

## Differential Processing of Propeptide Inhibitors of Rap Phosphatases in *Bacillus subtilis*†

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**In the phosphorelay signal transduction system for sporulation initiation in *Bacillus subtilis*, the opposing activities of histidine kinases and aspartyl phosphate phosphatases determine the cell's decision whether to continue with vegetative growth or to initiate the differentiation process. Regulated dephosphorylation of the Spo0A and Spo0F response regulators allows a variety of negative signals from physiological processes that are antithetical to sporulation to impact on the activation level of the phosphorelay. Spo0F~P is the known target of two related phosphatases, RapA and RapB. In addition to RapA and RapB, a third member of the Rap family of phosphatases, RapE, specifically dephosphorylated the Spo0F~P intermediate in response to competence development. RapE phosphatase activity was found to be controlled by a pentapeptide (SRNVT) generated from within the carboxy-terminal domain of the *phrE* gene product. A synthetic PhrE pentapeptide could (i) complement the sporulation deficiency caused by deregulated RapE activity of a *phrE* mutant and (ii) inhibit RapE-dependent dephosphorylation of Spo0F~P in in vitro experiments. The PhrE pentapeptide did not inhibit the phosphatase activity of RapA and RapB. These results confirm previous conclusions that the specificity for recognition of the target phosphatase is contained within the amino acid sequence of the pentapeptide inhibitor.**

Reversible protein phosphorylation mediated by kinases and phosphatases plays a cardinal role in regulating essentially all aspects of eukaryotic cell physiology (12). Similarly, protein phosphorylation in prokaryotes is a common mechanism utilized in signal transduction as a means of information transfer. The two-component signal transduction system is a widespread mechanism that couples a large variety of stimuli to a diverse array of adaptive responses through a signal-stimulated phosphotransfer pathway between two proteins: a histidine protein kinase and a response regulator (11, 22, 35). Moreover, it is now appreciated that in prokaryotes, as well as in eukaryotes, protein phosphatases with distinct specificities exist to counteract histidine kinase activities (3). Thus signal transduction must be viewed as a competitive process in which kinases and phosphatases are the instruments of positive and negative signals on the system. A complex example of such interplay is provided by the phosphorelay signal transduction system that governs the initiation of the developmental process of sporulation in *Bacillus subtilis*.

The phosphorelay is a more complex version of the typical two-component system. Since its original discovery in *B. subtilis* (4), phosphorelays have been described as regulating important and complex pathways such as pathogenesis in *Bordetella pertussis* (41), osmosensing in *Saccharomyces cerevisiae* (29), and anaerobic gene expression in *Escherichia coli* (6), among

others. In the *B. subtilis* phosphorelay, multiple kinases provide signal input into the system through an autophosphorylation reaction with subsequent transfer of the phosphoryl group to the Spo0A transcription factor via the Spo0F response regulator and the Spo0B phosphotransferase intermediates. The use of a multicomponent system, in place of the classic two-component system, was proposed to provide multiple entry levels to negative regulators for controlling the flow of phosphoryl groups in the system and the ultimate production of Spo0A~P (4). Negative regulation is carried out through controlled dephosphorylation at the level of Spo0F~P and Spo0A~P response regulators. The phosphorylation level of Spo0A is specifically and directly modulated by the Spo0E phosphatase in response to signals that remain unknown (21). Spo0F~P is the target for the RapA and RapB phosphatases (26). These response regulator aspartyl phosphate phosphatases provide access for negative signals to influence the cell's decision of whether to initiate the sporulation process or to continue with vegetative growth.

The expression of RapA and RapB phosphatases is known to be differentially activated by physiological processes alternative to sporulation, such as competence and growth (17, 26), thereby allowing the recognition of a variety of negative signals and providing a means to impact on the phosphorelay and its output product Spo0A~P. A further level of complexity is brought into the system by the mechanism modulating the Rap phosphatase activities. The RapA gene is transcriptionally coupled to a second gene, *phrA*, which encodes the phosphatase regulator protein PhrA. The Phr family of phosphatase regulators is comprised of seven members (PhrA, -C, -E, -F, -G, -I, and -K), each of which is associated with a corresponding Rap phosphatase (13, 25). The 44-amino-acid (aa) product of *phrA* is subject to a series of proteolytic events through an export-import control circuit that results in an active pentapeptide (ARNQT). This PhrA pentapeptide specifically and directly inhibits the phosphatase activity of RapA (24). The series of events that characterize the formation of the active PhrA pen-

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TABLE 1. *Bacillus subtilis* strains used in this study

Strain <sup>a</sup>	Relevant genotype	Source or reference(s) <sup>b</sup>
JH642	<i>trpC2 phe-1</i>	Laboratory stock
JH703	<i>spo0AΔ204</i>	Laboratory stock
JH12474	<i>Δspo0H::cat</i>	BH-1→JH642; 10
JH12546	<i>spo0A12 abrB::Tn917::mIs</i>	23a
JH12575	<i>abrB::Tn917::mIs</i>	23a
JH12834	<i>rapA::Tn917::mIs</i>	26
JH12954	<i>ΔphrA::cat</i>	28
JH11125	<i>rapE::cat</i>	pSK28→JH642
JH11435	<i>spo0FY13S</i>	26
JH11450	<i>ΔphrE::cat</i>	pSK34→JH642
JH11455	<i>ΔphrE::erm</i>	pCm::Erm→JH11450; 34
JH11499	<i>ΔphrA::cat ΔphrE::erm</i>	JH12954→JH11455
JH11505	<i>amyE::(rapE-lacZ cat)</i>	pSK35→JH642
JH11506	<i>amyE::(phrE-lacZ cat)</i>	pSK36→JH642
JH11534	<i>rapE::tet</i>	pCm::Tet→JH11125; 34
JH11542	<i>rapE::tet phrE::cat</i>	pSK34→JH11534
JH11751	<i>rapA::Tn917::mIs rapE::cat</i>	JH12834→JH11125
JH11760	<i>spo0FY13S ΔphrE::cat</i>	pSK34→JH11435
JH22141	<i>comA::cat amyE::(rapE-lacZ spc)</i>	BD1626→JH11505 Spc; 8 <sup>c</sup>
JH22143	<i>spo0A Δ204 amyE::(rapE-lacZ spc)</i>	11505 Spc→JH703 <sup>c</sup>
JH22144	<i>comA::cat amyE::(phrE-lacZ spc)</i>	BD1626→JH11506 Spc <sup>c</sup>
JH22145	<i>spo0H::cat amyE::(phrE-lacZ spc)</i>	JH12474→JH11506 Spc <sup>c</sup>
JH22146	<i>spo0A Δ204 amyE::(phrE-lacZ spc)</i>	JH11506 Spc→JH703; 8
JH22152	<i>spo0A Δ204 abrB::Tn917::mIs amyE::(rapE-lacZ spc)</i>	JH12575→JH22143
JH22154	<i>spo0A Δ204 amyE::(phrE-lacZ spc)</i>	JH12575→JH22146

