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Phosphate Starvation Induces the Sporulation Killing Factor of *Bacillus subtilis*

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Bacillus subtilis **produces and exports a peptide sporulation killing factor (SkfA) that induces lysis of sibling cells. *skfA* is part of the *skf* operon (*skfA-H*), which is responsible for immunity to SkfA, as well as for production and export of SkfA. Here we report that transcription of *skfA* is markedly induced when cells of *B. subtilis* are subjected to phosphate starvation. The role of PhoP in regulation of the *skf* operon was confirmed by in vitro gel shift assays, which showed that this operon is a new member of the PhoP regulon. A putative stem-loop structure in the *skfA-skfB* intergenic region is proposed to act as a stabilizer of an *skfA*-specific transcript.**

Phosphate starvation leads to marked changes in the expression of genes in the PhoP and SigB (σ^B) regulons of *Bacillus subtilis* (2, 4, 8, 12, 16, 27). Genes in the PhoP regulon provide a specific response to phosphate starvation stress, while genes in the SigB regulon provide a general response to the resulting energy stress. Additionally, the PhoP and SigB regulons interact to modulate the level to which each regulon is activated (4, 27). The activity of the PhoP regulon is also influenced by Spo0A; activated Spo0A (Spo0A~P) is responsible for the induction of sporulation and the repression of genes induced by the transition-phase regulator AbrB. The PhoP regulon is up-regulated in a *spo0A* null mutant that is unable to initiate sporulation (18, 25). Maximal induction of the PhoP regulon also requires an active ResD-ResE respiration signal transduction system (16). An *spo0A abrB resD* null mutant is not able to mount a specific response to phosphate starvation, showing that the induction of the PhoP regulon is dependent not only on the phosphate-specific PhoPR signal transduction system but also on this network of regulatory elements (30). If, despite these regulatory responses, phosphate starvation persists, Spo0A initiates sporulation and terminates the phosphate response by repressing the transcription of *phoPR* via AbrB and ResD-ResE (16, 17).

During the phosphate starvation-specific response, genes of the PhoP regulon are regulated by the PhoP-PhoR two-component signal transduction system (2, 25, 29). The PhoP response regulator is activated by its cognate sensor kinase, PhoR. Phosphorylated PhoP (PhoP~P) induces the expression of the *phoPR* operon about threefold from a low constitutive level of expression (17, 26, 27) and is required for the induction or repression of other members of the PhoP regulon (16).

Fawcett et al. (9) and Molle and colleagues (21) have shown that Spo0A regulates the *skf* operon, which encodes the sporulation killing factor (SkfA). SkfA induces the lysis of sibling *B. subtilis* cells that have not entered the sporulation pathway (i.e., Spo0A inactive), providing a source of nutrients to support this key differentiation process.

Reporter gene analysis of the response of *skfA* to phosphate starvation. Previous DNA array analysis of the response of *B. subtilis* to phosphate starvation indicated that *skfA* was induced in response to phosphate starvation in a *phoR*-dependent manner (2). This induction was transient, and the level typically returned to a noninduced level within 3 h. The induction of *skfA* in response to phosphate starvation was confirmed with strain 168-SKFA (Table 1), in which *skfA* is transcriptionally fused to a *lacZ* reporter (*skfA*::*lacZ*). Cultures of 168-SKFA grown in LPM (0.42 mM P_i) were sampled to monitor the alkaline phosphatase (APase) and β-galactosidase activities (24, 27). Three independent experiments gave comparable results, and a representative data set is shown in Fig. 1. APase, an intrinsic reporter of the PhoP regulon, was induced during the transition from the exponential phase to stationary phase (T_0), confirming that entry into stationary phase was due to phosphate starvation. *skfA* was induced (~60 to 70 nmol of *o*-nitrophenol [ONP]/min/unit of optical density at 600 nm [OD_{600}]) concomitantly with APase, as determined by the β-galactosidase activity of the reporter strain (Fig. 1).

