

A Conserved Two-Component Signal Transduction System Controls the Response to Phosphate Starvation in *Bifidobacterium breve* UCC2003

Pablo Alvarez-Martin,^{a*} Matilde Fernández,^{a*} Mary O'Connell-Motherway,^{a,b} Kerry Joan O'Connell,^{a,b} Nicolas Sauvageot,^{a*} Gerald F. Fitzgerald,^{a,b} John MacSharry,^a Aldert Zomer,^{a*} and Douwe van Sinderen^{a,b}

Alimentary Pharmabiotic Centre^a and Department of Microbiology,^b National University of Ireland, Cork, Ireland

This work reports on the identification and molecular characterization of the two-component regulatory system (2CRS) PhoRP, which controls the response to inorganic phosphate (P_i) starvation in *Bifidobacterium breve* UCC2003. The response regulator PhoP was shown to bind to the promoter region of *pstSCAB*, specifying a predicted P_i transporter system, as well as that of *phoU*, which encodes a putative P_i-responsive regulatory protein. This interaction is assumed to cause transcriptional modulation under conditions of P_i limitation. Our data suggest that the *phoRP* genes are subject to positive autoregulation and, together with *pstSCAB* and presumably *phoU*, represent the complete regulon controlled by the *phoRP*-encoded 2CRS in *B. breve* UCC2003. Determination of the minimal PhoP binding region combined with bioinformatic analysis revealed the probable recognition sequence of PhoP, designated here as the PHO box, which together with *phoRP* is conserved among many high-GC-content Gram-positive bacteria. The importance of the *phoRP* 2CRS in the response of *B. breve* to P_i starvation conditions was confirmed by analysis of a *B. breve phoP* insertion mutant which exhibited decreased growth under phosphate-limiting conditions compared to its parent strain UCC2003.

Bifidobacteria are Gram-positive, pleomorphic, anaerobic bacteria which are considered common inhabitants of the human and animal gastrointestinal tract (53). In recent years, bifidobacteria have received increased scientific interest due to perceived beneficial or probiotic properties which they impart to their hosts (21, 32, 33, 50, 59).

Commercially exploited probiotic bacteria are typically selected for fast growth, antimicrobial activity, good adhesion properties, and utilization of prebiotic substrates (32). In addition to these properties, they must also be able to resist the adverse conditions, such as acid and bile stress, encountered during large-scale production, product storage, and passage through the oral cavity, stomach, and small intestine (4, 43, 57). All these environmental challenges have a negative impact on bifidobacterial viability and, consequently, probiotic efficacy. Therefore, successful bifidobacterial delivery, colonization, and/or survival in the gastrointestinal tract will depend on physiological responses that allow adaptation to these hazardous conditions.

Phosphorus is an essential nutrient for all cells due to its involvement in many critical biological processes, e.g., nucleic acid and (lipo)teichoic acid biosynthesis and regulation of protein activity by phosphorylation. The most commonly assimilated P source is inorganic phosphate (PO₄³⁻, or P_i). For many bacteria, it has been shown that the mechanism by which P_i is taken up from the environment will depend on the extracellular P_i concentration (15, 37). When sufficient P_i is available to the cell (in the mM range), a low-affinity transport system named Pit (for “P_i transport”) is active. In *Escherichia coli*, Pit is represented by a single, constitutively expressed membrane-associated protein, which transports P_i in a proton motive force-dependent manner (11). However, when P_i becomes limiting (at a concentration below 20 μM), the expression of a high-affinity ABC-type transporter, designated Pst (for “P_i-specific transport”) and consisting of four different proteins, PstSCAB, is induced (38). For many bacteria,

such as *E. coli*, *Bacillus subtilis*, and *Corynebacterium glutamicum*, the P_i starvation response is governed by a two-component signal transduction system, such as PhoBR, PhoRP, PhoPQ, or PhoSR (14, 36, 45, 46, 63).

Many two-component regulatory systems (2CRS) are known to be responsible for gathering environmental information and translating such data into an appropriate physiological response, thus allowing adaptive responses that increase fitness and survival under adverse conditions (for reviews, see references 8, 12, 28, and 51). Typically, a 2CRS consists of a histidine protein kinase (HPK), usually a membrane-associated protein capable of sensing a specific environmental signal, and a cytoplasmic response regulator (RR), which translates the incoming signal directly or indirectly into a cellular adaptive response. A typical HPK contains an N-terminally located input or sensing domain and a C-terminal transmitter domain, which is autophosphorylated from ATP at a conserved histidine residue in response to fluctuations in chemical and/or physical conditions (sensed by the input domain). This phosphoryl group is then transferred to a conserved aspartate residue on the N-terminally positioned receiver domain of the cog-

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Address correspondence to Douwe van Sinderen, d.vansinderen@ucc.ie.

* Present address: Pablo Alvarez-Martin, Nestlé Research Center, Vers-chez-les-Blanc, Lausanne, Switzerland; Matilde Fernández, Bio-Iliberis R&D, Armilla, Granada, Spain; Nicolas Sauvageot, N.S. Laboratory of Environmental Microbiology, University of Caen-Basse Normandie, I.R.B.A., Caen, France; Aldert Zomer, Laboratory of Pediatric Infectious Diseases, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands.

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nate response regulator. This phosphorylation changes the biochemical properties of the RR's output domain, which can participate in DNA binding and transcriptional control, perform enzymatic activities, bind RNA, or engage in protein-protein interactions (8).

Although the number of 2CRS identified in a given bifidobacterial genome ranges from 5 to 17 (58, 25), their physiological function has remained elusive. In the current work, we report for the first time the molecular and functional characterization of a bifidobacterial 2CRS, designated here as PhoRP, from *Bifidobacterium breve* UCC2003, which is involved in the response to P_i starvation.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this study are listed in Table S1 in the supplemental material. *B. breve* UCC2003 was grown at 37°C in MRS medium (De Man, Rogosa, and Sharpe) or RCM (reinforced clostridial medium) (Oxoid, Hampshire, England) as standing cultures supplemented with 0.05% cysteine or on RCM agar plates containing 1.5% (wt/vol) agar under anaerobic conditions in a modular atmosphere controlled system (Davidson & Hardy Ltd., Dublin, Ireland). *E. coli* strains were grown in LB (lysogeny broth) medium under aerobic conditions on a rotary shaker (150 rpm) at 37°C or plated on LB agar plates. Where appropriate for plasmid maintenance, media were supplemented with ampicillin (Amp) (100 $\mu\text{g ml}^{-1}$), erythromycin (Er) (100 $\mu\text{g ml}^{-1}$), chloramphenicol (Cm) (20 $\mu\text{g ml}^{-1}$), kanamycin (Kn) (50 $\mu\text{g ml}^{-1}$), or tetracycline (Tet) (10 $\mu\text{g ml}^{-1}$) for *E. coli* and Cm (2 $\mu\text{g ml}^{-1}$) or Tet (5 $\mu\text{g ml}^{-1}$) for *B. breve*.

DNA techniques and transformation. The general procedures used for DNA manipulation were essentially those described previously (42). Restriction enzymes and T4 DNA ligase were obtained from Roche Diagnostics (Bell Lane, East Sussex, United Kingdom) and used according to the manufacturer's instructions. PCRs were performed using Taq PCR master mix (Qiagen GmbH, Hilden, Germany). Synthetic oligonucleotides were synthesized by MWG Biotech AG (Ebersberg, Germany) and are listed in Table S2 in the supplemental material. PCR products were purified by using a High-Pure PCR product purification kit (Roche). Plasmid DNA was introduced into *E. coli* and *B. breve* by electrotransformation; large-scale preparation of chromosomal DNA from *Bifidobacterium* spp. was performed as described previously (26). Plasmid DNA was obtained from *B. breve* and *E. coli* using a QIAprep spin plasmid miniprep kit (Qiagen GmbH, Hilden, Germany). An initial lysis step was performed using 30 mg ml^{-1} of lysozyme for 30 min at 37°C as part of the plasmid purification protocol for *B. breve*.