<sup>a</sup> All JH strains are derivatives of JH642 and therefore carry the *trpC2 phe-1* auxotrophic markers.

<sup>b</sup> →, construction by transformation.

<sup>c</sup> These strains were obtained by replacement of the chloramphenicol resistance gene with the spectinomycin resistance gene by means of plasmid pCm::Spc (34).

tapeptide, through export by the SecA-dependent system (5, 32) and reimportation by the oligopeptide permease (27, 30, 31), may be subject to a series of temporal and spatial regulatory mechanisms. Therefore, the production of the active Phr pentapeptides was postulated to be a regulatory mechanism required for timing coordination of alternative physiological events such as growth, competence, and sporulation (24).

In this communication, we characterized the RapE protein as the third member of the Rap family of phosphatases that specifically dephosphorylates the Spo0F~P response regulator of the phosphorelay. We showed that the phosphatase activity of RapE is specifically modulated by a pentapeptide generated from within the carboxy-terminal domain of the PhrE protein, which suggests a processing event distinct from the one postulated to produce the PhrA active pentapeptide.

#### MATERIALS AND METHODS

**Bacterial strains and growth conditions.** The *B. subtilis* strains used in this study are listed in Table 1. Sporulation assays were carried out in Schaeffer's sporulation medium or in Sterlini-Mandelstam resuspension medium (19). Cells were grown for the time indicated in the figure or tables and then treated with CHCl<sub>3</sub> before plating on Schaeffer's sporulation agar plates. Cultures for β-galactosidase assays were grown in Schaeffer's sporulation medium as previously described. β-Galactosidase activity was expressed in Miller units (15).

Antibiotics were used at the following concentrations: chloramphenicol, 5 μg/ml; spectinomycin, 50 μg/ml; erythromycin, 25 μg/ml (for strains carrying pHT315 and its derivatives) or 1 μg/ml (for strains carrying the macrolide-lincosamide-streptogramin B resistance gene from the Tn917 transposon). *E. coli* DH5α was used for plasmid construction and propagation.

**DNA manipulations.** The construction of the chromosomal library in the multicopy vector pHT315 was described previously (42). Plasmid pRM17 was subject to nucleotide sequence analysis at the 5' and 3' terminal ends. Plasmids pSK28 and pSK44 were derived from pRM17 by subcloning fragments in pJM103 and pHT315, respectively. The fragment carried by pSK38 was gener-

ated by PCR amplification of JH642 chromosomal DNA with oligonucleotides that introduced a *KpnI* site at the 5' end and a *BamHI* site at the 3' end. The fragment was first cloned in pJM103 (pSK31) and subjected to full-length sequence analysis. This revealed three nucleotide mismatches, only one of which resulted in an amino acid change (from G to E) at position 278 of the published sequence of the RapE protein (GenBank accession no. D32216) (36). The fragment from pSK31 was then transferred to pHT315, producing pSK38, and also digested and subcloned, producing the multicopy plasmids pSK39 and pSK43. The fragments carried by plasmids pSK33, pSK34, pSK35, and pSK36 were also generated by PCR amplification and subjected to sequence analysis. The fragment carried by pSK33 was transferred to the pHT315 multicopy vector, producing plasmid pSK40. The vectors used in this study were the integrative vector pJM103 (23); the multicopy vector pHT315 (2); the *lacZ* transcriptional fusion vectors pDH32 and pJM783 (23); the antibiotic cassette exchange plasmids pCm::Erm, pCm::Tet, and pCm::Spc (34); and the Cm cassette vector pJM105A, used for the construction of pSK34 (23).

**Protein expression and purification.** A fragment carrying the RapE coding sequence was generated by PCR amplification from JH642 chromosomal DNA with oligonucleotides that introduced a *BamHI* site at both the 5' and 3' ends. The fragment was cloned in the pET16b expression vector (Novagen) and verified by sequence analysis. This cloning generated an extension of 10 histidine codons to the 5' end of the *rapE* gene. Protein expression was obtained in *E. coli* BL21(DE3) pLysS (Novagen) by induction at an optical density at 600 nm of 0.7 with 2 mM isopropyl-β-D-thiogalactopyranoside. Cells were grown for 2 h at 37°C and the protein was purified by affinity chromatography on Ni-nitrilotriacetic acid agarose (Qiagen) as previously described (7). Purification of RapA, RapB, KinA, Spo0F and Spo0A was performed as previously described (7, 24). Spo0B~P was produced in a reaction mixture containing KinA, Spo0F, and [γ-<sup>32</sup>P]ATP and then purified as previously described (40).

