

Transition State Regulator AbrB Inhibits Transcription of *Bacillus amyloliquefaciens* FZB45 Phytase through Binding at Two Distinct Sites Located within the Extended *phyC* Promoter Region^{∇†}

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We have previously identified the *phyC* gene of *Bacillus amyloliquefaciens* FZB45, encoding extracellular phytase, as a member of the PhoP regulon, which is expressed only during phosphate starvation. Its σ^A -dependent promoter is positively and negatively regulated by the phosphorylated PhoP response regulator in a phosphate-dependent manner (O. Makarewicz, S. Dubrac, T. Msadek, and R. Borriss, *J. Bacteriol.* 188: 6953–6965, 2006). Here, we provide experimental evidence that the transcription of *phyC* underlies a second control mechanism exerted by the global transition-phase regulator protein, AbrB, which hinders its expression during exponential growth. Gel mobility shift and DNase I footprinting experiments demonstrated that AbrB binds to two different regions in the *phyC* promoter region that are separated by about 200 bp. One binding site is near the divergently orientated *yodU* gene, and the second site is located downstream of the *phyC* promoter and extends into the coding region of the *phyC* gene. Cooperative binding to the two distant binding regions is necessary for the AbrB-directed repression of *phyC* transcription. AbrB does not affect the transcription of the neighboring *yodU* gene.

Several *Bacillus* species produce phytases, extracellular degradative enzymes, which release free phosphates from myo-inositol hexakisphosphate, the main storage form of phosphate in plants. While the monocistronic *phyC* gene is silent in the laboratory strain *Bacillus subtilis* 168, probably due to the absence of functional PhoP binding sites of its transcriptional activator, PhoP, the *phyC* gene of *Bacillus amyloliquefaciens* FZB45 (*phyC*_{FZB45}) is well expressed (8). In vitro and in vivo studies with *phyC*_{FZB45} promoter *lacZ* reporter gene fusions expressed in the heterologous but closely related and genetically amenable *Bacillus subtilis* 168 host revealed that the phosphorylated response regulator PhoP~P affects the *phyC*_{FZB45} expression in a bifunctional manner. The *phyC* gene becomes activated by PhoP~P under phosphate starvation due to the binding of the response regulator to the PhoP box, positioned between –32 and –49. However, rising concentrations of PhoP~P cause the binding of the response regulator to a second site located near the –10 region, thereby reducing the efficiency of transcription by displacing the RNA polymerase (9).

In addition, our earlier studies indicated that the expression of *phyC*_{FZB45} underlies a second level of control. We observed that the induction of phytase under phosphate starvation is delayed until the transition from the exponential to the stationary growth phase (9). A mutant strain bearing a deletion of

the response regulator Spo0A, which governs the initiation of sporulation, was unable to express *phyC*_{FZB45} even under low-phosphate conditions (O. Makarewicz, unpublished observation). Spo0A is known to downregulate the global transition state regulator AbrB (6, 12, 17), which results in the relief of expression of numerous genes involved in functions such as the production of antibiotics (10, 11), the formation of biofilms (4, 7), the development of competence (6), the initiation of sporulation (11, 23), the production of extracellular proteases and other degradative enzymes (13), cannibalistic behavior (6), and, in *Bacillus anthracis*, the expression of toxin genes (15). During the transition state, phosphorylated Spo0A~P relieves AbrB-mediated repression by enhanced binding to the P2 promoter of *abrB*, thus lowering its transcription (12). On the other hand, during exponential growth AbrB indirectly controls the expression of Spo0A by repressing the transcription of the alternative sigma factor *sigH*, which is necessary for the full transcription of *spo0A* (22). In spite of the partial antagonism of both regulators, we decided to carry out *lacZ* reporter studies in a Spo0A- and AbrB-deficient background. Like other global transcriptional regulators shown to affect phytase gene expression (PhoP and Spo0A), AbrB is a highly conserved protein in *Firmicutes*. The 94-amino-acid AbrB proteins from *B. subtilis* and *B. amyloliquefaciens* are distinguished by only a single amino acid substitution. Since genetic studies are not feasible in FZB45 due to its low transformation frequency, we decided to use the related *B. subtilis* 168 as a heterologous host for in vivo studies with the *phyC*_{FZB45} promoter fragment.

Here, we present experimental evidence that in addition to the control exerted by the PhoPR system *phyC*_{FZB45}, transcription is directly repressed by the transition state regulator AbrB during exponential growth. Identical responses were found when using the AbrB protein isolated from *B. subtilis* and the

