using primers GG26 and DS17 (Table S4).
519

520 **Sporulation efficiency**

521 We assessed the effect of the overexpression of Rap proteins on sporulation efficiency in
522 Bt8741. Preinoculums were prepared by picking a single colony of each strain into 5 mL of
523 liquid media and grown overnight. Then, 1 mL of preinoculum was centrifuged, washed

524 and suspended in 1 mL of sterile PBS. Glass culture tubes (25 mm diameter) with 5 mL of
525 LB with erythromycin were inoculated with 50 μL (1% v/v) of preinoculum containing
526 $\approx 10^7$ cfu ml^{-1} and incubated for 72 h. All strains were cultured in triplicate, in LB with and
527 without the addition of xylose. To determine growth and sporulation, total and
528 thermoresistant CFU were calculated by plating 10-fold serial dilutions in nutrient agar. For
529 thermoresistant CFU, samples of 100 μL were incubated at 80 ºC for 20 min prior to
530 diluting and plating. Sporulation efficiency was calculated as the percentage of
531 thermoresistant CFU in total CFU.
532
533 **Biofilm formation assay**
534 We evaluated the effect of the overexpression of Rap proteins on the capacity of Bt874 to
535 form biofilms. For this assay, we used 13 x 100 mm glass tubes with 3 mL Nutrient Broth +
536 erythromycin, with and without the addition of xylose to a final concentration of 2 mM.
537 Three μL of preinoculum was added in triplicates, and the inoculated tubes were incubated
538 without agitation at 31 ºC ± 1 ºC for 48 hours. The culture media was then removed with a
539 syringe with needle. The biofilm and ring attached to the wall of the tube, composed of
540 cells from the biofilm, were resuspended in 1.5 mL of sterile PBS and the optical density
541 (OD_{600}) was measured. The OD_{600} was also measured from the removed liquid media to
542 address planktonic growth. At least 5 replicates of each treatment were performed.
543
544 **Extracellular proteolytic activity assay**
545 To evaluate the effect of Rap overexpression in extracellular proteolytic activity of Bt8741,
546 2 μL preinoculums of each Rap-overexpression strain, prepared as described above, were
547 spotted in triplicate on milk agar with and without the addition of xylose. The hydrolysis

548 halo area was measured after 24 h of incubation using the Image Lab™ Software
549 (BIORAD). To correct for differences in colony growth, we subtracted the colony area.
550
551 **Spreading phenotype assay**
552 The spreading phenotype of Rap-overexpression Bt8741 variants was followed in colonies
553 spotted on agar. For this assay, we used diluted nutrient agar (NA) (0.8 g L^{-1} Nutrient broth,
554 1.5 g L^{-1} agar) with erythromycin and with or without the addition of xylose. Plates were
555 air-dried inside a biological hood for 60 minutes prior to inoculation. Then, 5 µL of
556 preinoculum cultures were spotted in the center of the plate, dried for 5 minutes and
557 incubated at 30 ºC for 14 days. The inoculated agar plates were photographed at day 1, 3, 5,
558 7 and 14, using a gel documentation system (Gel Doc™ XR+, BIORAD). Colony area was
559 measured using the Image Lab™ Software (BIORAD) and radial growth was calculated.
560 For normalization of radial dispersion, we subtracted from all observations the colony
561 radius at day 1, which corresponds to the inoculated droplet area. Three replicates of each
562 treatment were performed.
563
564 **Phylogenetic Analysis**
565 To reconstruct the phylogeny of Rap proteins in the *B. cereus* group, we first selected one
566 representative strain of each species from NCBI GenBank, including *Bacillus cereus*
567 ATCC14579 (Accession NC_004722.1), *Bacillus anthracis* A0248 (NC_012659.1),
568 *Bacillus thuringiensis* 407 (NC_018877.1), *Bacillus mycoides* ATCC6442
569 (NZ_CP009692.1), *Bacillus pseudomycoides* DMS12442 (NZ_CM000745.1) and *Bacillus*
570 *cytotoxicus* NVH391-98 (NC_009674.1). Strains were selected based on the availability of
571 a complete genome (as of July of 2018) and thus, *Bacillus weihenstephanensis* was