**In vitro assay conditions.** RapE-dependent dephosphorylation of Spo0F~P was tested in a reaction mixture containing 0.1 μM KinA, 5 μM Spo0F, 1.0 mM ATP, and 1.8 mCi of [γ-<sup>32</sup>P]ATP (6,000 Ci/mmol; NEN) per ml. The reaction buffer was 50 mM *N*-(2-hydroxyethyl)piperazine-*N'*-(3-propanesulfonic acid) (EPPS) (pH 8.5), 20 mM MgCl<sub>2</sub>, 100 μM EDTA, and 5% glycerol. The reaction mixture was allowed to equilibrate for 30 min at room temperature. RapE was then added at a 2.5 μM final concentration. Time points were taken at the indicated times and the reactions were stopped by addition of sodium dodecyl sulfate (SDS) loading buffer. The Rap-Phr in vitro assays were carried out under the buffer conditions described above. KinA and Spo0F at the concentrations indicated in the figures were incubated for 1 h prior to the addition of Rap phosphatases or premixed Rap phosphatases and Phr peptides. The reactions were allowed to proceed for an additional 30 min and then stopped with SDS loading buffer. Purified Spo0B~P (1 μM) and Spo0B~P with Spo0A (2 μM) were incubated in the presence or absence of RapE (2 μM) for 30 min at room temperature in the reaction buffer described above. The reactions were run on SDS-glycine-15% polyacrylamide gels at constant current (25 mA) for 1.5 h. The gels were immediately exposed to Kodak X-Omat RP films at -80°C and then exposed to a Molecular Dynamics PhosphorImager and analyzed with ImageQuant software. The concentration of Phr peptides was determined by amino acid analysis.

#### RESULTS

**The product of *rapE* is a negative regulator of sporulation initiation.** Many negative regulators of the phosphorelay were found by their property of inhibiting sporulation when overexpressed on a multicopy plasmid. The KipI histidine kinase inhibitor was identified by screening a *B. subtilis* chromosomal library constructed in the shuttle vector pHT315 (42). This library yielded a series of sporulation-deficient clones, and plasmids isolated from these clones were subject to nucleotide sequence analysis. Among the plasmids isolated, pRM17 contained a 1,802-bp fragment of the *B. subtilis* genome from nucleotide 40974 to nucleotide 42776 (GenBank accession no. D32216) on the *skin* excisable element (36). An open reading frame (ORF) was identified on this fragment, ORF5, and the high level of similarity between the product of ORF5 and RapA (47% identity; 66% similarity) suggested this may be an additional member of the Rap family of phosphatases. ORF5 was renamed RapE.

The presence of plasmid pRM17 in the wild-type strain JH642 resulted in a sporulation-deficient phenotype (≈3-fold-fewer spores than in the wild-type strain carrying the vector pHT315) (Table 2). The sporulation deficiency was associated with the presence of an intact RapE coding sequence, since plasmids pSK43 and pSK44, which carried only the *rapE* pro-

TABLE 2. Sporulation efficiency of *B. subtilis* strains carrying multicopy plasmids<sup>a</sup>

Plasmid <sup>b</sup>	Insert	Viable cell count	Spore count	% Efficiency of sporulation
pRM17	<i>rapE</i>	2.2 × 10 <sup>8</sup>	2.3 × 10 <sup>7</sup>	10.4
pSK43	<i>rapE</i> promoter	4.2 × 10 <sup>8</sup>	1.8 × 10 <sup>8</sup>	42.8
pSK38	<i>rapE phrE</i>	3.9 × 10 <sup>8</sup>	1.2 × 10 <sup>8</sup>	30.7
pSK39	<i>phrE</i>	3.0 × 10 <sup>8</sup>	1.1 × 10 <sup>8</sup>	36.6
pSK40	<i>phrE</i> promoter	4.0 × 10 <sup>8</sup>	1.3 × 10 <sup>8</sup>	32.5
pHT315	Vector	3.2 × 10 <sup>8</sup>	1.2 × 10 <sup>8</sup>	37.5

<sup>a</sup> Representative of several independent experiments.

<sup>b</sup> JH642 derivatives harboring the multicopy plasmids were grown for 46 h at 37°C in Schaeffer's sporulation medium with the addition of erythromycin at a concentration of 5 µg/ml.

motor region and upstream sequences, did not inhibit sporulation (Fig. 1A and Table 2).

**The *phrE* gene.** Analysis of the nucleotide sequence of the region downstream of the *rapE* gene revealed the presence of an overlapping small ORF, *phrE* (Fig. 1). *phrE* encodes a 44-aa peptide with low primary sequence homology to the PhrA peptide regulating RapA but with similar structural features, i.e., a positively charged amino-terminal hydrophobic domain separated from a hydrophilic carboxy-terminal domain by a putative signal peptidase cleavage site (Fig. 1B) (20). Nucleo-

tide sequence analysis also revealed the presence of a putative sigma H promoter region buried within the *rapE* coding sequence and immediately upstream of the *phrE* ribosome binding site (not shown).

To test the possibility that the product of *phrE* regulated the activity of RapE, as is the case of PhrA with RapA, we constructed a multicopy plasmid carrying the entire *rapE phrE* operon. As shown in Table 2, the strain harboring plasmid pSK38 sporulated as efficiently as the control strain, indicating that the presence of *phrE* overcame the sporulation defect caused by *rapE* overexpression. Multicopy plasmids carrying the *phrE* gene (pSK39) or the *phrE* promoter alone (pSK40) did not significantly affect the efficiency of sporulation (Fig. 1A and Table 2).