To analyze *skfA* gene expression in a *phoR* null background, *B. subtilis* 168-SKFA was transformed with chromosomal DNA from 168-PR (a *phoR* null mutant) (26) to obtain strain 168-SKFA-PR. Strain 168-SKFA-PR was used to determine whether the response of *skfA* to phosphate starvation was dependent on the PhoPR regulatory system. Compared to the pattern of *lacZ* expression in 168-SKFA, the pattern of *lacZ* expression in 168-SKFA-PR was markedly different; low levels of β-galactosidase activity (>10 nmol ONP/min/OD_{600} unit) were observed during the transition phase, but the level increased to ~50 nmol ONP/

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TABLE 1. Bacterial strains, plasmids, and primers

Strain, plasmid, or primer	Relevant characteristic(s) or sequence (5′–3′)[a]	Position[b]	Reference or source
B. subtilis strains			
168	*trpC2*		3
168-PR	*trpC2 phoR*ΔBalI::Tc^r		26
168-SKFA	*trpC2 skfA*::pYBCOdd Em^r (previously YBCOdd)		T. Tanaka, Takai University
168-SKFA-PR	*trpC2 phoR*ΔBalI::Tc^r *skfA*::pYBCOdd Em^r		This study
SWV215	*trpC2 pheA1 spo0A*::Km^r		31
168-SKFA-OA	*trpC2 spo0A*::Km^r *skfA*::pYBCOdd Em^r		This study
168-SKFA-OA-PR	*trpC2 spo0A*::Km^r *phoR*ΔBalI::Tc^r *skfA*::pYBCOdd Em^r		This study
168-SKFB	*trpC2 skfB*::pYBCPdd Em^r (previously YBCPdd)		T. Tanaka, Takai University
Plasmids			
pET-PhoP	pET2816 containing a 722-bp insert of *phoP* Ap^r (6.386 kb)		25
pET-PhoR231	pET2816 containing a 1,049-bp insert of *phoR* Ap^r (6.713 kb)		25
Primers			
skfA-FOR	AAGAATGGGAATCTGTGA	213942–213960	
skfA-REV	**CTAATACGACTCACTATAGGGAG**ATAGCAGGATGCGGAAG	214081–214066	
skfB-FOR	CCAGAGTAGCGGTTCAT	214181–214166	
skfB-REV	**CTAATACGACTCACTATAGGGAG**TTACCTGAGCTGGCATAG	214550–214532	
skfH-FOR	CGTGACAAGTTGTGACGA	219909–219892	
skfH-REV	**CTAATACGACTCACTATAGGGAG**GTTGACTGAATGGCCAAG	219611–219628	
YbcO-FOR	TATAACATAATGGACCGTCT	213645–213664	
YbcO-REV	CACGTGTCATAGCAATACTC	214035–214055	
YhaX-FOR	TAACGATATTAGGGAGAATGGC	1056018–1056039	
YhaX-REV	GAAGCAGCGCTCCATCTATATT	1056207–1056228	

[a] Sequences in boldface type are specific for the T7 promoter used to initiate in vitro transcription.
[b] Positions of the primers specific for the *B. subtilis* 168 chromosome as indicated in the SubtiList database (http://genolist.pasteur.fr/SubtiList) (22).

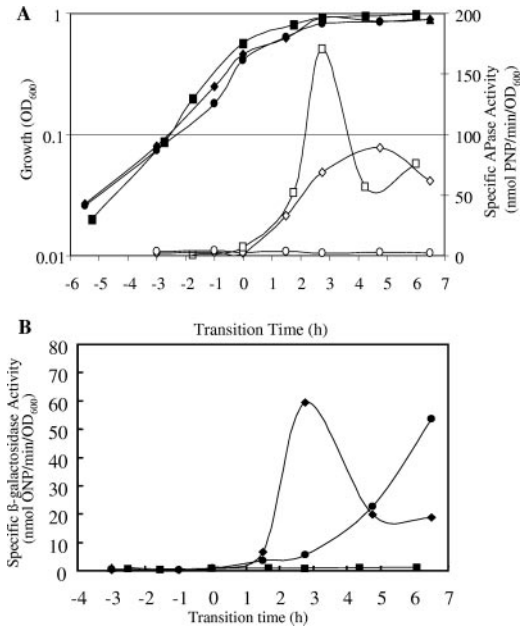

FIG. 1. Growth and reporter gene activities of *B. subtilis skfA-lacZ* reporter gene fusion strains grown in LPM. (A) OD_{600} of mutants 168-SKFA-PR (*phoPR* null mutant) (●), 168-SKFA (◆), and 168-SKFA-OA (*spo0A* null mutant) (■) and APase activities of strains 168-SKFA (◇), 168-SKFA-OA (□), and 168-SKFA-PR (○). (B) Transcriptional activities of *skfA-lacZ*: specific β-galactosidase activities in mutants 168-SKFA-PR (●), 168-SKFA-OA (■), and 168-SKFA (◆). PNP, *p*-nitrophenol.

min/OD_{600} unit at 6 h after entry into stationary phase (T_6) (Fig. 1).