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description ^a	Source or reference
<i>E. coli</i> strains		
DH5 α	<i>supE44</i> Δ <i>lacU169</i> (Φ 80 <i>dlacZ</i> Δ <i>M15</i>) <i>hsdR17 recA1 gyrA96 thi-1relA1</i>	Lab strain
BL21(DE3)	F ⁻ <i>ompT hsdS_B</i> (r _B ⁻ m _B ⁻) <i>gal dcm</i> (DE3)	Novagen
ECAbrB	F ⁻ <i>ompT hsdS_B</i> (r _B ⁻ m _B ⁻) <i>gal dcm</i> (DE3)::pPHOP	This work
<i>Bacillus amyloliquefaciens</i> strain		
FZB45	wt	FZB Berlin
<i>Bacillus subtilis</i>		
MF1	<i>trpC2 pheA1 rpoC_{His6} Neo^r</i>	M. Fujita
168	<i>trpC2</i>	Laboratory sock
1S13	<i>trpC2 spo0A1</i>	BGSC
OM61	<i>trpC2 amy::lacZ-phyC</i> (495 bp) <i>cat</i> (Cm ^r)	This work
OM61Km	<i>trpC2 amy::lacZ-phyC</i> (495 bp) <i>cat:aphA3</i> (Km ^r)	This work
OM71	<i>trpC2 amy::lacZ-phyC</i> (316 bp) <i>cat</i>	This work
OM545	<i>trpC2 amy::lacZ-phyC</i> (355 bp) <i>cat</i>	This work
OM57	<i>trpC2 amy::lacZ-phyC</i> (176 bp) Cm ^r	This work
OM64	<i>trpC2 spo0A1 amy::lacZ-phyC</i> (495 bp) <i>cat</i>	This work
OM64Km	<i>trpC2 spo0A1 amy::lacZ-phyC</i> (495 bp) <i>cat:aphA3</i>	This work
OM613	<i>trpC2 abrB::Cm^r amy::lacZ-phyC</i> (495 bp) <i>cat:aphA3</i>	This work
OM612	<i>trpC2 spo0A1 abrB::lacZ-phyC</i> (495 bp) <i>cat</i>	This work
OM74	<i>trpC2 spo0A1 amy::lacZ-phyC</i> (316 bp) <i>cat</i>	This work
OM45	<i>trpC2 spo0A1 amy::lacZ-phyC</i> (355 bp) <i>cat</i>	This work
OM457	<i>trpC2 spo0A1 amy::lacZ-phyC</i> (176 bp) <i>cat</i>	This work
EB21	<i>trpC2 amy::lacZ-phyC_{abrB2}</i> (495 bp) <i>cat</i>	This work
EB24	<i>trpC2 spo0A1 amy::lacZ-phyC_{abrB2}</i> (495 bp) <i>cat</i>	This work
EB31	<i>trpC2 amy::lacZ-phyC_{abrB31}</i> (495 bp) <i>cat</i>	This work
EB34	<i>trpC2 spo0A1 amy::lacZ-phyC_{abrB}</i> (495 bp) <i>cat</i>	This work
EB41	<i>trpC2 amy::lacZ-phyC_{abr312}</i> (495 bp) <i>cat</i>	This work
EB44	<i>trpC2 spo0A1 amy::lacZ-phyC_{abrB32}</i> (495 bp) <i>cat</i>	This work
Plasmids		
pDG268	Integrative vector <i>amy::lacZ cat bla</i> (Ap ^r)	C. Antoniewski
pOM6	pDG268/ <i>phyC</i> from -287 (Om01) to +208 (Om09) \rightarrow 495-bp insert	This work
pOM7	pDG268/ <i>phyC</i> from -287 (Om01) to +29 (Om14) \rightarrow 316-bp insert	This work
pCUT5	pDG268/ <i>phyC</i> from -147 (Cut5) to +208 (Om09) \rightarrow 355-bp insert	This work
pCUT57	pDG268/ <i>phyC</i> from -147 (Cut5) to +29 (Om14) \rightarrow 176-bp insert	This work
pEB2	pOM6, substitution of AbrB site 1 (<i>eb2</i>)	This work
pEB3	pOM6, substitution of AbrB site 1 (<i>eb3</i>)	This work
pEB4	pOM6, substitution of AbrB site 1 (<i>eb4</i>)	This work
pEB5	pOM6, substitution of AbrB site 2 (<i>eb5</i>)	This work
pEB6	pOM6, substitution of AbrB site 2 (<i>eb6</i>)	This work
pEB23	pOM6, double substitution of AbrB site 1 (<i>eb2</i> and <i>eb3</i>)	This work
pEB36	pOM6, double substitution of AbrB sites 1 (<i>eb3</i>) and 2 (<i>eb6</i>)	This work
pET15b	Expression vector, T7 <i>lac lacI bla</i>	Novagen
pABRB	pET15b/ <i>abrB</i> of <i>B. subtilis</i> \rightarrow 354-bp insert	This work NdeI/XhoI site
pABRBQ82K	pET15b/ <i>abrB</i> of <i>B. subtilis</i> \rightarrow 354-bp insert, substitution C:A (-245*)	This work NdeI/XhoI site
pPHOP	pET15b/ <i>phoP</i> of <i>B. subtilis</i> \rightarrow 728-bp insert	11
pPHOR231	pET28b(+)/C-terminal domain of <i>phoR</i> of <i>B. subtilis</i> \rightarrow 1,051-bp insert	11
pECE73	Cm ^r ::Km ^r	M. Steinmetz/R. Rich

^a *, position of the substitution of *abrB* is indicated relative to translation start. Neo^r; neomycin resistance.

mutant protein AbrBQ82K, corresponding to the *B. amyloliquefaciens* AbrB (EU549819).

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Bacterial strains and plasmids used in this study are listed in Table 1. Strains were grown in Luria-Bertani (LB) medium and low-phosphate medium, described previously (9). When appropriate, antibiotics were added in the following concentrations: for *Escherichia coli*, 100 mg/liter of ampicillin (Ap) and 50 mg/liter of kanamycin (Km); and for *B. subtilis*, 5 mg/liter of chloramphenicol (Cm) and 5 mg/liter of Km.

DNA manipulations and general methods. Isolation of plasmid and chromosomal DNA, restriction endonuclease digestion, agarose gel electrophoresis, PCR, and transformation of *E. coli* and *B. subtilis* were performed as described previously (16).