When a chromosomal inactivation of the *phrE* gene was obtained by the insertion of a chloramphenicol resistance cassette within the gene, the resulting strain, JH11450, showed a reduction in sporulation efficiency (Table 3). The sporulation defect of strain JH11450 was suppressed by the concurrent inactivation of the *rapE* gene (strain JH11542) (Table 3). The sporulation efficiency of strain JH11542 was comparable to the efficiency of strain JH11125 carrying the inactivated *rapE* alone; both strains sporulated at a higher level than the wild-type strain JH642. Furthermore, the sporulation defect of the *phrE* mutant was totally overcome by an *spo0F* mutation, Y13S, which renders the Spo0F response regulator insensitive

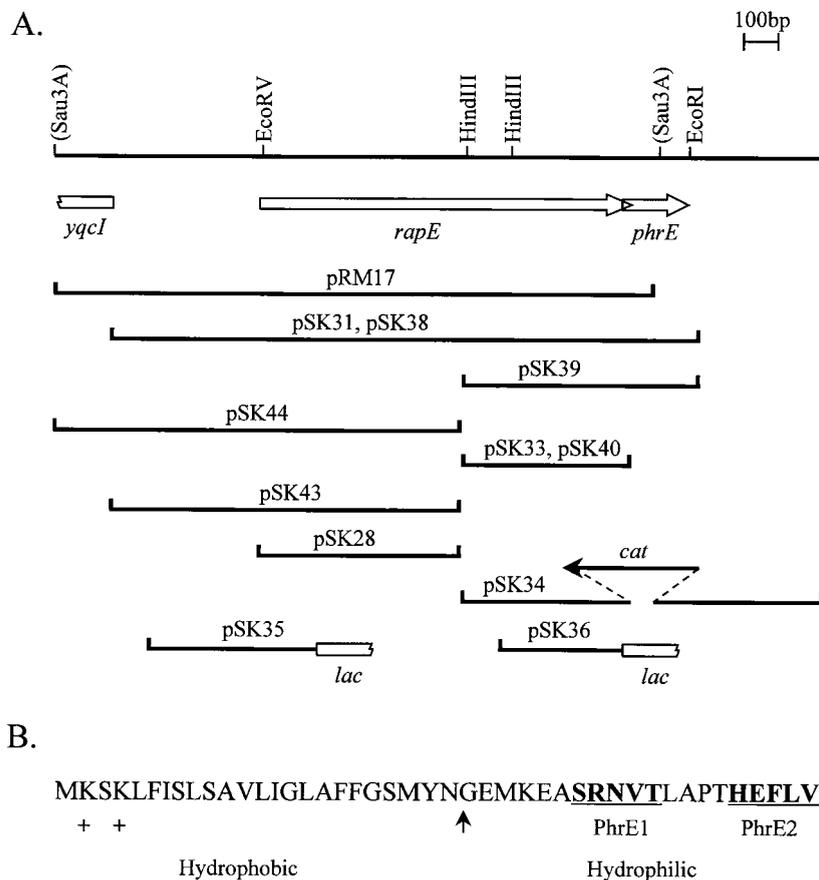


FIG. 1. (A) Restriction map of the chromosomal region containing the *rapE* and *phrE* loci. Fragments cloned in plasmids used in this study are indicated by lines. The fragments in plasmids pSK34, -35, -36, -38, and -40 were generated by PCR amplification from JH642 chromosomal DNA with oligonucleotides carrying restriction sites suitable for cloning. Restriction sites in parentheses are not unique. (B) Amino acid sequence of the PhrE protein. The arrow denotes the putative type I signal peptidase cleavage site, as determined by the SignalP program (20). The PhrE-1 and PhrE-2 pentapeptides are in boldface and underlined. +, positively charged residues.

TABLE 3. Sporulation efficiency of *rap* and/or *phr* mutant strains<sup>a</sup>

Strain <sup>b</sup>	Relevant phenotype	Viable cell count	Spore count	% of sporulation <sup>c</sup>
JH642	WT	$2.3 \times 10^8$	$1.35 \times 10^8$	58.7
JH11450	PhrE <sup>-</sup>	$4.8 \times 10^8$	$1.1 \times 10^8$	22.9
JH11542	RapE <sup>-</sup> PhrE <sup>-</sup>	$2.2 \times 10^8$	$1.5 \times 10^8$	68.2
JH11125	RapE <sup>-</sup>	$2.3 \times 10^8$	$1.5 \times 10^8$	65.2
JH11435	Spo0F (Y13S)	$1.5 \times 10^8$	$1.3 \times 10^8$	86.6
JH11760	Spo0F (Y13S) PhrE <sup>-</sup>	$1.2 \times 10^8$	$1.0 \times 10^8$	83.3
JH12954	PhrA <sup>-</sup>	$4.4 \times 10^8$	$4.0 \times 10^7$	9.1
JH11499	PhrA <sup>-</sup> PhrE <sup>-</sup>	$4.9 \times 10^8$	$3.6 \times 10^7$	7.3
JH12834	RapA <sup>-</sup>	$1.8 \times 10^8$	$1.4 \times 10^8$	77.7
JH11751	RapA <sup>-</sup> RapE <sup>-</sup>	$1.6 \times 10^8$	$1.2 \times 10^8$	75.0

<sup>a</sup> Representative of three independent experiments. WT, wild type.

<sup>b</sup> Strains were grown for 30 h at 37°C in Schaeffer's sporulation medium.

<sup>c</sup> Percentage of sporulation is expressed as the ratio between spore counts and viable cell counts. The significant differences observed in percentages, despite the limited differences in absolute spore counts, are due to the highly reproducible differences in viable counts between sporulating and nonsporulating cells.

to the activity of both RapA and RapB phosphatases (strain JH11760) (Table 3) (26). These observations strongly suggested that the PhrE peptide acts as a modulator of RapE activity and the target of RapE is the Spo0F~P response regulator intermediate of the phosphorelay.

**An exogenously provided synthetic PhrE pentapeptide complements the *phrE* mutant.** The structural features of the *phrE* gene product are reminiscent of the *phrA* and *phrC* gene products. Therefore, we investigated whether the 44-aa PhrE protein was subject to the same maturation process through the export-import control circuit that results in the formation of an active pentapeptide. It has recently been shown that the PhrA carboxy-terminal pentapeptide ARNQT is specifically active on RapA while the PhrC carboxy-terminal pentapeptide ERGMT weakly, but specifically, inhibits RapB (24). Although there is a very limited amino acid sequence homology among the members of the Phr family, there is a highly conserved arginine residue at position 2 and a threonine residue at position 5 of active carboxy-terminal pentapeptides. Analysis of the amino acid sequence of PhrE (Fig. 1) revealed that while the carboxy-terminal pentapeptide HEFLV (PhrE-2) did not contain any of the characteristic R or T residues, a pentapeptide corresponding to the sequence SRNVT (PhrE-1) was located 9 aa from the carboxy-terminal end. Thus, experiments were designed to determine which peptide was the active inhibitor.