Since *skfA* is a member of the Spo0A regulon (21), in order to determine whether Spo0A had a role in the induction of *skfA* during phosphate starvation, a *spo0A* null mutation was introduced into strain 168-SKFA (*skfA*::*lacZ*) (5). The *spo0A* null lesion eliminated the transcription of *skfA* but not the transcription of another member of the PhoP regulon, *phoA*, since alkaline phosphatase was induced normally during the transition and during early stationary phase (Fig. 1).

Transcription of *skfA* in response to phosphate starvation. Northern blot analysis (14, 25) was used to monitor the expression of genes in the *skf* operon in response to phosphate starvation, using RNA extracted from the wild type and a *phoR* null mutant as described previously (7). The extracted RNA was probed with antisense RNA specific for sequences in the *skfA*, *skfB*, and *skfH* genes. Probes were prepared by PCR with primer pairs (Table 1) by incorporating a promoter sequence recognized by T7 polymerase into the reverse primer. The resulting amplicons were used as substrates for in vitro T7 RNA polymerase-directed synthesis of digoxigenin-labeled *skf*-specific RNA probes (1).

Irrespective of the strain, little or no *skf*-specific RNA was detected in samples taken 2 h before the onset of phosphate starvation (Fig. 2), confirming the low level of expression of this operon under phosphate-replete conditions (P_i concentration, >0.1 mM). When the *skfA*-specific probe was used, a very intense ~0.25-kb transcript was detected during the transition to phosphate limitation (T_0) and 5 h later (T_5) in the wild-type strain (Fig. 2A). The size of this transcript is consistent with the

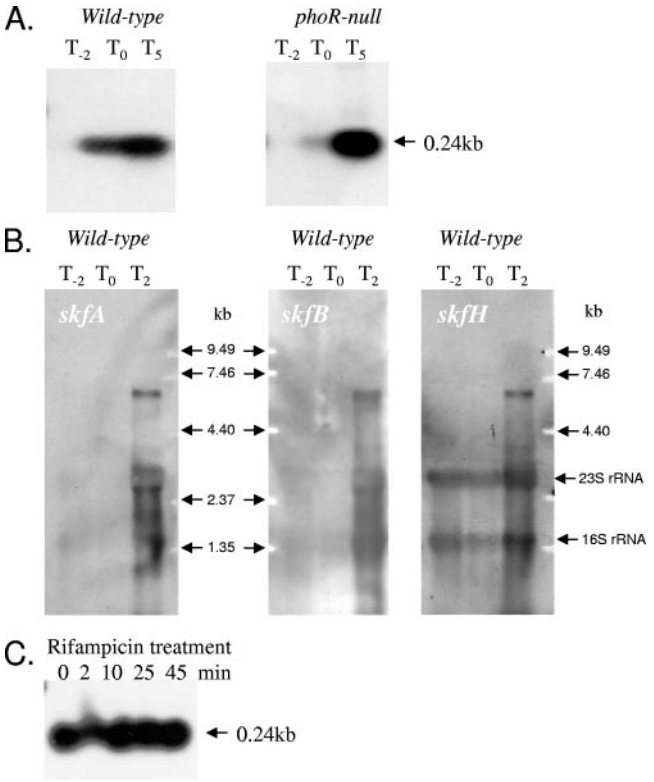

FIG. 2. (A) Northern blot analyses of the *skf* operon. Total RNA was isolated from wild-type strain *B. subtilis* 168 and a *phoR* null mutant. Bacteria were grown in LPM, and samples were taken 2 h before (T_{-2}), at the time of (T_0), and 5 h after (T_5) entry into stationary growth phase provoked by phosphate starvation. Five micrograms of RNA was applied to each lane, and then after capillary blotting the filters were hybridized to *skfA* gene-specific riboprobes. (B) Northern blot analyses of the *skf* operon. RNA was isolated from wild-type strain *B. subtilis* 168. Bacteria were grown in LPM, and samples were taken 2 h before (T_{-2}), at the time of (T_0), and 2 h after (T_2) entry into stationary growth phase provoked by phosphate starvation. Ten micrograms of RNA was applied per lane, and then after an alkaline-transblotting procedure the filters were hybridized to gene-specific riboprobes for *skfA*, *skfB*, and *skfH*. The 0.25-kb transcript was purposely run off the bottom of the gel to facilitate visualization of the weaker bands. (C) Transcriptional stability of *skfA* mRNA. RNA was isolated from wild-type strain *B. subtilis* 168, grown in LPM, and sampled 2 h after entry into stationary growth phase. RNA was isolated from samples harvested before and 2, 10, 25, 45 min after the addition of rifampin. Five micrograms of RNA was applied to each lane, and the membrane was hybridized to a *skfA*-specific riboprobe. Transcript sizes were determined by comparison with digoxigenin-labeled RNA size markers (Roche Diagnostics, Mannheim, Germany). An RNA molecular size ladder (0.24 to 9.5 kb) was purchased from Invitrogen.