572 excluded. We searched for Rap protein homologs in the selected genomes by querying the
573 amino acid sequence of *B. subtilis* RapA (NP_389125.1) and each of the eight Rap
574 sequences of *B. thuringiensis* 407: RapC (AFV21721.1), RapK (AFV22194.1), RapF
575 (AFV22088.1), RapF1 (AFV16731.1), RapF2 (AFV19251.1), RapI (AFV22208.1), RapI1
576 (AFV16776.1) and RapLike (AFV17466.1). Homologs were searched using BLAST tool
577 (71), the tBlastn tool and a local script designed for performing the blast search in an
578 assembled database of the selected genomes. To ensure the identity of the Rap protein
579 homologs, Blast hits were submitted manually to the Conserved Domain Search-NCBI tool
580 (75) in order to determine if they presented the characteristic TPR-containing domain. Rap
581 protein amino acid sequences were aligned in MAFFT version 7 (72) using the G-INS-i
582 iterative refinement method which incorporates pairwise alignment algorithms (73). RapA
583 and RapH from Bs168 were also included as outgroups for the phylogenetic reconstruction.
584 The selection of the best substitution evolutionary model (JTT+G+I+F) was made using the
585 Smart Model Selection with the Akaike Information Criterion in PhyML 3.0 (76, 77), as
586 well as the phylogeny reconstruction by the Maximum Likelihood method using 1000
587 bootstraps to support the phylogenetic prediction.
588

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

590 Additional to the identification of Rap homologs in the *B. cereus* group, we also analyzed
591 the putative *phr* genes, which code for pro-Phr, the precursor of the quorum sensing signal
592 peptide. For this, we performed a manual search targeting open reading frames (ORFs)
593 between 30 and 100 amino acids of length, downstream from the *rap* gene sequences.
594 When present, each Phr amino acid sequence was analyzed for the presence of a signal
595 peptide for secretion and a cleavage site using SignalP4.1 (78). The putative mature

596 signaling peptide (mature Phr) and pherogroup prediction were performed from the
597 exported Phr amino acid sequences (pro-Phr). For this, Phrs corresponding to Rap proteins
598 from different clades of the phylogenetic reconstruction were analyzed separately. The
599 amino acid sequences of the pro-Phr from each clade were aligned using ClustalW (79).
600 Pherogroups were identified by manually, by modifying the groups of aligned Phrs and
601 looking for consensus sequences in the alignments. For better identification of consensus
602 sequences, sequence Logos were created for each pherogroup using the Seq2Logo 2.0
603 online service (80).
604
605 **Statistics**
606 All the statistical analyses were performed using GraphPad Prism version 7.0a. Data
607 obtained from the extracellular proteolytic activity assay, spreading (at day 7) and biofilm
608 formation were analyzed with a multiple *t*-tests to search for differences between not
609 induced and induced Rap protein overexpression conditions of each strain. Significance of
610 0.05 was used in all statistical tests.
611
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619

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839 visualization of amino acid binding motifs and sequence profiles including sequence
840 weighting, pseudo counts and two-sided representation of amino acid enrichment
841 and depletion. Nucleic Acids Res 40:W281–W287.
842
843