Bioinformatics. Sequence data were obtained from the Artemis-mediated (41) annotations of the *B. breve* UCC2003 genome (31). Database searches were performed using the nonredundant sequence database accessible at the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov>) using TBLASTN, BLASTX, and BLASTP (1). Sequence analysis was performed using the Clone Manager professional suite (Sci-Ed Central), and multiple local alignments were carried out with ClustalW software (52). The presence of conserved sequences was examined using a hidden Markov model (HMM) with the HMMER tool (<http://hmmer.janelia.org/>). Searches were performed for sequences up to 400 bp upstream of the deduced translational start site of every identified gene of the *B. breve* UCC2003 genome. A graphical representation of the identified motif was obtained using WebLogo software (3).

Construction of a *phoP* insertion mutant. An internal 481-bp fragment of *phoP* was amplified by PCR using *B. breve* UCC2003 chromosomal DNA as the template and the oligonucleotide primers phoPKOf and phoPKOr (see Table S2 in the supplemental material). The generated 481-bp PCR product was cloned into pORI19, an Ori^+ RepA $^-$ integration plasmid (20), using the unique PstI and XbaI restriction sites that were incorporated into the primers, and introduced into *E. coli* EC101 by elec-

trporation. The expected structure of the recombinant plasmid, designated pORI19-*phoP*, was confirmed by restriction mapping and sequencing. The *tetW* gene, amplified by PCR using pAM5 plasmid DNA as the template (2) and primers tetWf and tetWr (see Table S2), thereby incorporating flanking Sall sites in the amplicon, was cloned into the Sall-cut pORI19-*phoP* plasmid to generate plasmid pORI19-tet-*phoP*. The latter plasmid was introduced into *E. coli* EC101 harboring pNZ-M.BbrII-M.BbrIII to facilitate methylation (30), and the resulting methylated pORI19-tet-*phoP* was then introduced into *B. breve* UCC2003 by electroporation and subsequent selection on RCA plates supplemented with Tet and Cm.

Growth analysis under P_i starvation conditions. P_i starvation experiments were performed in a chemically defined medium (CDM; for the composition, see Table S3 in the supplemental material), that can be modified as an essentially P_i -free chemically defined growth medium. In order to remove all P_i traces from the inoculum, cells were washed twice with P_i -free chemically defined medium (NP-CDM) before they were used to inoculate (4%) NP-CDM or NP-CDM containing various levels of P_i (final concentration of 0 mM, 1 mM, or 10 mM). Growth experiments were carried out in triplicate using a Power Wave 340 plate reader (Biotek Winooski), and obtained optical density (OD) readings were analyzed using Gen5 software (Biotek).

PhoP overexpression in *B. breve*. In order to achieve PhoP overexpression, the *phoP* gene was amplified by PCR from chromosomal DNA of *B. breve* UCC2003 using the primer combination RrFoefor and RrFoerev (see Table S2 in the supplemental material). The resulting PCR product was cloned in the pTOPO system (Invitrogen, Carlsbad, CA), sequenced, removed by NcoI/XbaI restriction, and then cloned into similarly digested pNZ44, which carries a strong constitutive promoter (27). The expected structure of the resulting plasmid, pNZ44:*phoP*, was verified by sequence analysis prior to its transfer to *B. breve* UCC2003 by electroporation. Microarray-based global transcription patterns of *B. breve* UCC2003 and *B. breve* UCC2003 pNZ44:*phoP* were determined employing bacterial cultures that had been grown to early exponential phase in MRS.

Gene expression analysis with *B. breve* DNA microarrays. In order to compare global transcription patterns of *B. breve* UCC2003 and the *B. breve* UCC2003 *phoP* insertion mutant under normal and phosphate starvation conditions, cells grown to early exponential phase were collected, washed twice in NP-CDM, and resuspended in the same medium containing either 20 mM P_i or no P_i . Samples were taken at 45 and 90 min after transfer. DNA microarrays containing oligonucleotide primers representing each of the 1,864 annotated genes in the genome of *B. breve* UCC2003 were obtained from Agilent Technologies (Palo Alto, CA). Methods for cell disruption, RNA isolation, RNA quality control, cDNA synthesis, and indirect labeling were performed as described previously (54). Labeled cDNA was hybridized using the Agilent gene expression hybridization kit (number 5188-5242) as described in the Agilent two-color microarray-based gene expression analysis v4.0 manual (publication number G4140-90050). Following hybridization, microarrays were washed as described in the manual and scanned using Agilent's DNA microarray scanner G2565A. The scans were converted to data files with Agilent's Feature Extraction software (version 9.5).

DNA microarray data were processed as previously described (9, 54). Differential expression tests were performed with the Cyber-T implementation of a variant of the *t* test (23). A gene was considered differentially expressed between a test condition and a control when an expression ratio of 3 or 0.33 relative to the result for the control was obtained with a corresponding *P* value that was equal to or less than 0.001.

Transcriptional fusions and GusA assays. The presumed *phoRP*, *pst*, and *pitA* promoter regions were amplified by PCR using the primer combinations p2CSFfor/p2CSFrev, pPstfor/pPstrev and pPitfor/pPitreve, respectively (see Table S2 in the supplemental material). The PCR products generated were first cloned into pTOPO vector, then recovered by digestion with BamHI/PstI, and subsequently cloned upstream of the promoterless *gusA* gene present in the similarly restricted pNZ272 reporter vector

(34). Ligation mixtures were independently introduced by electroporation in *E. coli* XL1-Blue competent cells. The resulting plasmids, once verified by restriction and sequence analysis, were then transferred to *B. breve* UCC2003 by electroporation.

GusA activity assays in *B. breve* UCC2003 or derivatives were carried out in triplicate as follows: cells were grown in CDM until an OD₆₀₀ of approximately 0.45 was reached and were then harvested, washed twice in NP-CDM, resuspended in the original volume of NP-CDM, and incubated at 37°C. Samples were then taken at different time points, harvested, and resuspended in 1 ml of P_i buffer (29). After 0.5 ml of culture was mixed with 12.5 μl of 0.1% sodium dodecyl sulfate (SDS) and 25 μl of chloroform (Sigma-Aldrich, Steinheim, Germany), the suspension was incubated for 5 min at 37°C, followed by the addition of 100 μl of 4 mg/ml of 4-nitrophenyl-*B*-D-glucuronide (Melford, United Kingdom). Reactions were stopped by the addition of 250 μl of 1 M sodium carbonate, and samples were then centrifuged to remove debris, after which the OD₄₂₀ and OD₅₅₀ values were determined. GusA activity was expressed in Miller units and calculated using the following equation: $(522 \times OD_{420}) / (t \times v \times OD_{600})$, where *t* is reaction time (min), *v* is cell volume, and OD₄₂₀ and OD₆₀₀ are optical densities at 420 nm and 600 nm, respectively (34). Optical measurements were carried out using a DU 530 Life Science UV/Vis spectrophotometer (Beckman Coulter, Brea, CA).

PhoP overproduction and purification. The entire coding region of the *phoP* gene was amplified by PCR using chromosomal DNA of *B. breve* UCC2003 as a template and primer combination Bbr1683fNcoI and Bbr1683rXbaI (see Table S2 in the supplemental material). NcoI and XbaI restriction sites were incorporated at the 5' ends of the forward and reverse primers, respectively. In addition, an in-frame His10-encoding sequence was incorporated into the forward primer to facilitate protein purification using a Ni-nitrilotriacetic acid system (Qiagen). The amplicon was digested with NcoI and XbaI and ligated into the similarly digested nisin-inducible translational fusion plasmid pNZ8048 (5). The ligation mixtures were introduced into *L. lactis* NZ9000 by electrotransformation, and transformants were selected based on Cm resistance (Cm^r). A single Cm^r transformant was shown by restriction and sequence analysis to contain the expected plasmid, which was designated pNZ8048:phoP.