Construction of plasmids and bacterial strains. Specific DNA fragments were amplified from the *phyC* promoter region of *B. amyloliquefaciens* FZB45 by use of the primer pairs listed in Table S1 in the supplemental material. Strains

containing promoter-*lacZ* fusions derived from *B. amyloliquefaciens* FZB45 were constructed as previously described (9). Mutagenesis of the AbrB binding sites *eb2*, *eb3*, and *eb4* was performed using the splicing by overlapping extension method. First, PCR with plasmid pOM6 and the following primer pairs were used to generate overlapping fragments: AbrB2for/Om09 and Om01/AbrB2rev, AbrB3for/Om09 and Om01/AbrB3rev, and AbrB4for/Om09 and Om01/AbrB4rev. The fragments were fused in a second PCR in which 10 cycles were run without primers by use of 200 ng of the overlapping fragments and a further 15 cycles were run in the presence of the additional primers Om01 and Om09. The double substitution for *eb24* was performed as for *eb2* but by use of pEB4 as the template. The resulting PCR products were cloned into the EcoRI and BamHI sites of plasmid pDG268 and the mutations were verified by sequencing using sequencing primer Cy5-Om16. Two-base pair substitutions of the AbrB binding sites *eb5*, *eb6*, and *eb36* were introduced by using the QuikChange XL site-directed mutagenesis kit (Stratagene). Plasmid pOM61 was used as the template for mutagenizing *eb5* and *eb6* and pEB3 for *eb36*. The primers used were AbrB5for/AbrB5for and AbrB6for/AbrB6rev. After linearization by XhoI, the resulting plasmids were transformed into competent cells prepared from *B.*

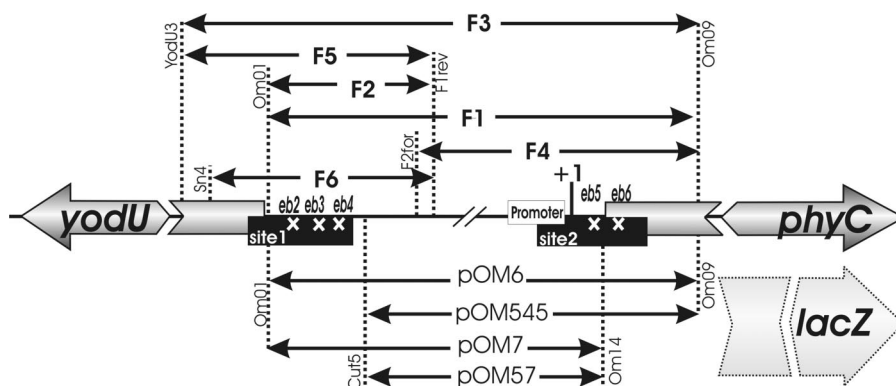


FIG. 1. Schematic representation of the *yodU-phyC* intergenic region of *B. amyloliquefaciens* FZB45. The position of the *phyC*_{FZB45} promoter and the initiation point of *phyC* transcription (+1) are indicated. The two AbrB binding regions (sites 1 and 2) are indicated as filled boxes. The positions of the mutated binding areas (*eb2*, *eb3*, *eb4*, *eb5*, and *eb6*) within regions 1 and 2 are marked by white crosses. Double-headed arrows indicate DNA fragments amplified from parts of the entire *phyC* promoter region. DNA fragments used for *lacZ* fusions are listed at bottom, while DNA fragments used for DNase I footprinting, gel shift, and *in vitro* transcription are listed at top. DNA primers used for amplifying the respective DNA fragments are also shown at the vertical dotted lines.

subtilis 168 and 1S13 (*spo0A* derivative). Plasmids and resulting strains are compiled in Table 1. A schematic representation of the fusions obtained is shown in Fig. 1.

Exchange of the antibiotic-resistant markers was accomplished with the plasmid pECE73 (BGSC). It was transformed into strains OM61 and OM64 to generate strains OM61KM (wild type [wt]) and OM64KM (*spo0A* derivative), in which the Cm resistance cassette was replaced by the Km resistance cassette. To obtain the clones OM612 (*spo0A abrB*) and OM613 (*abrB*), OM61KM and OM64KM were transformed with chromosomal DNA isolated from the *abrB*-negative *B. subtilis* mutant strain JH12586 (*pheA1 trpC2 abrB::cat*) generously supplied by Tarek Msadek, Institut Pasteur. The *abrB::Cm* genotype was verified by PCR using primers AbrB1 and AbrB4. To construct the expression plasmid pABRB, the *abrB* gene was amplified using primers AbrB3 and AbrB4. The PCR product was cloned into the NdeI and BamHI sites of pET15b. To obtain the AbrBQ82K protein, corresponding to AbrB of *B. amyloliquefaciens*, the Q amino acid residue of *B. subtilis* AbrB (AbrB_{sub}) was replaced by K by use of the QuikChange XL site-directed mutagenesis kit with primers AbrBQ82Kfor and AbrBQ82Krev and template pABRB DNA. The sequences of the primers used in this study are compiled in Table S1 in the supplemental material.

Overexpression and purification of proteins. PhoP, PhoR231-His₆, and RNA polymerase were overexpressed and purified as described previously (1, 5, 9, 14). The His₆-AbrB proteins were overexpressed in *E. coli* C41 (BL21) according to the protocol of Novagen. The cells were lysed by sonication in buffer A, consisting of 50 mM Tris-HCl (pH 7.5), 300 mM NaCl, 10 mM β-mercaptoethanol, 10% glycerol, and 1 mM phenylmethylsulfonyl fluoride. His₆-AbrB_{sub} and His₆-AbrBQ82K were purified by Ni-agarose chromatography and dialyzed against 10 mM Tris-HCl (pH 7.5), 300 mM NaCl, and 50% glycerol. Protein concentrations were determined at 280 nm.

Enzyme assays. The assays for alkaline phosphatase (APase) and β-galactosidase were performed as described previously (9), except for a slight modification of the β-galactosidase assay: a 100-μl cell suspension was mixed with 800 μl of Z buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, 1 mM dithiothreitol, 0.5 U/ml benzonase, 100 μg/ml Cm, 0.3 mg/ml lysozyme, 0.005% Triton X-100) and incubated for 10 min at 30°C.

DNase I footprinting assay. DNase I footprinting experiments were performed essentially as described previously (9). Two separate DNA fragments, F6 and F4, covering the extended *phyC* promoter region (Fig. 1), were amplified using the primer pairs Sn4/F1rev and F2for/Om09, respectively, and purified with the QIAquick PCR purification kit (Qiagen). The PCR products were labeled either at the coding or at the noncoding strand as described previously (9). The DNA-binding reactions were performed for 20 min at room temperature in binding buffer with different AbrB concentrations (0, 1.2, 2.4, 4.8, 9.6, 14.4, and 20 μM). The labeled F6 fragment (100,000 cpm) and 5 nM of the nonlabeled F4 fragment and vice versa were used.

