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SÃO JOÃO EVANGELISTA-MG

Novembro-2020

Horários de 1º/2020 perfazendo 19 aulas no IFMG – SJE.

Professor Fernanda Fazion

IFMG, Campus São João Evangelista, MG

	1 M	2 M	Intervalo Manhã	3 M	4 M	5 M	6 M	Almoço	1 T	2 T	Intervalo Tarde	3 T	4 T	5 T	6 T	Intervalo Vespertino	1N	2N	Intervalo Noite	3N	4N	5N
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DECLARAÇÃO

Declaro, para os fins que se fizerem necessários, que o(a)s professores(as) abaixo relacionado(a)s atuaram como colaboradores(as) no Projeto de Ensino Hora do Enem, em sua(s) respectiva(s) disciplina(s). Trata-se de projeto organizado e proposto pela Coordenação Geral de Ensino Médio e Técnico, entre 25 de maio e 31 de julho de 2020, para atender a demanda do(a)s estudantes das terceiras séries dos cursos técnicos integrados, interessado(a)s na preparação para o Exame Nacional do Ensino Médio (ENEM).

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Declaro para os devidos fins que **Fernanda Aparecida Pires Fazion** co-orientou o(s) discente(s) Luis Carlos da Silva Soares, e atuou como coordenador do Projeto "Potencial do uso de óleos essenciais no controle in vitro de Xanthomonas axonopodis, causadora da mancha bacteriana do eucalipto.", na modalidade PIBIC, submetido ao edital 10/2020, registrado sob o n° SJEPE06/2020, com vigência no período de Outubro de 2020 a Março de 2021.

São João Evangelista, 05 de Novembro de 2020

Alisson José Eufrásio de Carvalho
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PORTARIA Nº 131 DE 15 DE JULHO DE 2019

Dispõe sobre designação de membros do Colegiado do Curso de Licenciatura em Ciências Biológicas do IFMG - *Campus* São João Evangelista.

O DIRETOR-GERAL SUBSTITUTO DO INSTITUTO FEDERAL DE EDUCAÇÃO, CIÊNCIA E TECNOLOGIA DE MINAS GERAIS – CAMPUS SÃO JOÃO EVANGELISTA, no uso das atribuições que lhe são conferidas pela Portaria IFMG nº 833, de 12 de julho de 2019, publicada no Diário Oficial da União de 16 de julho de 2019, Seção 2, página 19; e considerando a Portaria IFMG nº 475, de 06 de abril de 2016, publicada no DOU de 15 de abril de 2016, Seção 2, pág.17, retificada pela Portaria IFMG nº 805, de 04 de julho de 2016, publicada no DOU de 06 de julho de 2016, Seção 2, pág. 22, e pela Portaria IFMG nº 1078, de 27 de setembro de 2016, publicada no DOU de 04 de outubro de 2016, Seção 3, pág. 20,

RESOLVE:

Art. 1º. DESIGNAR os membros do Colegiado do Curso de Licenciatura em Ciências Biológicas do IFMG - *Campus* São João Evangelista, conforme segue:

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Art. 3º. Esta Portaria entra em vigor na data de sua publicação, com efeitos retroativos a 30 de maio de 2019.

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PORTARIA Nº 111 DE 04 DE JUNHO DE 2019

Dispõe sobre a designação de servidores como membros do Colegiado da Área de Ciências da Natureza do IFMG – *Campus* São João Evangelista.

O DIRETOR-GERAL SUBSTITUTO DO INSTITUTO FEDERAL DE EDUCAÇÃO, CIÊNCIA E TECNOLOGIA DE MINAS GERAIS – CAMPUS SÃO JOÃO EVANGELISTA, no uso das atribuições que lhe são conferidas pela Portaria IFMG-SJE nº 102, de 28 de julho de 2016, publicada no Diário Oficial da União de 02 de agosto de 2016, Seção 2, página 19; e considerando a Portaria IFMG nº 475, de 06 de abril de 2016, publicada no DOU de 15 de abril de 2016, Seção 2, pág.17, retificada pela Portaria IFMG nº 805, de 04 de julho de 2016, publicada no DOU de 06 de julho de 2016, Seção 2, pág. 22, e pela Portaria IFMG nº 1078, de 27 de setembro de 2016, publicada no DOU de 04 de outubro de 2016, Seção 3, pág. 20,

Considerando o Despacho DDE nº 27 de 04 de junho de 2019,

RESOLVE:

Art. 1º. DESIGNAR os servidores docentes **GIUSLAN CARVALHO PEREIRA**, Matrícula SIAPE nº 1752710; **ALBERTO VALADARES NETO**, Matrícula SIAPE nº 2322575; **CHARLES DE ASSIS OLIVEIRA ROCHA**, Matrícula SIAPE nº 1885035; **CLÁUDIO JÚNIOR ANDRADE RIBEIRO**, Matrícula SIAPE nº 189900; **CLEONIR COELHO SIMÕES**, Matrícula SIAPE nº 1890709; **DERLI BARBOSA DOS SANTOS**, Matrícula SIAPE nº 3124440; **FÁBIO WELITON JORGE LIMA**, Matrícula SIAPE nº 2419080; **FERNANDA APARECIDA PIRES FAZION**, Matrícula SIAPE nº 1414985; **GERALDINO MOURA DOS SANTOS**, Matrícula SIAPE nº 1247728; **GRAZIELE WOLFF DE ALMEIDA CARVALHO**, Matrícula SIAPE nº 1870907; **MARCELO AUGUSTO FILARDI**, Matrícula SIAPE nº 3123842; **MATEUS RAMOS DE ANDRADE**, Matrícula SIAPE nº 1325162; **MICHELLE PIRES TANNURE**, Matrícula SIAPE nº 1122752 para, sob a presidência do primeiro citado, constituírem o Colegiado da Área de Ciências da Natureza do IFMG – *Campus* São João Evangelista.

Art. 2º. Revogar a Portaria nº 44 de 12 de março de 2018.

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PORTARIA Nº 132 DE 16 DE JULHO DE 2019

Dispõe sobre a designação de servidores como membros do Núcleo Docente Estruturante (NDE) do Curso de Licenciatura em Ciências Biológicas do IFMG – *Campus* São João Evangelista.

O DIRETOR-GERAL SUBSTITUTO DO INSTITUTO FEDERAL DE EDUCAÇÃO, CIÊNCIA E TECNOLOGIA DE MINAS GERAIS – CAMPUS SÃO JOÃO EVANGELISTA, no uso das atribuições que lhe são conferidas pela Portaria IFMG nº 833, de 12 de julho de 2019, publicada no Diário Oficial da União de 16 de julho de 2019, Seção 2, página 19; e considerando a Portaria IFMG nº 475, de 06 de abril de 2016, publicada no DOU de 15 de abril de 2016, Seção 2, pág.17, retificada pela Portaria IFMG nº 805, de 04 de julho de 2016, publicada no DOU de 06 de julho de 2016, Seção 2, pág. 22, e pela Portaria IFMG nº 1078, de 27 de setembro de 2016, publicada no DOU de 04 de outubro de 2016, Seção 3, pág. 20,

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Art. 1º. DESIGNAR os servidores docentes **GIUSLAN CARVALHO PEREIRA**, Matrícula SIAPE nº 1752710; **DERLI BARBOSA DOS SANTOS**, Matrícula SIAPE nº 3124440; **FERNANDA APARECIDA PIRES FAZION**, Matrícula SIAPE nº 1414985; **GRAZIELE WOLFF DE ALMEIDA CARVALHO**, Matrícula SIAPE nº 1870907; **MARCELO AUGUSTO FILARDI**, Matrícula SIAPE nº 3123842; **MATEUS RAMOS DE ANDRADE**, Matrícula SIAPE nº 1325162; **MICHELLE PIRES TANNURE**, Matrícula SIAPE nº 1122752 para, sob a presidência do primeiro citado, constituírem o Núcleo Docente Estruturante (NDE) do Curso de Licenciatura em Ciências Biológicas do IFMG - *Campus* São João Evangelista.

Art. 2º. Revogar a Portaria nº 106, de 02 de maio de 2018.

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INVERTEBRATE MICROBIOLOGY

Rap-Phr Systems from Plasmids pAW63 and pHT8-1 Act Together To Regulate Sporulation in the *Bacillus thuringiensis* Serovar kurstaki HD73 Strain

AQ: au

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Priscilla Cardoso, Fernanda Fazion, and Stéphane Perchat contributed equally to this work. Author order was determined alphabetically.

ABSTRACT *Bacillus thuringiensis* is a Gram-positive spore-forming bacterium pathogenic to various insect species. This property is due to the Cry toxins encoded by plasmid genes and mostly produced during sporulation. *B. thuringiensis* contains a remarkable number of extrachromosomal DNA molecules and a great number of plasmid *rap-phr* genes. Rap-Phr quorum-sensing systems regulate different bacterial processes, notably the commitment to sporulation in *Bacillus* species. Rap proteins are quorum sensors acting as phosphatases on Spo0F, an intermediate of the sporulation phosphorelay, and are inhibited by Phr peptides that function as signaling molecules. In this study, we characterize the Rap63-Phr63 system encoded by the pAW63 plasmid from the *B. thuringiensis* serovar kurstaki HD73 strain. Rap63 has moderate activity on sporulation and is inhibited by the Phr63 peptide. The *rap63-phr63* genes are cotranscribed, and the *phr63* gene is also transcribed from a σ^H-specific promoter. We show that Rap63-Phr63 regulates sporulation together with the Rap8-Phr8 system harbored by plasmid pHT8_1 of the HD73 strain. Interestingly, the deletion of both *phr63* and *phr8* genes in the same strain has a greater negative effect on sporulation than the sum of the loss of each *phr* gene. Despite the similarities in the Phr8 and Phr63 sequences, there is no cross talk between the two systems. Our results suggest a synergism of these two Rap-Phr systems in the regulation of the sporulation of *B. thuringiensis* at the end of the infectious cycle in insects, thus pointing out the roles of the plasmids in the fitness of the bacterium.