Protein production and purification was carried out as previously described (29). A 400-ml portion of M17 broth supplemented with 0.5% glucose was inoculated with a 2% inoculum of *L. lactis* strain NZ9000 harboring pNZ8048:phoP, followed by incubation at 30°C until an OD₆₀₀ of 0.5 was reached, at which point protein overexpression was induced by the addition of purified nisin (5 ng ml⁻¹) and cultures were incubated at 30°C for 90 min. Cells were harvested by centrifugation, washed, and concentrated 40-fold in lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole [pH 8.0]). Cell extracts were prepared using 106-μm glass beads and a Mini-BeadBeater-8 cell disrupter (Biospec Products, Bartlesville, OK). After homogenization, the glass beads and cell debris were sedimented by centrifugation, and the supernatant containing the cytoplasmic fraction was retained. Protein purification from the cytoplasmic fraction was performed using Ni-nitrilotriacetic acid matrices in accordance with the manufacturer's instructions (Qiagen). Elution fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), as described by Laemmli (19), on a 12.5% polyacrylamide gel. After electrophoresis, the gels were fixed and stained with Coomassie brilliant blue to identify fractions containing the purified protein. Rainbow-prestained low-molecular-weight protein markers (New England Biolabs, Hertfordshire, United Kingdom) were used to estimate the molecular weights of the purified proteins.

Phosphorylation of the response regulator protein PhoP. Acetyl phosphate-dependent phosphorylation was achieved by incubating 2 μg of purified His-PhoP protein with 4 μCi of acetyl [γ -³²P]phosphate (DuPont NEN) in 20 μl of phosphorylation buffer (50 mM Tris-HCl [pH 7.5], 100 mM KCl, 1 mM MgCl₂). This mixture was incubated at 25°C, and samples were taken at regular intervals. Phosphorylation reactions were stopped with SDS loading buffer, and samples were run on a 12.5% SDS-

polyacrylamide gel. After electrophoresis, gels were dried and autoradiographed in order to detect acetyl phosphate-dependent phosphotransfer to His-PhoP.

Electrophoretic mobility shift assay (EMSA). DNA fragments encompassing overlapping fragments of the *pstSCAB*, *phoRP*, and *phoU* promoter regions were prepared by PCR using primers described in Table S2 in the supplemental material. EMSAs were performed as described previously (55), with the following modifications: PhoP was phosphorylated by incubating 50 μM purified protein with 50 mM acetyl phosphate at 25°C for 1 h; the PCR products were generated using IRD-800-labeled fluorescent primers; and protein and probe were mixed on ice and subsequently incubated for 30 min at 37°C. Samples were loaded onto a 6% nondenaturing polyacrylamide gel prepared with 1× TAE (40 mM Tris-acetate [pH 8.0], 2 mM EDTA) and run in a 0.5×-to-2.0× gradient of TAE at 100 V for 90 min in a mini-Protein electrophoresis system (Bio-Rad Laboratories). Following electrophoresis, the presence and mobility position of the fluorescent PCR products in the gel were detected using an Odyssey instrument (LI-COR Biosciences UK Ltd., Cambridge, United Kingdom).

qRT-PCR amplifications. Real-time quantitative RT-PCR (qRT-PCR) assays and *de novo* cDNA preparations were performed as previously described (35, 56). PCR primers (see Table S2 in the supplemental material) and probes were designed using the Universal ProbeLibrary Assay Design Centre (Roche). The *rnpA* gene (encoding the RNase P protein component) was used as a housekeeping gene with an assumed constant level of transcription to correct for variability in the initial amount of total RNA. Amplification reaction mixtures contained 1 μl of 6.7-fold-diluted cDNA, 10 μl of 2× FastStart TaqMan Probe Master (Roche), a 900 nM concentration of each primer, and 250 nM probe mix and were brought to a total volume of 20 μl by the addition of RNase-free water. All qRT-PCRs were performed in triplicate by means of a Light-Cycler 480 system (Roche) using 384-well plates. Thermal cycling conditions were as recommended by the manufacturer (Roche). The 2^{-ΔΔCT} method (22) was used to calculate relative changes in gene expression determined from real-time quantitative PCR experiments. Pooled cDNA from all samples was used as a reference calibrator for analysis of differential gene expression. Results were calculated from at least two independent RNA extractions. The qRT-PCR expression data, following 2^{-ΔΔCT} analysis, were subjected to a Mann-Whitney *t* test to compare all groups using GraphPad Prism 4 software (GraphPad Software). Data are reported as means and standard errors of the means. *P* values of <0.05 were considered significant.

Primer extension analysis. Total RNA was isolated from *B. breve* UCC2003 grown to early exponential phase and incubated under phosphate starvation conditions for 45 min. RNA samples were treated with RNase-free DNase (Ambion). Primer extension was performed by annealing 1 pmol of IRD800 synthetic 18-mer oligonucleotides to 20 μg of RNA as described by Ventura et al. (60). Sequence ladders of the presumed *pstS* or *phoR* promoter regions, which were run alongside the primer extension products, were produced using the same primer as the primer extension reaction using a Thermo Sequenase primer cycle sequencing kit (Amersham). Separation was achieved on a 6.5% Li-Cor Matrix KB Plus acrylamide gel. Signal detection and image capture were performed with a Li-Cor sequencing instrument (Li-Cor Biosciences).

Microarray data accession number. All microarray data have been deposited in the GEO database (accession number GSE34983).

RESULTS

Transcriptome analysis shows increased expression of a presumed P_i uptake system under P_i starvation conditions. In order to investigate the genome-wide response of *B. breve* UCC2003 to P_i starvation, changes in gene expression were analyzed following transfer of *B. breve* UCC2003 to a medium without P_i (see Materials and Methods). Total RNA samples were ob-

TABLE 1 Summary of gene expression changes in *B. breve* UCC2003 grown under P_i limitation or subject to PhoP overexpression

Locus tag	Annotation	Ratio ^a					
		Phosphate starvation		PhoP overexpression	<i>phoP</i> knockout vs wild-type phosphate starvation		<i>phoP</i> knockout phosphate starvation
		45 min	90 min		45 min	90 min	
Bbr_0087	Hemolysin III homolog	3.1	6.8	0.9	1.8	2.2	0.8
Bbr_0191	Membrane protein	3.5	8.7	1.0	0.7	0.5	1.0
Bbr_0323	<i>atpB</i>	4.5	6.3	0.6	1.2	1.2	1.4
Bbr_0330	<i>atpC</i>	3.0	6.1	0.6	1.7	1.2	1.7
Bbr_0824	Fic family protein	3.1	8.4	0.9	0.3	0.5	0.9
Bbr_0876	Peptidase E	3.4	5.1	0.8	0.7	0.7	4.7
Bbr_1460	Helicase	3.1	3.6	1.2	1.7	1.6	0.8
Bbr_1506	CFA synthase	4.1	12.7	1.0	0.8	1.2	1.2
Bbr_1573	<i>phoU</i> homolog	1.6	2.3	1.5	0.5	0.5	0.9
Bbr_1679	<i>pstB</i>	3.1	12.1	2.2	1.6	1.5	0.9
Bbr_1680	<i>pstA</i>	3.9	12.4	3.2	1.5	1.8	0.9
Bbr_1681	<i>pstC</i>	5.4	15.3	3.8	1.7	1.9	0.9
Bbr_1682	<i>pstS</i>	9.8	25.9	9.6	3.4	4.6	1.0
Bbr_1683	<i>phoP</i>	2.0	5.9	164.1	0.9	1.0	1.0
Bbr_1684	<i>phoR</i>	1.7	3.5	2.9	0.9	0.8	1.1
Bbr_1811	Multidrug transporter	4.8	11.9	0.8	1.0	1.3	1.2
Bbr_0865	Low-affinity inorganic phosphate transporter	0.8	0.7	0.9	1.3	1.0	1.0

^a Calculated as average mRNA level of a given gene under phosphate starvation or PhoP overexpressing/average mRNA level of the same gene under normal growth conditions or no PhoP overexpression, respectively.

tained from cultures grown in a medium containing either a high level of P_i (20 mM) or no P_i for 45 min (early response) or 90 min (late response). Following cDNA synthesis and labeling, the samples were hybridized to DNA microarrays representing all identified open reading frames (ORFs) of the *B. breve* UCC2003 genome.

