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

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ORIGINAL ARTICLE

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

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Abstract

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

Keywords Rap–Phr · *Bacillus cereus* group · RNPP · Sporulation · Phosphatase

Introduction

Several bacterial processes are regulated by quorum sensing, a cell–cell communication that enables bacteria to regulate their fate with regard to the population density. The Rap proteins and their cognate Phr peptide inhibitors are quorum-sensing systems present in the *Bacillus cereus* group but not extensively studied in these bacteria. The *B. cereus* group comprises at least seven species (*Bacillus cereus* sensu stricto, *Bacillus thuringiensis*, *Bacillus anthracis*, *Bacillus weihenstephanensis*, *Bacillus mycoides*, *Bacillus pseudomycoides* and *Bacillus cytotoxicus*) of rod-shaped, spore-forming, Gram-positive bacteria that are found in diverse ecological niches and able to colonize different hosts (Liu et al. 2015). Due to the complex phylogeny of the group, as phylogenetic clades are polyphyletics and species are paraphyletics, its taxonomy continues to be debated (Bazinet 2017; Guinebretière et al. 2008; Helgason et al. 2000; Liu et al. 2015; Raymond, 2017; Tourasse et al. 2011).

The three main species of the *B. cereus* group have a significant impact on human activity. *B. cereus* is a ubiquitous and opportunistic bacterium and includes strains that cause food poisoning with vomiting or diarrhea and severe local infections, such as endophthalmitis or periodontitis (Callegan et al. 2003; Ehling-schulz et al. 2006; Stenfors Arnesen et al. 2008). *B. thuringiensis* is the world's most

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used biopesticide due to its production of insecticidal toxins (designated as Cry proteins) specifically pathogenic to a wide range of insects (Schnepf et al. 1998). *B. anthracis* is a mammal pathogen, including humans, and is the causative agent of anthrax (Liu et al. 2014). Although phenotypically different, these species are closely related genetically (Rasko et al. 2005), and the species determinants are encoded by plasmid genes (Vilas-Boas et al. 2007). For *B. cereus*, the enzymatic complex involved in cereulide (emetic toxin) synthesis is encoded by pCER270 (Ehling-Schulz et al. 2006). Strains are identified as *B. thuringiensis* if they produce a crystal inclusion during sporulation due to the presence of plasmids carrying genes encoding Cry toxins, generally active against insects or nematodes (Deng et al. 2014; Schnepf et al. 1998). The high toxicity of *B. anthracis* is due to toxins and its capsule, which are encoded by genes located on the plasmids pXO1 and pXO2, respectively (Kolstø et al. 2009).

Several microorganisms behaviors, such as biofilm formation, sporulation, motility, genetic exchange (competence and conjugation), and virulence factor production, are regulated by quorum sensing (QS), a cell–cell communication process that allows bacteria and eukaryotic microorganisms to coordinate their biological processes based on the population density (Polke and Jacobsen 2017; Rutherford and Bassler 2012). In Gram-positive bacteria, this communication is done by signaling oligopeptides that are recognized by cognate regulators, such as the QS systems of the RNPP family (from Rap, NprR, PlcR, and PrgX) (Declerck et al. 2007). These regulators are formed by tetratricopeptide repeat (TPR) domains that are structural motifs of degenerated residues that mediate protein–protein and protein–peptide interactions (D'Andrea and Regan 2003). The activity of these cytoplasmic regulators is activated (NprR and PlcR) or inhibited (Rap) by secreted, matured, and re-imported peptides that function as signaling molecules (Perchat et al. 2011; Perego and Hoch 1996; Pottathil and Lazazzera 2003; Slamti and Lereclus 2002). The genes encoding these signaling peptides are located directly downstream from the coding sequence of their cognate RNPP regulator, and the two genes are transcribed in the same orientation (Declerck et al. 2007). Except for the Rap proteins, RNPP regulators have an HTH (helix-turn-helix) DNA-binding domain, allowing them to function as transcriptional regulators (Declerck et al. 2007). During the infectious cycle of *B. thuringiensis* in insect larvae, three QS systems are successively activated (Slamti et al. 2014): (i) PlcR–PapR regulates the virulence stage by controlling the expression of virulence genes; (ii) NprR–NprX regulates the necrotrophic stage, allowing bacteria to survive and to sporulate in the insect cadaver; and (iii) Rap–Phr regulates the initiation of the sporulation process.

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

Eleven *rap* genes (from *rapA* to *rapK*) were identified on the chromosome of the *B. subtilis* 168 strain. Functional studies have shown that RapA, RapB, RapE, RapH, RapI, and RapJ can dephosphorylate Spo0F (Jiang et al. 2000b; 2011, Parashar et al. 2011, 2013a; Perego et al. 1996; Smits et al. 2007). RapC, RapD, RapF, RapH, and RapK regulate competence by inhibiting ComA (Auchtung et al. 2006; Bongiorni et al. 2005; Core and Perego 2003; Ogura and Fujita 2007; Smits et al. 2007), RapG regulates extracellular protease production by inhibiting DegU (Ogura et al. 2003), and RapI also regulates the mobility of the ICE*Bs1* genetic element (Auchtung et al. 2005). *rap–phr* genes have also been identified in *B. subtilis* plasmids. These plasmid systems are involved in the regulation of proteolytic enzyme production (Koetje et al. 2003), sporulation, competence, biofilm formation (Parashar et al. 2013b), and plasmid conjugation (Singh et al. 2013). The activities of Rap proteins are inhibited by their cognate Phr peptides. Phr-encoding genes are located downstream from the *rap* genes and are generally co-transcribed, although many *phr* genes have a secondary promoter (Perego and Brannigan 2001; McQuade et al. 2001). The pro-Phr are secreted and processed in the extracellular environment. The mature Phr are then internalized within the bacterial cells by oligopeptide permeases and bind to Rap proteins to inhibit their activity (Perego 1997).

