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Phosphate Starvation-Inducible Proteins of *Bacillus subtilis*: Proteomics and Transcriptional Analysis

HAIKE ANTELMANN, CHRISTIAN SCHARF, AND MICHAEL HECKER*

Institut für Mikrobiologie, Ernst-Moritz-Arndt-Universität, 17487 Greifswald, Germany

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The phosphate starvation response in *Bacillus subtilis* was analyzed using two-dimensional (2D) polyacrylamide gel electrophoresis of cell extracts and supernatants from phosphate-starved cells. Most of the phosphate starvation-induced proteins are under the control of σ^B, the activity of which is increased by energy depletion. In order to define the proteins belonging to the Pho regulon, which is regulated by the two-component regulatory proteins PhoP and PhoR, the 2D protein pattern of the wild type was compared with those of a *sigB* mutant and a *phoR* mutant. By matrix-assisted laser desorption ionization–time of flight mass spectrometry, two alkaline phosphatases (APases) (PhoA and PhoB), an APase-alkaline phosphodiesterase (PhoD), a glycerophosphoryl diester phosphodiesterase (GlpQ), and the lipoprotein YdhF were identified as very strongly induced PhoPR-dependent proteins secreted into the extracellular medium. In the cytoplasmic fraction, PstB1, PstB2, and TuaD were identified as already known PhoPR-dependent proteins, in addition to PhoB, PhoD, and the previously described PstS. Transcriptional studies of *glpQ* and *ydhF* confirmed the strong PhoPR dependence. Northern hybridization and primer extension experiments showed that *glpQ* is transcribed monocistronically from a σ^A promoter which is overlapped by four putative TT(A/T)ACA-like PhoP binding sites. Furthermore, *ydhF* might be cotranscribed with *phoB* initiating from the *phoB* promoter. Only a small group of proteins remained phosphate starvation inducible in both *phoR* and *sigB* mutant and did not form a unique regulation group. Among these, YfhM and YjbC were controlled by σ^B-dependent and unknown PhoPR-independent mechanisms. Furthermore, YtxH and YvyD seemed to be induced after phosphate starvation in the wild type in a σ^B-dependent manner and in the *sigB* mutant probably via σ^H. YxiE was induced by phosphate starvation independently of σ^B and PhoPR.

Phosphate starvation induces the specific Pho regulon as well as the σ^B-dependent general stress regulon in *Bacillus subtilis*. σ^B-dependent general stress proteins (Gsps) are thought to provide nongrowing cells with nonspecific, multiple, and prospective stress resistance. These proteins seem to be involved in the protection of DNA, membranes, and proteins against oxidative stress and appear to contribute to the survival of extreme environmental conditions, such as heat or osmotic stress as well as acid or alkaline shock of starved cells (3, 11, 14, 15).

The expression of the Pho regulon genes requires the two-component regulatory proteins PhoP and PhoR and enables cells to use limiting phosphate resources more efficiently or to make accessible alternative phosphate sources. These Pho regulon genes include the two major vegetative alkaline phosphatase (APase) structural genes, *phoA* and *phoB* (5, 18), which account for 98% of total APase activity; a gene encoding an APase-alkaline phosphodiesterase (APDase), *phoD* (9), which has a putative role in cell wall teichoic acid turnover; the high-affinity phosphate transport operon, *pstSACB1B2* (12, 33); the *tuaABCDEFGH* operon, which is responsible for the synthesis of teichuronic acid, which replaces the teichoic acid in the cell walls of phosphate-starved cells (23); the teichoic acid biosynthesis operons, *tagAB* and *tagDEF*, whose transcription was shown to be repressed by PhoP and PhoR (22); and the *phoPR* operon, encoding PhoP and PhoR (35, 36). The activation or repression of Pho regulon gene transcription require PhoP-phosphate (PhoP-P), which binds to four TT(A/T)ACA-like sequences repeated at intervals of 11 bp and separated by approximately 5 bp in the promoter regions of *phoA*, *phoB*, *phoD*, *pstS*, *tuaA*, and *tagAD* (8, 21, 22, 23, 32). Gel retardation assays suggested that all four repeats were required for PhoP-P binding and transcriptional activation; therefore, this conserved sequence arrangement was termed the core binding region (32). It has been shown that a dimer of PhoP-P is able to bind two consensus repeats in a stable fashion (8). Interestingly, the stronger Pho regulon *phoA* and *pstS* promoters contain secondary PhoP binding sites which consist of fewer than four TT(A/T/C)ACA-like repeats within the coding region and which are required for promoter activation (24). The *phoD* promoter was characterized as the strongest Pho regulon promoter and contains the core binding region and a 5′ secondary binding region which is important for coordinated PhoP binding to the core binding region (8). It was hypothesized that PhoP binding to the core and secondary binding regions results in DNA loop formation to activate transcription from the stronger Pho regulon promoters (8).