The obtained results revealed significant changes in the transcription profiles after 45 min, with a further increase of such changes following prolonged P_i starvation. From a total of 1,864 tested open reading frames, as identified on the *B. breve* UCC2003 genome, microarray analysis showed a significantly increased transcriptional activity for 118 genes (>3-fold; P value \leq 0.001), while 134 genes exhibited significantly decreased levels of transcription (<3-fold; P value \leq 0.001). Genes showing at least 3-fold upregulation in both conditions are shown in Table 1.

Among the various gene clusters upregulated by P_i starvation, a predicted 2CRS, represented by Bbr_1683/Bbr_1684 (referred to here as *phoRP*), was identified. Bbr_1684 or *phoR* is predicted to encode a histidine protein kinase, while the protein product of Bbr1683 (designated *phoP*) specifies a presumed response regulator. PhoR contains a phosphoacceptor and an ATPase domain (PFAM003709), whereas PhoP resembles a typical response regulator with a signal receiver and an effector domain (PFAM00486). The *B. breve* UCC2003 *phoRP* genes are representatives of homologous systems present in all currently available bifidobacterial genomes and in other high-GC-content Gram-positive genera, such as *Streptomyces*, *Corynebacterium*, and *Mycobacterium* (Fig. 1). Immediately downstream of *phoRP*, a cluster of four genes showed strong transcriptional upregulation under P_i starvation conditions (Table 1). This gene cluster displays significant homology to the well-characterized P_i -specific transport system of *Streptomyces coelicolor* and *E. coli* (44, 39). We refer to these four genes

as the *pst* gene cluster, represented by *pstSCAB*, as illustrated in Fig. 1.

In order to identify genes whose expression is under *phoRP* control, a *B. breve* UCC2003 PhoP-overexpressing derivative was generated by cloning the *phoP* gene into the pNZ44 vector to generate pNZ44:phoP, which was then introduced into *B. breve* UCC2003 (see Materials and Methods). Microarray analysis showed that the level of *phoP* transcription in *B. breve* UCC2003 (pNZ44:phoP) was 164-fold higher than that in the *B. breve* control strain containing the empty pNZ44 vector (Table 1), indicating that cloning of *phoP* on pNZ44 leads to a significant overexpression of this gene. A number of genes showed different levels of upregulation in their expression, some of them corresponding to different cell stress proteins, although the gene showing the highest level of increased transcription was *pstS*. The remaining genes of the *pst* cluster, as well as *phoR*, also exhibited significant ($P < 0.001$) transcriptional upregulation (Table 1).

P_i -dependent control of the *pst* and *phoRP* promoters. In order to further analyze P_i -dependent regulation, transcriptional fusions were constructed by cloning specific DNA fragments encompassing the prospective promoter regions upstream of *phoRP*, *pstSCAB*, and *pitA*, upstream of the promoterless *gusA* reporter gene in pNZ272 (see Materials and Methods). The *pitA* gene (Bbr_0865) on the *B. breve* UCC2003 genome specifies the presumed low-affinity inorganic phosphate transporter, which did not exhibit any appreciable increase in transcription under P_i starvation conditions or upon *phoP* overexpression (Table 1). *B. breve* UCC2003 strains harboring the *gusA* fusion plasmids pNZ272:phoP, pNZ272:pst, and pNZ272:pit were used for GusA assays under P_i abundance and P_i starvation conditions. Growth under P_i -limiting conditions clearly induced the promoters present in the upstream regions of *phoRP* and *pst*, while the promoter up-

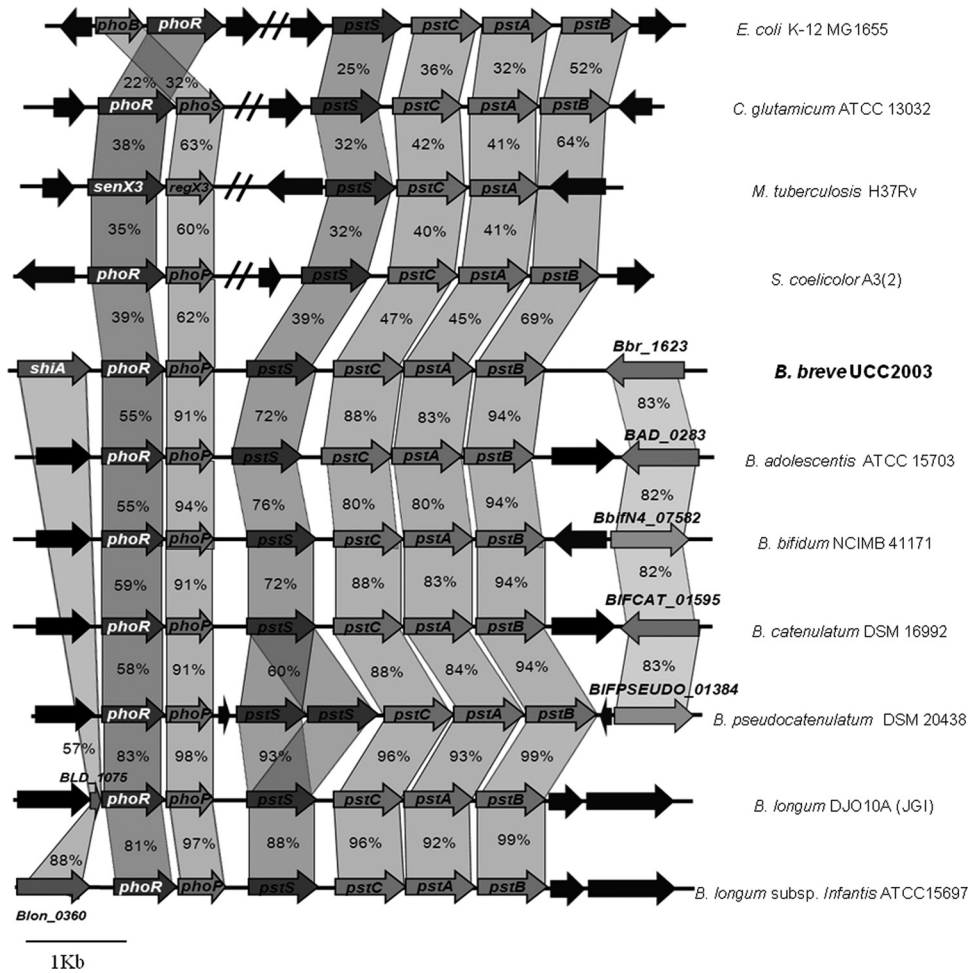


FIG 1 Comparison of the *phoRP* and *pstSCAB* loci of *B. breve* UCC2003 with corresponding (putative or proven) 2CRS and P_i uptake loci from other *Bifidobacterium* and high-GC-content Gram-positive bacteria. Each solid arrow indicates an open reading frame (ORF). The lengths of the arrows are proportional to the length of the predicted ORF, and the gene locus name, which is indicative of its putative function, is given within the arrow. Orthologous genes are linked, and the amino acid identity of each predicted protein is indicated as a percentage relative to its equivalent protein encoded by *B. breve* UCC2003.

stream of *pit* appeared to be negatively affected by P_i -limiting conditions (Fig. 2; the array results were consistent with this finding, as the observed downregulation of *pit* was 1.2-fold at 45 min and 2.2-fold at 90 min).