Gel shift assay. 5′-γ³²P-labeled DNA fragments corresponding to the *phyC* promoter region were synthesized using the primer pairs Om01/[γ³²P]Om09 (F1), [γ³²P]Om01/F1rev (F2), and F2for/[γ³²P]Om09 (F4) and then purified with the QIAquick PCR purification kit (Qiagen). The binding reaction was carried

out in binding buffer [20 mM Tris-HCl buffer (pH 8), 100 mM KCl, 5 mM MgCl₂, 1 mM dithiothreitol, 10% glycerol, and 0.1 mg/ml poly(dI-dC) as a competitive nonspecific DNA] with labeled DNA (~5 nM) and various AbrB concentrations (0.25 μM to 2 μM) for 20 min at room temperature. The entire *phyC* promoter fragment (pOM6 [Fig. 1]) and the *appA* gene of *E. coli* were used for competition experiments. Concentrations of AbrB (1 μM) and labeled *phyC* (5 nM) were kept constant, and the amounts of the competitor DNA varied between 1 nM and 5 nM. The reaction mixtures were then separated on 6% nondenaturing polyacrylamide gels in 1× Tris-borate-EDTA buffer at 100 V.

In vitro transcription assay. Three linear templates were amplified using the following primer pairs: YodU3/Om09, yielding product F3; F2for/Om09, yielding product F4; and Yod3/F1rev, yielding product F5 (Fig. 1). *Pfu* DNA polymerase and chromosomal DNA of *B. amyloliquefaciens* FZB45 were used for PCR. The *in vitro* transcription was performed using 10 nM DNA, 0.25 μM previously phosphorylated PhoP~P (9), 0.1 μM RNA polymerase, and various concentrations of AbrB or AbrBQ82K (0.25 μM to 2 μM) in transcription buffer (20 mM Tris-HCl, pH 8, 10 mM NaCl, 10 mM MgCl₂, 50 mM KCl, 1 mM CaCl₂, 0.02 mM EDTA, 1 mM dithiothreitol, and 2% glycerol). The reaction mixtures were incubated at 37°C for 20 min, and then the reactions were stopped by the addition of 5 μl stop solution (95% deionized formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol); finally, products were separated on a 6% polyacrylamide gel containing 7 M urea.

Sequence determination. All substitutions were confirmed by sequence analysis. The Thermo Sequenase Cy5 dye terminator kit (Amersham Biosciences) was used to perform the sequencing reactions. The samples were run on ALFexpress II (Amersham Biosciences) using ReproGel high resolution (Amersham Biosciences) and analyzed by DS Gene (Accelrys) and NCBI BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>).

RESULTS

Effects of *spo0A* and *abrB* mutations on the *phyC* promoter activity. To analyze how the antagonistically acting transcriptional regulators AbrB and Spo0A affect phytase expression *in vivo*, we fused the extended FZB45 *phyC* promoter DNA sequence ranging from -289 to +221 with the *lacZ* reporter gene as described previously (9). The resulting construct, pOM6, consisted of the whole *yodU-phyC* intergenic region and the 5′ part of the *phyC* coding region (Fig. 1). Linearized plasmid pOM6 was ectopically integrated into the *amyE* site of the heterologous host *B. subtilis* 168 and its respective mutant strains (see Materials and Methods). We analyzed the *phyC* promoter-driven reporter activities in low-phosphate medium in the genetic backgrounds of *B. subtilis* wt and the *ΔabrB*,

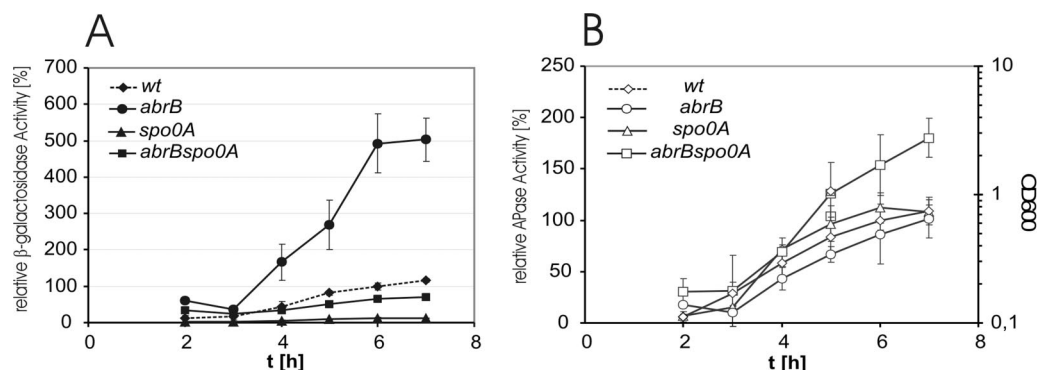


FIG. 2. Effects of *abrB*, *spo0A*, and *abrB spo0A* mutations on the expression of *phyC(pOM6)::lacZ* fusions. (A) Relative β -galactosidase activities were measured from cells grown in low-phosphate medium. The activity of the wt cells grown for 6 h was defined as 100%. (B) The APase activity was measured under the same conditions.

$\Delta spo0A$, and $\Delta abrB spo0A$ mutants. The β -galactosidase activity was abolished in the *spo0A* mutant but was five times enhanced in the *abrB* mutant. The introduction of the *abrB* mutation in the *spo0A* background restored 80% of the β -galactosidase activity compared to what was seen for the wt (Fig. 2A). In contrast, PhoP-dependent expression of the APase was not dramatically altered in the wt and in the respective mutant strains (Fig. 2B). From these results, we conclude that AbrB negatively affects the expression of the *phyC* gene and that Spo0A antagonizes this effect. Since AbrB acts as a positive regulator of the *phoPR* operon (19), the *phyC* expression is only partially restored in the double mutant.