IMPORTANCE The life cycle of *Bacillus thuringiensis* in insect larvae is regulated by quorum-sensing systems of the RNPP family. After the toxemia caused by Cry insecticidal toxins, the sequential activation of these systems allows the bacterium to trigger first a state of virulence (regulated by PlcR-PapR) and then a necrotrophic lifestyle (regulated by NprR-NprX); ultimately, sporulation is controlled by the Rap-Phr systems. Our study describes a new *rap-phr* operon carried by a *B. thuringiensis* plasmid and shows that the Rap protein has a moderate effect on sporulation. However, this system, in combination with another plasmid carrying a *rap-phr* operon, provides effective control of sporulation when the bacteria develop in the cadavers of infected insect larvae. Overall, this study highlights the important adaptive role of the plasmid Rap-Phr systems in the developmental fate of *B. thuringiensis* and its survival within its ecological niche.

KEYWORDS *Bacillus*, Rap-Phr, plasmids, quorum sensing, sporulation

Bacillus thuringiensis belongs to the *Bacillus cereus* group of Gram-positive, rod-shaped, spore-forming bacteria and is distinguished from the other, closely related species of this group by the production of crystal inclusions toxic to the larvae of

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various insects (1). Due to this entomopathogenic property, *B. thuringiensis* is widely used as a biopesticide to control agricultural pests, mainly lepidopteran and coleopteran insects, or human disease vectors, such as mosquitoes (2, 3). Crystal inclusions consist of insecticidal proteins encoded by *cry* and *cyt* genes located on plasmids and generally transcribed by sporulation sigma factors (4). *B. thuringiensis* strains have been shown to carry a complex plasmid pattern, with as many as 17 different extrachromosomal elements with sizes ranging from 2 to 600 kb (5–7). Because of their biotechnological relevance, plasmids harboring *cry* genes have been the most studied (8–10). Recent studies have shown that a number of *Bacillus* plasmids harbor genes encoding various Rap-Phr systems (11–16). Rap-Phr quorum-sensing systems were first described in *Bacillus subtilis*, in which they regulate various processes, such as sporulation, competence, transfer of mobile genetic elements, production of proteases, and biofilm formation (17).

The Rap proteins belong to the RNPP family of quorum-sensing regulators from Gram-positive bacteria, which consist of a response regulator with TPR (tetratricopeptide repeat) domains forming a hydrophobic pocket able to bind the signaling peptide that induces a conformational change and modulates regulator activities (17–19). The infectious cycle of *B. thuringiensis* in the insect is sequentially regulated by three RNPP regulators and their cognate peptides (20): first, PlcR-PapR regulates the virulence stage; next, NprR-NprX controls the necrotrophic stage and the transition to sporulation; and ultimately, Rap-Phr controls the initiation of the sporulation process.

AQ: A

The commitment to sporulation is regulated by the phosphorylation state of the major response regulator Spo0A (21, 22). Different signals, such as nutritional deprivation, are recognized by sporulation kinases, which are able to autophosphorylate (23). These kinases phosphorylate Spo0F, which is used as a substrate by the phosphotransferase Spo0B to phosphorylate Spo0A (24). Response regulator aspartate phosphatases (Rap) inhibit this signal transduction pathway by dephosphorylating the Spo0F-P response regulator (25). Rap protein activity is inhibited by its cognate Phr peptide, which is translated in a premature form that needs to be secreted, processed, and reimported by oligopeptide permeases to be active (26–28). The mature Phr peptides contain 5, 6, or 7 amino acids (12, 27, 29, 30).

A number of Rap-Phr systems are found in bacteria of the *B. cereus* group, particularly in *B. thuringiensis* plasmids, and mainly have an effect on sporulation (31, 32). The pXO1 pathogenicity plasmid from *B. anthracis* hosts a Rap-Phr system that regulates the sporulation process (11). A recent study revealed that plasmid Rap-Phr systems of *B. thuringiensis* strain Bt8741 regulate sporulation, biofilm formation, spreading, and extracellular proteolytic activity (33). Moreover, the Rap8-Phr8 system of pHT8_1, a cryptic plasmid of *B. thuringiensis* serovar kurstaki strain HD73, inhibits sporulation and biofilm formation (12). The effect of this system on sporulation was particularly evident in bacteria developing in insect larvae, the presumed ecological niche of *B. thuringiensis* (12). The serovar kurstaki strain HD73 harbors six other plasmids, among which pHT77 and pAW63 also carry *rap-phr* genes (31, 34). The Rap-Phr system from pHT77 does not affect sporulation, and its function remains unknown (31). The pAW63 plasmid harbors a Rap-Phr system (35) whose involvement in sporulation has been predicted *in silico* (31). pAW63 is a theta-replicating conjugative plasmid of 72 kb (36, 37) that efficiently conjugates and mobilizes nonconjugative plasmids in food matrices and under adverse conditions (38, 39). Furthermore, pAW63 shares a common backbone with the pXO2 pathogenic plasmid of *B. anthracis* and with plasmid pBT9727 from the pathogenic *B. thuringiensis* serovar konkukian strain 97-27, including replication and transfer modules (35).

In this study, we characterized the Rap63-Phr63 quorum-sensing system from the pAW63 plasmid. Our results show that it acts together with Rap8-Phr8 to synergistically regulate sporulation *in vitro* and *in vivo*. However, despite important Phr sequence similarities, no cross talk was detected between these closely related Rap-Phr systems, revealing a high specificity of the Phr peptides for their cognate Rap proteins. These

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results highlight the importance of plasmid-borne Rap-Phr systems in improving the fitness of the bacteria under naturalistic conditions.

(This research was conducted partly by P. Cardoso in partial fulfillment of the requirements for a doctoral degree from the Université Paris-Saclay, Jouy-en-Josas, France, and the Universidade Estadual de Londrina, Londrina, Brazil [40]).

RESULTS

Transcriptional analysis of the *rap* and *phr* genes. In *B. subtilis*, the *rap* and *phr* genes have been shown to form a transcriptional unit with a promoter located upstream of the *rap* gene (27). As generally described for these genes, the *phr63* gene overlaps the *rap63* gene by 1 bp. To determine whether the *rap63* and *phr63* genes are cotranscribed, reverse transcription-PCR (RT-PCR) was performed on RNA extracted 3 h after the onset of the stationary phase in a sporulation medium (HCT). The result demonstrates that the *rap63* and *phr63* genes are cotranscribed from a promoter located upstream of the *rap63* gene and that these two genes form a transcriptional unit (Fig. 1A and B).

To monitor the expression of the *rap63-phr63* operon, a 659-bp DNA fragment upstream of the *rap63* gene was fused to the *lacZ* reporter gene on the low-copy-number plasmid pHT304-18Z. In the wild-type HD73 strain, transcription from this promoter region (P_{rap63}) started 2 h after the onset of the stationary phase (t2), increased sharply, and reached a maximum at t4 (Fig. 1C). In the *sigH* mutant strain, the expression level was higher and started earlier (t1) than in the wild-type strain. In the *spo0A* mutant strain, expression started at t2 but with a slope slightly lower than that of the wild-type strain. These results demonstrate that expression of the *rap63-phr63* operon is slightly activated by Spo0A and repressed by the sigma factor SigH. No Spo0A boxes and no SigH consensus sequences were found in the P_{rap63} promoter region, suggesting an indirect effect of Spo0A and SigH on the transcription of the *rap63-phr63* operon.

The transcription of *phr* genes is often regulated by an additional specific promoter situated upstream of the *phr* gene and inside the *rap* gene, and generally controlled by the alternative sigma factor sigma H (σ^H, or SigH) (27, 41). A 562-bp DNA region (P_{phr63}) upstream of *phr63* was cloned into plasmid pHT304-18Z in order to determine whether this gene is transcribed from such a specific promoter. In the wild-type HD73 strain, expression from P_{phr63} started during late-exponential growth and was activated from t2 to t4 (Fig. 1D). In the HD73 Δ*sigH* and Δ*spo0A* mutant strains, $P_{phr63'}$-*lacZ* expression was significantly reduced, and the activation observed at t2 in the wild-type strain was lacking. Sequences corresponding to a −35 (GCAGGAATT) and a degenerated -10 (AAAGAAG) SigH consensus box (42) were identified in the P_{phr63} promoter region. These sequences, predicted to be recognized by SigH, are located 142 bp upstream of the start codon of the *phr* gene. These results demonstrate that the *phr63* gene is also transcribed from a specific promoter that might be regulated by the SigH factor. The highly SigH-dependent expression of *phr63* may boost the production of signaling peptides to repress Rap63 activity in stationary phase. However, $P_{phr63'}$-*lacZ* expression was not abolished in the HD73 Δ*sigH* mutant strain (Fig. 1D), indicating that *phr63* can also be transcribed during stationary phase from a SigH-independent promoter.

