



# Binase-like guanyl-preferring ribonucleases are new members of *Bacillus* PhoP regulon

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## ABSTRACT

Extracellular low-molecular weight guanyl-preferring ribonucleases (LMW RNases) of *Bacillus* sp. comprise a group of hydrolytic enzymes that share highly similar structural and catalytic characteristics with barnase, a ribonuclease from *Bacillus amyloliquefaciens*, and binase, a ribonuclease from *Bacillus intermedius*. Although the physical-chemical and catalytic properties of *Bacillus* guanyl-preferring ribonucleases are very similar, there is considerably more variation in the environmental conditions that lead to the induction of the genes encoding these RNases. Based on structural differences of their genes the guanyl-preferring ribonucleases have been sub-divided into binase-like and barnase-like groups. Here we show the ability of the key regulator of phosphate deficiency response, PhoP, to direct the transcription of the binase-like RNases but not barnase-like RNases. These results, together with our demonstration that binase-like RNases are induced in response to phosphate starvation, allow us to categorise this group of ribonucleases as new members of *Bacillus* PhoP regulon. In contrast, the barnase-like ribonucleases are relatively insensitive to the phosphate concentration and the environmental conditions that are responsible for their induction, and the regulatory elements involved, are currently unknown.

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## 1. Introduction

Representatives of the soil-living bacterial genus *Bacillus* secrete a variety of macromolecular hydrolases that allow them to utilize the complex carbohydrates, proteins and nucleic acids in their environment as sources of nutrients. Nucleic acids provide sources of carbon, nitrogen and phosphate and consequently, bacteria often secrete nucleases to facilitate the recovery of these essential nutrients from environmental DNA and RNA. Several species of *Bacillus*, including *Bacillus amyloliquefaciens* (Bam RNase, barnase), *Bacillus pumilus* (Bpu RNase), *Bacillus thuringiensis* (Bth RNase), *Bacillus intermedius* (Bin RNase, binase) and *Bacillus circulans* (Bci RNase) [Hartley and Barker, 1972; Aphanasenko et al., 1979; Struminskaia et al., 1992; Dement'ev et al., 1993a; Dementiev et al., 1993b], secrete low-molecular weight guanyl-preferring ribonucleases

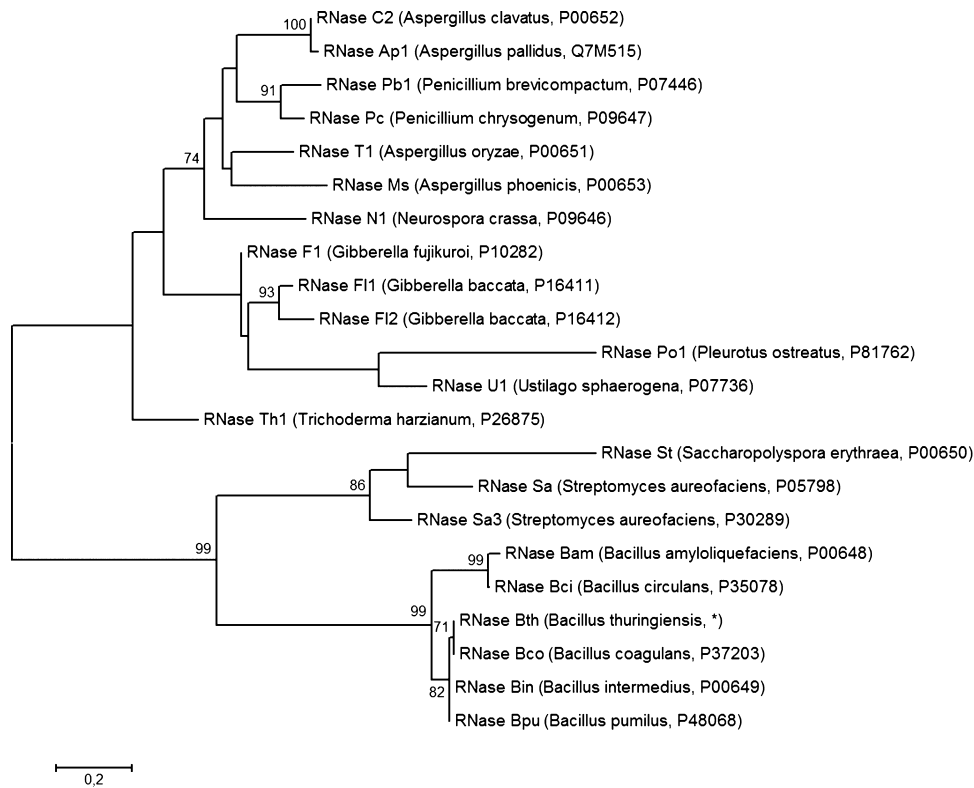
(LMW RNases), that belong to N1/T1 ribonuclease family (Fig. 1). These basic proteins of *Bacillus* are between 109 and 110 amino acid residues in length with a molecular mass of about 12 kDa. Their primary structures share more than 80% identity, with similar secondary and tertiary structures [Bycroft et al., 1991; Reibarkh et al., 1998]. Although the physical-chemical and catalytic properties of *Bacillus* guanyl-preferring ribonucleases are similar, there is considerable variation in the environmental conditions that lead to the induction of the genes encoding these RNases [Ulyanova et al., 2011]. This variability is reflected in differences in their upstream regulatory sequences (Fig. 2A). Based on these and other features, LMW RNases of *Bacillus* can be divided into two groups – binase-like RNases that include Bin, Bpu and Bth and barnase-like RNases that include Bam and Bci (Fig. 1). As an example, these RNases respond to phosphate availability differently. Generally, binase-like RNases are repressed at high phosphate concentrations and strongly induced at low concentrations. In contrast barnase-like RNases are less sensitive to the concentration of phosphate in the medium, indicating that they may have an ecological role that is distinct to that of binase-like RNases [Znamenskaya et al., 1995; Znamenskaia et al., 1998; Shul'ga et al., 2000; Ulyanova et al., 2011].