844 **Tables**

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

846 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

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848

Figures

Figure 1

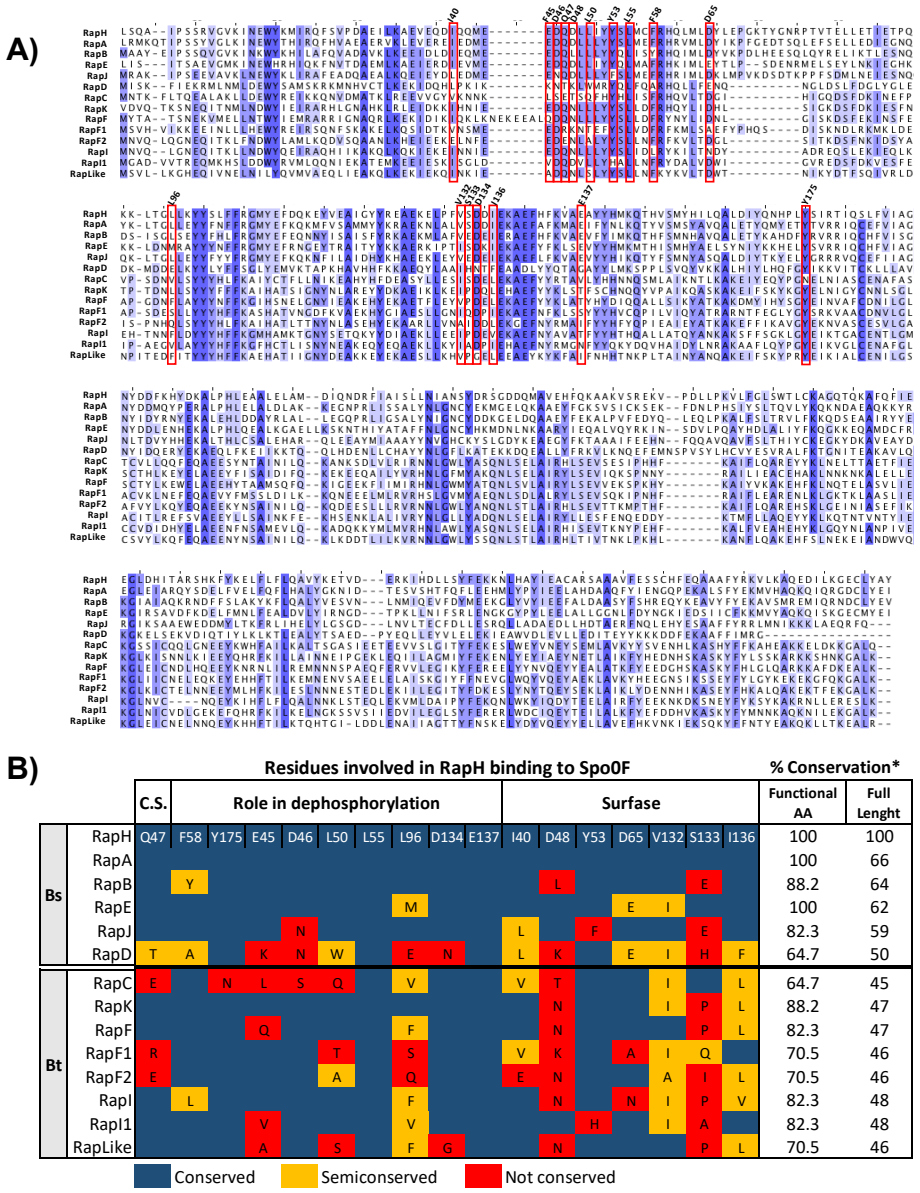
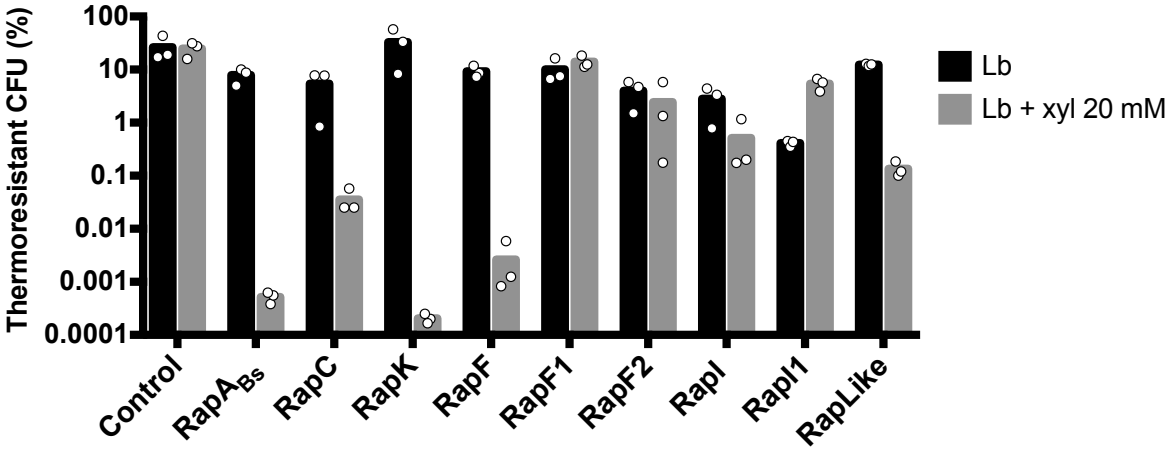