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

In this study, we performed an overview of the Rap–Phr systems in the *B. cereus* group, including their identification, distribution, and prediction of their sporulation activity. We show that the Rap–Phr systems are widespread in all strains, in both chromosomes and plasmids, and with great sequence variability. A comparison between the *B. cereus*

and *B. thuringiensis* strains showed that plasmid Rap–Phr systems are more frequently present in *B. thuringiensis* than in *B. cereus*. One-third of the Rap proteins were predicted to have a sporulation function and these Rap proteins are preferentially located on plasmids and, therefore, are mainly present in *B. thuringiensis*.

Materials and methods

Bacterial genomes

The *B. cereus* group strains with a complete genome sequence available in the NCBI Genome database (http://www.ncbi.nlm.nih.gov/genome/) on April 2015 were selected. The species classification was considered as found in the database in the moment of data collection. The main available features (chromosome size and GC content, MLST sequence type, size and number of plasmids, and the proportion of the total genome they represent) of the genomes from the 49 selected strains are presented in Table 1 and Online Resource Table 1.

Construction of the *rap–phr* database

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

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

A 20 kb region around the *rap* genes (10 kb upstream and 10 Kb downstream) was analyzed using the ISfinder database (Siguier et al. 2005) to verify the presence of mobile elements within these genomic regions.

Rap protein clustering

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

Multilocus sequencing type (MLST) tree

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

Plasmids construction and growth conditions

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

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

Strain (named as in NCBI database)	Release date	Assembly	Chromosome		Plasmids			STs (MLST)
			Size (Mb)	%GC	Nb	Size (Mb)	% ↑ genome	
B. cereus								
ATCC 10987	2002	GCA_000008005.1	5.22	35.6	1	0.21	4%	2
ATCC 14579	2003	GCA_000007825.1	5.41	35.3	1	0.01	0%	33
E33L	2004	GCA_000011625.1	5.30	35.4	5	0.55	10%	57
AH187	2008	GCA_000021225.1	5.27	35.6	4	0.33	6%	3
B4264	2008	GCA_000021205.1	5.42	35.3	0	–	–	– 2
G9842	2008	GCA_000021305.1	5.39	35.3	2	0.35	6%	120
AH820	2008	GCA_000021785.1	5.3	35.4	3	0.28	5%	39
Q1	2009	GCA_000013065.1	5.21	35.6	2	0.29	6%	40
03BB102	2009	GCA_000022505.1	5.27	35.4	1	0.18	3%	122
anthracis CI	2010	GCA_000143605.1	5.2	35.4	3	0.28	5%	153
NC7401	2011	GCA_000283675.1	5.2	35.6	5	0.33	6%	3
F837/76	2011	GCA_000239195.1	5.22	35.4	2	0.07	1%	182
FRI-35	2012	GCA_000292415.1	5.08	35.6	4	0.3	6%	188
FT9	2014	GCA_000724585.1	5.22	35.5	0	–	–	191
03BB87	2014	GCA_000789315.1	5.46	35.3	2	0.26	5%	58
D17	2015	GCA_000832385.1	5.38	35.4	1	0.21	4%	179
FM1	2015	GCA_000832525.1	5.3	35.5	1	0.40	8%	186
3a	2015	GCA_000832765.1	5.27	35.4	3	0.37	7%	124
G9241	2015	GCA_000832805.1	5.27	35.4	3	0.45	9%	58
ATCC 4342	2015	GCA_000832845.1	5.27	35.4	1	0.04	1%	4
03BB108	2015	GCA_000832865.1	5.34	35.3	7	0.73	14%	119
Al Hakam[a]	2015	GCA_000832885.1	5,23	35,8	6	0.45	9%	173
S2-8	2015	GCA_000835185.1	5.27	35.4	2	0.37	7%	124
FORC_005	2015	GCA_000978375.1	5.35	35.3	0	–	–	187
B. cereus average			5.29	35.4	2.5	0.31	6%	
B. thuringiensis								
konkukian 97-27	2004	GCA_000008505.1	5.24	35.4	1	0.07	1%	59
Al Hakam	2006	GCA_000015065.1	5.26	35.4	1	0.06	1%	89
BMB171	2010	GCA_000092165.1	5.33	35.3	1	0.31	6%	152
finitimus YBT-020	2011	GCA_000190515.1	5.36	35.5	2	0.33	6%	155
chinensis CT-43	2011	GCA_000193355.1	5.49	35.4	10	0.66	12%	44
HD-771	2012	GCA_000292455.1	5.89	35.2	8	0.56	10%	75
HD-789	2012	GCA_000292705.1	5.5	35.3	6	0.84	15%	136
MC28	2012	GCA_000300475.1	5.41	35.4	7	1.28	24%	231
Bt407	2012	GCA_000306745.1	5.5	35.4	9	0.65	12%	44
kurstaki HD73	2013	GCA_000338755.1	5.65	35.3	7	0.27	5%	115
thuringiensis IS5056	2013	GCA_000341665.1	5.49	35.4	14	1.3	24%	44
YBT-1518	2013	GCA_000497525.2	6	35.4	6	0.68	11%	261
kurstaki YBT-1520	2014	GCA_000688795.1	5.6	35.3	11	0.98	18%	115
kurstaki HD-1	2014	GCA_000717535.1	5.63	35.3	13	1.13	20%	115
galleriae HD-29	2014	GCA_000803665.1	5.7	35.3	10	1.04	18%	211
HD1011	2015	GCA_000832485.1	5.23	35.5	4	0.86	16%	71
HD571	2015	GCA_000832825.1	5.26	35.4	1	0.06	1%	89
HD682	2015	GCA_000832925.1	5.21	35.5	3	0.08	2%	212
HD1002	2015	GCA_000835025.1	5.49	35.3	7	1.08	20%	136
morrisoni BGSC 4AA1	2015	GCA_000940785.1	5.65	35.3	6	0.53	9%	112
B. thuringiensis average			5.49	35.4	6.4	0.64	12%	