In this study, the phosphate starvation response in *B. subtilis* was analyzed using two-dimensional (2D) protein gel electrophoresis (proteome analysis) to identify new phosphate starvation-inducible (Psi) proteins. Because the APases are secreted into the extracellular space, we also analyzed the 2D pattern of extracellular proteins (secretome analysis). By comparison of the *B. subtilis* wild type with a *sigB* mutant and a *phoR* mutant, the Psi proteins were allocated to the respective regulons. By matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry, the glycerophosphoryl diester phosphodiesterase GlpQ and the lipoprotein YdhF were identified as new members of the Pho regulon.

* Corresponding author. Mailing address: Institut für Mikrobiologie, Ernst-Moritz-Arndt-Universität, 17487 Greifswald, Germany. Phone: 49 (3834) 864200. Fax: 49 (3834) 864202. E-mail: hecker@microbio7.biologie.uni-greifswald.de.

TABLE 1. Primers

Purpose	Primer	Sequence
Amplification of the internal gene fragments (5′-3′)	GlpQ1	CTAATACGACTCACTATAGGGAGAGTAAGGATGAAGCAGGAGG
	GlpQ2	GTTCTGTCGCTGGGCTTAC
	YdhF1	CTAATACGACTCACTATAGGGAGAAAGACGTCACTTGTCGTG
	YdhF2	GCTATCATGCTTGCGGGC
	YjbC1	CTAATACGACTCACTATAGGGAGATTTCTTCATCGATGATTTCC
	YjbC2	CAATGAACTGGTATGAAAAG
	YfhM1	CTAATACGACTCACTATAGGGAGAAGATGAGATGTCCATTCGG
	YfhM2	AAGTGGACGGAGTTAAATGC
	YxiE1	CTAATACGACTCACTATAGGGAGACTTTATGGCTGACGCTTCC
	YxiE2	ATGTTAGTAGCGATTGACG
Amplification of the promoter regions (5′-3′)	GlpQvor	CCCAAGCTTGGGAACGGCGGCTTTATCATG
	GlpQrev	CGGGATCCCGGTAAGGATGAAGCAGGAGG
	YjbCvor	GCAGGAAGTTATTCCCGAGC
	YjbCrev	CATCGATGATTTCCAAGACCTC
Sequencing and primer extension reaction (5′-3′)	GlpQ-PE	TGCCGACACTGGCGTTACC
	YjbC-PE	TCAATTGGAAAGTACTCGCTAAGC

MATERIALS AND METHODS

Bacterial strains, growth conditions, and APase measurement. The bacterial strains used were *B. subtilis* 168 (*trpC2*) (1), *B. subtilis* ML6 (*trpC2 sigB*::Δ*Hin*dIII-*Eco*RV::*cat*) (20), and *B. subtilis phoR* (*trpC2 phoR*Δ*Bal*I::*tet*) (12). *B. subtilis* strains were cultivated under vigorous agitation at 37°C in a synthetic medium described previously (3). Starvation for phosphate was provoked by cultivating the bacteria in a medium containing 0.16 mM KH_2PO_4.

Units of APase activity were calculated as the amount that hydrolyzed 1 nmol of *p*-nitrophenyl phosphate in 1 min at 30°C and pH 8.0 in 1 M Tris-Cl. Units per milliliter of culture were defined as 2/3 [(A_{410} × 352)/reaction time (minutes)] (27).

Preparation of the cytoplasmic protein fraction. Cells grown under phosphate starvation conditions were labeled with 5 μCi of L-[^{35}S]methionine per ml for 5 min at different times along the growth curve. The control was labeled at an optical density at 500 nm (OD_{500}) of 0.4, and the other samples were taken beginning at the transient phase (t_0) at time intervals of 30 min. L-[^{35}S]methionine incorporation was stopped by the addition of chloramphenicol and an excess of cold methionine as well as by transfer of the culture to ice. Cells were disrupted by sonication, and crude protein extracts were separated from the cell waste by centrifugation. The supernatant containing the whole-cell soluble fraction was separated by analytical 2D polyacrylamide gel electrophoresis (PAGE).