AbrB binds to two different sites within the *phyC* promoter region. To investigate whether AbrB interacts directly with the *phyC* promoter sequence, we expressed and isolated the AbrB protein from *B. subtilis* and the AbrBQ82K protein, corresponding to the *B. amyloliquefaciens* AbrB protein (see Materials and Methods). Sequence comparison revealed that the *abrB* gene products derived from *B. subtilis* 168 and *B. amyloliquefaciens* FZB45 are nearly identical in sequence except for one Gln \rightarrow Lys substitution at position 82.

Two binding sites of AbrB_{sub} at the *phyC*_{FZB45} promoter separated by a spacer region of about 185 bp were identified by DNase I footprinting, despite the fact that the protection patterns of the coding and the noncoding strands were slightly different. To obtain sufficient resolution in the DNase I footprinting assay, we used two different *phyC*_{FZB45} fragments: F6, amplified by primers Sn4 and F1rev, covering the upstream *phyC*_{FZB45} promoter sequence ranging from -392 to -76 ; and F4, amplified by primers F2for and Om09, covering the core promoter region and the 5' part of the *phyC*_{FZB45} coding region ranging from -104 to $+221$ (Fig. 1). One of the two AbrB binding sites (site 2), ranging from -18 to approximately $+107$, overlaps with the -10 region, the transcription start, and extends into the *phyC* coding region. The second site, site 1, is located upstream of the *phyC* core promoter, covering the sequence from -180 to approximately -354 relative to the *phyC* transcription start. Both regions bound by AbrB appeared to be heterogeneous, with protected areas flanked or interrupted by hypersensitive sites (Fig. 3).

Gel retardation studies performed with AbrB_{sub} and AbrBQ82K corroborated the existence of the two AbrB binding sites revealed by DNase I footprinting analysis. Again, we used different DNA fragments either bearing both putative recognition areas (F1) or containing only one of the two binding sites (F2 and F4). The sizes and positions of the fragments are shown in Fig. 1. Both AbrB proteins were able to shift the F1 fragment in a concentration-dependent manner. At low AbrB concentrations of between $0.375 \mu\text{M}$ and $0.875 \mu\text{M}$, an unstable DNA-protein complex was visible. A more stable and larger complex was detected at higher concentrations of AbrB, i.e., those exceeding $1 \mu\text{M}$ (Fig. 4A and B). The addition of the *phyC* promoter DNA as a cold competitor to the DNA-protein complex resulted in the dissociation of the complex and the release of unbound labeled DNA. This effect did not occur when nonspecific competitor DNA derived from the *E. coli appA* gene was used (see Fig. S1 in the supplemental material). AbrB was also able to shift fragments F2 and F4, which contained only one of the two AbrB binding sites. However, the affinities of the AbrB proteins were slightly reduced in those fragments, suggesting that AbrB might possess a higher affinity to the *phyC* promoter when occupying both sites present in the F1 fragment simultaneously.

Three AT-rich elements, *eb2*, *eb3*, and *eb4*, located within the AbrB binding region 1 (Fig. 1), were replaced by the corresponding GC-rich sequences (Fig. 3 and 2). Gel retardation experiments performed with the mutated F2 and F4 fragments revealed decreased affinity to AbrBQ82K. The F2 fragment bearing the mutations *eb2*, *eb3*, and *eb4* began to interact with AbrBQ82K at concentrations above $1 \mu\text{M}$, while the non-mutated fragments were shifted as early as $0.5 \mu\text{M}$ (Fig. 4C). AbrBQ82K did not shift the F2 fragment *eb24*, in which two putative AbrB binding sites within binding region 1, *eb2* and *eb4*, have been eliminated (Fig. 4D). Similarly, the F4 fragments bearing the mutated binding sites *eb5* and *eb6*, located within binding region 2, shifted less in the presence of AbrBQ82K than the corresponding wt sequences, underlining the importance of both regions for AbrB binding.

Mapping of functional AbrB binding sites. To map functional regions important for the AbrB interactions, we introduced deletions at the upstream and downstream termini