Figure 1. Prediction of the capacity of Rap proteins from Bt8741 to bind and dephosphorylate Spo0F. A) Multiple sequence alignment of the complete amino acid sequences of RapH, A, B, E, J and D from *B. subtilis* 168, and eight Rap proteins from

856 Bt8741. Blue highlights indicate highly conserved amino acids. Residues involved in the
857 RapH-Spo0F binding are indicated in red rectangles and its position in RapH is shown on
858 top of the alignment. B) Conservation of residues involved in RapH binding to Spo0F.
859 Residues were considered as semiconserved when a functional amino acid of RapH was
860 substituted with another amino acid with similar characteristics. Bs, *Bacillus subtilis* 168;
861 Bt, *Bacillus thuringiensis* 407; C.S., Catalytic Site; *Percentage of conserved and
862 semiconserved amino acids in pairwise alignment to RapH.
863
864

865 **Figure 2**

866



867

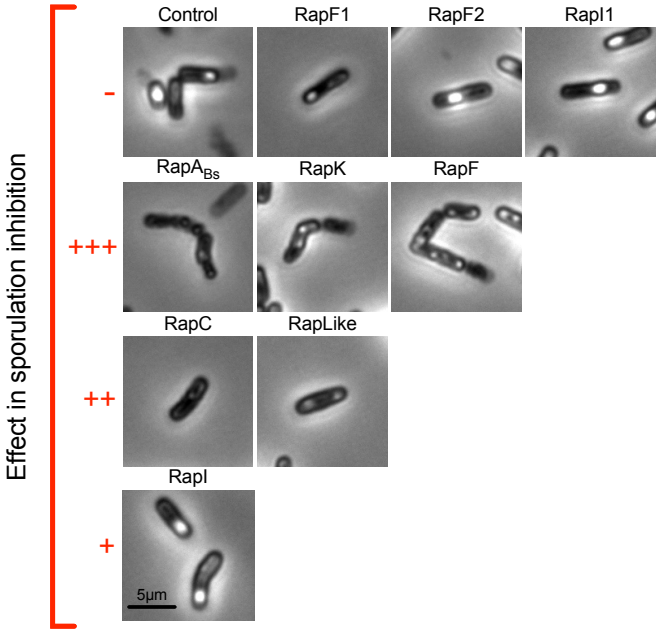
868 **Figure 2.** Sporulation efficiency of Bt8741 carrying overexpression plasmids for Rap

869 proteins, with and without addition of inducer. Columns represent average of three

870 individual measurements, shown as dots.

871

872 **Figure 3**



873

874 **Figure 3.** Cell morphology in strains with induced Rap protein overexpression at 72 h.