Preparation of the extracellular protein fraction. *B. subtilis* cells were grown in 1 liter of minimal medium under phosphate starvation conditions and harvested at an OD_{500} of 0.4 for the control and 1 h after entry into the stationary phase (OD_{500} = 0.8) for the other samples. The cells were harvested by centrifugation for 20 min at 4°C. The extracellular proteins in the supernatant were precipitated with 10% (wt/vol) trichloroacetic acid overnight on ice and centrifuged for 2 h. The resulting protein pellet was washed with 96% ethanol (vol/vol) three times and dried.

Analytical and preparative 2D PAGE. Analytical 2D PAGE was performed using the immobilized pH gradient (IPG) technique described by Bernhardt et al. (4). The protein samples were separated using IPG strips (Amersham Pharmacia Biotech, Piscataway, N.J.) in the pH range of 3 to 10. For identification of the proteins by mass spectrometry, protein samples of 400 μg were separated by preparative 2D PAGE and the gels were stained with Coomassie blue R-250.

Peptide mass fingerprinting. In-gel tryptic digestion was performed using a peptide-collecting device (29). Peptide solution (0.5 μl) was prepared with equal volumes of saturated α-cyano-4-hydroxycinnamic acid solution in 50% acetonitrile–0.1% trifluoroacetic acid (vol/vol) to a sample template for a MALDI-TOF mass spectrometer (Voyager DE-STR; PerSeptive Biosystems). Peptide mass fingerprints were analyzed using MS-Fit software (http://prospector.ucsf.edu.).

Analysis of transcription. Total RNA of the *B. subtilis* strains was isolated from cells at different times along the growth curve under phosphate starvation conditions by the acid-phenol method of Majumdar et al. (25). The control sample was taken at an OD_{500} of 0.4, and the other samples were taken beginning at t_0 at time intervals of 30 min. Northern blot analyses were performed as described previously (39). Hybridization specific for *glpQ*, *ydhF*, *yjbC*, *yfhM*, and *yxiE* was tested with digoxigenin-labeled RNA probes synthesized in vitro with T7 RNA polymerase from T7 promoter-containing internal PCR products of the respective genes using the primers listed in Table 1.

The synthetic oligonucleotide primers complementary to the N terminus-encoding region of the *glpQ* and *yjbC* genes (shown in Table 1) were 5′ end labeled with [γ-^{32}P]ATP and used as primers for primer extension analysis as described previously (39). The corresponding promoter region was sequenced using as a template PCR products containing the promoter region of the respective genes and the primers shown in Table 1 as described by Sanger et al. (34).

RESULTS

Analysis of the cytoplasmic proteins from phosphate-starved *B. subtilis* cells. Proteome analysis is an excellent tool for the comprehensive understanding of gene expression regulation in response to changing environmental conditions. Previous studies done by Eymann et al. (12) and based on the carrier ampholyte isoelectric focusing technique showed that nearly 20 proteins were induced after phosphate starvation. The induction of nine of these was dependent on the two-component system PhoPR. Among these proteins, the main protein was identified as PstS, the binding component of the high-affinity phosphate transport system. In this study, the IPG technique was used and resulted in improved resolution and reproducibility of the 2D gels. To allocate the phosphate starvation-inducible (Psi) proteins to different regulons, we compared the 2D pattern of the wild type (Fig. 1) with the 2D patterns of the *phoR* mutant (Fig. 2) and the *sigB* mutant (Fig. 3).

Cytoplasmic proteins belonging to the Pho regulon were identified as the APase PhoB; the APase-APDase PhoD; the phosphate transport system components PstS, PstB1, and PstB2; and the UDP-glucose 6-dehydrogenase TuaD, which is involved in the biosynthesis of teichuronic acid. These proteins were strongly induced in the wild type as well as in the *sigB* mutant but failed to be induced in the *phoR* mutant after phosphate starvation. However, there was low-level induction of PstS in the *phoR* mutant, a result which was also obtained in previous transcriptional studies (33). Only small fractions of the APase PhoB and the APase-APDase PhoD were found in the cytoplasm. The majority of these APases-APDases are secreted into the medium.