The adaptation of *Bacillus subtilis* to phosphate-starvation conditions involves the activation of the Pho stimulon, comprising at least the specific PhoP regulon, regulated by the PhoP-PhoR

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**Fig. 1.** A maximum-likelihood phylogenetic tree constructed based on amino acid sequences of ribonucleases from N1/T1 family using a Poisson model with bootstrap values determined by 1000 replicates in the MEGA6 software [Tamura et al., 2013]. The UniProtKB/Swiss-Prot accession numbers of the ribonucleases are indicated in parentheses. (\*) Amino acid sequence of *B. thuringiensis* ribonuclease was reported by Dement'ev et al. (1993a).

two-component system, and the general stress regulon, regulated by the alternative sigma factor, SigB [Antelman et al., 2000; Allenby et al., 2005]. Consensus sequences homologous to the PhoP-binding sites of *B. subtilis* (Pho boxes) were only identified in the upstream regulatory sequences of the binase-like RNase genes (Fig. 2A) [Znamenskaya et al., 1999]. Consequently, by cloning these genes into wild type and *phoPR*-null ( $\Delta phoP$  and  $\Delta phoR$ ) mutant strains of *B. subtilis*, it was shown that the expression of binase-like RNase genes was under the positive control of the PhoP-PhoR two-component system [Znamenskaya et al., 1999; Morozova et al., 2001].

In this work we studied the *in vitro* interactions between the PhoP response regulator and the upstream regulatory regions of genes encoding Bin-like guanyl-preferring ribonucleases. We also show that these genes are not members of the SigB regulon.

## 2. Materials and methods

### 2.1. Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used in the study are listed in the Table 1. *Escherichia coli* BL21  $\lambda$ DE3 was the host strain for the production of *B. subtilis* PhoP-His<sub>6</sub> and PhoR231-His<sub>6</sub> proteins from expression vectors pET-PhoP and pET-PhoR231. Genomic DNA of *B. amyloliquefaciens* H2, *B. circulans* BCF247, *B. thuringiensis* var. *subtoxicus* B388, *B. intermedius* 7P, *B. pumilus* KMM62 was used for the amplification of the upstream regulatory regions of the guanyl-preferring ribonuclease genes. The roles of SigB, PhoP and PhoR were studied in *B. subtilis* strains JH642 (wild type) and *B. subtilis* PB73 (*sigB::cat*) MH5117 ( $\Delta phoP$ ) and MH5124 ( $\Delta phoR$ ) carrying plasmids pMZ55 or pMZ59 encoding, respectively, RNases Bin or Bci.