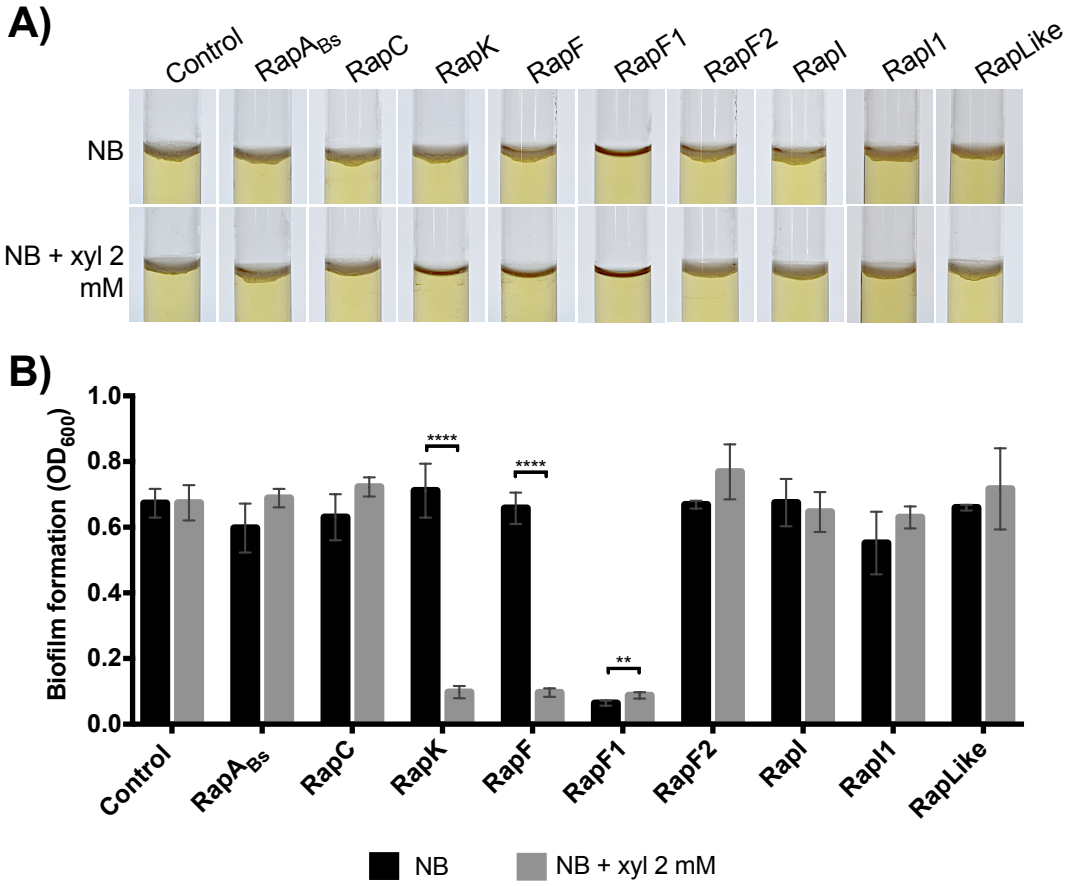
875 Phase contrast microscopy 63X and 1.8X magnification. -, no effect; +, decreasement under

876 10-fold; decreasement between 90 and 160-fold; +++, decreasement >1,000-fold.

877

878

879 **Figure 4**



880

881 **Figure 4.** Biofilm formation of Rap-overexpression strains at 48 h. A) Biofilms formed in

882 the liquid-air interphase in 13 x 100 mm glass tubes at 48 h. Biofilms are identified as a

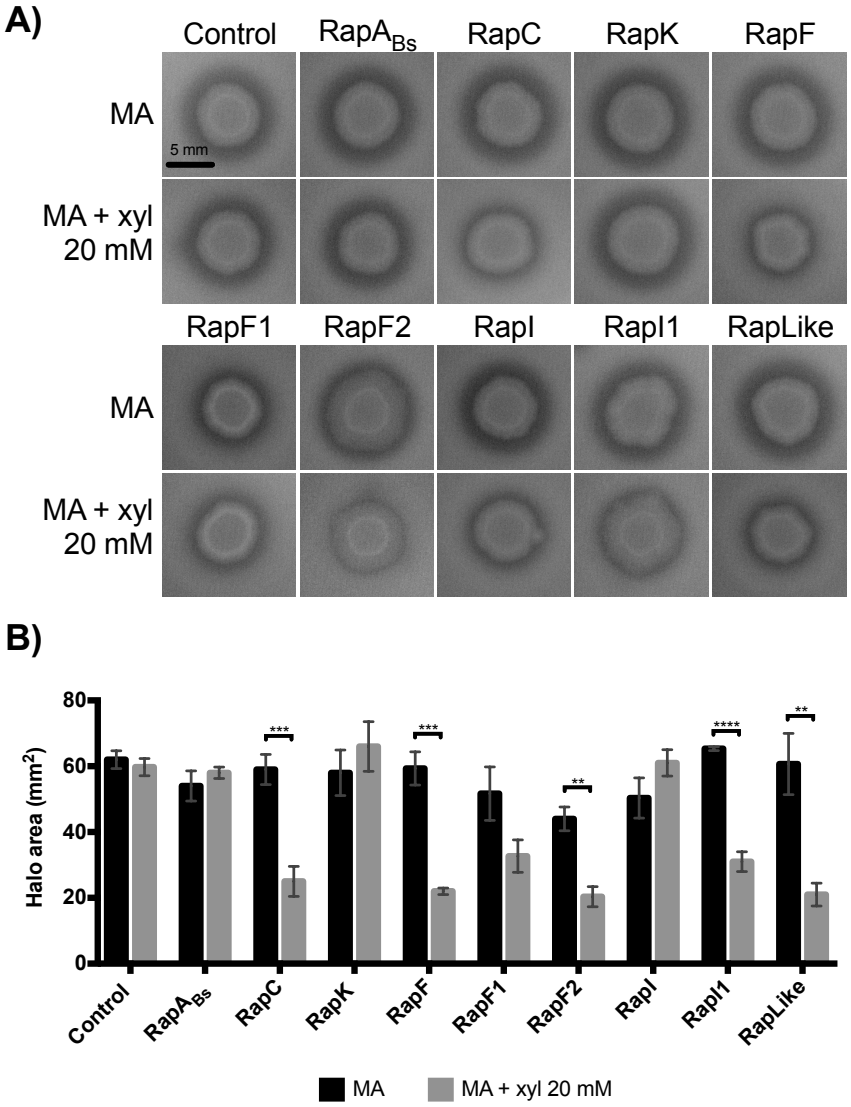
883 white layer on the surface. B) Biofilm formation quantification of Rap-overexpression