Rap63 negatively affects sporulation. Our previous *in silico* analyses of Rap-Phr systems from the *B. cereus* group predicted that Rap63 could affect sporulation (31). To verify this prediction, *rap63*, *rap63-phr63*, and *phr63* genes were introduced into plasmid pHT315xyl (43), a multicopy plasmid in which the expression of the cloned genes is under the regulation of a xylose-inducible promoter (P_{xylA}). These constructions were transformed into the *B. thuringiensis* HD73 wild-type strain. The control strain, bearing the empty pHT315xyl plasmid, sporulated efficiently (82% of spores) after 48 h at 30°C in HCT medium supplemented with xylose (Fig. 2; see also Table S1 in the supplemental material). In sharp contrast, the strain expressing the *rap63* gene presented a reduced sporulation efficiency (38% versus 82%) and a 5-fold reduction in the production of heat-resistant spores (5.80E+07 versus 3.25E+08 in the control

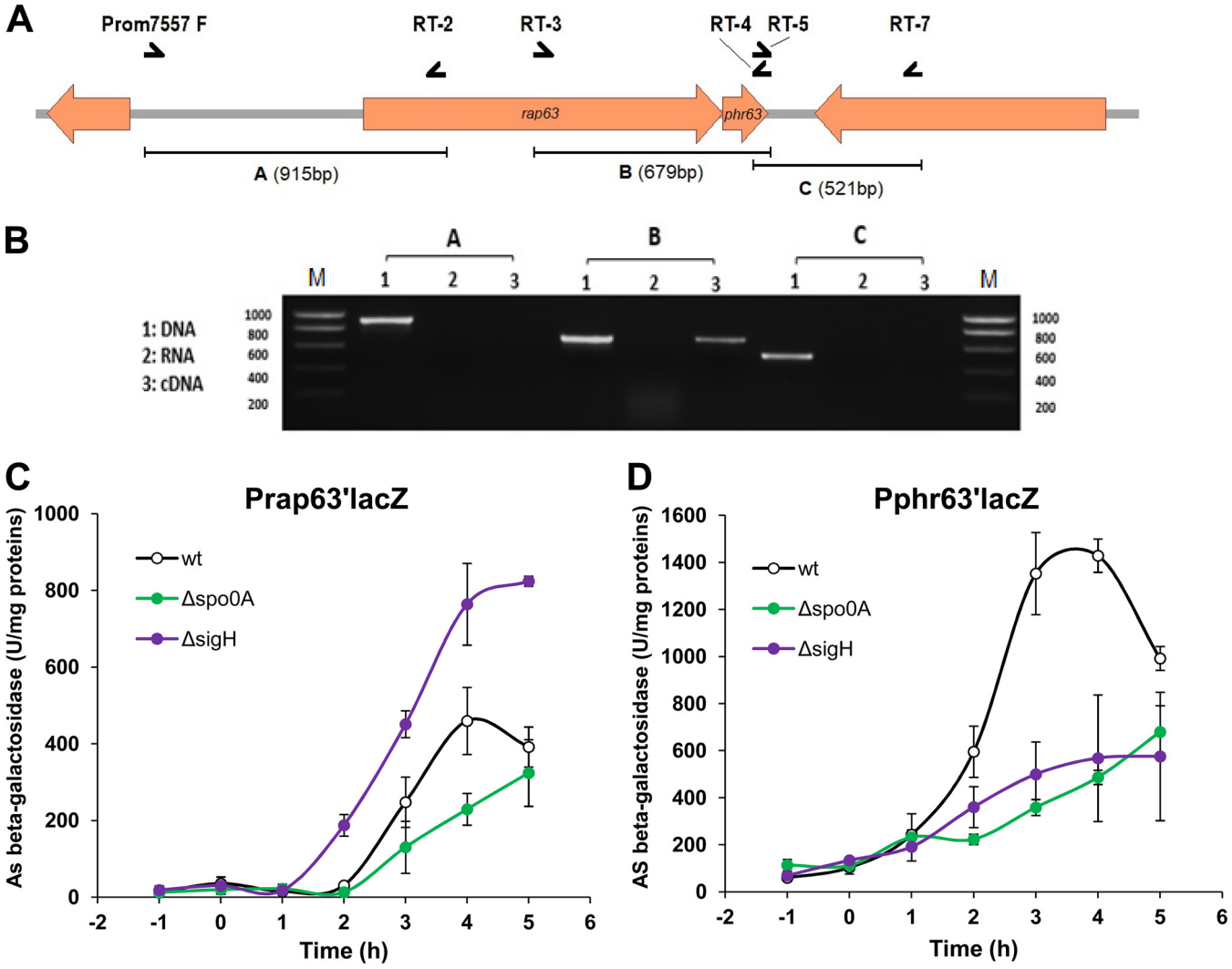

FIG 1 The *rap63-phr63* transcription unit. (A) Schematic representation of the *rap63-phr63* locus in the pAW63 plasmid. Arrowheads correspond to the primers used to amplify the three RT-PCR fragments (fragments A, B, and C). (B) RT-PCR experiment. Total RNA was extracted from a t3 culture in HCT medium at 37°C. Genomic DNA (1), RNA (2), and cDNA (3) were used as templates for PCR amplification, analyzed in a 1% agarose gel, and compared to molecular weight markers (M) (SmartLadder small fragments; Eurogentec). (C) Kinetics of *rap63-phr63* expression. Shown are the β-galactosidase activities of the HD73 wild-type (wt), Δ*spo0A*, and Δ*sigH* strains carrying $P_{rap63'}$-*lacZ* in HCT medium at 37°C. (D) Kinetics of *phr63* expression. Shown are the β-galactosidase activities of the HD73 wild-type, Δ*spo0A*, and Δ*sigH* strains carrying $P_{phr63'}$-*lacZ* in HCT medium at 37°C. Time zero corresponds to the entry into stationary phase. Error bars represent the standard errors of the means.

strain). This greater reduction is not due to an effect of *rap63* overexpression on growth (Fig. S1) but reflects the low viability of the bacteria that do not sporulate. The strains expressing the *rap63-phr63* or *phr63* gene presented sporulation rates similar to that of the control strain (84% and 85%, respectively). Therefore, these results confirm that Rap63 inhibits sporulation moderately and that Rap63 activity is counteracted by its cognate Phr63 peptide.

Rap63 delays the expression of Spo0A-regulated genes. Rap proteins prevent the phosphorylation of Spo0A, and the phosphorylated form of Spo0A (Spo0A-P) activates the expression of several genes related to sporulation, such as the *spoIIE* gene, which is transcribed at the onset of stationary phase in *B. subtilis* (21) and *B. thuringiensis* (44). This gene can therefore be used as a reporter to measure the presence of Spo0A-P in the bacterial cell. The promoter region of *spoIIE* (P_{spoIIE}) was fused to the *yfp* fluorescent reporter gene (encoding yellow fluorescent protein [YFP]) and inserted into the pHT315xylΩ*rap63* and pHT315xylΩ*rap63-phr63* plasmids to yield plasmids pHT315xylΩ*rap63*_$P_{spoIIE'}$-*yfp* and pHT315xylΩ*rap63-phr63*_$P_{spoIIE'}$-*yfp*, respectively

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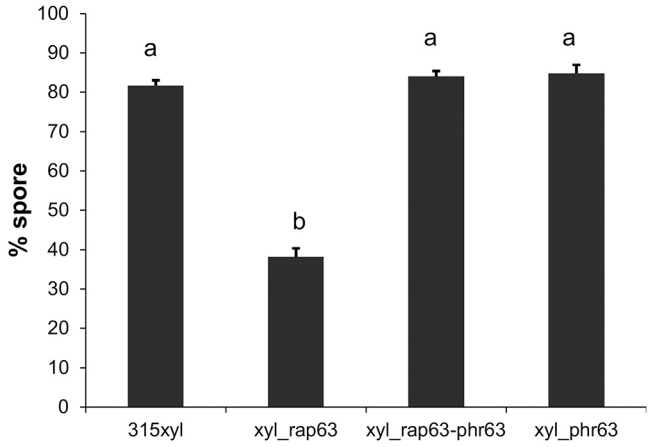

FIG 2 Rap63 negatively affects sporulation. Sporulation efficiency was measured in the HD73 control strain (315xyl) and in HD73 strains expressing *rap63* (xyl_*rap63*), *rap-phr63* (xyl_*rap63-phr63*), or *phr63* (xyl_*phr63*) in HCT medium at 30°C. After 48 h of growth, the percentage of spores was calculated as 100 multiplied by the ratio between the number of heat-resistant spores per milliliter and the number of total viable cells per milliliter. Error bars represent standard errors of the means. Experimental values are detailed in Table S3. The letters "a" and "b" above the bars indicate significant differences in mean values ($P < 0.001$).

(Table 1). In the control strain (harboring plasmid pHT315xyl_P$_{spoIIE'}$-*yfp*), the transcription of *spoIIE* started 2 h after the onset of the stationary phase (Fig. 3). In the strain harboring the *rap63* gene, expression from P$_{spoIIE}$ was strongly delayed, beginning around t5. When both Rap63 and Phr63 were produced, expression from P$_{spoIIE}$ was restored to the same kinetics and level as in the control strain. These results demonstrate that Rap63 delays the expression of Spo0A-regulated genes and that Phr63 inhibits Rap63 activity. This is in accordance with the sporulation experiments, suggesting that the moderate effect of Rap63 on the control of the sporulation process might be due to the delayed transcription of Spo0A-regulated genes.