predicted length of *skfA*. In the case of the *phoR* null mutant, an ~0.25-kb *skfA*-specific transcript was detected at T_5, but little or no transcript was detected at T_0, confirming that the expression of this gene during phosphate starvation-induced entry into the transition phase is under the influence of the PhoPR two-component signal transduction system. When the gels were run long enough to remove the ~0.25-kb transcript (the intensity of which obscured a significant portion of the gel), a larger less intense transcript was observed (Fig. 2B). The size of this transcript, ~6.5 kb, was consistent with the hypothesis that it carried the entire *skfA-H* operon. When hybridization reactions were carried out with *skfB*- and *skfH*-specific probes, a similar ~6.5-kb transcript was observed (Fig. 2B), but the ~0.25-kb transcript was not observed. Additional bands at intermediate sizes could have been either processing products or mRNA entrapped in the rRNA bands (1).

DNA microarray analysis (11) of the mRNA decay rates of ~1,500 *B. subtilis* mRNA transcripts in early-stationary-phase cultures indicated that about 80% of them had a half-life of less than 7 min. However, the *skfA* transcript was among ~30 mRNA species that were found to have a half-life of >15 min. We therefore attempted to determine the half-life of the phosphate starvation-induced *skfA*-specific transcript following treatment with rifampin, an inhibitor of transcription initiation. *B. subtilis* was grown in LPM, and rifampin was added to the culture 2 h after the transition to the phosphate starvation-induced transition phase (T_2). Samples were harvested immediately before the addition of rifampin (zero time) and at 2, 10, 25, and 45 min after the addition of the antibiotic. The samples were hybridized with the *skfA*-specific probe. As shown in Fig. 2C, the *skfA* mRNA transcript was extremely stable, and no observable decrease in the amount of transcript was detected 45 min after the addition of rifampin. Analysis of the *skfA-skfB* intergenic region using the MFOLD program (32) predicted that the RNA in this region is able to form a stable stem-loop structure ($\Delta G = -23.3$ kcal/mol) (data not shown).

Binding of PhoP and PhoP~P to the region of the *skfA* promoter. The *B. subtilis* genome sequence was interrogated using the pattern-matching function of SubtiList (http://genolist.pasteur.fr/SubtiList/) (22) to identify potential PhoP binding sequences. A 1-bp deviation from the PhoP consensus sequence ($TTHACA_{3-7}TTHACA$, where H is A, C, or T) was allowed for each element of the search sequence, and only targets within 200 bp of a start codon were reported. The promoter region of *skfA* was found to contain at least one PhoP consensus sequence, and a possible second sequence was located immediately downstream (data not shown).

To determine whether the putative PhoP binding sequences identified were active in vitro, gel shift assays we used to analyze the binding of PhoP to the promoter region of *skfA*. Proteins PhoP-His_6 and PhoR231-His_6 were produced from *Escherichia coli* BL21(λD3) carrying pET-PhoP or pET-PhoR231 (25), as described previously (6). Fragments of the *skfA* and *yhaX* promoter regions were amplified with primers YbcO-FOR and YbcO-REV and with primers YhaX-FOR and YhaX-REV (Table 1). In the gel shift reactions, 4 μM PhoR231 and 0.1 μg of poly(dI-dC) (Sigma) per μl were incubated with 0, 24, 47, 71, and 95 μM PhoP in the presence or absence of 5 mM ATP for 15 min at room temperature (20). After addition of the DNA the mixture was incubated for a further 30 min. The samples were analyzed on a 6% native polyacrylamide gel by using Tris-glycine buffer (28) and were stained with Sybr GOLD (Molecular Probes).