Overexpression, purification, and phosphorylation of PhoP.

For functional characterization of the DNA binding activity of PhoP, this protein was expressed in *L. lactis* and purified as an N-terminally His-tagged version using the lactococcal nisin-controlled expression (NICE) system (5). The recombinant H10-PhoP protein was purified as a single protein of approximately 27 kDa, as determined by SDS-PAGE (see Fig. S1 in the supplemental material). Purified H10-PhoP was shown to become phosphorylated upon incubation with acetyl phosphate, a known phosphoryl donor of response regulators (24), proving that it contains a functional phosphoacceptor domain (Fig. 3).

PhoP specifically binds the *pstS*, *phoR*, and *phoU* promoters.

In order to obtain evidence for the expected regulatory function of *phoP* in the cellular response to phosphate starvation, the ability of this presumed response regulator to bind to the promoter regions of *pstS*, *phoR*, and *phoU* (*Bbr_1573*) was analyzed *in vitro* by

EMSA. Although *phoU* transcription was only 2-fold upregulated upon P_i starvation (Table 1), it is known that the *phoU* homologue in *E. coli* is induced by low levels of P_i , where its gene product acts as a negative regulator of the Pho regulon (39). Three overlapping IRD800-labeled amplicons were generated for each promoter region, namely, *pstS*-1, *pstS*-2, and *pstS*-3, *phoR*-1, *phoR*-2, and *phoR*-3, and *phoU*-1, *phoU*-2, and *phoU*-3 (Fig. 4). EMSAs were carried out with phosphorylated or nonphosphorylated H10-PhoP protein, which revealed that phosphorylation of PhoP is essential for binding of this protein to the *phoR*, *phoU*, and *pstS* promoter regions (data not shown). Furthermore, these experiments revealed that phosphorylated H10-PhoP interacts with particular segments of each promoter region, indicating the presence of specific binding sequences within DNA fragments that exhibit mobility shifts (Fig. 4).

No interaction between other tested DNA fragments (representing presumed promoter regions of genes that were either up- or downregulated upon P_i starvation as determined by transcriptome analysis) and phosphorylated H10-PhoP was observed in EMSAs (data not shown).

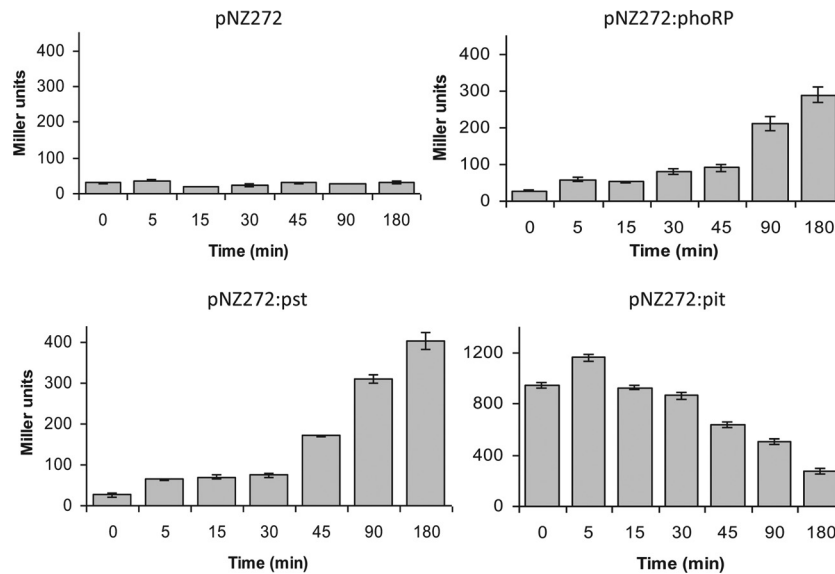


FIG 2 GusA assays testing phosphate starvation effect on *phoRP*, *pst*, and *pit* promoters. Enzyme activity was measured after the indicated times under phosphate starvation. Data are means from three independent experiments, and error bars show standard deviations.

PHO box identification and its relative distance to the transcriptional start site of the *pstS* and *phoR* promoters. The EMSA results (Fig. 4) indicate that the PhoP binding sites in the *pstS*, *phoR*, and *phoU* promoter regions are localized within DNA fragments between positions -127 and $+112$, -66 and $+51$, and -121 and $+105$ (relative to the deduced translational start position of *pstS*, *phoR*, and *phoU*), respectively. Analysis of these PhoP-binding sequences within the three promoter regions revealed a commonly present direct repeat, GTTCAYY-N₄-GTTCAYY, referred to here as the PHO box, shown to be highly conserved and present in the intergenic regions upstream of genes encoding proteins that are presumed to be involved in P_i uptake, P_i release, and P_i regulation in various *Bifidobacterium* species, like *B. longum*, *B. adolescentis*, *B. dentium*, and *B. animalis* subsp. *lactis*, as well as in other Gram-positive bacteria, such as *Streptomyces coelicolor*, *Propionibacterium acnes*, and *Brevibacterium linens* (Table 2). Interestingly, in the genome of *B. adolescentis* and those of various other bifidobacteria, this motif was detected upstream of a gene encoding a putative alkaline phosphatase (BAD_1548 in *B. adolescentis*).

In order to investigate how the PHO box, as a binding motif for the presumed transcriptional regulator PhoP, is positioned relative to the promoters of its target genes, the transcription start sites of the *pstS* and *phoR* promoters were determined by primer extension analysis using RNA extracted from *B. breve* UCC2003 grown

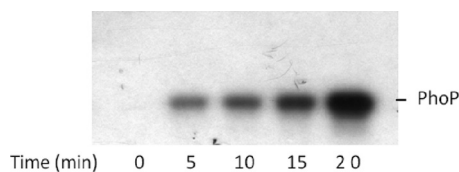


FIG 3 Time-dependent PhoP-His phosphorylation. Purified PhoP-His protein (2 mg) was incubated with $4 \mu\text{Ci}$ of $[\gamma\text{-}^{32}\text{P}]\text{acetyl phosphate}$, and samples were taken every 5 min and subjected to SDS-PAGE followed by autoradiography.

under phosphate-limiting conditions. An extension product was identified 64 nucleotides 5' of the predicted translational start site for the *pstS* gene, while for *phoR* gene the transcription initiation site was observed 53 bp upstream of its predicted translational start site (Fig. 5). Analysis of the *pstS* promoter region revealed potential promoter recognition sequences resembling consensus -10 and -35 hexamers, while the PHO box was located 43 bp upstream of the transcription start site. For the *phoR* promoter, a potential -10 sequence could be identified, with no obvious -35 sequence present within the expected range of this -10 sequence, although a potential -35 sequence is present further upstream (Fig. 5). Interestingly, the PHO box was found to overlap the deduced -10 promoter recognition sequence.