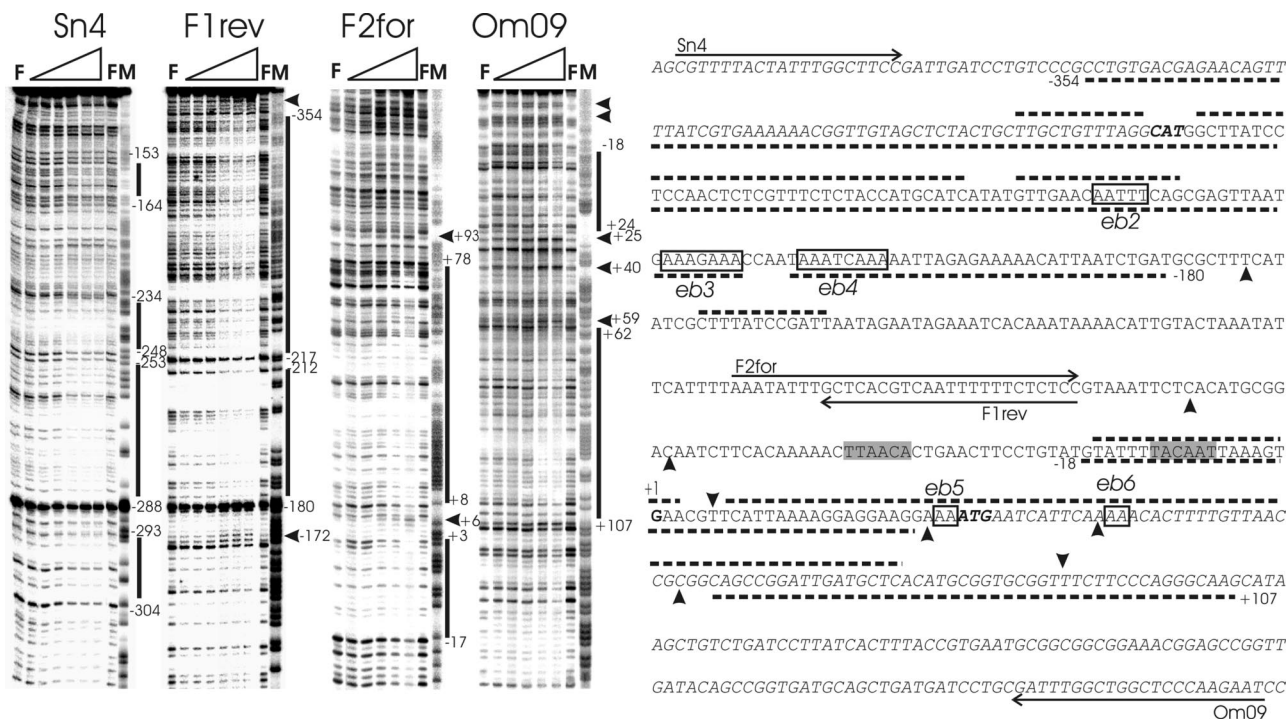


FIG. 3. DNase I footprinting analysis of *AbrB_{sub}* at the *phyC_{FZB45}* promoter region. The footprints of the coding and noncoding strands obtained with fragments F6 (Sn4 and F1rev) and F4 (F2for and Om09) in the presence of increasing concentrations of *AbrB* can be seen in the four panels on the left. The *AbrB* concentrations were 0 μ M (for lanes F) and 0.5 μ M, 1 μ M, 2 μ M (missing for fragment F4), 4 μ M, 6 μ M, and 8 μ M (from left to right for the lanes marked with gradients). M indicates the corresponding A+G (Maxam-Gilbert) sequencing reaction. The protected and hypersensitive sites are marked with bars and arrowheads, respectively. The corresponding sequence of the *phyC* intergenic region is shown on the right. Protected areas of *AbrB* are delineated by dotted lines, and hypersensitive sites are shown by filled arrowheads (top, coding strand; bottom, noncoding strand). The *phyC* and *yodU* coding regions are indicated in italic letters, and the -10 and -35 promoter sequences are shaded. Binding sites within *AbrB* binding regions 1 and 2 (*eb2*, *eb3*, *eb4*, *eb5*, and *eb6*) are framed and labeled.

of the *phyC_{FZB45}* promoter region and fused them with the *lacZ* reporter gene. The resulting integrative plasmids, pOM545, pOM7, and pOM57, missing either of *AbrB* binding sites 1 and 2 (Fig. 1), were ectopically integrated as single copies into the chromosomes of *B. subtilis* 168 and its *spo0A* derivative, 1S13. The *lacZ* reporter activities were measured in low-phosphate medium under *phyC*-inducing conditions. Both the upstream and downstream truncations

yielded similar effects. Each deletion resulted in a fourfold increase in the reporter gene activity compared to what was seen for the full-length promoter fragment, pOM6 (Table 2). Double truncations at both sites did not further augment the activity of the reporter gene. β -Galactosidase activity remained in the same range as that determined for the single truncations. Interestingly, the repressive effect on *phyC* expression observed for pOM6 in the *spo0A* back-

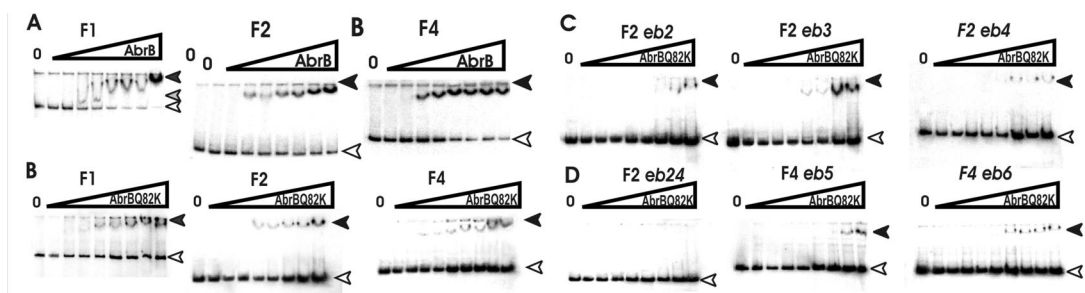


FIG. 4. Gel retardation mobility shift assays in the presence of increasing concentrations of *AbrB* and *AbrBQ82K*. *AbrB* concentrations used were 0 (for lanes 0) and 0.25, 0.5, 0.75, 1, 1.25, 1.5, and 2 μ M (from left to right for the lanes marked with gradients). (A) Gel shift analysis performed with DNA fragments of different lengths bound to *AbrB*. (B) Assay corresponding to that for panel A but performed with *AbrBQ82K*. Fragment F1 harbors the entire *phyC* promoter region with both *AbrB* binding regions, fragment F2 harbors region 1, and fragment F4 harbors region 2. (C and D) Effect of mutations *eb2*, *eb3*, *eb4*, and *eb24* introduced into *AbrB* binding region 1 on the mobility of the F2 fragment and effect of mutations *eb5* and *eb6* within *AbrB* binding region 2 on the mobility of the F4 fragment. White arrowheads, free DNA; filled arrowheads, protein-DNA complex.