Table 1 (continued)

Strain (named as in NCBI database)	Release date	Assembly	Chromosome		Plasmids			STs (MLST)
			Size (Mb)	%GC	Nb	Size (Mb)	% ↑ genome	
B. anthracis 'Ames Ancestor' A2084	2004	GCA_000008445.1	5.23	35.4	2	0.27	5%	1
B. weihenstephanensis KBAB4[b]	2007	GCA_000018825.1	5.26	35.6	4	0.61	12%	118
B. mycoides ATCC 6462	2015	GCA_000832605.1	5.26	35.5	3	0.38	7%	133
B. pseudomycoides DSM 12442	2012	GCA_000161455.1	5.78	35.4	–	–	–	132
B. cytotoxicus NVH 391-98	2007	GCA_000017425.1	4.09	35.9	1	0.01	0%	117
B. cereus group Average			5.35	35.4	4.1	0.46	9%	

STs (Sequence Types) data was obtained from the University of Oslo's *Bacillus cereus* group MultiLocus and MultiData Typing website (http://mlstoslo.uio.no). Plasmid 'Nb' is the sum of different plasmids sequenced for each strain and ' % ↑ genome' is how much all these plasmids increase the genome size of that strain (in relation to chromosome size alone)

[a]This strain was first annotated as *B. cereus* (Johnson et al. 2015), and is now classified as *B. pseudomycoides* (strain BTZ)

[b]Nowadays, *B. weihenstephanensis* strains are considered as heterotypic synonym of *B. mycoides* (Liu et al. 2018)

Dam$^-$ Dcm$^-$ *E. coli* strain ET12567 (Stratagene, La Jolla, CA, USA) by thermal shock. Finally, each constructed plasmid was transformed by electroporation (Lereclus et al. 1989) in the acrystalliferous (Cry$^-$) *B. thuringiensis* var. *kurstaki* HD73 strain (Wilcks et al. 1998). Luria–Bertani (LB) medium was used to cultivate *E. coli* and *B. thuringiensis* at 37 °C for DNA preparation. The medium HCT was used to optimize the sporulation of *B. thuringiensis* (Lereclus et al. 1982). Antibiotics were used at the following concentration: ampicillin 100 µg/mL for *E. coli* and erythromycin 10 µg/mL for *B. thuringiensis*.

DNA manipulation

Genomic DNA from the three *B. thuringiensis* strains (HD-1, Bt407, and HD73) and the *B. cereus* strain (ATCC14579) was extracted using the Puregene Yeast/Bact. Kit B (Qiagen, France). PCRs were performed in an Applied Biosystems 2720 Thermal cycler (Applied Biosystem, USA) with Phusion High-Fidelity or Taq DNA Polymerase (New England Biolabs, USA) and oligonucleotides (Online Resource Table 2) were synthesized by Eurofins Genomics (Germany). The QIAquick PCR Purification Kit (Qiagen, France) was used to purify the amplified DNA fragments that were subsequently treated with appropriated restriction enzymes (New England Biolabs). Digested DNA fragments were separated on 1% agarose gels and purified from gels using the QIAquick gel extraction kit (Qiagen, France). T4 DNA ligase and restriction enzymes were used following the manufacturer's recommendations (New England Biolabs). *E. coli* plasmid DNA extractions were performed using the QIAprep Spin Miniprep Kit (Qiagen, France). DNA sequencing was carried out by GATC Biotech (Konstanz, Germany).