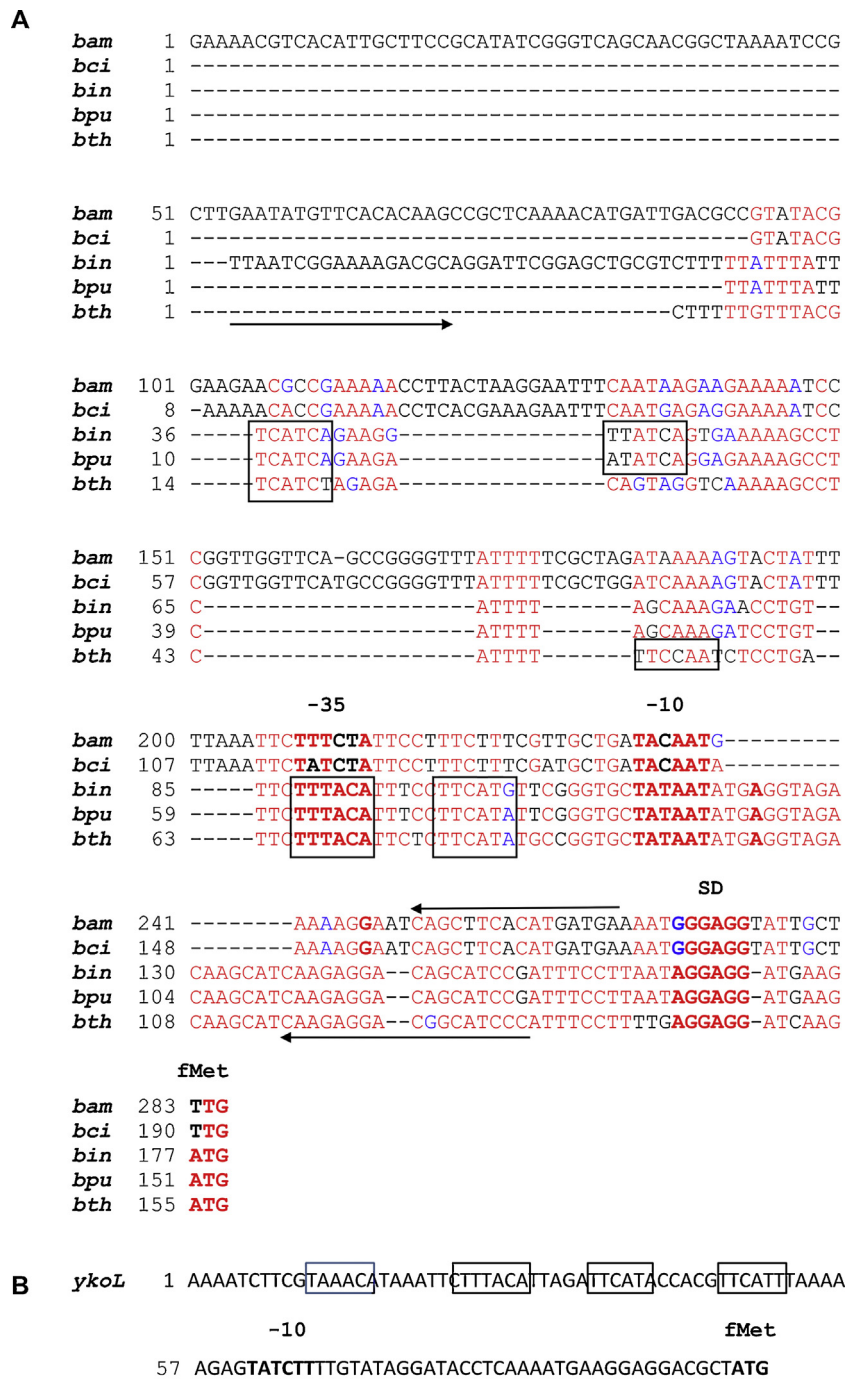
Strains were grown in Luria Bertani (LB) medium or low phosphate medium (LPM; 0.042 mM Pi) contained (g/l): Tris, 6.05; KCl, 5.0; NaCl, 1.0; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2.0; sodium citrate, 1.0; pH 8.5 with separate addition of MgSO<sub>4</sub> × 7H<sub>2</sub>O, 0.2; yeast extract, 0.5; glucose, 5.0. When required, antibiotics were added at the following concentrations: for *E. coli*, 100  $\mu$ g ml<sup>-1</sup> ampicillin (Ap); for *B. subtilis*, 10  $\mu$ g ml<sup>-1</sup> chloramphenicol (Cm), 10  $\mu$ g ml<sup>-1</sup> kanamycin (Km), 10  $\mu$ g ml<sup>-1</sup> tetracycline (Tet). Phenylalanine and tryptophan were added to a final concentration of 50  $\mu$ g ml<sup>-1</sup>.

### 2.2. DNA manipulations

Extraction of plasmid and chromosomal DNA, restriction endonuclease digestion, agarose gel electrophoresis, transformation of *E. coli* and *B. subtilis* cells, and PCR analysis were carried out according to Sambrook and Russell (2001) and Harwood and Cutting (1990).

### 2.3. Production PhoP-His<sub>6</sub> and PhoR231-His<sub>6</sub>

His-tagged versions of PhoP and a soluble (N-terminally truncated) PhoR were prepared from *E. coli* BL21  $\lambda$ DE3 carrying expression plasmids pET-PhoP or pET-PhoR231 [Prágai et al., 2004]. Cultures of these strains were grown to exponential phase (OD ~0.5) in LB medium at 30 °C and heterologous protein production induced by the addition of 0.5 mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG). PhoP-His<sub>6</sub> or PhoR231-His<sub>6</sub> proteins were purified from crude cell extracts on a Ni-NTA agarose column as described previously [Chastanet et al., 2003]. The purification of PhoP-His<sub>6</sub> or PhoR231-His<sub>6</sub> was monitored by SDS-PAGE using a 10% polyacrylamide gel and Tris-glycine buffer (pH 8.8) [Laemmli, 1970]. The gels were stained with Coomassie Blue and



**Fig. 2.** (A) Multiple alignment of promoter regions of guanyl-preferring ribonuclease genes from *B. amyloliquefaciens* H2 (*bam*), *B. circulans* BCF247 (*bci*), *B. intermedius* 7P (*bin*), *B. pumilus* KMM62 (*bpu*) and *B. thuringiensis* B388 (*bth*). Putative PhoP-binding sites are boxed. Arrows indicate positions of forward and reverse primers upon PCR amplification of barnase-like (upper arrows) and binase-like (lower arrows) promoters. (B) Promoter region of *ykoL* gene. PhoP-binding sites [Robichon et al., 2000] are shown in boxes. (C) Sequence logo of PhoP-binding consensus site. Logo was generated on the basis of a multiple alignment of known Pho boxes represented in a database of transcriptional regulation in *B. subtilis* (DBTBS, <http://dbtbs.hgc.jp/>) using WebLogo 3.3 tool [Crooks et al., 2004].