TABLE 2. β -Galactosidase activities of *phyC* promoter-*lacZ* fusions expressed in the wt (*B. subtilis* 168) and the *spo0A* mutant (*B. subtilis* 1S13)

Plasmid	Promoter range/substitution ^a	% Relative promoter activity (\pm SD) for:	
		wt	<i>spo0A</i> mutant
pOM6	-287 to +208	100 (\pm 10)	5 (\pm 0.5)
pOM545	-147 to +208	370 (\pm 20)	350 (\pm 20)
pOM7	-287 to +29	410 (\pm 20)	420 (\pm 15)
pOM57	-147 to +29	375 (\pm 25)	470 (\pm 20)
pEB2	-241 to -236 (<u>AAATTT</u> : <u>CCCCC</u>)	207 (\pm 15)	247 (\pm 17)
pEB3	-223 to -216 (<u>AAAGAAA</u> : <u>CCCGCCC</u>)	326 (\pm 23)	356 (\pm 25)
pEB4	-211 to -203 (<u>AAATCAAAA</u> : <u>CCCTCCCC</u>)	372 (\pm 25)	302 (\pm 20)
pEB5	+25 to +27 (<u>AA</u> : <u>CC</u>)	98 (\pm 7)	103 (\pm 7)
pEB6	+40 to +42 (<u>AA</u> : <u>CC</u>)	311 (\pm 22)	278 (\pm 19)
pEB24	-241 to -236 (<u>AAATTT</u> : <u>CCCCC</u>)	278 (\pm 20)	417 (\pm 19)
pEB36	-211 to -203 (<u>AAATCAAAA</u> : <u>CCCTCCCC</u>)	263 (\pm 13)	378 (\pm 15)
	+40 to +42 (<u>AA</u> : <u>CC</u>)		
	-223 to -216 (<u>AAAGAAA</u> : <u>CCCGCCC</u>)		

^a The nucleotide substitutions of the mutations *eb2*, *eb3*, *eb4*, *eb5*, and *eb6* are indicated with underlining.

ground was completely abolished (Table 2). This indicates that simultaneous binding of AbrB to both sites is necessary for full repression.

These findings were corroborated by the results obtained with *lacZ* fusion strains bearing nucleotide substitutions within one of the AbrB binding regions, i.e., regions 1 (*eb2*, *eb3*, *eb4*, and *eb24*) and 2 (*eb5* and *eb6*), or within both of them (*eb36*). The substitutions of the AT-rich sequences located either between -241 and -203 (AbrB binding region 1) or between +8 and +78 (AbrB binding region 2) by the corresponding GC-rich sequences relieved the inhibition of *phyC*_{FZB45} expression by AbrB. The activity of the *lacZ* reporter gene was at least two times enhanced in the mutant strains. The effect of the mutations introduced within the AbrB binding region was more pronounced in the Δ *spo0A* background. Despite the continuous production of AbrB in the absence of Spo0A, *phyC*_{FZB45} promoter activity was found to be enhanced by three to five times (Table 2), suggesting reduced repressor affinity for AbrB binding sites 1 and 2. Remarkably, our attempt to relieve the AbrB-dependent inhibitory effect by introducing a corresponding substitution, *eb5*, in AbrB binding region 2 resulted in comparably reduced reporter gene activities in both wt and *spo0A* mutants. We assume that altering the nucleotide sequence at +25 and +26 affects translation initiation at the Shine-Dalgarno sequence, which is in close vicinity to the substituted sequence. On the other hand, the introduction of a mutated sequence within binding site *eb6* (+40 and +41) resulted in a complete abolition of the repressive effect. No dependence on the genetic background (wt or *spo0A*) was registered (Table 2).

Both AbrB binding sites are required for efficient transcriptional repression. Genetic and in vitro analyses revealed that the repressive effect of AbrB on *phyC* gene expression is due to the binding of AbrB to the *phyC*_{FZB45} promoter region. In vitro transcription performed in the presence of 0.2 μ M phosphorylated PhoP together with F3, the full-length DNA fragment harboring both AbrB binding sites (Fig. 1), confirmed this idea. Rising AbrB concentrations gradually repressed the synthesis of the 221-bp *phyC* gene fragment. A faint second transcript, most likely indicating weak transcription of the divergently orientated *yodU* gene, was also visible. The transcript

was more strongly expressed when using fragment F5 in the transcription assay. In any case, AbrB did not affect its expression.

No inhibition of *phyC* transcription by AbrB was detected when fragment F4 was used as the template. F4 contains only promoter-proximal AbrB binding site 2 (Fig. 1), again suggesting that direct contact of AbrB with one binding area is not sufficient to inhibit *phyC* expression. Interestingly, the repressing effect exerted by AbrB was gradually restored in the presence of increasing amounts of DNA fragment F5, containing promoter-distal AbrB binding site 1, which is in close vicinity to the divergently orientated *yodU* gene (Fig. 1). On the other hand, the addition of increasing amounts of fragment F4 to the F5 template did not restore the inhibitory effect of AbrB, which was registered when the entire fragment was incubated with increasing AbrB concentrations. No differences of the effects exerted by AbrB_{sub} and AbrBQ82K on in vitro transcription were detected (Fig. 5).

DISCUSSION

Using an array of in vivo and in vitro experiments, we have shown here that the growth phase-dependent expression of the FZB45 *phyC* gene is directly controlled by the global transition state regulator AbrB, a transcription factor known to control the expression of more than 60 different genes during late exponential and early stationary phase (18). We detected the repression of the *phyC*_{FZB45} promoter activity in the *spo0A* mutant, while *phyC*_{FZB45} gene transcription was found to be strikingly enhanced in the *abrB*-negative background. In the *abrB spo0A* double mutant, the *phyC*_{FZB45} promoter-driven expression of the *lacZ* reporter was nearly completely restored, suggesting that *phyC* underlies repression by AbrB. DNase I footprinting demonstrated that AbrB binds to two remote regions flanking the core *phyC* promoter which are separated by about 200 bp. One binding region, site 1, lies near the mainly silent *yodU* gene and is located approximately between -300 and -180 relative to the *phyC* transcription initiation site. This area consists of three AT-rich core binding sites of 15 bp, 18 bp, and 27 bp in size. The second AbrB binding region, site 2, overlaps the +1 transcription initiation site and extends into