The Δ*phr8* Δ*phr63* double mutation negatively affects the commitment to sporulation. The complete infectious cycle—pathogenesis, necrotrophism, and sporulation—of *B. thuringiensis* in insect larvae has been shown to be controlled by quorum-sensing systems of the RNPP family (20). Hence, we analyzed the role of Rap63-Phr63 under such naturalistic conditions, in larvae of the lepidopteran insect *Galleria mellonella*. For this purpose, HD73 mutant strains with *rap63-phr63* or *phr63* deletions were constructed, and we also used the Δ*rap8* Δ*phr8* and Δ*phr8* mutant strains constructed previously (12). A double *phr* mutant strain (Δ*phr8* Δ*phr63*) was also constructed to investigate the cumulative effect of the two Rap proteins in the absence of their cognate signaling peptides. The sporulation efficiency of each strain was evaluated in dead larvae 96 h after intrahemocoelic injection (Fig. 4A; Table S3), a mode of infection that bypasses the intestinal barrier and therefore does not require the presence of the Cry toxins (1). The wild-type strain (HD73) and the Δ*rap63* Δ*phr63*, Δ*phr63*, and Δ*rap8* Δ*phr8* mutant strains had similar sporulation efficiencies (22.3%, 24.5%, 16.7%, and 23.6% of spores, respectively). However, sporulation efficiency was significantly reduced in the Δ*phr8* mutant strain (3.1%) and to a greater extent in the Δ*phr8* Δ*phr63* double mutant strain (0.23%). The results obtained *in vitro* (Fig. 4B; Table S3), in HCT medium, showed that the Δ*rap63* Δ*phr63* and Δ*phr63* strains sporulated like the wild-type strain, whereas sporulation was slightly but significantly affected in the Δ*phr8* Δ*phr63* strain. Under both conditions, the effect on sporulation caused by the loss of the two *phr* genes was greater than the sum of the loss of the single *phr* genes. These results suggest a synergistic effect of the Rap8-Phr8 and Rap63-Phr63 systems to regulate the sporulation process in insect larvae.

Determination of the active form of Phr63. Rap proteins are inhibited by Phr oligopeptides, whose active form generally corresponds to the C-terminal end of the

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TABLE 1 Plasmid constructions used in this study

Plasmid	Description
pHT315xylΩ*rap63* (xyl_*rap63*)	The *rap63* gene was amplified using primer set Rap7557-F/Rap7557-R with *B. thuringiensis* HD73 genomic DNA as the template. The fragment was inserted between the BamHI and HindIII sites downstream of the inducible P_{xylA} promoter carried by plasmid pHT315xyl (42).
pHT315xylΩ*phr63* (xyl_*phr63*)	The *phr63* gene was amplified using primer set Phr7557-F/Phr7557-R with *B. thuringiensis* HD73 genomic DNA as the template. The fragment was inserted between the BamHI and HindIII sites of the pHT315xyl plasmid.
pHT315xylΩ*rap63-phr63* (xyl_*rap63-phr63*)	The *rap-phr63* genes were amplified using primer set Rap7557-F/Phr7557-R with *B. thuringiensis* HD73 genomic DNA as the template. The fragment was inserted between the BamHI and HindIII sites of the pHT315xyl plasmid.
pHT315xylΩ*rap63-phr63*_R3 (xyl_*rap63-phr63*_R3)	The *rap63* gene, together with a truncated *phr63* gene, was amplified using primer set Rap7557-F/Phr7557R3 with *B. thuringiensis* HD73 genomic DNA as the template. The fragment was inserted between the BamHI and HindIII sites of the pHT315xyl plasmid.
pHT304-18-$P_{rap63'}$-*lacZ*	Primers Prom7557-F and Prom7557-R were used to amplify the promoter region of the *rap63* gene, using *B. thuringiensis* HD73 genomic DNA as the template. The fragment of 659 bp was inserted between the HindIII and BamHI sites of pHT304-18*Z* (65).
pHT304-18-$P_{phr63'}$-*lacZ*	Primers Prom7557Phr-F and Prom7557Phr-R were used to amplify the promoter region of the *phr63* gene, using *B. thuringiensis* HD73 genomic DNA as the template. The fragment of 562 bp was inserted between the HindIII and BamHI sites of pHT304-18Z.
pHT315xyl-$P_{spoIIE'}$-*yfp*	Plasmid described by Fazion and colleagues (12)
pHT315xylΩ*rap63*_$P_{spoIIE'}$-*yfp*	The $P_{spoIIE'}$-*yfp* fragment was amplified using primer set PU-*Eco*RI/YFP-R with the pHT315xyl-$P_{spoIIE'}$-*yfp* plasmid as the template, and the fragment was inserted into the EcoRI site of the pHT315xylΩ*rap63* plasmid. To avoid the influence of the P_{xylA} promoter, the orientation of the inserted fragment was verified by PCR using primer set PspoIIE-F/xylRout3′.
pHT315xylΩ*rap63-phr63*_$P_{spoIIE'}$-*yfp*	The $P_{spoIIE'}$-*yfp* fragment was amplified using primer set PU-*Eco*RI/YFP-R with the pHT315xyl-$P_{spoIIE'}$-*yfp* plasmid as the template, and the fragment was inserted into the EcoRI site of the pHT315xylΩ*rap63-phr63* plasmid. To avoid the influence of the P_{xylA} promoter, the orientation of the inserted fragment was verified by PCR using primer set PspoIIE-F/xylRout3′.
pMADΩ*rap63-phr63*::*spec*	The 5′ and 3′ regions of *rap63-phr63* genes were amplified using primer sets 7557Amont1-F/7557Amont1-R and 7557Aval-F/Aval7557-R, respectively, with *B. thuringiensis* HD73 genomic DNA as the template. The 5′ end was purified as an NcoI/KpnI fragment and the 3′ end as an XbaI/EcoRI fragment. The spectinomycin resistance gene was purified as a KpnI/XbaI fragment from pUC18Ω*spec* (laboratory stock) and was inserted together with the 5′ and 3′ regions of *rap63-phr63* between the NcoI and EcoRI sites of the thermosensitive plasmid pMAD (66).
pMADΩ*phr63*::*spec*	The 5′ and 3′ regions of *phr63* genes were amplified using primer sets 7557Amont2-F/7557Amont2-R and 7557Aval-F/Aval7557-R, respectively, with *B. thuringiensis* HD73 genomic DNA as the template. The 5′ end was purified as an NcoI/KpnI fragment and the 3′ end as an XbaI/EcoRI fragment. The spectinomycin resistance cassette was purified as a KpnI/XbaI fragment from pUC18Ω*spec* and was inserted together with the 5′ and 3′ regions of *phr63* between the NcoI and EcoRI sites of pMAD.
xyl_*rap8*	Plasmid pHT-*xyl*R from the work of Fazion and colleagues (12)
xyl_*rap8-phr8*	pHT-*xyl*RP from the work of Fazion and colleagues (12)

Phr peptide (27). To determine whether the mature form of Phr63 corresponds to the C-terminal end of the propeptide, we constructed a plasmid carrying the *rap63* gene and a 3′-end-truncated *phr* gene (xyl_*rap63-phr63*_R3′) expressing a Phr peptide lacking the six C-terminal amino acids (Fig. 5A). The sporulation efficiency of the strain harboring this plasmid (32%) was similar to that of the strain expressing the *rap63* gene (Fig. 5B; Table S3). This result suggests that the active form of Phr63 is included in the C-terminal part of the premature Phr.

To define the Phr63 active form, various Phr63 peptides corresponding to the C-terminal end were synthesized: GETI (Phr63-4), HGETI (Phr63-5), AHGETI (Phr63-6), YAHGETI (Phr63-7), and QYAHGETI (Phr63-8) (Fig. 5A). To evaluate the abilities of these synthetic peptides to inhibit Rap63 activity, they were separately added to the culture medium of the strain expressing the *rap63* gene (xyl_*rap63*). Phr63-4 was not able to inhibit Rap63 activity (30% of spores), while Phr63-5 (77%), Phr63-6 (84%), Phr63-7 (87%), and Phr63-8 (87%) efficiently counteracted the effect of Rap63 on sporulation

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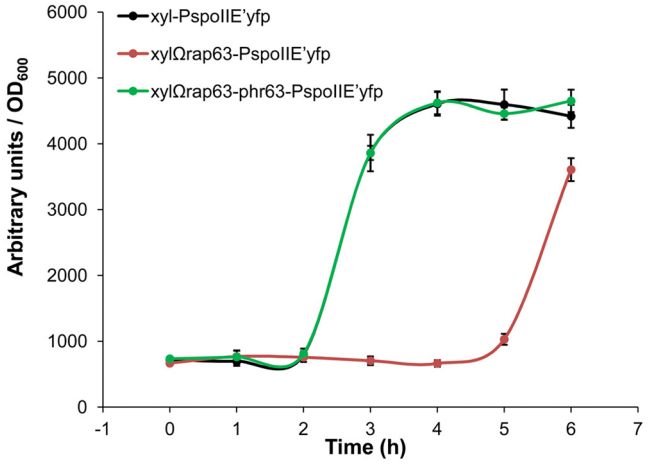

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FIG 3 Rap63 delays the expression of a Spo0A-regulated gene. Shown is the kinetics of *spoIIE* expression in HD73 wild-type strains carrying the pHT315xyl_P$_{spoIIE'}$-*yfp*, pHT315xylΩ*rap63*_P$_{spoIIE'}$-*yfp*, or pHT315xylΩ*rap63-phr63*_P$_{spoIIE'}$-*yfp* plasmid. YFP fluorescence was measured during growth in HCT medium at 30°C in the presence of 20 mM xylose added at t0 (entry into stationary phase). The results are expressed in arbitrary units per OD_{600} unit.