Gel shift assays showed that both PhoP and PhoP~P decreased the mobility of the DNA fragment encoding the *skfA* promoter region (Fig. 3A). As described previously (25), there was very little difference in the observed retardation of the probe when PhoP was phosphorylated, indicating either that PhoP and PhoP~P bind to the *skfA* promoter region in vitro with similar efficiencies or that PhoP is spontaneously phosphorylated in *E. coli* (19). As a negative control, a gel shift

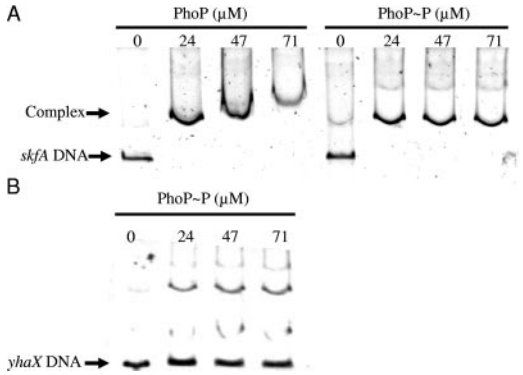

FIG. 3. Gel shift assays of the *skfA* and *yhaX* promoter regions using PhoP and PhoP~P. PhoR231 (4 μM) was incubated with PhoP (0, 24, 47, and 71 μM) in the absence or presence of ATP at room temperature for 15 min. *skfA* (A) and *yhaX* (B) promoter probes were added, and after binding (30 min), the samples were loaded onto a 6% native polyacrylamide gel to separate free DNA probe from DNA-protein complexes. The gels were then stained with Sybr GOLD used according to the manufacturer's instructions and were visualized by UV illumination. The amounts of PhoP and PhoP~P added to the reaction mixtures are indicated above the lanes.

assay was performed for the promoter region of *yhaX* (Fig. 3B), a gene that appears to be induced indirectly by PhoP. As shown previously (25), the mobility of *yhaX* was not influenced by PhoP~P.

Conclusions. *B. subtilis* has a range of adaptive responses which are induced under adverse conditions to maintain viability and, in some cases, to restore growth (1, 2, 4, 15). The role of SkfA is to bring about the lysis of cells that have not been induced to enter the sporulation pathway. This provides cells with activated Spo0A (Spo0A~P) with a source of nutrients (including phosphorus from the cell wall, nucleic acids, membranes, and proteins) (9, 10, 21). The induction of *skfA* during the phosphate starvation-induced transition phase (T_0 to T_3) is dependent on PhoPR. This response is transient and is presumably terminated by Spo0A (18). A later induction event (T_3 to T_6) (Fig. 2) is PhoPR independent and likely to be associated with the onset of sporulation since *skfA* is a member of the Spo0A regulon (9, 21).

The binding of PhoP to the *skfA* promoter region was compared with the binding to the SigE-dependent *yhaX* promoter region (25). In contrast to the binding to *yhaX*, PhoP binds directly to the promoter region upstream of *skfA*, indicating that the *skf* operon is a member of the PhoP regulon. Northern blot hybridization (Fig. 2) detected a weak band corresponding to a full-length transcript of the *skf* operon (6.5 kb) and a much stronger band for the ~0.25-kb transcript corresponding to *skfA*. Our results extend previous data (11) showing that the *skfA* transcript is extremely stable, with an estimated half-life of more than 45 min. Since the kinetics of induction and the relative amounts of β-galactosidase synthesized by strains 168-SKFA (*skfA*::*lacZ*) (Fig. 1) and 168-SKFB (*skfB*::*lacZ*) (not shown) in response to phosphate starvation were similar, it is likely that the genes are transcribed from the same promoter. By analogy with the *pst* operon (1), we propose that the stem-loop acts primarily as a barrier to 3′-5′ exoribonuclease activity with the *skfA* transcript and is therefore responsible for its extraordinary stability (13, 23). This provides a mechanism for coordinating the transcription of all the genes in the *skf* operon, while it allows preferential translation of *skfA* mRNA. Functionally, this should allow a large molar excess of the killing factor peptide compared with the other products of the *skf* operon.

Phosphate limitation is a common feature of life in the soil, the natural environment of *B. subtilis*. Induction of the sporulation killing factor by cells that have entered the sporulation pathway and the subsequent lysis of the noninduced portion of the population provide the former organisms with an important source of nutrients. The resulting influx of nutrients increases the survival potential of these cells and delays the onset of sporulation (9, 10, 21). We showed that the *skf* operon is induced in response to phosphate starvation in a PhoPR-dependent manner. This induction is distinct from that previously reported for Spo0A. An interesting aspect of this induction is its absolute dependence on Spo0A. This observation reinforces previous data which showed that the presence of fully functional PhoPR is not sufficient for maximal expression of the PhoP regulon, since an *spo0A abrB resD* null mutant does not show PhoP regulon activity (16). In the case of the *skf* operon, the requirement for Spo0A presumably arises from the need to coordinate its expression with sporulation since the sporulation killing factor's role is to provide nutrients for the subpopulation of cells that are committed to this differentiation process.

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