884 strains in induced and not induced media after 48 h. Columns represent average of 5

885 replicates, ± SD. NB, Nutrient Broth; **, $p<0.005$; ****, $p<0.0001$.

886

887 **Figure 5**



888

889 **Figure 5.** Extracellular proteolytic activity of Rap-overexpression strains. A) Effect of Rap

890 protein overexpression in the hydrolysis halo of Rap-overexpression strains colonies. B)

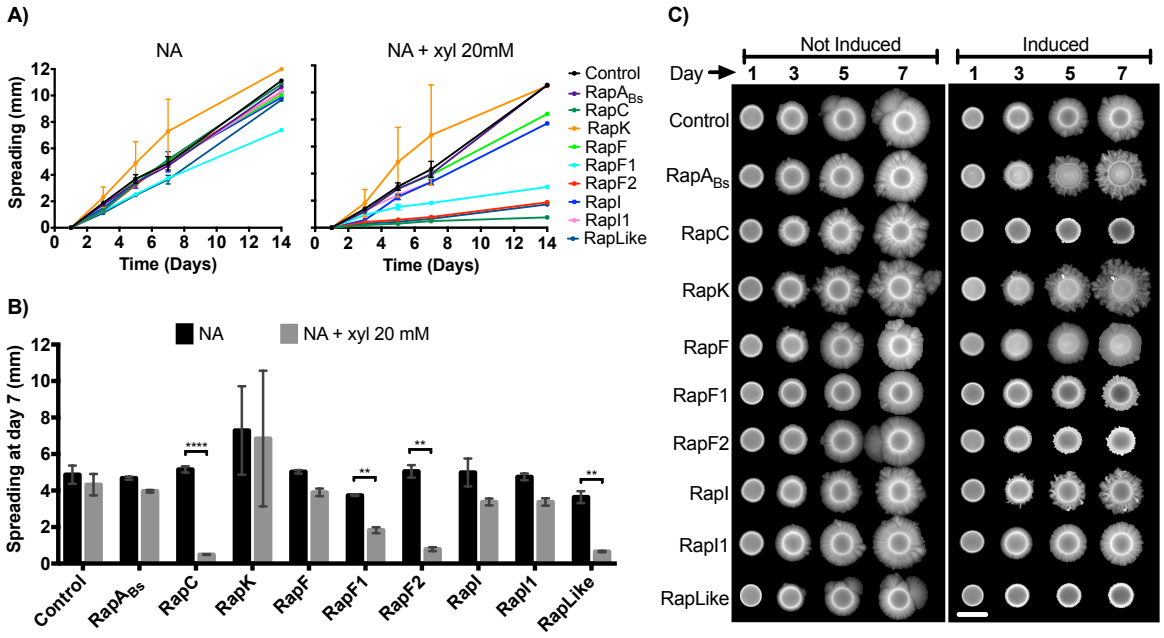
891 Hydrolysis halo area with and without Rap-overexpression induction. Columns represent

892 average of 3 replicates ± SD. MA, milk agar; **, $p<0.005$; ***, $p< 0.0005$; ****, $p<0.0001$.