(Fig. 5B; Table S3). These results demonstrated that the minimal active form of Phr63 is the pentapeptide HGETI.

Specificity of the Rap-Phr63 and Rap-Phr8 systems. The C-terminal parts of the Phr8 and Phr63 peptides, which include the mature peptides, are closely related (Fig. 6A), with only 2 divergent residues among the last 8 amino acids. Due to these sequence similarities and the sporulation results of the Δ*phr8* Δ*phr63* mutant strain compared to those of the Δ*phr8* and Δ*phr63* single mutant strains, we investigated the possibility of cross talk between the Rap8-Phr8 and Rap63-Phr63 systems. We constructed the Δ*rap8* Δ*phr8* Δ*rap63* Δ*phr63* mutant strain to avoid the effect of intrinsic systems. This mutant strain was transformed with the pHT315xyl (control strain), pHT315xylΩ*rap8*, or pHT315xylΩ*rap63* plasmid, and the sporulation of the resultant strains (in whose designations the Δ*rap8* Δ*phr8* Δ*rap63* Δ*phr63* mutant is indicated by ΔΔ) was assessed (Fig. 6B; Table S3). The control strain (ΔΔ 315xyl) sporulated efficiently (84% of spores). The ΔΔ xyl-*rap63* mutant strain, expressing *rap63*, presented reduced sporulation efficiency (32%), like the wild-type strain expressing *rap63* (Fig. 2). The addition of the Phr63-7 peptide restored sporulation efficiency to a level similar to that of the control strain (Fig. 6B). However, in the presence of Phr8-6, Phr8-7, or Phr8-8, sporulation was similar to that of the strain expressing *rap63* alone, indicating that none of the Phr8 peptides used was able to counteract the negative effect of Rap63 on sporulation. The sporulation of the ΔΔ xyl-*rap8* strain, expressing *rap8*, was strongly reduced, and addition of the Phr8-7 peptide inhibited Rap8 activity (Fig. 6B), as already described for the wild-type strain (12). In contrast, none of the Phr63 peptides was able to counteract the negative effect of Rap8 on sporulation (Fig. 6B). Taken together, these results showed that despite the sequence similarities between Phr8 and Phr63, there is no cross talk between the Rap8-Phr8 and Rap63-Phr63 systems.

DISCUSSION

The complete genome sequence of the serovar kurstaki HD73 Cry^+ strain (34) includes seven plasmids (pHT7, pHT8_1, pHT8_2, pHT11, pAW63, pHT73, pHT77). Among these, three harbor a Rap-Phr system (pHT8_1, pAW63, and pHT77). The Rap8-Phr8 quorum-sensing system from pHT8_1 is involved in biofilm formation and in the regulation of sporulation (12), but in contrast, Rap-Phr from pHT77 does not affect sporulation, and its role remains unknown (31). In this study, we characterized the Rap63-Phr63 system carried by plasmid pAW63.

Our results show that in sporulation medium, the *rap63-phr63* operon is transcribed

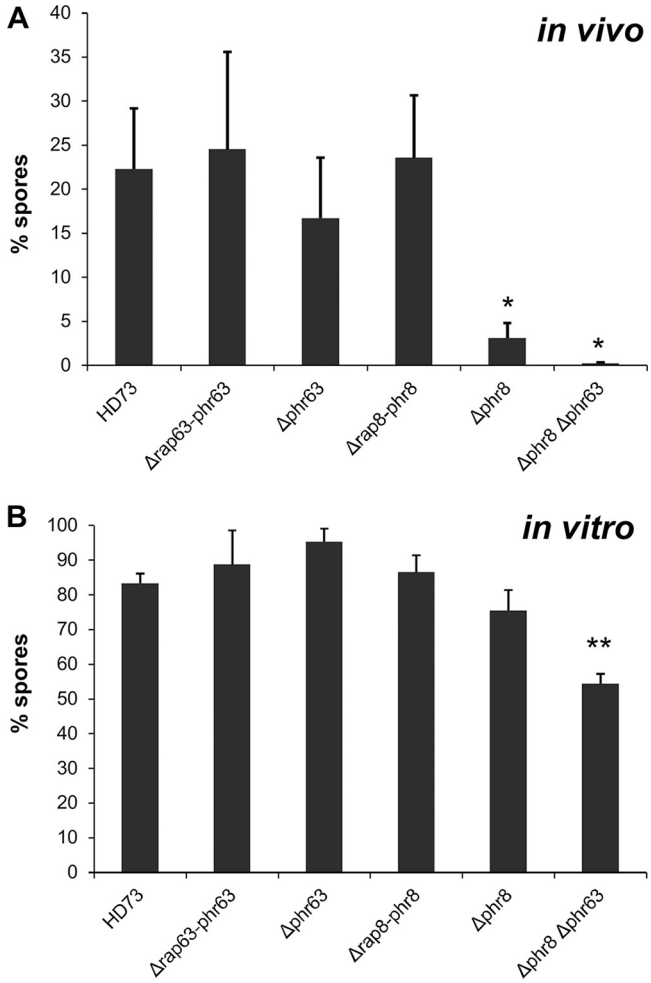

FIG 4 Synergistic activity of the Rap63-Phr63 and Rap8-Phr8 systems on sporulation. Shown are the sporulation efficiencies of the HD73 wild-type strain and of Δ*rap63* Δ*phr63*, Δ*phr63*, Δ*rap8* Δ*phr8*, Δ*phr8*, and Δ*phr8* Δ*phr63* mutant strains. (A) In dead larvae of *G. mellonella* (*in vivo*). Viable cells and heat-resistant spores were counted in dead larvae 4 days postinfection at 30°C. (B) In HCT medium (*in vitro*), viable cells and heat-resistant spores were counted after 48 h of growth at 30°C. The percentages of spores were calculated as 100 multiplied by the ratio between the number of heat-resistant spores per milliliter and the number of total viable cells per milliliter. Error bars represent standard errors of the means. Experimental values are detailed in Table S3. Asterisks indicate significant differences from values for the wild type (*, $P < 0.05$; **, $P < 0.01$).

from a promoter activated during the stationary phase and partially repressed by SigH. The absence of a sequence resembling a SigH promoter upstream of the *rap63* gene suggests indirect repression by a SigH-dependent repressor. In contrast, the expression measured in the *spo0A* mutant strain is similar to that observed in the wild-type strain. In *B. subtilis*, SigH is produced in a *spo0A* mutant, but to a lesser extent than in a wild-type strain (45). Therefore, SigH could be sufficiently produced in the *B. thuringiensis spo0A* mutant strain to activate the expression of the repressor. The *phr63* gene is also transcribed separately from a SigH-dependent promoter located in the *rap63* coding sequence. This regulation is similar to the expression of *B. subtilis phr* genes, which are also activated by SigH (41). This additional expression of the *phr* genes boosts the production of the signaling peptides and allows bacteria to trigger sporulation by inhibiting the Rap proteins. Transcriptional analysis of pAW63 coding sequences was performed previously at the mid-exponential-growth phase by comparing the expression pattern of the wild-type serovar kurstaki HD73 Cry^+ strain with that of the same strain cured of the pAW63 plasmid (46). The results showed that the *rap63* gene was expressed at a moderate level, while the *phr63* gene was found at a high level.

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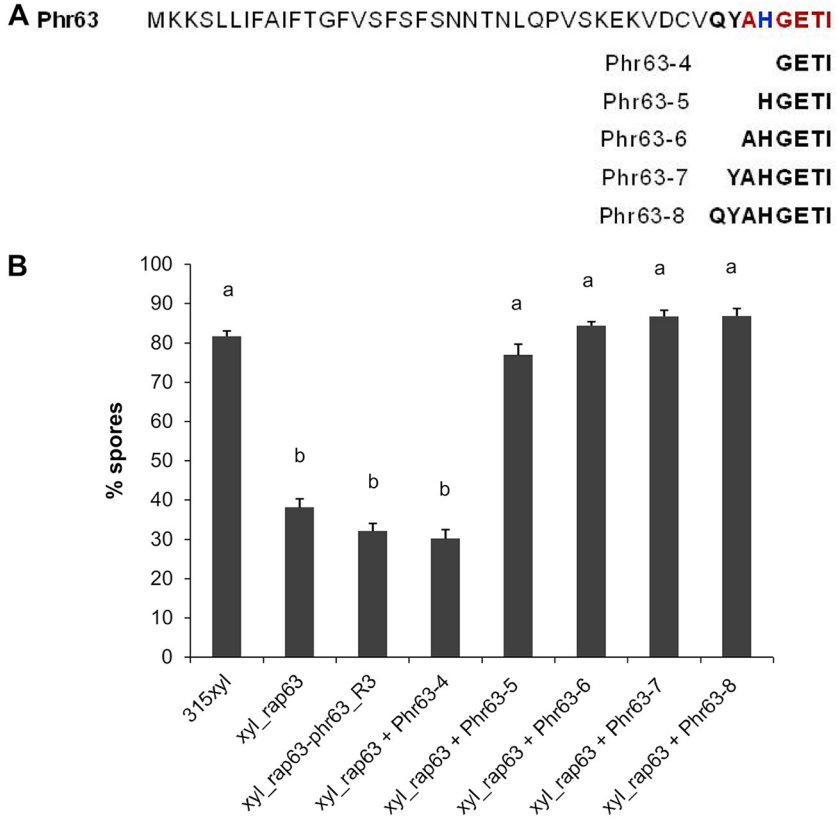