Sporulation assay

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

Statistical analyses

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

Results

Genomic overview of the *B. cereus* group strains

To study the occurrence, the prevalence and the distribution of Rap–Phr systems in the *B. cereus* group, those genome sequences with complete assembly level available in NCBI Genome section until April 2015 were selected. Most of these genomes belong to *B. cereus*, *B. thuringiensis* or *B. anthracis*, considering the relevance

of these species. All *B. cereus* and *B. thuringiensis* available strains were selected. However, as *B. anthracis* is a clonal species (Helgason et al. 2000; Rasko et al. 2005), only one representative genome sequence (Ames Ancestor strain) was selected, even if around 30 genomes of this species were available. Moreover, our preliminary results revealed identical Rap–Phr profiles in all the *B. anthracis* isolates, thus confirming the clonal aspect of the *B. anthracis* strains (data not shown). For the other species (*B. mycoides*, *B. pseudomycoides*, *B. weihenstephanensis*, and *B. cytotoxicus*), only one genome for each species was chosen. When the same strain was sequenced twice, only one was selected. Following these criteria, 49 genomes were used in this study (Table 1).

The GC content of these genomes was around 35%, with minor differences among the strains. In the *B. cereus* strains, the chromosomal size ranged from 5.08 to 5.46 Mb, while in *B. thuringiensis* the variation was from 5.21 to 6 Mb. All the other species showed chromosomes larger than 5 Mb, except *B. cytotoxicus* NVH 391-98, which was 4.09 Mb (Table 1). The analysis of the plasmid content of the 49 selected strains revealed a total of 197 plasmids with a size ranging from 2.1 to 502 Kb (Online Resource Table 3). These plasmids can provide a significant increase in genome size (Table 1). In *B. cereus*, the average increase of the genome size is 5% with a maximum of 12% in the strain *B. cereus* 03BB108. In *B. thuringiensis*, the average increase of the genome size was 12%, with a maximum of 24% in the *B. thuringiensis* MC28 and *B. thuringiensis* IS5056 strains.

rap–phr genes distribution

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

From 2 to 16 *rap–phr* genes were identified in all strains (Fig. 1a, Online Resource Table 4). Two to eight chromosomal *rap–phr* systems were found by strain, representing 70% of all the identified *rap–phr* genes (Fig. 1b). Plasmid *rap–phr* genes were found in 27 of the 49 strains and on 65 of the 197 plasmids. Some strains harbor a large number of plasmid *rap–phr* genes, up to 12 plasmid systems for the *B. thuringiensis* strain IS 5056 (Online Resource Table 3). The size of plasmids harboring *rap–phr* genes varied from 6.88 to 502 kb. However, the occurrence of these genes is most common (70%) in plasmids larger than 70 kb (Online Resource Table 3). Also, large plasmids (> 200 kb) might contain several *rap–phr* genes, up to five, such as the pBMB422 plasmid (422.7 kb) of the *B. thuringiensis* YBT-1520 strain (Online Resources Table 3 and 4).

The average number of chromosomal *rap–phr* genes is similar between *B. cereus* and *B. thuringiensis* (4.04 and 4.45, respectively). However, the average number of plasmid *rap–phr* genes is sixfold higher in *B. thuringiensis* than in *B.*

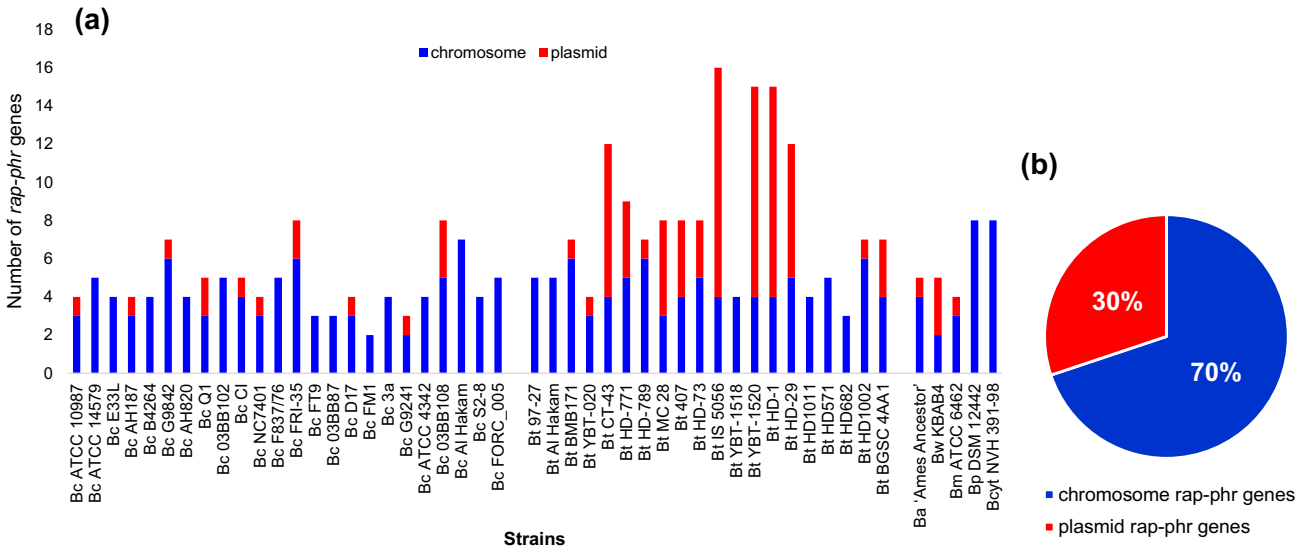

Fig. 1 Distribution of the identified *rap–phr* genes in the *B. cereus* group. **a** The number of *rap–phr* genes by strain with the chromosomal systems in blue and the plasmid systems in red. **b** Percentage of the *rap–phr* genes by replicon with chromosomes in blue and plasmids in red