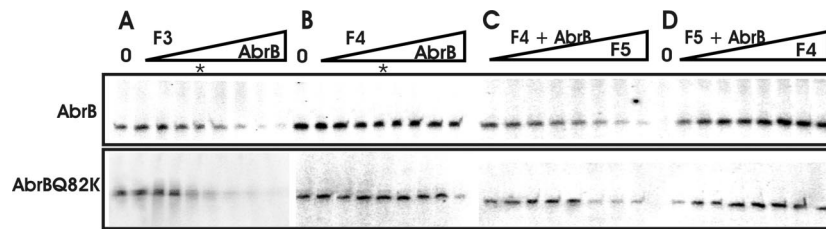


FIG. 5. In vitro transcription analysis of different *phyC* fragments. The transcription reactions were carried out in 20- μ l volumes in transcription buffer by use of 200 fmol of the template, 0.25 μ M PhoP~P, and 0.1 μ M RNA polymerase (*B. subtilis*). The F3 fragment, harboring the entire *phyC* promoter region with AbrB binding sites (A), and the F4 fragment, harboring the promoter proximal AbrB binding site 2 (B), were transcribed in the presence of various AbrB or AbrBQ82K concentrations as described for Fig. 4. The concentration of 1 μ M AbrB or AbrBQ82K is indicated by an asterisk. (C) In vitro transcription of F4 in the presence of 1 μ M AbrB and increasing concentrations of the complementary F5 DNA fragment (Fig. 1). (D) In vitro transcription of fragment F5, which does not contain the *phyC* coding region, in the presence of 1 μ M AbrB or AbrBQ82K and increasing concentrations of the complementary fragment F4. The DNA fragments were added at amounts from 0 fmol to 400 fmol in 50-fmol steps.

the *phyC* coding region. It consists of two core binding sites of about 21 and 23 bp (Fig. 3).

Although AbrB interacts with numerous DNA targets, no general consensus sequence for AbrB binding sites could be defined. Local variations of the DNA helix configurations (e.g., minor groove width and degree of propeller twisting, etc.) contribute to the differential binding proclivities of AbrB (3). Here, we demonstrated that the replacement of local AT-rich sequence stretches within the AbrB binding region by GC-rich oligonucleotides decreases affinity toward AbrB. Simultaneously, the repressing effect of AbrB on *phyC*_{FZB45} gene transcription was abolished. This effect was especially pronounced in *spo0A* mutants, in which AbrB is permanently expressed (Table 2). The functional form of AbrB has been described as a homotetramer rather than as a homodimer (2, 20). DNA-binding and dimerization functions are located in the N-terminal domain (AbrBN), and the C-terminal domain is responsible for the tendency towards multimerization (2, 3, 20, 21). In this study, we have not addressed the oligomeric state of AbrB, but gel filtration suggested that AbrB could form oligomers containing more than four subunits (see Fig. S2 in the supplemental material), at least in vitro.

In its tetrameric state, AbrB possesses two oppositely oriented DNA-binding sites at its N terminus. This fact allows us to speculate that the two remote AbrB recognition sites may be bound and held together simultaneously by the AbrB tetramer. However, in spite of the large distance between the two sites, it seems that AbrB molecules of a higher oligomeric state, or, more likely, multiple AbrB tetramers, are involved in the binding. In addition, given the extensive nature of the AbrB protected sites in DNase I footprinting (Fig. 3), it is unlikely that one AbrB tetramer causes such an effect. The full functionality of AbrB is accomplished only if both AbrB target sites are occupied. This idea is supported by the following experimental data. (i) Leftward and rightward deletions of the promoter fragment fused with the *lacZ* reporter gene relieved AbrB-dependent repression. Similarly, mutation of each of the two AbrB binding regions relieved the regulatory effect exerted by AbrB. Only if both binding sites were available was *phyC*_{FZB45} promoter expression repressed. (ii) Gel retardation experiments revealed that the affinity of AbrB for the *phyC*_{FZB45} promoter fragment was diminished in DNA fragments consisting of only one binding region. A similar observation was made

when one of the two AbrB binding regions had been mutated. (iii) The in vitro transcription of *phyC* in the presence of PhoP~P and AbrB confirmed that no repression occurs if shortened *phyC* DNA fragments containing only one of the AbrB binding sites are used as templates. Remarkably, by providing the promoter-distal AbrB binding region 1 by use of a separate DNA fragment, the inhibitory effect of AbrB on *phyC* transcription could be restored (Fig. 5C).

What are the physiological consequences of our finding that AbrB negatively affects *phyC* gene expression in vivo and in vitro? Gene expression in response to phosphate depletion during vegetative growth is a well-known phenomenon. Genes which meet this criterion and which are also dependent on the PhoP-PhoR two-component system have been defined as members of the Pho regulon. We have shown previously that the *phyC* gene also belongs to this group (9). However, repression exerted by the transition state global regulator AbrB hinders an adequate response to phosphate depletion during vegetative growth. If we assume that the main function of phytase lies in its ability to overcome phosphate limitation by making an additional source of this important nutrient available, we must revise the simple model that the limitation of phosphate leads directly to the expression of the target gene via activation of the PhoPR two-component signal transduction system. The AbrB-dependent reduction of *phyC* gene expression even in the presence of the phosphorylated response regulator suggests that lowering the growth rate is a basic necessity for the action of PhoPR on *phyC* gene expression. As a positive regulator of the *phoPR* operon, AbrB leads to the synthesis of sufficient amounts of the *phoPR* gene products before transition to the stationary growth phase. This ensures that the activated response regulator PhoP~P is ready to act immediately when growth reduction due to phosphate limitation occurs and AbrB expression is relieved by Spo0A-P.

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