**Table 1**  
List of strains and plasmids used in the study.

Strain or plasmid	Relevant characteristics	Source or reference
<b>Strains</b>		
<i>E. coli</i> BL21 $\lambda$ DE3	F <sup>-</sup> <i>ompT gal dcm lon hsdS<sub>B</sub> (r<sub>B</sub> m<sub>B</sub><sup>-</sup>)</i> $\lambda$ DE3 ( <i>lacI lacUV5-T7 ind1 sam7 nin5</i> )	Novagen
<i>B. subtilis</i> JH642	<i>pheA1, trpC2</i>	BGSC, USA
<i>B. subtilis</i> PB73	<i>furB1 sigB::cat trpC2</i>	BGSC, USA
<i>B. subtilis</i> MH5117	<i>pheA trpC2 <math>\Delta</math>phoP EcoRlmdh::Tet<sup>r</sup></i>	M. Hulett, The University of Illinois at Chicago, USA
<i>B. subtilis</i> MH5124	<i>pheA1 trpC2 <math>\Delta</math>phoR Ballmdh::Tet<sup>r</sup></i>	M. Hulett, The University of Illinois at Chicago, USA
<i>B. amyloliquefaciens</i> H2	Wild isolate	R.W. Hartley, NIH, USA
<i>B. circulans</i> BCF247	Wild isolate	Bioengineering Center of Russian Academy of Sciences
<i>B. thuringiensis</i> var. <i>subtoxius</i> B388	Wild isolate	Bioengineering Center of Russian Academy of Sciences
<i>B. intermedius</i> 7P	Wild isolate	All-Russian Collection of Microorganisms – VKM
<i>B. pumilus</i> KMM62	Wild isolate	Pacific Institute of Bioorganic Chemistry, Russia
<b>Plasmids</b>		
pET-PhoP	pET2816 containing a 722-bp insert of <i>phoP</i> , Ap <sup>r</sup>	Prágai et al., 2004
pET-PhoR231	pET2816 containing a 1049-bp insert of <i>phoR</i> , Ap <sup>r</sup>	Prágai et al., 2004
pMZ55	pUB110 containing full-length <i>binase</i> and <i>barstar</i> genes, Km <sup>r</sup>	Znamenskaya et al., 1999
pMZ59	pUB110 containing full-length RNase Bci and <i>barstar</i> genes, Km <sup>r</sup>	Znamenskaya et al., 1999

low-molecular weight unstained protein standards were used as molecular size markers (Bio-Rad).

#### 2.4. Gel shift assay

Gel shift assays were used to determine the binding of PhoP to the regulatory regions upstream of the guanyl-preferring ribonuclease genes. The DNA probes (encoding the promoter and upstream regulatory regions of guanyl-preferring ribonuclease genes, Fig. 2A) were generated using the following primers: Ba-for 5'GAAAACGTCACATTGC3', Ba-rev 5'TCATCATGTGAAGCTG3', Bp-for 5'TTAATCGGAAAAGACG3', Bp-rev 5'GAAGCTGTCTCTTG3'. The DNA probes were incubated with PhoP-His<sub>6</sub> and PhoR231-His<sub>6</sub> in the presence or absence of ATP as described previously [Allenby et al., 2006]. Samples were analysed on 5% native polyacrylamide gels using Tris-glycine buffer and stained with SYBR Gold (Molecular Probes).

#### 2.5. Enzyme assay

The ability of the recombinant *B. subtilis* strains to produce the required ribonucleases was assessed qualitatively after growth on LPM agar medium supplemented with yeast RNA (5 mg ml<sup>-1</sup>) [Jeffris et al., 1957]. For the quantitative determination of RNase activity, overnight cultures in LB were diluted into pre-warmed

LPM to an OD<sub>590</sub> of between 0.1 and 0.15 and growth continued at 37 °C with shaking at 200 rpm. Samples (1 ml) were collected for the determination of growth (OD<sub>590</sub>) and the measurement of RNase activity, as described previously [Znamenskaya et al., 1999].

#### 2.6. Bioinformatical analysis

Manually annotated and reviewed sequences of ribonucleases from N1/T1 family were extracted from UniProtKB/Swiss-Prot database. A search for orthologues of *B. subtilis* PhoP was made using tools at MicrobesOnline database at the Virtual Institute of Microbial Stress and Survival (<http://www.microbesonline.org>) and the BLAST algorithm at the National Center for Biotechnology Information NCBI (<http://www.ncbi.nlm.nih.gov/>). PhoP-binding sites were retrieved from DBTBS: a database of transcriptional regulation in *B. subtilis* (<http://dbtbs.hgc.jp/>). Multiple alignments were accomplished in MEGA6 software [Tamura et al., 2013] using built-in Muscle algorithm. Similar regions in the alignments were highlighted using Boxshade ver. 3.21 from ExpASy Bioinformatics Resource Portal ([http://www.ch.embnet.org/software/BOX\\_form.html](http://www.ch.embnet.org/software/BOX_form.html)). A phylogenetic tree of N1/T1 ribonucleases was constructed based on amino acid sequences using a maximum-likelihood algorithm and a Poisson model with bootstrap values determined by 1000 replicates in the MEGA6 software [Tamura et al., 2013]. Sequence logo of PhoP-binding sites was made using WebLogo 3.3 tool [Crooks et al., 2004].