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

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

Rap protein clustering

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

Three phylogenetic trees were constructed using Rap protein sequences, according to the location of their encoding genes in the genome (all sequences, chromosome or plasmid sequences) (Fig. 2 and Online Resource Fig. 1). These trees were built by the Neighbor-Joining method with corrections based on the Jones–Taylor–Thornton matrix model that enables the assessment of the overall divergence among Rap proteins. The phylogenetic tree with all sequences showed a high number of branches with a dispersion of plasmid sequences in several branches. Closely related Rap sequences are encoded by similar kind of plasmids distributed in eight major sets (Fig. 2, sets A–H). Set A comprises *rap* genes harbored by an identical 8.5 kb plasmid present in different strains and set B by plasmids higher than 70 kb. Set C includes pXO1-like plasmids; set D plasmids larger than 200 kb, while plasmids from set E are smaller than 20 kb. Set F includes plasmids around 70 kb and set G plasmids larger than 200 kb that can also harbor Rap sequences from set B. The set H is the most versatile since it comprises two distinct subsets: one with plasmids larger than 400 kb and another with plasmids ranging from 75 to 235 kb.

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

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

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

Statistical analyses: [a]unpaired *t* test; [b]Mann–Whitney test; [c]Fisher's exact test; [d]unpaired *t* test with Welch correction. *ns* not significant; *$P < 0.05$; **$P < 0.01$; ***$P \leq 0.001$