Disruption of the *phoP* gene in *B. breve* UCC2003. In order to test if disruption of *phoP* in UCC2003 would result in the loss of this strain's capacity to appropriately respond to P_i limitation, a *phoP* insertion mutant was generated, designated *B. breve* UCC2003::*phoP* (see Materials and Methods). To verify the expected phenotype of the *B. breve* UCC2003::*phoP* mutant strain, both wild-type and mutant strains were analyzed for their ability to grow in NP-CDM alone or NP-CDM to which P_i had been added at 1 mM and 10 mM, the latter representing the approximate P_i concentration present in complex medium. Growth experiments were carried out in triplicate, and results are presented in Fig. 6. As expected, both UCC2003 and UCC2003::*phoP* exhibited little or no growth in the absence of P_i in the medium (NP-CDM). *B. breve* UCC2003 was able to grow in CDM with various P_i concentrations, although its growth capacity was directly proportional to the concentration of P_i , reaching a maximum OD_{600} of 0.99 when grown in CDM containing 10 mM P_i . The growth ability of the UCC2003::*phoP* strain was markedly lower than that of wild-type UCC2003 in CDM with 1 mM P_i , while the observed growth pattern of these two strains was similar in CDM containing 10 mM P_i , at least for the first 9 h. These results demonstrate that *phoRP* plays an important role in the adaptive response of *B. breve* UCC2003 to phosphate limitation. In order to determine the

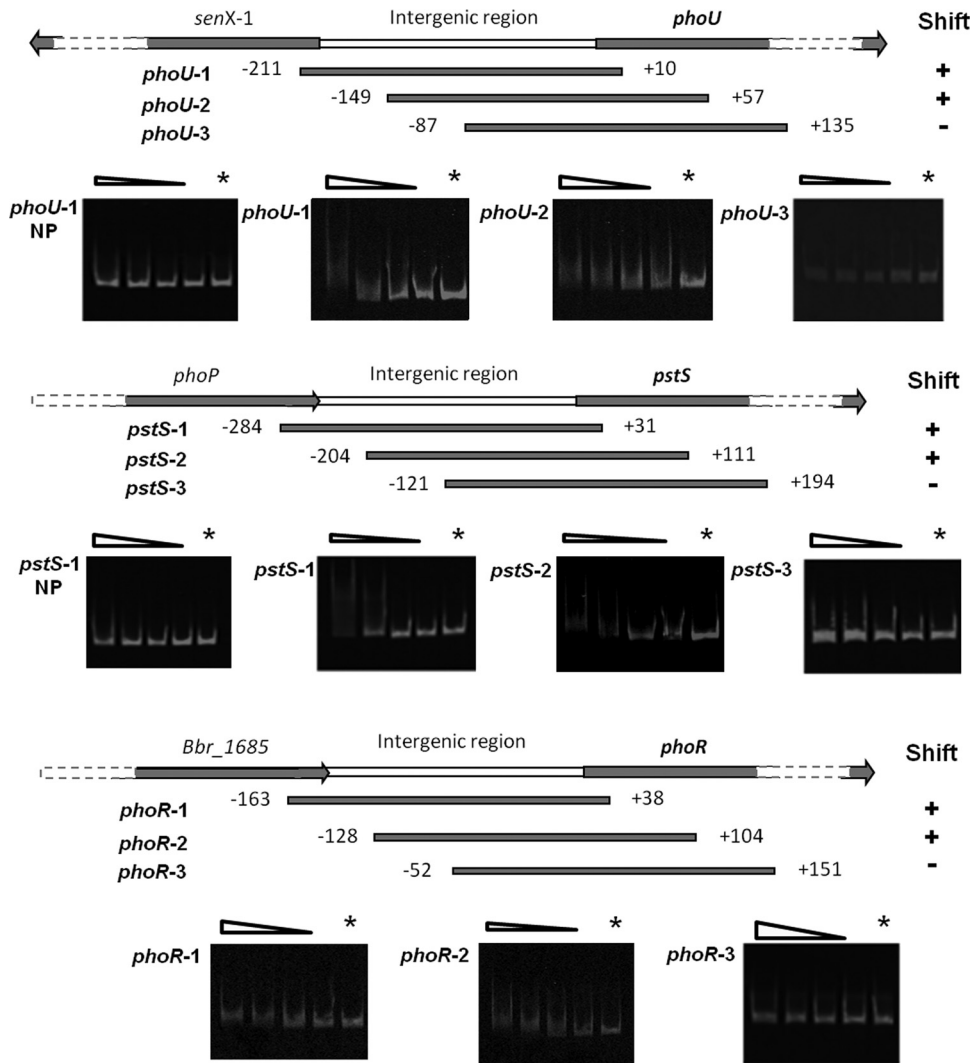


FIG 4 PhoP binding assays to various promoter regions. Shown is a schematic representation of amplified DNA fragments used in gel mobility shift assays; the numbers correspond to the ends of the fragments relative to the presumed translation start site. Plus and minus signs indicate observed binding or no binding, respectively, of PhoP to specific sections of the *phoU*, *pstS*, or *phoR* promoter regions. The terms *phoU-1-NP* and *pstS-1-NP* represent binding assays with unphosphorylated PhoP, and the remainder of the bindings assays were performed using phosphorylated PhoP. PhoP was phosphorylated for 1 h at 25°C in the presence of acetyl phosphate. Each gel represents a binding assay with a particular DNA fragment as indicated in the left margin and decreasing amounts of (phosphorylated) PhoP (lanes 1 to 5 correspond to 400, 200, 100, 50, and 0 mM PhoP, respectively).

effects of the *phoP* insertional mutation, transcriptional profiling was performed for this mutant using DNA microarray technology (see Materials and Methods). The obtained results revealed a total absence of induction of any of the genes of the *pstSCAB* cluster, consistent with the observed reduced growth of this mutant when subjected to P_i starvation (Table 3). qRT-PCR was performed in order to confirm these differences in gene expression; primers were designed for *pstS*, as this gene displayed the most significant transcription difference of this cluster. Results indeed confirmed increased transcription in the wild-type strain, while in the *phoP* mutant strain there was effectively no change in *pstS* transcription, although this gene seems to exhibit a basal transcription level (data not shown).

DISCUSSION

Two-component regulatory systems are employed extensively in nature by microorganisms to modify their cellular physiolo-

gy in response to alterations in environmental conditions. In this work we describe the first characterization of a bifidobacterial 2CRS and provide evidence that the *B. breve* UCC2003 PhoRP proteins constitute an autoregulatory, P_i -responsive 2CRS, which regulates the expression of the P_i starvation response operon *pstSCAB*. The PhoRP system shows high homology to corresponding 2CSs in other members of the genus *Bifidobacterium*, while it also appears to be conserved among many other members of the *Actinobacteridae*.

The histidine protein kinase PhoR contains a single predicted transmembrane domain (TMD) in its N-terminal domain that allows it to be anchored to the membrane while also enabling it to sense the presence of extracellular P_i . PhoR contains a classical phosphoacceptor domain with a conserved histidine residue, as well as an ATPase motif (7). Although HPKs commonly have two TMDs, HPKs with a single TMD have been reported, e.g., for

TABLE 2 Alignment of probable PhoP binding sites in *B. breve* UCC2003 with putative PhoP binding sites in high-GC-content Gram-positive species