### 3. Results

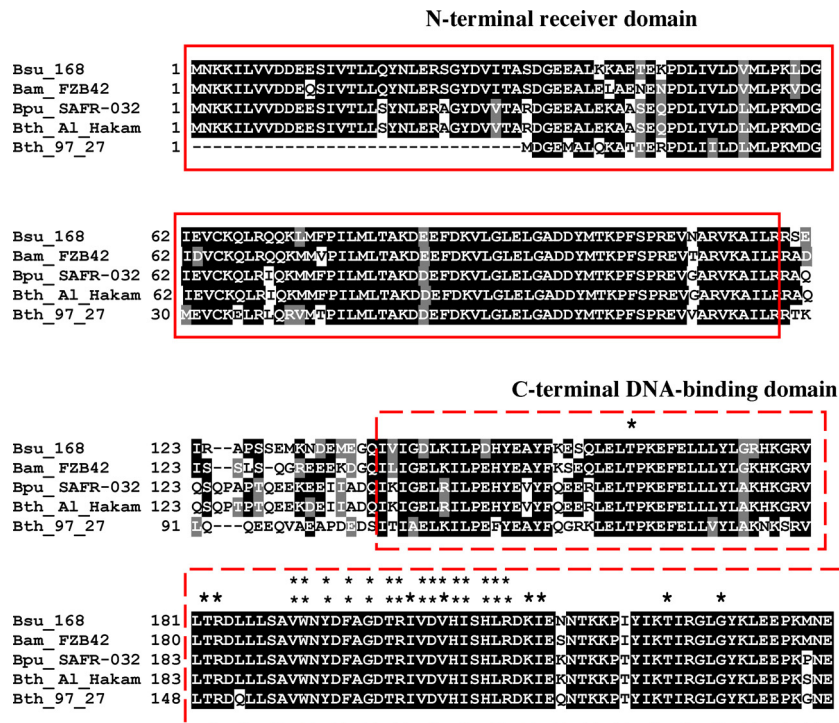
#### 3.1. Orthologues of *B. subtilis* *phoP*

The PhoP response regulator, controlling the phosphate-starvation response in *B. subtilis*, is well conserved among representatives of the genus *Bacillus*, and orthologues are encoded by all currently sequenced *Bacillus* genomes, as confirmed by the Basic Local Alignment Search Tool (blastn) searching the nucleotide database at the National Center for Biotechnology Information (NCBI). Moreover, the *phoP* genes are located in similar genetic neighbourhoods, as viewed using the Genome Browser at MicrobesOnline ([www.microbesonline.org](http://www.microbesonline.org)). The degree of identity of the PhoP protein sequences ranges of 68–92%. To locate more precisely their conserved and variable regions, we compared the amino acid sequences of PhoP proteins from *B. amyloliquefaciens*, *B. pumilus* and *B. thuringiensis* with that of *B. subtilis* (Fig. 3). This analysis showed that the functionally significant residues associated with the DNA-binding output domains were conserved, whereas the regions linking the N- and C-terminal domains were more variable. In view of the conserved nature of the output domain, we concluded that we could use the *B. subtilis* PhoP protein for experiments aimed at demonstrating *in vitro* interactions with the promoter regions of guanyl-preferring RNase genes from these related species.

#### 3.2. PhoP binding to promoters of *Bacillus* guanyl-preferring ribonuclease genes

Electromobility shift assays (EMSA) were used to identify interactions between the PhoP response regulator and the regulatory regions upstream of the various ribonuclease genes. In order to be able to test the binding of both non-phosphorylated and phosphorylated forms of PhoP, His-tagged versions of *B. subtilis* PhoP and PhoR (PhoR231) were purified from *E. coli* BL21 ( $\lambda$ DE3) with vectors pET-PhoP and pET-PhoR231. PhoR231 is a soluble version of PhoR that lacks the N-terminal transmembrane domains, and therefore





**Fig. 3.** Amino acid sequences of PhoP proteins from *Bacillus* species. Bsub.168 – *B. subtilis* subsp. *subtilis* str. 168, Bamy.FZB42 – *B. amyloliquefaciens* FZB42, Bpum.SARF-032 – *B. pumilus* SAFR-032, Bthu.A1.Hakam – *B. thuringiensis* str. Al Hakam, Bthu.97-27 – *B. thuringiensis* serovar *konkukian* str. 97-27. Single large asterisks indicate residues required for the binding of the OmpR-like response regulators to their target sites (CDD: 29475). Amino acid residues of *B. subtilis* the PhoP protein involved in binding of target DNA and interaction with RNA polymerase are marked with twin small asterisks [Chen et al., 2004].

starts at amino acid residue 231. The purity of the proteins was confirmed by SDS-PAGE (Fig. 4).

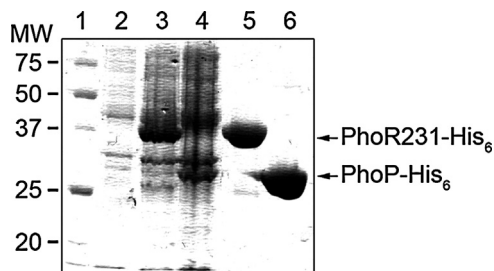
The upstream regulatory regions of the target ribonuclease genes were amplified from chromosomal DNA of the corresponding *Bacillus* strains. Two sets of consensus sequence primers were designed, one set for the binase-like RNase genes (Bp\_for and Bp\_rev) and the other for the barnase-like RNase genes (Ba\_for and Ba\_rev) (Fig. 2A). The upstream regulatory region of the *B. subtilis* *ykoL* gene (Fig. 2B), an established member of the PhoP regulon [Allenby et al., 2005], used as a positive control for the DNA-binding activity of the PhoP, was generated with primers YkoL\_for, 5'TGAAATGCTGGAGACGTTTATG3', and YkoL\_rev, 5'TTTTCTAAAGCGGATTCAATA3' [Prágai et al., 2004].