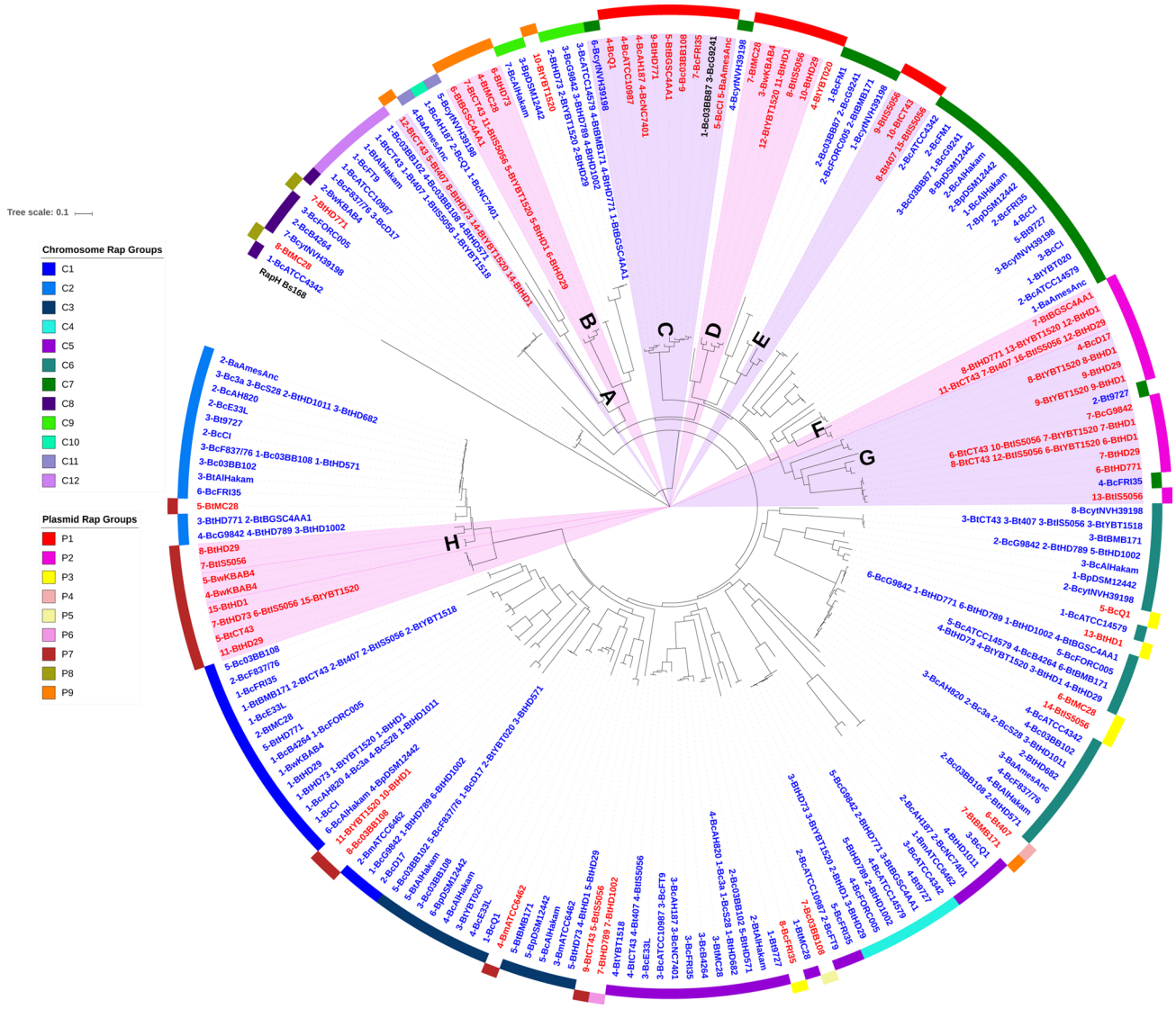

Fig. 2 Phylogenetic tree of all Rap protein sequences (plasmid and chromosome sequences) inferred by Neighbor-Joining method (conducted in MEGA6) and visualized by iTOL (Letunic and Bork 2019). RapH from *B. subtilis* 168 was used as outgroup. The evolutionary distances were computed using the JTT matrix-based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

chromosomal tree, many groups are composed of several sequences, such as Group P2 with 26 Rap proteins. However, Group P4 and Group P5 are composed of unique sequences and Group P6 by two identical sequences.

Phr peptides

In silico determination of the mature Phr sequences is complex due to the high variability of these sequences within the *B. cereus* group. Indeed, the mature Phr already described from the *B. cereus* group revealed some differences in size and location within the pro-peptide. The BXA0205Phr from the pXO1 plasmid (Phr5-*B. anthracis*) is a pentapeptide while the Phr8 from the pHT8_1 plasmid (Phr8-*B. thuringiensis* HD73) is an heptapeptide but both are located in the C-terminal end. Moreover, the active form of the BA3791Phr from *B. anthracis* (Phr3-*B. anthracis*) is located within the C-terminal region of its precursor, but its exact sequence was not determined (Bongiorni et al. 2006; Fazion et al. 2018). However, all these mature peptides present a positively charged residue, the typical feature of Phr active form (Pottathil and Lazazzera 2003). The Phr sequences from the *B. cereus* group present a great variability in their amino acid sequences and in their sizes (Online Resources Data 1).

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

The relationship between MLST phylogenetic tree and Rap distribution

The 49 selected strains are distributed into six of the seven phylogenetic clusters determined by Guinebretière et al. (2008). These clusters, established from the *Bacillus cereus* group MultiLocus and MultiData Typing website (Tourasse et al. 2011), are supported by recent results of a pangenomic study of this clade (Bazinet 2017). Moreover, the MLST data are still effective in discriminating variation of biology, ecology and host association among this group strains (Raymond and Federici 2017). The cluster III, including *B. anthracis* strains, emetic strains, and other pathogenic strains (mainly composed by *B. cereus* strains), and the cluster IV with *B. cereus* and *B. thuringiensis* strains from diverse environmental sources (mainly formed by *B. thuringiensis* strains) are the more extensively represented (Fig. 3). The Rap profile of each strain was analyzed in relation to the MLST tree of the *B. cereus* group. We observed that any Rap group was not present in all strains and that a Rap group was not exclusively related to an MLST cluster. None of the strains has more than five different Rap chromosomal groups, and a same Rap group can be present more than once in the same strain. As expected, the Cluster IV (mainly composed of *B. thuringiensis* strains) has a higher number of plasmid Rap. Additionally, phylogenetically related strains show a similar Rap profile both for chromosomal and plasmid groups. For example, the closely related strains *B. thuringiensis* Bt407 Cry^-, *B. thuringiensis* CT-43, and *B. thuringiensis* IS5056 show an identical chromosomal profile and similar plasmid profile. These three strains might derive from a same parental strain, for example the *B. thuringiensis* strain 407 Cry^+ from the serotype 1 (Lereclus et al. 1989).