FIG 5 Characterization of the Phr63 active form. (A) Amino acid sequences of the pre-Phr63 peptide and the five synthetic peptides used in complementation experiments. The C-terminal end sequence truncated in xyl_*rap63-phr63*_R3 is shown in red, with the positively charged histidine residue in blue. (B) Sporulation efficiencies of the HD73 control strain (315xyl) and of HD73 expressing *rap63* (xyl-*rap63*) and *rap63-phr63* producing C-terminally truncated Phr63 (xyl_*rap63-phr63*_R3). The culture medium of the strain expressing *rap63* was supplemented with the synthetic peptide Phr63-4, Phr63-5, Phr63-6, Phr63-7, or Phr63-8 added independently 1 h after the onset of the stationary phase at a 50 μM final concentration. The percentages of spores were calculated as 100 multiplied by the ratio between the number of heat-resistant spores per milliliter and the number of total viable cells per milliliter after 48 h in HCT medium at 30°C in the presence of 20 mM xylose. Error bars represent standard errors of the means. Experimental values are detailed in Table S3. Different letters above the bars correspond to significant differences in values ($P < 0.001$).

However, the Phr signal was also present at a high level in the cured strain, suggesting that the probe used to detect the *phr63* gene was not specific and revealed the presence of another, related *phr* gene in the *B. thuringiensis* HD73 genome, possibly *phr8*. Both this previous transcriptional analysis and our results point out that the expression of *rap63-phr63* genes—and presumably that of other *rap-phr* genes—is modulated by variations in environmental conditions. For example, the *rap63* and *phr63* genes are expressed from the mid-exponential-growth phase in Luria-Bertani (LB) medium (46), whereas they are expressed from the beginning of the stationary-growth phase in the sporulation-specific medium HCT.

Strains belonging to the *B. cereus* group have, on average, six Rap-Phr systems (31, 32). Even if there are redundant regulatory operons among these systems, they might not act simultaneously, or they might be regulated by different signals (31–33). A comparison with the results obtained by Fazion and colleagues (12) revealed that the transcription of *rap63-phr63* genes occurs concomitantly with that of *rap8-phr8* genes from the pHT8_1 plasmid, under the same conditions. Both Rap proteins from *B. thuringiensis* plasmids are able to inhibit the sporulation process, but to different extents. While Rap8 strongly inhibits sporulation (12), Rap63 induces a moderate inhibition of sporulation but significantly delays the expression of the *spoIIE* gene

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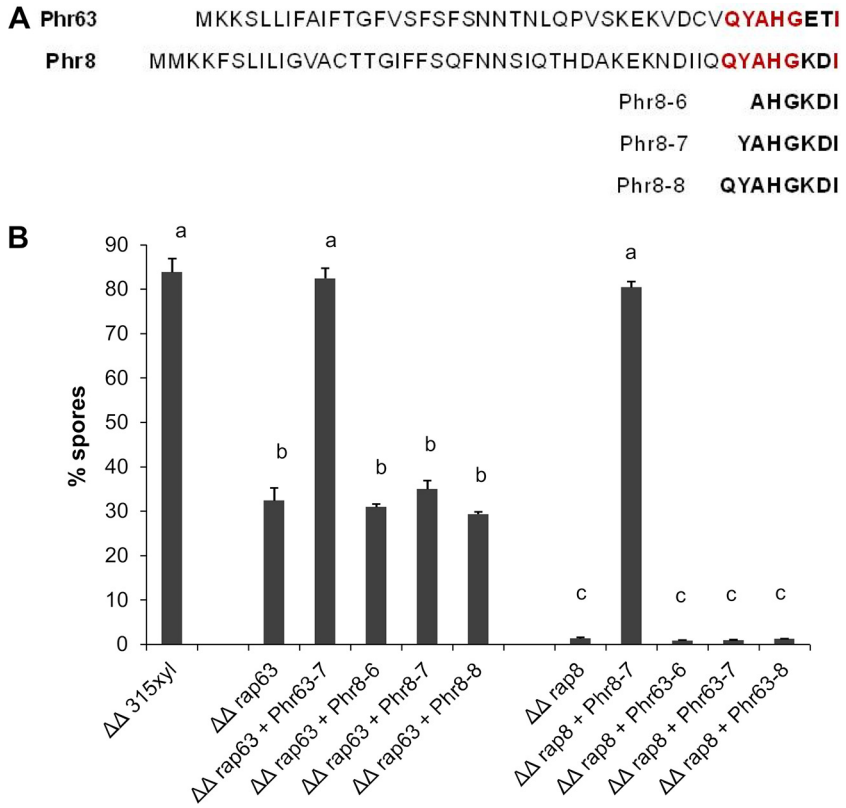

FIG 6 Evaluation of cross talk between the Rap-Phr8 and Rap-Phr63 systems. (A) Comparison of the amino acid sequences of the Phr63 and Phr8 peptides. C-terminal end similarities are highlighted in red, and the two divergent residues are shown in black boldface. Phr8-6, Phr8-7, and Phr8-8 correspond to the synthetic Phr8 peptides used by Fazion and colleagues (12). (B) Sporulation efficiency of the HD73 Δ*rap8* Δ*phr8* Δ*rap63* Δ*phr63* control strain (ΔΔ 315xyl) or of this strain expressing *rap63* (ΔΔ *rap63*) or *rap8* (ΔΔ *rap8*) in HCT medium at 30°C in the presence of 20 mM xylose. The culture medium of the strain expressing *rap63* was supplemented with the Phr8-6, Phr8-7, or Phr8-8 synthetic peptide, and the culture medium of the strain expressing *rap8* was supplemented with the Phr63-6, Phr63-7, or Phr63-8 synthetic peptide. Peptides were added independently 1 h after the onset of the stationary phase at 50 μM final concentrations. The percentages of spores were calculated as 100 multiplied by the ratio between the number of heat-resistant spores per milliliter and the number of total viable cells per milliliter. Error bars represent standard errors of the means. Experimental values are detailed in Table S3. Different letters above the bars correspond to significant differences in the values ($P < 0.001$).

controlled by Spo0A. Various reasons may explain these different levels of inhibition: the copy numbers of the plasmids carrying the *rap-phr* genes (see below), the levels of gene expression, and the intrinsic properties of the Rap proteins, including affinity with their substrate (i.e., Spo0F).

In agreement with the analysis of Even-Tov and colleagues, which predicted that the dominant repression feature of Rap proteins enables the accumulation of multiple quorum-sensing systems with a synergistic effect (47), the Rap63-Phr63 and Rap8-Phr8 systems act together to regulate the initiation of sporulation. In shaken HCT medium, only strains lacking both *phr* genes sporulated less efficiently than the wild-type HD73 strain. The synergistic effect of the two Rap-Phr systems was more prominent under *in vivo* conditions. Indeed, the sporulation of the Δ*phr63* mutant strain was not significantly reduced, but the deletion of the two *phr* genes in the Δ*phr8* Δ*phr63* mutant strain strongly impaired the commitment to sporulation *in vivo*. In insect larvae, the sporulation efficiency of the Δ*phr8* strain was lower than that of the HD73 strain (about 8-fold), while in the double mutant strain, sporulation efficiency dropped by a factor of 100, highlighting a synergistic effect of the two quorum-sensing systems. We suggest that this apparent synergy of the two Rap-Phr systems depends on the cumulative effect of each Rap protein on the phosphorylation of Spo0A. Thus, the combined action

zam-aem/zam01820/zam9989d20z | xppws | S=5 | 7/24/20 | 13:46 | 4/Color Fig: 1,3,5,6 | ArtID: 01238-20 | DOI:10.1128/AEM.01238-20 | CE: CNV-jmm

of Rap8 and Rap63 reduces the concentration of Spo0A-P below a threshold that has a greater impact on sporulation than that expected on the basis of the independent action of each Rap. As a result, in the absence of the two signaling peptides Phr8 and Phr63, Rap8 and Rap63 appear to act synergistically.