893

894

895 **Figure 6**



896

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

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

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

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

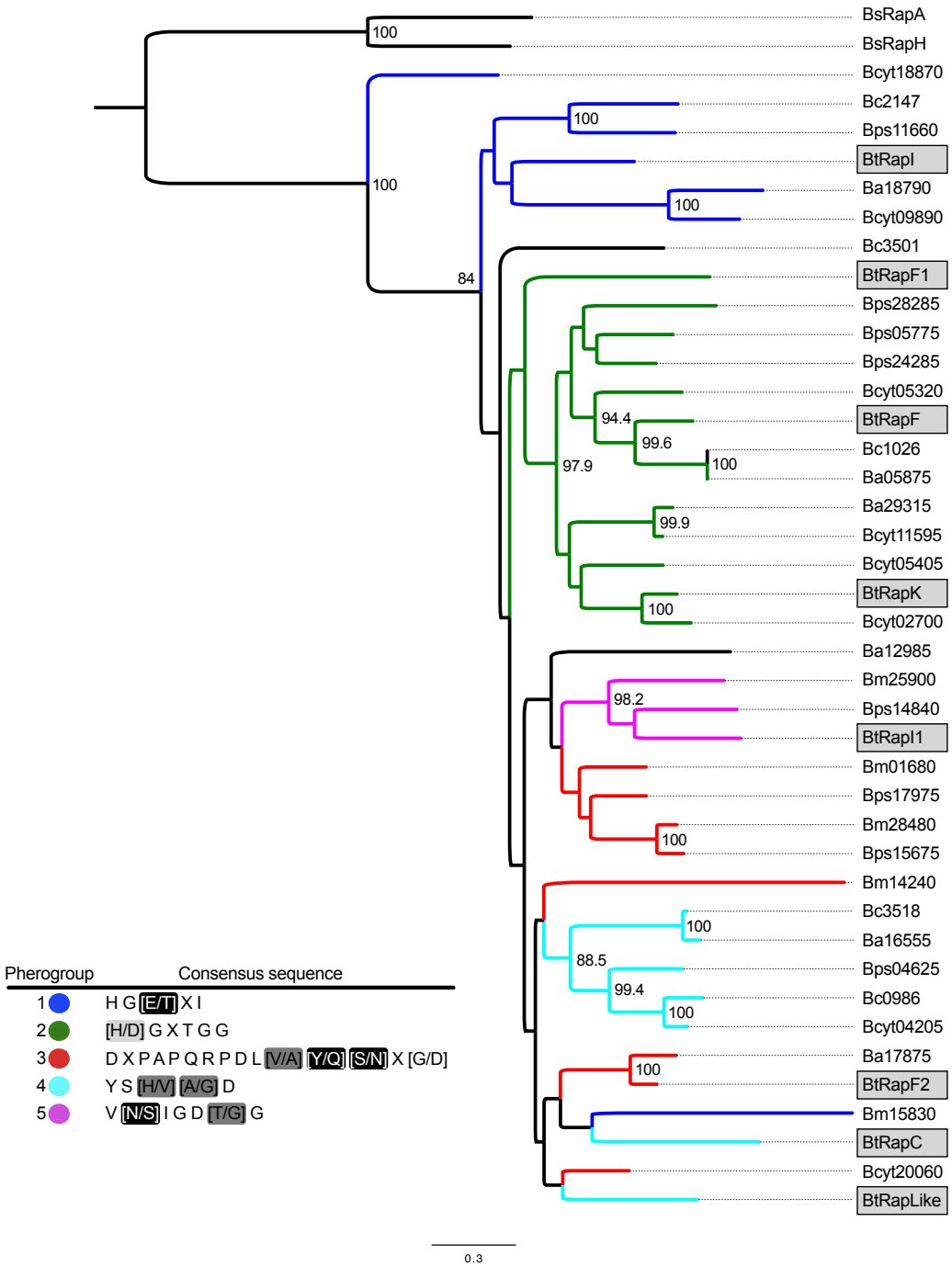
901 representative Rap-overexpression strains spreading during 7 days. Scale bar indicates 5

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

903

904

905 **Figure 7**



906

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

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

909 identified in colors. Bootstraps higher than 80% are shown in each node. Insert table:

910 pherogroups and consensus sequences of mature Phr. Semiconservations in the consensus
911 sequences are highlighted: black, polar residues; gray, hydrophobic residues; silver, polar
912 and charged residues.