Sporulation activity prediction

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

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

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

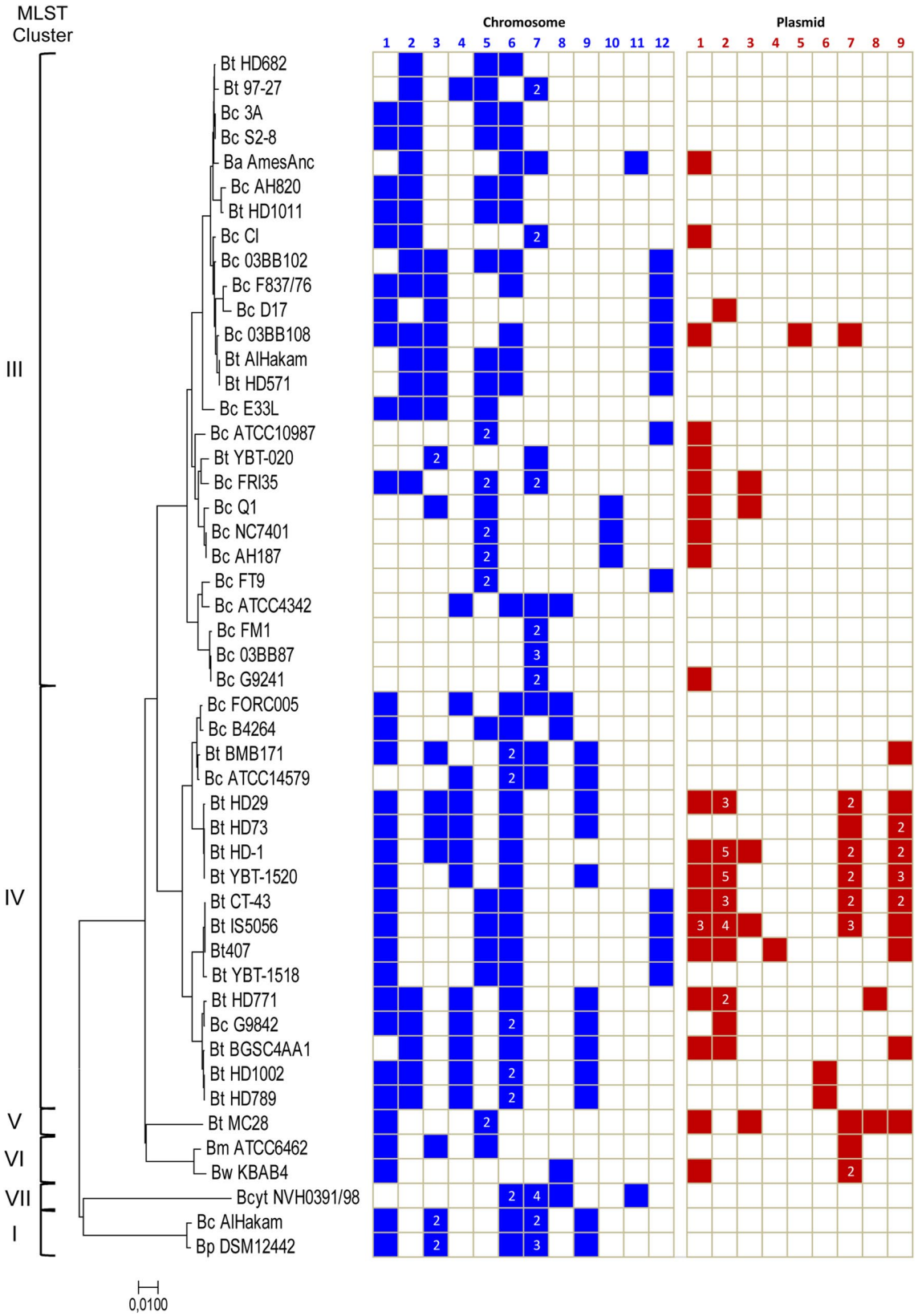

Fig. 3 Distribution of the chromosomal and plasmid Rap–Phr systems groups in relation to the MLST phylogenetic tree (*Bacillus cereus* group MultiLocus and MultiData Typing website—http://mlstoslo.uio.no). MLST clusters proposed by Guinebretière et al. (2008) are showed on the left. Chromosomal systems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

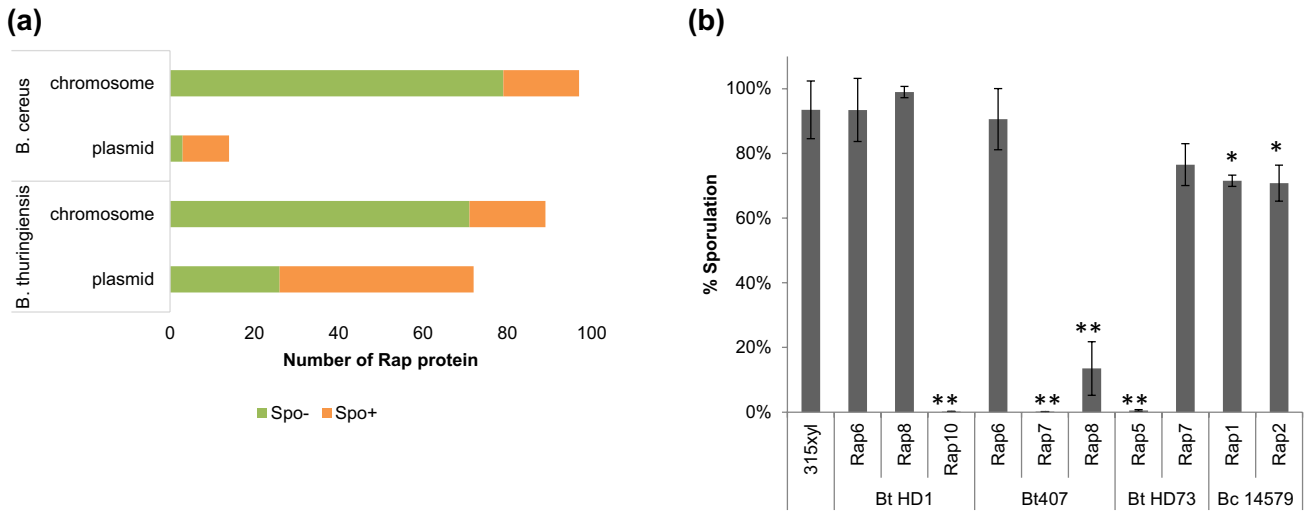

Fig. 4 Activity of Rap from *B. cereus* group in sporulation. **a** Distribution of the Rap protein predicted as Spo+ (orange) or Spo − (green), difference between species are statistically significant ($P<0.01$—Fisher's Exact test); **b** efficiency of sporulation, calculated as 100 × the ratio between heat-resistant spores per milliliter and total viable cells per milliliter. The data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey–Kramer Multiple Comparisons Test ($P<0.01$). * ($P<0.01$) and ** ($P<0.001$): % of spores is statistically different from control (315xyl)

thuringiensis HD73 model strain in this growth condition. Hence, the sporulation results allow us to confirm the predicted phenotype for nine of the ten tested Rap.