As shown in Fig. 5, both the unphosphorylated and phosphorylated (PhoP~P) forms of PhoP interacted with and retarded the

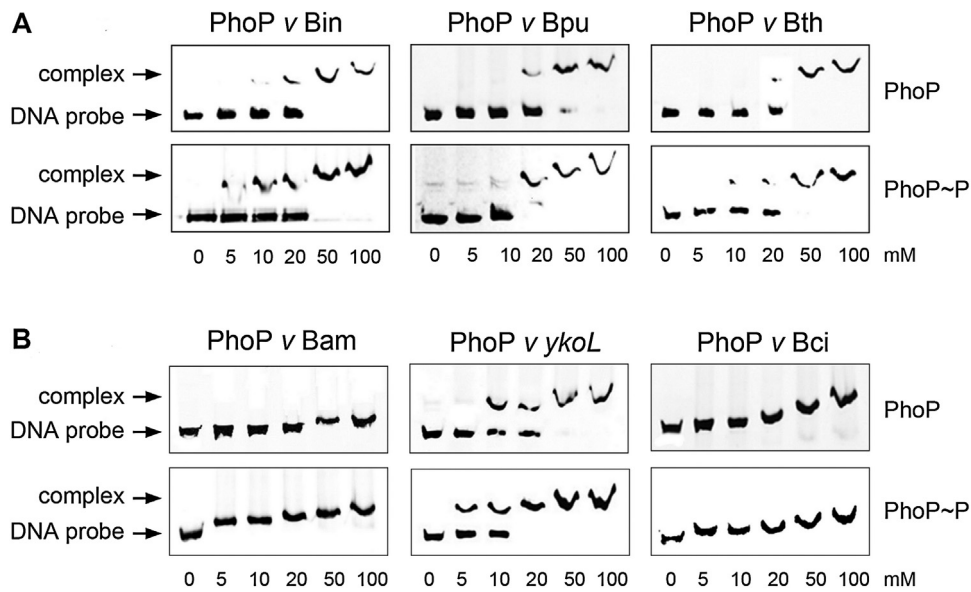
mobility of the *B. intermedius*, *B. pumilus* and *B. thuringiensis* RNase upstream regulatory regions, in a manner similar to that of the known Pho regulon gene *ykoL*. However, in each case DNA-protein complex formation was slightly more efficient when PhoP was phosphorylated by its cognate sensor kinase, PhoR. The degree of binding was dependent on the concentration of PhoP. In contrast, the mobility of the regulatory regions upstream of the *B. amyloliquefaciens* and *B. circulans* RNase genes was not affected by PhoP as revealed by combination of DNA and protein staining (Figs. 5 and 6). It was demonstrated that the PhoP~P protein fraction was associated with Bin DNA and not with Bci DNA (Fig. 6), indicating that the promoters of the latter are PhoP independent.

### 3.3. Role of the SigB factor in control of RNase gene expression

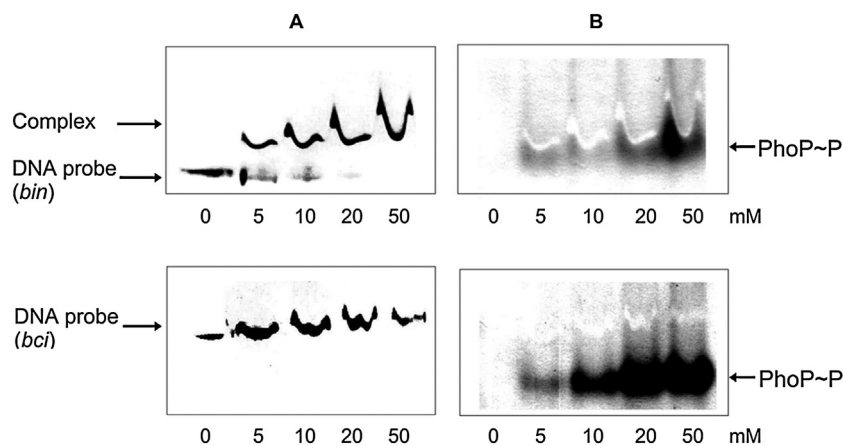
The SigB regulon is a component of the Pho stimulon and responds to the depletion in energy that accompanies phosphate starvation. We therefore determined whether this alternative sigma factor influences the regulation of the RNase genes in response to phosphate starvation stress. We studied the effect of a mutation in *sigB* on the activity of representative ribonucleases genes encoding the *B. intermedius* binase-like and the *B. circulans* barnase-like RNase. *B. subtilis* strain 168 lacks a LMW guanyl-preferring ribonucleases and therefore the wild type (JH642) and a *sigB*-null (PB73) strain were transformed with plasmids pMZ55 and pMZ59, encoding the *Bin* and *Bci* RNase genes, respectively. The ribonuclease activities in culture supernatants of the pMZ55 and pMZ59 transformants grown in LPM medium containing 5 or 100 mg/l phosphate were determined by quantifying the acid-soluble products of RNA hydrolysis (Table 2). Analogous experiments were carried out with the *phoP*- and *phoR*-null mutants as a control. The yield of the cultures with