The results of the concomitant activity of the two Rap-Phr systems raise two main questions about the difference between Rap8 and Rap63 activities and between results obtained under *in vitro* and *in vivo* conditions. First, the copy numbers of the plasmids carrying these *rap-phr* genes are different and should impact their levels of expression in bacterial cells. The copy number of pHT8_1 (75 per chromosome) is certainly much higher than that of the low-copy-number plasmid pAW63 (7, 36). Thus, more Rap8 than Rap63 is likely produced, which could explain the dominant effect of Rap8 on the sporulation of the bacteria. This might be further investigated by Western blot analysis using antibodies targeting Rap8 and Rap63. Moreover, the HD73 strain harbors six other Rap-Phr systems, two of which are predicted to have effects on sporulation (31). The sporulation activities of these two systems could mask the moderate Rap63 activity, or the Rap63 activity could be inhibited by a chromosomal Phr peptide. Second, not all cells in a bacterial population sporulate, as previously reported for *B. subtilis* (48) and *B. thuringiensis* (49, 50). This is partly due to the heterogeneous activation pattern of the master sporulation regulator Spo0A (51). In *B. thuringiensis* populations, it has been shown that sporulation efficiency and the differentiation pathway differ according to environmental conditions (49, 50). In HCT medium, an optimized medium for *B. thuringiensis* sporulation, the transition phase between the exponential phase and sporulation is reduced from that in LB medium or in insects, the bacterial population is homogeneous, and sporulation is triggered synchronously (52). In sharp contrast, a high degree of heterogeneity was observed in a complex culture medium, such as LB medium, or in insect cadavers (50). Moreover, in insect larvae, the distribution of Phr peptides should be less homogeneous than that in shaken cultures, accentuating the heterogeneity of the bacterial population and preventing synchronous entry into sporulation. Taken together, these reasons could explain the difference in sporulation efficiency observed between *in vitro* and *in vivo* experiments.

Rap proteins are inhibited by their cognate Phr oligopeptides (30), and the mature signaling peptide generally corresponds to the C-terminal end of the Phr peptide sequence and contains a positively charged residue (27, 53). In accordance, we showed that the expression of the *phr63* gene prevents the negative effect of Rap63 on sporulation (Fig. 2). Furthermore, we demonstrated that the active form of Phr63 is part of the C-terminal end and that its minimal form is a pentapeptide. However, the exact physiological size of the mature Phr63 was not determined. Indeed, synthetic oligopeptides Phr63-5, Phr63-6, Phr63-7, and Phr63-8 successfully inhibited the activity of Rap63 on sporulation. This suggests that Rap63 could be more versatile for binding Phr63 than Rap8, which is effectively inhibited only by the heptapeptide Phr8 (12). In *B. subtilis*, the mature Phr signaling peptides are penta- or hexapeptides (27, 29), while mature peptides of the RNPP regulators in *B. cereus* group bacteria are commonly heptapeptides, such as NprX (54), PapR (55), and Phr8 (12).

The C-terminal ends of Phr8 and Phr63 present high sequence similarity, with six of eight amino acids identical, including a histidine residue (positively charged) at position 4 of the octapeptides [QYAHG(E/K)(T/D)I]. Only the residues in positions 6 and 7 are different between Phr8 and Phr63 (Fig. 6A). Previous studies demonstrated that residues located at similar positions in PapR are critical for the interaction with PlcR and for activation of the PlcR regulon (55–58). In contrast, amino acid variations at similar positions in NprX do not affect the affinity of the peptide for NprR or the activation of the NprR regulon (44, 54, 59). Based on the relationship between NprX and the peptides of the Phr family (27), we hypothesized possible cross talk between the Rap63-Phr63 and Rap8-Phr8 plasmid systems. Our results show that Phr8 is not able to inhibit Rap63 and that Phr63 cannot prevent Rap8 activity. Even-Tov and colleagues (32) revealed high orthogonality between Rap and Phr; that is, a given Rap protein is inhibited primarily by its cognate Phr peptide. Indeed, no cross talk was observed among a large

number of Rap-Phr systems from the *B. subtilis* group species, and the absence of cross talk among RapA-PhrA, RapC-PhrC, and RapE-PhrE has been demonstrated (30, 60). As with these quorum-sensing systems, the absence of cross talk between Rap8-Phr8 and Rap63-Phr63 highlights the specificity of the Rap and Phr interactions, supporting the hypothesis of a coevolution of the Rap-Phr system components (17). In the other two *B. thuringiensis* RNPP systems (PlcR-PapR and NprR-NprX), cross-activation has been reported between different pherotypes of the same quorum-sensing system, but not between different quorum-sensing systems within a given strain (54–56). However, a notable exception has been observed recently with the *B. thuringiensis* PlcRa regulator, a structural paralog displaying 29% sequence identity with PlcR (61). This RNPP regulator, involved in the oxidative stress response and cysteine metabolism, is activated during stationary phase by PapR, the cognate signaling peptide of PlcR, suggesting a high and unexpected versatility of PlcRa (62).

The RNPP quorum-sensing systems are largely reported to play a role in the tight control of physiological and developmental processes of *B. cereus* group bacteria in response to environmental changes (20, 49, 50, 52, 63, 64). Taken together, our results reinforce the role of Rap-Phr systems in this fine-tuned regulation. Concomitant activity of different Rap-Phr systems allows the bacteria to better adapt their developmental processes to various environmental conditions.

Horizontal gene transfer can increase the variability of the genetic set of a bacterial species, allowing the bacteria to better adapt to environmental changes. The *B. thuringiensis* plasmids had been widely studied, mainly due to the *cry* and *cyt* genes coding for the insecticidal toxins. However, *B. thuringiensis* strains harbor a wide range of plasmids, many of which contain Rap-Phr systems predicted to regulate sporulation (31). In the present study, the activities of two plasmid-borne Rap-Phr systems have been tested in the same strain, and we show that Rap8-Phr8 and Rap63-Phr63 work synergistically to control the commitment to sporulation in insect larvae. Since *B. thuringiensis* is an invertebrate pathogen, it is expected that this species will have a rich repertoire of mechanisms that facilitate its development and survival in insects. Our results reinforce the relevance of plasmid Rap-Phr quorum-sensing systems and highlight the fact that plasmids other than the *cry*-harboring plasmids play important roles in *B. thuringiensis* infectious-cycle regulation and bacterial survival.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All strains used in this study were derived from the *B. thuringiensis* serovar kurstaki HD73 acrystalliferous (Cry$^-$; cured of the pHT73 plasmid) strain (36). *Escherichia coli* K-12 strain TG1 was used as the host strain for plasmid construction. *E. coli* strain ET12567 (Dam$^-$ Dcm$^-$) was used to prepare plasmids to transform *B. thuringiensis* strains by electroporation (65). *E. coli* strains were transformed by thermal shock and were cultivated in Luria-Bertani (LB) medium at 37°C. *B. thuringiensis* strains were grown in LB medium or in the sporulation-specific medium HCT (5) at 30°C or 37°C. Liquid cultures were performed with shaking at 175 rpm. For bacterial selection, antibiotics were used at the following concentrations: 100 μg/ml ampicillin and 50 μg/ml spectinomycin for *E. coli* and 10 μg/ml erythromycin, 200 μg/ml spectinomycin, and 200 μg/ml kanamycin for *B. thuringiensis*. LB plates with 100 μg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) were used for LacZ colorimetric screening during pMAD mutagenesis. When required, xylose was used at a concentration of 20 mM. The strains used in this study are described in Table 2. T2

Plasmid and strain constructions. The GenBank accession number of plasmid pAW63 is CP004072.1. The *rap63-phr63* locus is located between nucleotides 51638 and 53496. Rap63 corresponds to NCBI Protein accession number AGE81704.1, and Phr63 corresponds to NCBI Protein accession number AGE81705.1.

Plasmid pHT315xyl, a multicopy vector with a xylose-inducible promoter (43), was used to express *rap63* and/or *phr63*. Promoter regions of *rap63* or *phr63* genes were inserted into plasmid pHT304-18*Z* (66) in order to determine their expression kinetics. Plasmid pMAD (67) was used for gene disruption by homologous recombination.

The plasmids used in this study are described in Table 1.

DNA manipulations. Genomic DNA from *B. thuringiensis* strains was extracted using the Puregene Yeast/Bact. kit (Qiagen, France), and plasmid DNA from *E. coli* was extracted with the QIAprep Spin Miniprep kit (Qiagen, France). Phusion High-Fidelity DNA polymerase, standard *Taq* DNA polymerase, restriction enzymes, and T4 DNA ligase were used according to the manufacturer's recommendations