Peptides corresponding to the sequences of PhrE-1 and PhrE-2 were chemically synthesized and used to determine their ability to complement the *phrE* deficiency in vivo. Strain JH11450 (PhrE<sup>-</sup>) was grown in Sterlini-Mandelstam medium, as described in Materials and Methods. At resuspension time, the synthetic PhrE-1 and PhrE-2 peptides were added at increasing concentrations and the cells were allowed to sporulate for 12 h. The results of the sporulation assay (Fig. 2) indicated that the PhrE-1 peptide (SRNVT) restored the sporulation capability of the *phrE* mutant strain JH11450 when used at a 1 μM concentration, while the PhrE-2 peptide (HEFLV) was totally inactive even at the highest concentration tested (10 μM). The assay also showed that at low concentrations, the PhrA and PhrC synthetic pentapeptides did not complement the *phrE* defect, confirming the observation that no significant cross-reactivity exists in vivo among Phr peptides (24).

**RapE dephosphorylates Spo0F~P and PhrE-1 inhibits its activity.** The results of the genetic analysis prompted us to carry out in vitro biochemical assays in order to confirm the target of RapE activity and the role of the PhrE pentapeptides.

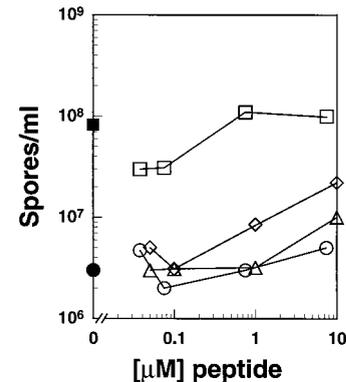


FIG. 2. In vivo complementation of the *phrE* mutant by synthetic Phr pentapeptides. The assay was carried out by the Sterlini-Mandelstam resuspension method, as described in Materials and Methods. Cells were grown for 12 h at 37°C. The efficiency of sporulation of strains JH642 (wild type) (■) and JH11450 (*phrE*) (●) is indicated. □, PhrE-1; ○, PhrE-2; △, PhrA; ◇, PhrC.

The RapE protein was purified from an overexpressing *E. coli* strain and tested in vitro. The results of a time course of RapE-dependent dephosphorylation of Spo0F~P are shown in Fig. 3A. RapE is specifically active on Spo0F~P and it does not directly affect the phosphorylation level of Spo0A~P or Spo0B~P, as previously observed for RapA and RapB (Fig. 3B).

The ability of the synthetic PhrE-1 peptide to inhibit RapE activity, compared to the in vivo-inactive PhrE-2 peptide, was then tested. PhrE pentapeptide concentrations ranging from 50 to 400 μM were utilized and the results of the in vitro assays are reported in Fig. 4A. Increasing concentrations of the PhrE-1 peptide (SRNVT) inhibited the RapE-dependent dephosphorylation of Spo0F~P, while the PhrE-2 peptide (HEFLV) was totally inactive and actually seemed to stimulate phosphatase activity. These results corroborate the in vivo properties of the PhrE-1 and PhrE-2 peptides.

In order to test the specificity of RapE inhibition by Phr peptides, a RapE-dependent dephosphorylation assay of Spo0F~P was carried out in the presence of PhrA (ARNQT) or PhrC (ERGMT). PhrA showed some inhibitory activity (a

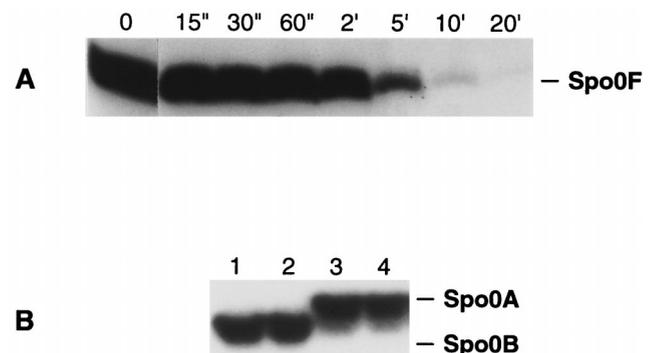


FIG. 3. (A) Time course of Spo0F~P dephosphorylation by RapE. The reaction mixture containing KinA (0.1 μM), Spo0F (5 μM), and RapE (2.5 μM) was incubated in the presence of [<sup>32</sup>P]ATP as described in Materials and Methods, and aliquots were taken at the indicated times. (B) RapE does not dephosphorylate Spo0B~P or Spo0A~P. Purified Spo0B~P (1 μM) (lanes 1 and 2) or Spo0B~P and Spo0A (2 μM) (lanes 3 and 4) were incubated without (lanes 1 and 3) or with (lanes 2 and 4) RapE (2 μM) for 30 min at room temperature.