**Fig. 4.** SDS-PAGE analysis of the production and purification of *B. subtilis* PhoP-His<sub>6</sub> and PhoR231-His<sub>6</sub> proteins from *E. coli*. 1, Molecular size standards (kDa); 2, supernatant fraction of the whole-cell lysate from *E. coli* BL21(ΔDE3) (negative control); 3, supernatant fraction of the whole-cell lysate from IPTG-induced *E. coli* cells encoding PhoR231-His<sub>6</sub>; 4, supernatant fraction of the whole-cell lysate from IPTG-induced *E. coli* encoding PhoP-His<sub>6</sub>; 5, affinity purified PhoR231-His<sub>6</sub>; 6, affinity purified PhoP-His<sub>6</sub>.



**Fig. 5.** Electromobility shift assays of the upstream regions of *Bacillus* ribonuclease genes with phosphorylated and non-phosphorylated PhoP proteins. (A) Promoter regions of *B. intermedius* (*Bin*), *B. pumilus* (*Bpu*) and *B. thuringiensis* (*Bth*) RNase genes. (B) Promoter regions of *B. amyloliquefaciens* (*Bam*) and *B. circulans* (*Bci*) RNase gene, and the *B. subtilis ykoL* gene (positive control). The amounts of PhoP and PhoP~P added to the reaction mixtures are indicated below the individual gels.



**Fig. 6.** Electromobility shift assays of the upstream regions of *Bacillus intermedius* (*bin*) and *B. circulans* (*bci*) ribonuclease genes with phosphorylated PhoP protein. (A) Gels stained by SYBR Gold. (B) Gels stained by Coomassie Blue. The amounts of PhoP added to the reaction mixtures are indicated below the individual gels.

100 mg/l phosphate was significantly higher than that of the cultures containing 5 mg/l phosphate, confirming that the latter were phosphate limited. The data confirm that the specific activities of the RNases in the culture medium of the wild type were induced in response to phosphate starvation. Similar results

were obtained with the *sigB*-null mutant, indicating that the production of both binase and barnase RNases are SigB independent. In contrast, binase-like production was dependent on PhoPR, whilst barnase-like RNase production was PhoPR independent.

**Table 2**  
Yield (OD units) and specific activity ([optical units/ml/h]/OD unit) of cultures of *B. subtilis* wild type and *sigB*-, *phoP*- and *phoR*-null mutants grown in LPM with limiting (5 mg/l) and excess (100 mg/l) concentrations of phosphate.

Strain	Plasmid	OD590		RNase (opt.u/ml/h)/OD	
		Pi (5 mg/l)	Pi(100 mg/l)	Pi (5 mg/l)	Pi(100 mg/l)
JH642	None	0.89 ± 0.06	1.44 ± 0.11	96 ± 4.0	73 ± 2.0
	pMZ55 (RNase Bin)	0.76 ± 0.07	1.57 ± 0.08	22806 ± 2026	990 ± 95
	pMZ59 (RNase Bci)	0.80 ± 0.07	1.52 ± 0.07	20560 ± 1700	7615 ± 810
<i>sigB</i> -null	pMZ55 (RNase Bin)	0.79 ± 0.09	1.37 ± 0.10	18085 ± 2575	935 ± 55
	pMZ59 (RNase Bci)	0.78 ± 0.09	1.23 ± 0.08	21400 ± 2400	7800 ± 760
<i>phoP</i> -null	pMZ55 (RNase Bin)	0.95 ± 0.05	1.40 ± 0.18	40 ± 2.0	18 ± 1.5
	pMZ59 (RNase Bci)	1.20 ± 0.08	1.73 ± 0.22	26,570 ± 2250	14628 ± 960
<i>phoR</i> -null	pMZ55 (RNase Bin)	1.10 ± 0.09	1.22 ± 0.08	90 ± 3.5	45 ± 1.5
	pMZ59 (RNase Bci)	1.24 ± 0.13	1.47 ± 0.13	24850 ± 2770	13780 ± 845

#### 4. Discussion

To cope with the stress caused by phosphate limitation *B. subtilis* induces at least two sets of genes; members of the PhoP regulon reduce the cell's demand for Pi and induce enzymes to recover phosphate from organic sources in the environment, whilst members of the SigB regulon help to protect the cells from collateral damage [Prágai and Harwood, 2002; Allenby et al., 2005]. Guanyl-preferring ribonucleases, secreted by a variety of *Bacillus* species (but not *B. subtilis*), hydrolyze polymeric RNA into mono- and oligonucleotides. Nucleosides and nitrogen bases derived from degraded RNA can either be used directly for *de novo* RNA synthesis or provide substrates for phosphatases and phosphorylases that release Pi for subsequent uptake by the Pst high affinity phosphate transporter [Allenby et al., 2004]. Guanyl-preferring ribonucleases are therefore good potential candidates for inclusion in the PhoP regulon.