TABLE 2 Strains used in this study

Strain	Description	Source or reference
HD73	*B. thuringiensis* serovar kurstaki HD73 Cry$^-$, cured of the pHT73 plasmid. This strain was designated as the wild-type strain and was used to construct all the other strains described below.	36
HD73($P_{rap63'}$-*lacZ*)	HD73 strain carrying the transcription fusion plasmid pHT304-18_$P_{rap63'}$-*lacZ*	This study
HD73 Δ*spo0A*($P_{rap63'}$-*lacZ*)	HD73 *spo0A*-deficient strain (70) carrying plasmid pHT304-18_$P_{rap63'}$-*lacZ*	This study
HD73 Δ*sigH*($P_{rap63'}$-*lacZ*)	HD73 *sigH*-deficient strain (71) carrying plasmid pHT304-18_$P_{rap63'}$-*lacZ*	This study
HD73($P_{phr63'}$-*lacZ*)	HD73 strain carrying the transcription fusion plasmid pHT304-18_$P_{phr63'}$-*lacZ*	This study
HD73 Δ*spo0A*($P_{phr63'}$-*lacZ*)	HD73 *spo0A*-deficient strain (70) carrying plasmid pHT304-18_$P_{phr63'}$-*lacZ*	This study
HD73 Δ*sigH*($P_{phr63'}$-*lacZ*)	HD73 *sigH*-deficient strain (71) carrying plasmid pHT304-18_$P_{phr63'}$-*lacZ*	This study
HD73 315xyl	HD73 strain harboring the empty plasmid pHT315xyl carrying the xylose-inducible promoter P_{xylA}	12
HD73 xyl-*rap63*	HD73 strain harboring plasmid pHT315xylΩ*rap63* and expressing *rap63* from P_{xylA}	This study
HD73 xyl-*phr63*	HD73 strain harboring plasmid pHT315xylΩ*phr63* and expressing *phr63* from P_{xylA}	This study
HD73 xyl-*rap63-phr63*	HD73 strain harboring plasmid pHT315xylΩ*rap63-phr63* and expressing *rap63* and *phr63* from P_{xylA}	This study
HD73 xyl-*rap63-phr63*_R3	HD73 strain harboring plasmid pHT315xylΩ*rap63-phr63*–R3 and expressing *rap63* and truncated *phr63* from P_{xylA}	This study
HD73 xyl_$P_{spoIIE'}$-*yfp*	HD73 strain carrying the transcription fusion plasmid pHT315xyl_$P_{spoIIE'}$-*yfp*	12
HD73 xylΩ*rap63*_$P_{spoIIE'}$-*yfp*	HD73 strain carrying the transcription fusion plasmid pHT315xylΩ*rap63*_$P_{spoIIE'}$-*yfp* expressing *rap63*	This study
HD73 xylΩ*rap63-phr63*_$P_{spoIIE'}$-yfp	HD73 strain carrying the transcription fusion plasmid pHT315xylΩ*rap63-phr63*_$P_{spoIIE'}$-*yfp* expressing *rap63* and *phr63*	This study
HD73 Δ*rap63* Δ*phr63*	HD73 strain with *rap63* and *phr63* deleted	This study
HD73 Δ*phr63*	HD73 strain with *phr63* deleted, constructed using plasmid pMADΩ*phr63*::*spec*	This study
HD73 Δ*rap8* Δ*phr8*	HD73 strain with *rap8* and *phr8* deleted	12
HD73 Δ*phr8*	HD73 strain with *phr8* deleted	12
HD73 Δ*phr8* Δ*phr63*	HD73 strain with *phr8* and *phr63* deleted	This study
HD73 ΔΔ	HD73 strain with the *rap8-phr8* and *rap63-phr63* genes deleted	This study
HD73 ΔΔ 315xyl	HD73 ΔΔ strain carrying the empty plasmid pHT315xyl	This study
HD73 ΔΔ xyl-*rap63*	HD73 ΔΔ strain harboring plasmid pHT315xylΩ*rap63* expressing Rap63 from P_{xylA}	This study
HD73 ΔΔ xyl-*rap63-phr63*	HD73 ΔΔ strain harboring plasmid pHT315xylΩ*rap63-phr63* expressing Rap63 and Phr63 from P_{xylA}	This study
HD73 ΔΔ xyl-*rap8*	HD73 ΔΔ strain harboring plasmid pHT315xylΩ*rap8* expressing Rap8 from P_{xylA}	This study

(New England Biolabs, USA). PCRs were performed in an Applied Biosystems 2720 thermal cycler using the oligonucleotides listed in Table 3, synthesized by Eurofins Genomics (Germany). The amplified DNA fragments were purified using a QIAquick PCR purification kit (Qiagen, France), and the QIAquick gel extraction kit (Qiagen, France) was used to purify digested DNA fragments separated on 1% agarose gels. All constructs were verified by DNA sequencing (GATC Biotech, Germany).

RT-PCR experiments. Growth conditions, RNA extraction, and reverse transcriptase reactions were performed as described elsewhere (12). Three different fragments were amplified by PCRs with oligonucleotides PromRapF7557 and RT7557-2 for the *rap63* gene and its upstream region, with RT7557-3 and RT7557-4 for the *rap63* and *phr63* genes, and with RT7557-5 and RT7557-7 for the *phr63* gene and its downstream region. The sequences of the oligonucleotides are given in Table 3.

***β*-Galactosidase assays.** Expression from the P_{rap63} and P_{phr63} promoter regions was analyzed by measuring β-galactosidase activity. Strains containing plasmids pHT304-18_$P_{rap63'}$-*lacZ* and pHT304-18_$P_{phr63'}$-*lacZ* were grown in HCT medium at 37°C with shaking at 175 rpm. The assays were performed as described previously (54). Specific activities are expressed in β-galactosidase units per milligram of protein. The assays were independently repeated three times.

Fluorescence analysis. YFP fluorescence produced from the strain carrying plasmid pHT315xyl_$P_{spoIIE'}$-*yfp* was measured from bacterial cultures grown in HCT medium at 37°C. Cells were harvested at determined time points and were fixed as described elsewhere (68). Cultures were supplemented with 20 mM xylose at the onset of the stationary-growth phase (t0). Fixed cells were kept at 4°C until analysis. Samples were distributed into a 96-well black polystyrene microplate (Greiner) and were measured with an Infinite 200 Pro microplate reader device (Tecan, Switzerland), applying an excitation wavelength of 485 nm and an emission wavelength of 535 nm. Data were recovered by Tecan i-control software (Tecan, Switzerland), and results at each time point were expressed in arbitrary units per OD_{600} (optical density at 600 nm) unit. Promoter analyses were carried out in triplicate, and mean values were calculated.

Synthetic oligopeptides. Phr peptides, corresponding to the C-terminal end of the *phr63* gene product, were synthesized, purified, and identified by mass spectrophotometry by GenScript (USA). To determine the active oligopeptide, the synthetic peptides were tested in sporulation assays (see below) at 50 μM final concentrations.

***In vitro* sporulation assays.** *In vitro* sporulation efficiency tests were carried out in the sporulation-specific medium HCT. *B. thuringiensis* strains were grown at 30°C for 48 h, and serial dilutions were plated

TABLE 3 Primers used in this study

Primer name	Sequence	Restriction site[a]
Rap7557-F	CGC**GGATCC**GAATGAGGGGATTAAATATGAATGTG	BamHI
Rap7557-R	CCC**AAGCTT**TCATTATTTTAAAGCTCCTTTCTCGG	HindIII
Phr7557-F	CGC**GGATCC**TGATAAAAAGGCTTCCGAGAAAG	BamHI
Phr7557-R	CCC**AAGCTT**GGTGTTAAATAGTTTCACCATGTGC	HindIII
7557Amont1-F	CATG**CCATGG**CGCCTTTATTGTCAAGATACATCTACTC	NcoI
7557Amont1-R	CGG**GGTACC**ACATTCATATTTAATCCCCTCATTC	KpnI
7557Amont2-F	CATG**CCATGG**TATCAATCCATCATTTCACAACATG	NcoI
7557Amont2-R	CGG**GGTACC**ATTATTTTAAAGCTCCTTTCTCGG	KpnI
7557AvaI-F	CG**TCTAGA**CACCATAAAGTACTAAAAAGTTATGTCATTAC	XbaI
7557AvaI-R	CCG**GAATTC**CAATTTTGACCAAAGTCAATCCAC	EcoRI
Prom7557-F (Prap F)	CCC**AAGCTT**CGTTACTTATAAGAAACAAACAAGAGCC	HindIII
Prom7557-R (Prap R)	CGC**GGATCC**ACATTCATATTTAATCCCCTCATTC	BamHI
Prom7557Phr-F (Pphr F)	CCC**AAGCTT**GCTGCTTGTAATAACACACTAGG	HindIII
Prom7557Phr-R (Pphr R)	CGC**GGATCC**ATTATTTTAAAGCTCCTTTCTCGG	BamHI
Phr7557R3	CCC**AAGCTT**AATATTGAACACAGTCTACTTTTTCTTTTG	HindIII
RT7557-2 (RT-2)	GAAGGCATCTGCTTGATCAGGTATAC	
RT7557-3 (RT-3)	GCTTGTAATAACACACTAGGTCTTGC	
RT7557-4 (RT-4)	CCATGTGCATATTGAACACAGTCTAC	
RT7557-5 (RT-5)	GTAGACTGTGTTCAATATGCACATGG	
RT7557-7 (RT-7)	CTTCAAGACATAGAAGACCAACATGTG	
PU-EcoRI	CG**GAATTC**GCCAGGGTTTTCCCAGTCACGAC	EcoRI
YFP-R	CG**GAATTC**TTATTTGTATAGTTCATCCATGC	EcoRI
PspoIIE-F	AACTGCAGCTGGCTAGAGCGTACGG	
xylRout3′	GGAATGTCCTCCATTGTGATTGATC	

[a]Restriction sites are shown in boldface in primer sequences.

before and after heat treatment (12 min at 80°C). When required, xylose (20 mM) and synthetic peptides were added to the culture at the beginning of the stationary-growth phase (t0). The sporulation percentage was calculated as 100 multiplied by the ratio between the number of heat-resistant spores per milliliter and the number of total viable cells per milliliter. Experiments were done at least in triplicate, and mean values were calculated. Results were analyzed statistically by analysis of variance (ANOVA), followed by Tukey's test ($P < 0.05$).

***In vivo* sporulation assays.** Experiments in insect larvae were performed as described elsewhere (12, 69). Briefly, larvae (last instar) of the lepidopteran insect *G. mellonella* were infected by intrahemocoelic injection of 2×10^4 vegetative bacteria and were kept at 30°C. Dead larvae were crushed 96 h after infection, serially diluted in 0.9% NaCl solution, and plated onto LB agar before and after heat treatment for 12 min at 80°C. The sporulation efficiencies of the harvested *B. thuringiensis* cells were calculated as described for the *in vitro* assays. At least three independent replicates were performed for each strain, and results were statistically analyzed by an unpaired *t* test with Welch's correction ($P < 0.05$).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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We declare no conflict of interest.

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