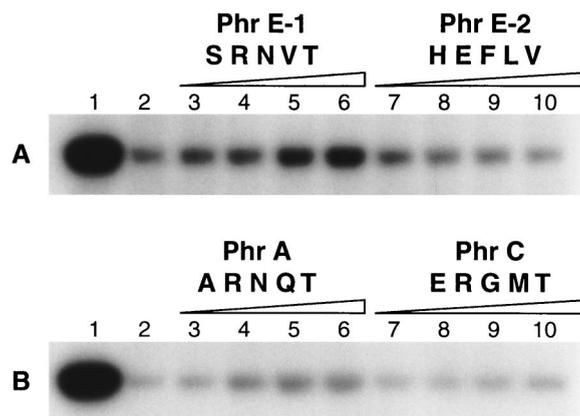


FIG. 4. Inhibition of RapE activity by Phr peptides. The reaction mixture containing KinA (0.1 μM), Spo0F (5 μM), and [ $\gamma$ - $^{32}$ P]ATP was incubated for 1 h at room temperature. Aliquots were then incubated with RapE (2.5 μM) (lanes 2) or RapE premixed with PhrE-1 and PhrE-2 (A) or PhrA and PhrC (B) synthetic pentapeptides at 50, 100, 200, and 400 μM (lanes 3 to 6 and 7 to 10, respectively). The control level of Spo0F phosphorylation is shown in lanes 1.

threefold lower level of activity than PhrE-1 at the highest concentration used) while PhrC was totally inactive (Fig. 4B). Despite the weak inhibitory activity observed with PhrA, the *in vitro* and *in vivo* data indicate that RapE is most likely inhibited specifically by the PhrE-1 peptide. Furthermore, PhrE-1 and PhrE-2 did not show any inhibitory activity toward RapA or RapB (Fig. 5). Therefore, we concluded that the PhrE-1 pentapeptide specifically inhibits the RapE phosphatase activity on Spo0F~P both *in vivo* and *in vitro*.

**Transcription regulation of *rapE* and *phrE*.** The RapA and RapB proteins are known to be phosphorelay regulators that each prevent sporulation in response to specific and unique physiological conditions (26). Transcription of *rapA* is dependent upon the ComA-ComP two-component system for competence development (17), whereas *rapB* is under control of the AbrB transition state regulator and is induced by vegetative growth conditions (26; our unpublished data). Examination of the nucleotide sequence of the *rapE* promoter region revealed the presence of a putative ComA binding site (18) followed by -35 and -10 consensus sequences for  $\sigma^A$ -containing RNA

polymerase. A *rapE* promoter fusion to the *E. coli lacZ* gene was constructed in the transcriptional fusion vector pJM115 and  $\beta$ -galactosidase assays were carried out in various genetic backgrounds. As shown in Fig. 6A, when wild-type cells were grown under sporulation conditions, transcription from the *rapE* promoter was induced approximately 2 h before the transition from vegetative growth to sporulation. The *rapE* gene was transcribed at a very low level (10 to 15 Miller units) and the induction was dependent upon an active ComA protein. The transcription of *rapE* was also inhibited by a *spo0A* mutation and this inhibition was released by inactivation of the *abrB* gene. The effect of the *spo0A* and *abrB* mutations, however, was most likely indirect and a result of the Spo0A and AbrB regulatory role in ComA-ComP activation and competence development (9).

The genetic organization of the *rapE* and *phrE* genes (Fig. 1) was suggestive of an operon structure in which *phrE* is transcriptionally coupled to *rapE* in a manner similar to that in the *rapA-phrA* operon. However, examination of the nucleotide sequence within the *rapE* coding region that immediately precedes the *phrE* gene revealed the presence of -35 and -10 consensus sequences for  $\sigma^A$ - and  $\sigma^H$ -containing RNA polymerase (16). This suggested that in addition to being coupled to *rapE* transcription, *phrE* could be independently transcribed from its own promoter. A *phrE* promoter-*lacZ* fusion was constructed and integrated in the *amyE* locus of wild-type strain JH642.  $\beta$ -Galactosidase assays (Fig. 6B) confirmed that *phrE* is transcribed independently of *rapE*. The transcription of *phrE* is also induced approximately 2 h before the transition time (To), as observed for *rapE* transcription, but at a slightly and reproducibly higher level than *rapE*. Induction of *phrE*, however, is not dependent upon ComA and is totally inhibited in a *spo0A* background, owing to repression by AbrB. When *phrE* transcription was analyzed in a *spo0H* background, the level of induction was approximately 50% lower than that in the wild-type strain, suggesting that the putative  $\sigma^H$  promoter might have a limited, if any, role in *phrE* transcription.

$\beta$ -Galactosidase assays were also carried out on JH642 derivative strains carrying the same *rapE-lacZ* and *phrE-lacZ* fusions integrated at the *rapE* and *phrE* loci. The results (data not shown) indicated that, although the patterns of transcription were comparable to the ones observed in the *amyE* locus, the levels of transcription at the isotopic position were 50% lower than the ones obtained at the ectopic integration site.

**Relative contribution of RapA-PhrA and RapE-PhrE to the modulation of phosphorelay activity.** Transcription of *rapE* and *rapA* is similarly controlled by the ComA-ComP signal transduction system. However, *rapA* is transcribed at a very high level (approximately 1,500 Miller units at To) (our unpublished data) (17) while *rapE* expression never exceeds 15 Miller units, as determined by measuring the  $\beta$ -galactosidase activity of *rapA-lacZ* and *rapE-lacZ* fusion constructs. In order to assess the relative contribution of RapA and RapE in modulating the level of Spo0F~P in the phosphorelay *in vivo*, we carried out a sporulation efficiency test on various *rap* or *phr* mutant strains. As shown in Table 3, a deletion of *phrE* that results in the deregulation of RapE had a minor effect on sporulation efficiency, compared to the deletion of *phrA* (40% of residual sporulation versus 15%). The double mutant *phrA phrE* did not display a significantly additive phenotype. Furthermore, while the deletion of the RapA coding gene resulted in an increase of sporulation efficiency (35% more spores than in the wild type), a deletion of *rapE* only resulted in a 12% increase in spore formation. Once again, the double mutant *rapA rapE* did not exhibit an additive phenotype. Furthermore, the sporulation defect of an oligopeptide transport mutant