Organism	Locus	Putative gene name	Sequence ^a	Strand relative to gene
<i>Bifidobacterium breve</i> UCC2003	Bbr_1682	<i>pstS</i>	CTGTT CATCTTCTGTTT ACTGAT	+
	Bbr_1684	<i>phoR</i>	AAGTT CGCTATTGTT CATTTTT	-
	Bbr_1573	<i>phoU</i>	AAGTT GTTCTACTGTT CATTGGG	-
<i>Bifidobacterium longum</i> NCC2705	BL0315	<i>pstS</i>	G TGTT CATCTTCTGTTTACTGAT	+
	BL0317	<i>phoR</i>	AAGTT CGTCTATTGTT CATTTTT	-
	BL1658	<i>phoU</i>	AAGTT GTTCTACTGTT CATTGGG	-
<i>Bifidobacterium adolescentis</i> ATCC 15703	BAD_0278	<i>pstS</i>	CTGTT CATCTTCCGTT CACCGTT	+
	BAD_0276	<i>phoR</i>	G CGTT CACCTCCGATAATCCTC	-
	BAD_1548	<i>phoA</i>	G TGTT CATCTGCCGTTCTTCTGT	+
<i>Bifidobacterium dentium</i> Bd1	BDP_0387	<i>pstS</i>	CTGTT CATCTACCGTT CACCGAT	+
	BDP_0385	<i>phoR</i>	G CGTTA ACCTCCGATAATCCAG	-
<i>Bifidobacterium animalis</i> BB12	BIF_01097	<i>pstS</i>	ACGTT CACGCTAAAT CACCGTT	+
	BIF_01099	<i>senX3</i>	TGG CTCACCTTCTG CGGCGTGTG	-
<i>Brevibacterium linens</i> BL2	BlinB_010200010959	<i>pstS</i>	TCAT T CATCTTCCAT T CACTTAG	+
<i>Arthrobacter aureus</i> TC1	AAur_0195	<i>pstS</i>	TCGTT CACCTTCCGTT CACCTTT	+
<i>Streptomyces coelicolor</i> A3(2)	SCO1845	<i>pitH</i>	CAGTT CATCTTCCGTT CACCGCTG	+
	SCO4142	<i>pstS</i>	GGGTT CACCCGGGTT CATTAC	+
<i>Frankia alni</i> ACN14a	FRAAL6535	<i>pstS</i>	TTGTT CATCTTTCGTT CACTCCG	+
<i>Leifsonia xyli</i> subsp. <i>xyli</i> CTCB07	Lxx18420	<i>pstS</i>	TCGTT AACCTTCCGTT TACCACT	+
<i>Mycobacterium tuberculosis</i> H37Rv	Rv0928	<i>pstS3</i>	AAGTT CGCCGACCGTTA ACCTAG	+
	Rv2984	<i>ppk</i>	CCGTT GGTCTGCCGTT CACCCCC	-
<i>Propionibacterium acnes</i> SK137	HMPREF0675_3383	<i>pstS</i>	G TGTT CACGTTGCCGTT CACCT TG	+
Consensus			NGGTT CAYY-Y4-GTT CAYNNN	

^a A consensus sequence for *B. breve* could be deduced. Conserved positions are highlighted in bold. Numbers indicate positions with respect to the translational point. R, purine; Y, pyrimidine; N, any nucleotides.

Streptomyces lividans (48). We show in this report that heterologously expressed H10-PhoP is readily and stably phosphorylated using acetyl phosphate as a phosphoryl donor, while it also exhibits *in vitro* DNA binding ability. Generally, response regulators exhibit DNA binding activity when phosphorylated, although specific DNA binding activity of unphosphorylated RRs has been described previously (40).

Microarray analysis data obtained from *B. breve* cells subjected to P_i limitation revealed a significant change in global gene transcription, where transcription of around 10% of the total *B. breve* gene content was affected. Similar results have previously been obtained for other bacteria, such as *B. subtilis* (13) and *C. glutamicum* (16). The transcriptional fusion results as well as microarray experiments convincingly show that transcription of *phoRP* and the *pst* operon is induced under conditions of P_i starvation. In addition, among the P_i starvation-induced genes, we identified some genes that correspond to characterized stress proteins like GroEL, ClpB, DnaK, different ABC transporters, while expression of such stress response proteins is even higher in the *phoP* insertion mutant strain (data not shown). It is also remarkable that P_i starvation reduces the transcription of genes encoding ribosomal proteins. This extensive alteration in the observed transcription profile

can be explained by significant growth rate delay as a result of P_i scarcity, which is consistent with our finding that under P_i starvation conditions, *B. breve* UCC2003::phoP showed clearly altered growth behavior compared with the wild-type strain.

In many bacteria whose P_i starvation response has been studied in detail, e.g., *E. coli* (62), *B. subtilis* (13) or *Corynebacterium* (16), PhoRP homologues activate additional operons related to the acquisition and metabolism of alternative P sources. Compared to PhoRP-dependent genes found in *C. glutamicum*, a homologous *glpQ* gene (glycerophosphoryldiester phosphodiesterase) is present in the *B. breve* genome, while the *ugpA/EBC* (ABC-type sn-glycerol 3-phosphate uptake system) is absent. Homologous genes coding for PhoC (putative cell wall-associated phosphatase), UshA (UDP sugar hydrolase and 5' nucleotidase activity), and NucH (extracellular nuclease) appear to be absent from the *B. breve* UCC2003 genome. In contrast to its equivalent in *C. glutamicum* (46), the *pitA* promoter in *B. breve* does not appear to be regulated by PhoRP. Transcriptional fusions of the *pitA* promoter indicated that this gene is highly expressed when cells are actively growing. The observed drop in transcription following transfer to P_i-limiting medium is possibly caused by an arrest of metabolic processes, as is evident from the cessation of growth. Presumably, the PitA system is used for P_i uptake under normal physiological

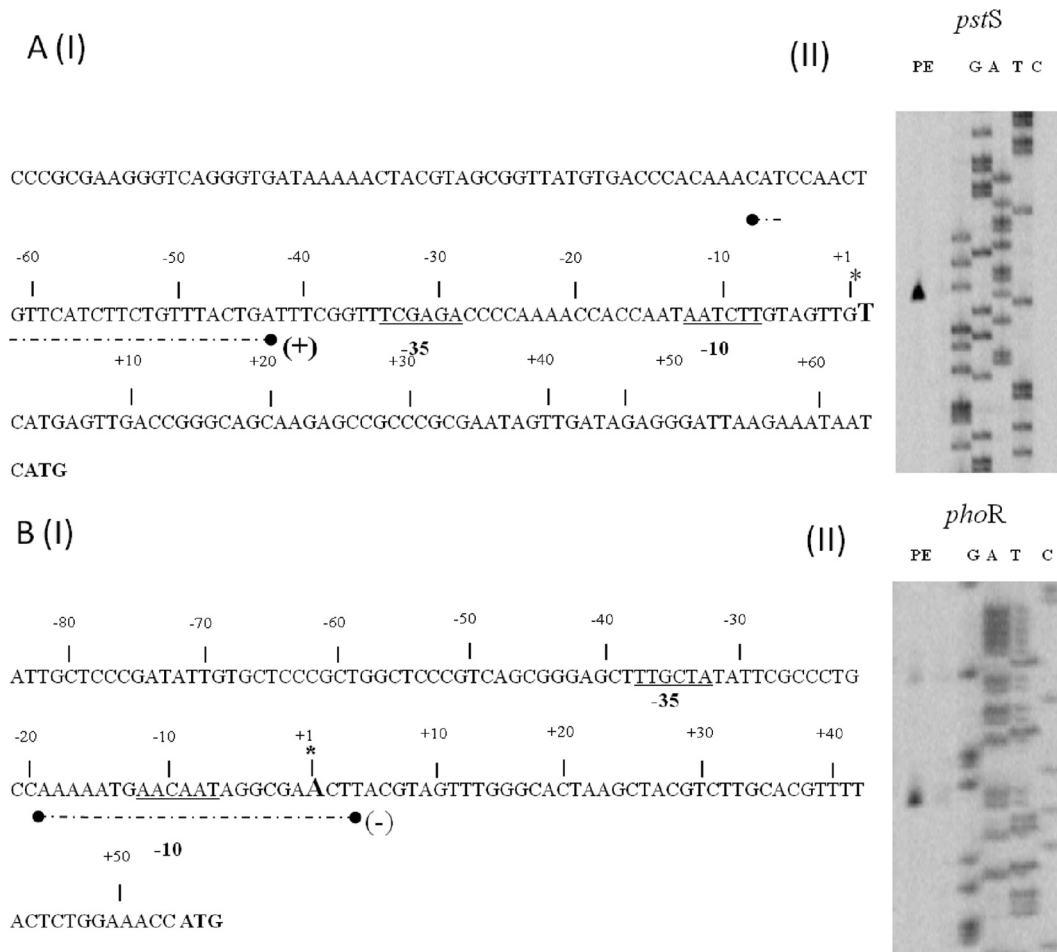


FIG 5 Schematic representation of the *B. breve* UCC2003 *ptsS* [A(I)] and *phoR* [B(I)] promoter regions. Underlined sequences indicate the -10 and -35 hexamers as deduced from the primer extension results [A(II) and B(II)]; the transcriptional start sites (TSS) are indicated by asterisks; broken black lines underneath the respective sequence indicate the PhoP binding sequence (PHO box).