The biosynthesis of guanyl-preferring ribonucleases from *B. pumilus*, *B. thuringiensis*, *B. intermedius* and *B. circulans* increased when these bacteria were grown on low phosphate media, whilst the synthesis of *B. amyloliquefaciens* guanyl-preferring RNase was unaffected by changes in the extracellular phosphate concentration [Znamenskaya et al., 1995]. We therefore carried out studies to determine the difference in the behaviour of the RNases. Because many of these species are refractile to genetic manipulation, it was not possible to study the responses of these genes *in situ*. We therefore set out to establish whether the PhoP orthologues from other *Bacillus* species were sufficiently conserved with respect to *B. subtilis* PhoP to enable us to use the latter for DNA binding assays. The amino acid sequences of PhoP orthologues exhibited a greater than 70% identity to that of *B. subtilis* PhoP. In particular, the C-terminal output domains and their functional residues were conserved, indicating that this protein was likely to be functional in the other *Bacillus* species (Fig. 3).

To change the expression of its target genes, the PhoP response regulator interacts with TT(A/T/C)ACA- like direct repeats separated by 3–7 non-conserved nucleotides (Fig. 2C). In electromobility shift assays, PhoP was shown to form a complex only with the upstream regulatory regions of binase-like RNases that contained putative Pho-boxes (Fig. 5). Phosphorylated PhoP with its cognate sensor kinase PhoR only enhanced binding slightly, as reported previously [Allenby et al., 2005]. Although phosphorylation is necessary for transcription initiation, dimers of non-phosphorylated PhoP are still able to interact with their target binding sites [Birck et al., 2003; Abdel-Fattah et al., 2005]. As a result of the observed binding, the binase-like genes are likely to be members of the PhoP regulons of their respective organisms. In contrast, the barnase-like genes were refractile to PhoP/PhoP~P binding (Figs. 5 and 6) and this is consistent with previous observations that the *B. amyloliquefaciens* barnase is not induced in response to phosphate starvation [Znamenskaya et al., 1995]. However, the *B. circulans* RNase synthesis does respond to low phosphate and, since we have ruled out an involvement of the general stress sigma factor, SigB, it would be interesting to determine which regulatory system is involved. For example, a proteome analysis of *B. subtilis* under various stress conditions, showed that, in addition to PhoP and SigB, the CodY and SigH regulators were responsible for inducing proteins in response to phosphate deficiency [Tam le et al., 2006]. CodY- and SigH-dependent proteins were induced under both phosphate and ammonium starvation. This is consistent with the induction of RNase Bci since its biosynthesis was shown to increase under nitrogen limitation [Kharitonova and Vershinina, 2009]. This would suggest that the RNase of *B. circulans* is involved in a more general system for adapting to nutrient depletion in contrast to the more specific response of binase-like RNases to phosphate starvation.

Differences in the expression of homologous genes in closely-related species have also been observed in the case of genes encoding phytase, an enzyme involved in the degradation of phytic acid, the main phosphorous-containing storage compound in plants. In *Bacillus licheniformis* and *B. amyloliquefaciens* the phytase genes belongs to the PhoP regulon, whilst the *B. subtilis* phytase gene does not [Allenby et al., 2005; Hoi le et al., 2006; Makarewicz et al., 2006]. Consequently, the regulatory behaviour of the RNases and phytases in related *Bacillus* sp. provide additional evidence that gene expression is highly niche-specific [Rediers et al., 2005].

Genes of the *B. subtilis* Pho regulon are controlled by a regulatory network consisting of at least three interconnected two-component signal transduction systems: PhoP-PhoR, the key regulator of phosphate-deficient response; ResD-ResE, the main function of which is control of aerobic and anaerobic respiration; and Spo0A, the initiator of sporulation [Birkey et al., 1998; Hulett, 1996]. The ResD-ResE two-component system, together with transition state regulator AbrB, is required for the full activation of the PhoP regulon under phosphate starvation [Sun et al., 1996; Schau et al., 2004], whilst Spo0A has a negative effect on the PhoP regulon [Prágai et al., 2004; Sun et al., 1996]. The roles of these regulators on the regulation of LMW RNases from *B. circulans*, *B. intermedius*, *B. pumilus* and *B. thuringiensis*, genes have been studied previously in corresponding mutants of *B. subtilis* [Znamenskaya et al., 1999; Morozova et al., 2001; Ulyanova et al., 2007; Ulyanova et al., 2008]. It was shown that, with the exception of Bci RNase, the patterns of expression of these ribonuclease genes correspond to that of known Pho regulon genes [Hulett, 1996].

The results indicate that the extracellular low-molecular weight guanyl-preferring ribonucleases from *B. intermedius*, *B. pumilus* and *B. thuringiensis* are new members of PhoP regulon and likely to be indispensable for adaptation to phosphate starvation stress. However, despite being induced in response to low phosphate, the mechanism involved in the regulation of the *B. circulans* guanyl-preferring ribonuclease gene remains unclear. Whilst it might have been assumed that this gene is a member of the SigB regulon, we have now shown that this is not the case. It has been shown previously that, in addition to the PhoP and SigB regulons, a third group of phosphate-dependent genes has been identified [Antelman et al., 2000; Allenby et al., 2005; Tam le et al., 2006]. However, the mechanism controlling their response to phosphate stress is currently not understood and it is possible that the gene encoding the guanyl-specific ribonuclease of *B. circulans* belongs to this third group of phosphate-dependent genes.

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