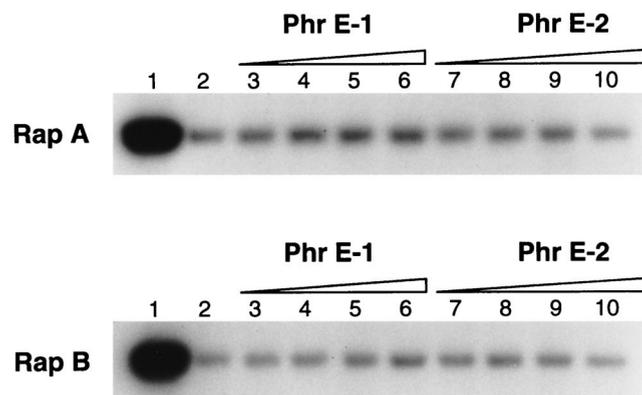


FIG. 5. PhrE peptides do not inhibit RapA or RapB. PhrE-1 (lanes 3 to 6) or PhrE-2 (lanes 7 to 10) at 50, 100, 200, and 400 μM was incubated with RapA or RapB at 2.5 μM and the reaction mixture containing KinA (0.1 μM), Spo0F (5 μM), and [ $\gamma$ - $^{32}$ P]ATP. Lanes 1 contain the control level of Spo0F phosphorylation. Lanes 2 show the control level of Spo0F~P dephosphorylation by RapA or RapB.

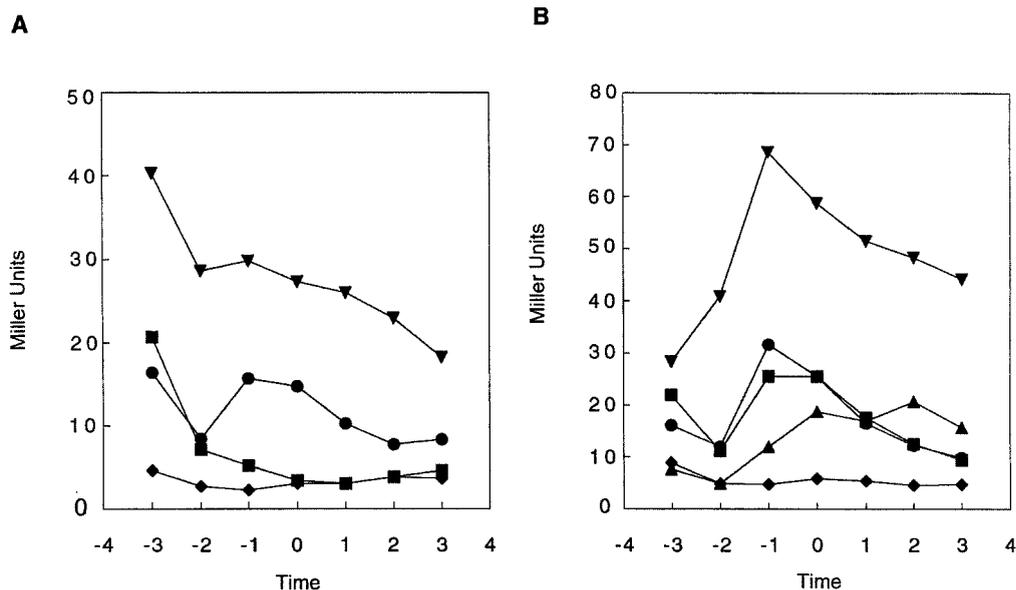


FIG. 6. Time course of  $\beta$ -galactosidase activity of *rapE-lacZ* (A) and *phrE-lacZ* (B) fusion constructs integrated at the *amyE* locus. Time points were taken hourly before and after the transition (To) from exponential to stationary phase. Cells were grown in Schaeffer's sporulation medium. Symbols: ●, wild-type; ◆, *spo0A*; ▼, *spo0A*, *abrB*; ■, *comA*; ▲, *spo0H*.

which is unable to transport either the *phrA* or *phrE* peptide is overcome by a deletion of *rapA*, but it is not significantly suppressed by a deletion of *rapE* (Table 4).

Altogether, these results suggest that the major role in modulating the phosphate flow through the phosphorelay by the dephosphorylation of Spo0F~P is played by the RapA phosphatase, while RapE has an accessory role. Indeed, the location of the *rapE* and *phrE* genes on the *B. subtilis* *skin* excisable element supports this view. The *skin* element is seen as a cryptic remnant of an ancestral temperate phage and it is reportedly absent from other *B. subtilis* stock strains at the University of Tokyo (Y. Kobayashi, personal communication) (36) or from closely related bacilli such as *Bacillus thuringiensis* (1). We analyzed various natural isolates of *B. subtilis* for the presence of the *rapE* gene with the *rapA* gene as a control. PCR amplifications were carried out on chromosomal DNA isolated from the following sporulating *Bacillus* strains: *B. subtilis* "Polish" (J. A. Hoch strain collection), *Bacillus natto*, *B. subtilis* 23<sup>SR</sup>, ATCC 10783, ATCC 12139, ATCC 14593, and ATCC 10774. The results showed that a fragment of the size of the *rapA* coding sequence (1.1 kb) was generated by the *rapA* oligonucleotides in all the strains tested, while a *rapE* fragment was detected only in the JH642 laboratory strain and in *B. subtilis* "Polish" (data not shown).

These observations confirm the hypothesis that RapE and

PhrE play a dispensable role in the overall context of sporulation initiation and support previous findings about the nonubiquitous presence of the *skin* element in *Bacillus* strains (1, 36).

## DISCUSSION

The RapE member of the Rap family of response regulator aspartyl phosphate phosphatases was found to promote the dephosphorylation of Spo0F~P, the response regulator intermediate of the phosphorelay. RapE contributes, with RapA and RapB, to the integration of negative signals into the phosphorelay for sporulation initiation, in response to physiological conditions antithetical to the developmental process. Transcription of the RapE-coding gene is under control of the ComA-ComP two-component system for competence development, which also activates transcription of *rapA* (17). Expression of the *rapB* gene, on the contrary, is induced by conditions that favor vegetative growth. Since vegetative growth and competence are processes that cannot occur in a sporulating cell, the induction of *rap* phosphatases prevents sporulation from interfering with these processes.