conditions, while the more energy-demanding Pst system is utilized during conditions of P_i starvation. In contrast, expression of the *phoU* gene in *E. coli* is induced in response to low levels of environmental phosphate, where PhoU acts as a negative regulator of the Pho regulon, although it is unclear exactly how PhoU accomplishes this. Recently it was postulated that in *E. coli*, PhoU controls the activity of the PstSCAB₂ transporter, as well as its abundance within the cell, through the PhoBR two-component signaling pathway (39). A *phoU* homolog, designated Bbr_1573, is present on the *B. breve* UCC2003 genome, although microarray analysis of data obtained from *B. breve* UCC2003 subjected to P_i starvation, PhoP overexpression, or *phoP* mutation indicated that *phoU* transcription is only moderately affected (and always below the selected cutoff value). However, PhoP was shown to specifically bind the presumed *phoU* promoter region, thus indicating that *phoU* is part of the PhoRP regulon.

No information is available concerning regulatory sequences involved in P_i starvation response in *Bifidobacterium* species, although PHO boxes have been described for other bacteria as two tandem direct repeats of 11 bp in which the first five nucleotides of the repeat are highly conserved, while the following six positions are much less or not at all conserved (2,

6, 46, 49, 63, 64). A similar situation was found in the *phoRP*, *pst*, and *phoU* promoters in *B. breve* UCC2003, as well as in upstream regions of genes related to P_i metabolism from other *Bifidobacterium* species and various high-GC-content Gram-positive bacteria, which allows us to propose a consensus PHO motif in high-GC-content Gram-positive bacteria: GTTCAYY-Y4-GTTCAYY. Gel retardation experiments have demonstrated PhoP's ability to bind to its own promoter as well as the *pst* promoter; however, the position of these PHO boxes relative to the transcriptional start site of the operons is different: the PHO box in the *pst* promoter region is located 43 bp upstream of the transcription start site, while in the *phoRP* promoter, it overlaps the deduced -10 sequence. Differences in the position of PHO boxes in promoters that are subject to PhoP regulation have been reported for several species. For example, the *E. coli* *phoBR* operon is transcriptionally autoregulated via the direct binding of PhoB to a single PHO box which is 10 bp upstream of the putative -10 region. The *E. coli* Pho regulon consists of about 40 genes, and PhoB-binding sites can be found in two or more copies within some promoter regions (17, 18). The presence of multiple PHO boxes within a single promoter region has also been found: the *Vibrio cholerae* *phoBR* operon is autoregulated by a novel and

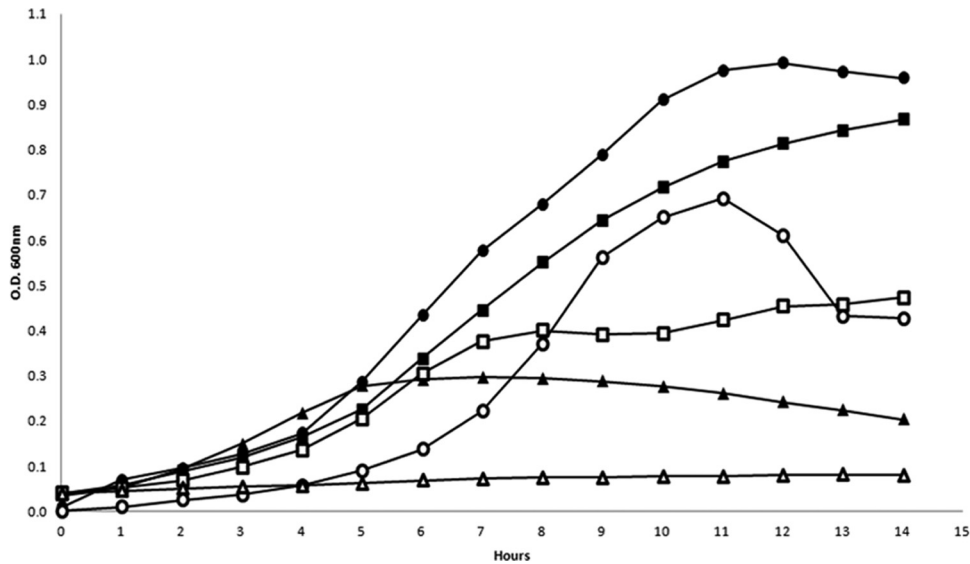


FIG 6 Growth rates of *B. breve* UCC2003 and UCC2003::phoP in CDM with different P_i concentrations: ●, 10 mM P_i , wild type; ■, 1 mM P_i , wild type; ▲, no P_i , wild type; ○, 10 mM P_i , mutant; □, 1 mM P_i , mutant; △, no P_i , mutant.

complex mechanism that involves PhoB binding to three Pho boxes, at 35 (box 1), 60 (box 2), and 80 (box 3) base pairs upstream of the putative *phoB* translation start site. These boxes are located in the sense (box 1) or antisense (boxes 2 and 3) strands, and PhoB interacts with them in a sequential and cooperative manner and with different affinities (6).

B. breve UCC2003::phoP was shown to exhibit reduced growth rates under P_i -limiting conditions compared to the wild-type strain, and this result is consistent with our genetic data and substantiates the notion of a direct correlation between *phoRP* and the response to P_i starvation in *B. breve* UCC2003.

Contradictory studies about limiting P_i concentrations for bacteria in the gut have been reported (61, 63), and an explanation for this may be the multiplicity of different microhabitats and metabolic niches associated with the gut. We have found that the *phoRP* genes are highly conserved among available bifidobacterial genomes, whose single known habitat is the gastrointestinal tract. Nevertheless, only a small number of genes appear to be directly under PhoRP control, perhaps re-

flecting a rather degenerated coping strategy for, at least as far as these gut commensals are concerned, a seemingly rare environmental condition.

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TABLE 3 Summary of differential transcription behavior of *phoRP* and *pstSCAB* in *B. breve* UCC2003 and UCC2003::phoP grown under P_i limitation

ORF	Annotation	Ratio ^a			
		UCC2003		UCC2003::phoP	
		45 min	90 min	45 min	90 min
Bbr_1679	<i>pstB</i>	5.3	8	0.6	0.7
Bbr_1680	<i>pstA</i>	6.2	8.8	0.6	0.5
Bbr_1681	<i>pstC</i>	7.8	12.3	0.6	0.5
Bbr_1682	<i>pstS</i>	14.4	20	0.3	0.2
Bbr_1683	<i>phoP</i>	3.3	4.1	ND	ND
Bbr_1684	<i>phoR</i>	2.5	2.8	ND	ND

^a Calculated as average mRNA level of a given gene under phosphate starvation conditions/average mRNA level of the same gene under non-starvation conditions. The relative mRNA levels are averages from three independent experiments. ND, not determined.

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