Discussion

Despite the importance of the Rap–Phr systems in the regulation of various essential pathways, they have been poorly studied in the bacteria of the *B. cereus* group (Bongiorni et al. 2006; Fazion et al. 2018; Slamti et al. 2014). Here, we provide a complete and detailed overview of these systems in this group, concerning their prevalence, sequence diversity, relevant association to plasmids and their role in sporulation. We show that the *rap* genes are widespread in all the studied strains of the *B. cereus* group and that a putative *phr* gene is always present immediately downstream from all *rap* genes. The *rap–phr* genes are always encoded on the same DNA strand but in different transcription frames, a characteristic of the RNPP family (Declerck et al. 2007). Genes coding for the Phr peptides were diverse in size and the occurrence of *phr* genes two times longer than the average could be explained by the duplication of the region coding for the mature signaling peptide, important for the evolutionary diversification of Rap–Phr specificity (Even-Tov et al. 2016). The *rap–phr* genes are located in all chromosomes and numerous plasmids. The total number of chromosome *rap–phr* genes is similar between *B. cereus* and *B. thuringiensis* species. However, chromosome size seems unrelated to the amount of chromosomal *rap–phr* genes as the *B. cytotoxicus* NVH 391-98 strain (distantly related to other strains of the *B. cereus* group) presents the smallest chromosome and the largest number of chromosomal *rap–phr* genes.

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

(Perchat et al. 2016a). This hypothesis could also explain the difference in the number of *rap–phr* genes between *B. subtilis* and *B. cereus*.

Our analysis revealed that 30% of the identified *rap–phr* genes were plasmid-born. The plasmid *rap–phr* genes have been described in diverse *Bacillus* species (Koetje et al. 2003; Parashar et al. 2013b; Singh et al. 2013; Yang et al. 2015), including the *B. cereus* group (Bongiorni et al. 2006; Chao et al. 2007; Fazion et al. 2018; Slamti et al. 2014; Van der Auwera et al. 2005). These plasmid *rap–phr* genes are carried by a wide range of plasmids but are mainly located in conjugative plasmids greater than 70 kb and are more abundant in *B. thuringiensis* than in *B. cereus*. This difference is not only the consequence of a higher number of plasmids in *B. thuringiensis*, which has only twice as many plasmids as *B. cereus* and six-times as many *rap–phr* plasmid genes. Moreover, this difference might be explained by the higher presence of mobile elements close to plasmid *rap* genes in *B. thuringiensis* than in *B. cereus*. We have shown that plasmids larger than 200 kb can host multiple *rap–phr* genes, and it is also remarkable that *rap–phr* genes were found on virulence plasmids, such as pXO1 in *B. anthracis* and pCER270 in *B. cereus*, as well as in some Cry plasmids in *B. thuringiensis*. However, we did not find specific *rap–phr* genes associated to the Cry plasmids. The multiplicity of the *rap–phr* genes in the *B. cereus* group creates appropriate conditions for their diversity and evolution, as demonstrated by Even-Tov et al. (2016). Thus, different *rap–phr* genes can respond to various signals and be regulated differently at transcriptional level.

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

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

One-third of the Rap proteins of the *B. cereus* group are predicted to have phosphatase activity on Spo0F. However, some strains did not have any predicted Rap Spo + (four *B. cereus* strains, one *B thuringiensis* strain, *B. mycoides* and *B. pseudomycoides*). This absence of Rap Spo + could be compensated by NprR, which has a Rap-like activity on the sporulation phosphorelay and which is conserved in all the strains of the *B. cereus* group (Perchat et al. 2016b). In these strains, Rap proteins might have undergone genetic variations leading to the loss of sporulation function and the acquisition of new undetermined functions. Interestingly, Rap proteins predicted to regulate sporulation are mainly encoded by plasmid genes and, therefore, are more abundant in *B. thuringiensis*. These plasmid Rap–Phr systems could help *B. thuringiensis* to adapt and survive in its complex ecological niche, the insect. Moreover, many Rap–Phr plasmid systems are located on cryptic plasmids less than 16 kb in size. These plasmid genes could regulate different beneficial functions leading to the maintenance of these plasmids in the bacterial cell. Recently, the plasmid pHT8_1 from the *B. thuringiensis* HD73 strain has been characterized, and the role of its Rap–Phr system in the regulation of the sporulation process in insect larvae has been demonstrated (Fazion et al. 2018).

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

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

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