Inhibition of RapE phosphatase activity both in vivo and in vitro occurs by action of a pentapeptide, SRNVT, generated from the central portion of the C-terminal half of the *phrE* gene product. Transcription of *phrE* occurs independently of the *rapE* promoter and is controlled by the Spo0A-AbrB pair of transcription regulators. This may represent a mechanism ensuring sufficient production of PhrE peptide to inhibit RapE activity when the level of phosphorylated Spo0A in the cells is high enough to prevent *abrB* transcription, therefore allowing the initiation of the transition phase to sporulation.

PhrE, like PhrA, has the characteristics of a protein exported by the SecA-dependent system (5, 28). Key features are the positively charged amino end followed by a stretch of hydrophobic residues, a putative type I signal peptidase cleavage site, and a slightly hydrophilic carboxy-terminal portion. Buried within this latter region is the PhrE-1 pentapeptide (SRNVT) that specifically inhibits RapE activity. The C-ter-

TABLE 4. Suppression of the *oppD* sporulation phenotype by *rapA* and *rapE* mutations<sup>a</sup>

Strain <sup>b</sup>	Relevant phenotype	% of spores <sup>c</sup>
JH642	WT	65.3
JH12795	OppD <sup>-</sup>	5.3
JH11110	OppD <sup>-</sup> RapE <sup>-</sup>	6.5
JH11053	OppD <sup>-</sup> RapA <sup>-</sup>	66.0
JH11108	OppD <sup>-</sup> RapA <sup>-</sup> RapE <sup>-</sup>	70.0

<sup>a</sup> The results are the average of two independent experiments. WT, wild type.

<sup>b</sup> Strains were grown in Schaeffer's sporulation medium for 30 h.

<sup>c</sup> Calculated as the ratio between viable cells and CHCl<sub>3</sub>-resistant spores.

minal PhrE-2 pentapeptide (HEFLV) was inactive both in vivo and in vitro. The PhrE-1 pentapeptide does not act in vitro or in vivo on RapA or RapB. Similarly, synthetic PhrA or PhrC pentapeptides do not inhibit RapE activity in vitro when used at low concentrations. These results support previous observations on the highly specific recognition of phosphatase targets by Phr peptides (24). It was reported that single amino acid substitutions within a pentapeptide can severely affect its inhibitory activity and/or its target specificity. Thus, it is not surprising that, despite the fact that the PhrA pentapeptide shares 3 aa with PhrE-1, no cross-reactivity is observed in vivo and very little in vitro. Moreover, the PhrC pentapeptide is inactive on RapE in vitro and the partial complementation in vivo is most likely due to inhibition of RapB phosphatase activity, which would result in increased sporulation frequency (24, 33).

Production of active Phr pentapeptide inhibitors was postulated to occur through an export-import control circuit (24). The major unanswered question is the identity of the proteases responsible for the liberation of the active Phr pentapeptides from the *phr* gene products. The first modification of the primary gene product is a SecA-dependent export process associated with proteolytic cleavage by type I signal peptidases, as suggested by the primary structure and organization of Phr proteins (5, 28). Five type I signal peptidase-coding genes (*sipS*, *-T*, *-U*, *-V* and *-W*) have been identified by the *B. subtilis* genome sequencing project (13, 38). However, none of them seems to be specifically involved in processing Phr peptides, since single inactivation of the *sip* genes does not result in a sporulation defective phenotype (M. Jiang and M. Perego, unpublished data). Such phenotype would be expected if the control circuit leading to the production of the active PhrA and PhrE pentapeptides were interrupted.

It has been reported that a *sipS-sipT* double deletion is lethal to the cells (39) and we have observed that a *sipV-sipT* double deletion results in a severe sporulation defect that cannot be rescued by deletion of Rap phosphatases (our unpublished work). All this suggests that some processing specificity must exist in signal peptidases and more than one signal peptidase must be involved in processing the Phr proteins, unless a still-unidentified processing enzyme exists with signal peptidase enzymatic activity. However, the sporulation-deficient phenotype of the *sipV-sipT* mutant is the result of a more severe defect than the inability to process Phr peptides.

After the initial signal peptidase cleavage of Phr proteins, a second proteolytic event was postulated to occur in the extra cytoplasmic compartment in order to generate the active inhibitor pentapeptide from the inactive proinhibitor of presumably 19 aa. This step is necessary to generate a peptide of a size (5 aa) suitable for reimportation by the oligopeptide transport system (37). If one proteolytic event was required to generate the PhrA and PhrC pentapeptides from the C-terminal ends of their respective precursors, two processing events are needed to generate the PhrE-1 peptide from within its precursor. These differential processing events among Phr peptide maturation processes raise intriguing questions about how the specificity of proteolytic events is achieved. Are the determinants for protease recognition embedded within the sequence of the pentapeptides or do they extend within the inactive propeptide inhibitor? Are the conserved R and T residues at positions 2 and 5 of active pentapeptides involved in recognition by proteolytic enzymes? The R and T residues were previously proposed to define the sites of Rap-Phr interaction by providing the correct orientation for binding, while the remaining amino acids may determine specificity through the interactions established by their side chains (24).

A remarkable feature of Rap phosphatases is their specificity for target recognition. RapA, RapB, and RapE are specifically active on Spo0F~P and do not promote the dephosphorylation of Spo0A~P or other response regulators tested (our unpublished data). Likewise, Spo0F~P is not dephosphorylated by RapF or by RapC (our unpublished data); the latter phosphatase is known from genetic studies to affect competence development (14, 33). Nevertheless, RapC and RapF residues share 44 and 41% identity, respectively, and 62% identity with RapA. Furthermore, the remaining chromosomally coded Rap phosphatases all share between 25 to 45% identity with RapA but none of them seem to affect sporulation, based on genetic analysis (our unpublished data). The stringent substrate specificity, despite the high level of homology of Rap phosphatases, and the conserved structural features of response regulators raise challenging questions about molecular recognition. Answering these questions will significantly move forward our understanding of the mechanisms governing signal transduction.

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