Besides the proteins belonging to the phosphate-specific Pho regulon, the σ^B-dependent general stress proteins (Gsps) are induced after phosphate starvation. As shown in Fig. 1, the σ^B-dependent proteins were induced in the wild type as well as in the *phoR* mutant. However, we observed that the induction of the σ^B-dependent proteins was stronger in the *phoR* mutant than in the wild type. Most of these Gsp proteins have been identified in previous 2D studies (2, 4, 30) or by other approaches, such as oligonucleotide hybridization (31) and DNA array technology (A. Petersohn, J. Hoheisel, and M. Hecker,

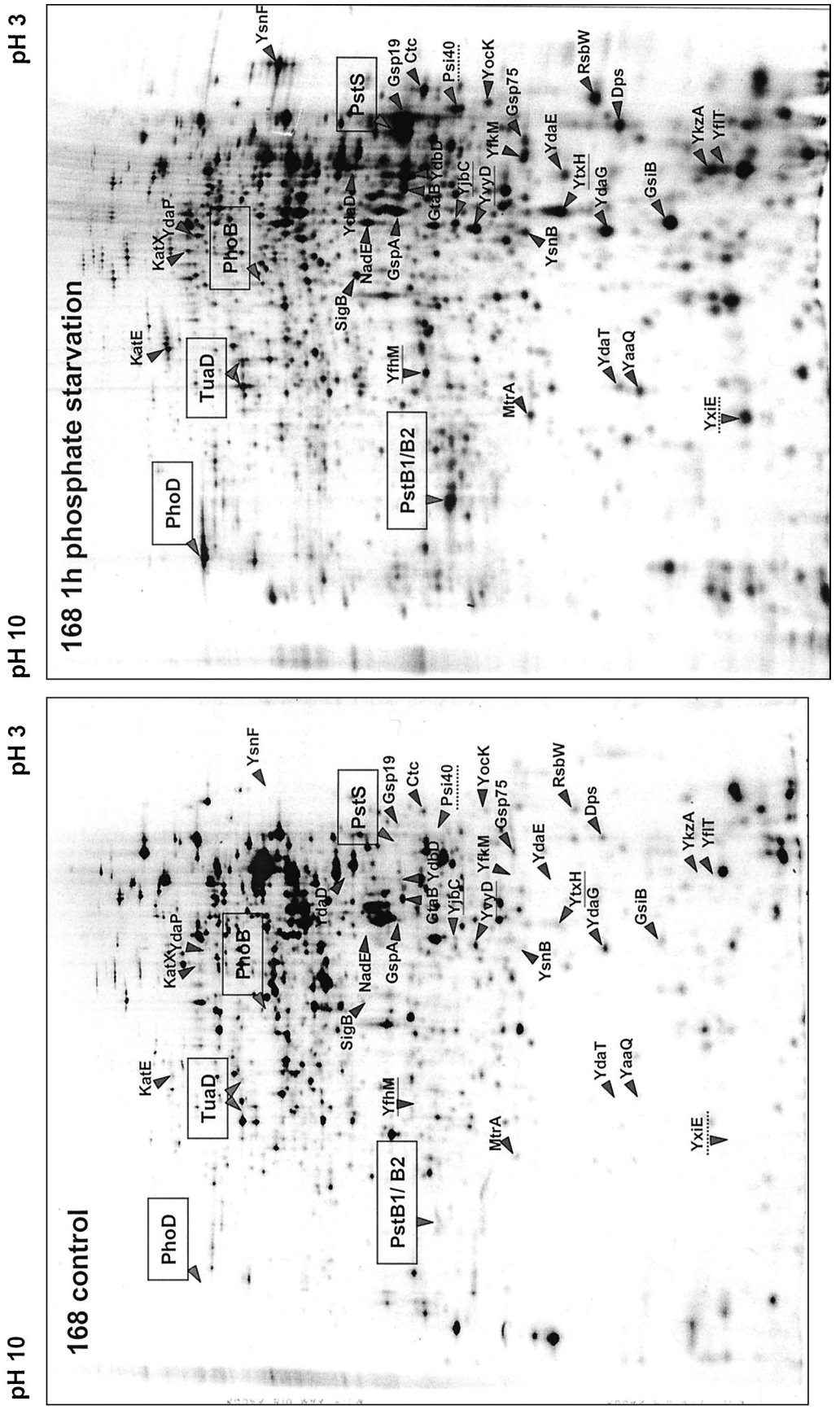

FIG. 1. Synthesis of cytoplasmic phosphate starvation-inducible proteins in the *B. subtilis* wild type labeled with L-[^{35}S]methionine before (168 control) and 1 h after entry into the stationary phase provoked by phosphate starvation (168 1h phosphate starvation). PhoPR-dependent proteins are indicated by boxes, PhoPR-independent proteins are indicated by broken underlining, and proteins which are controlled by both σ^B and other sigma factors are indicated by solid underlining. The remaining proteins are σ^B-dependent Gsp proteins.

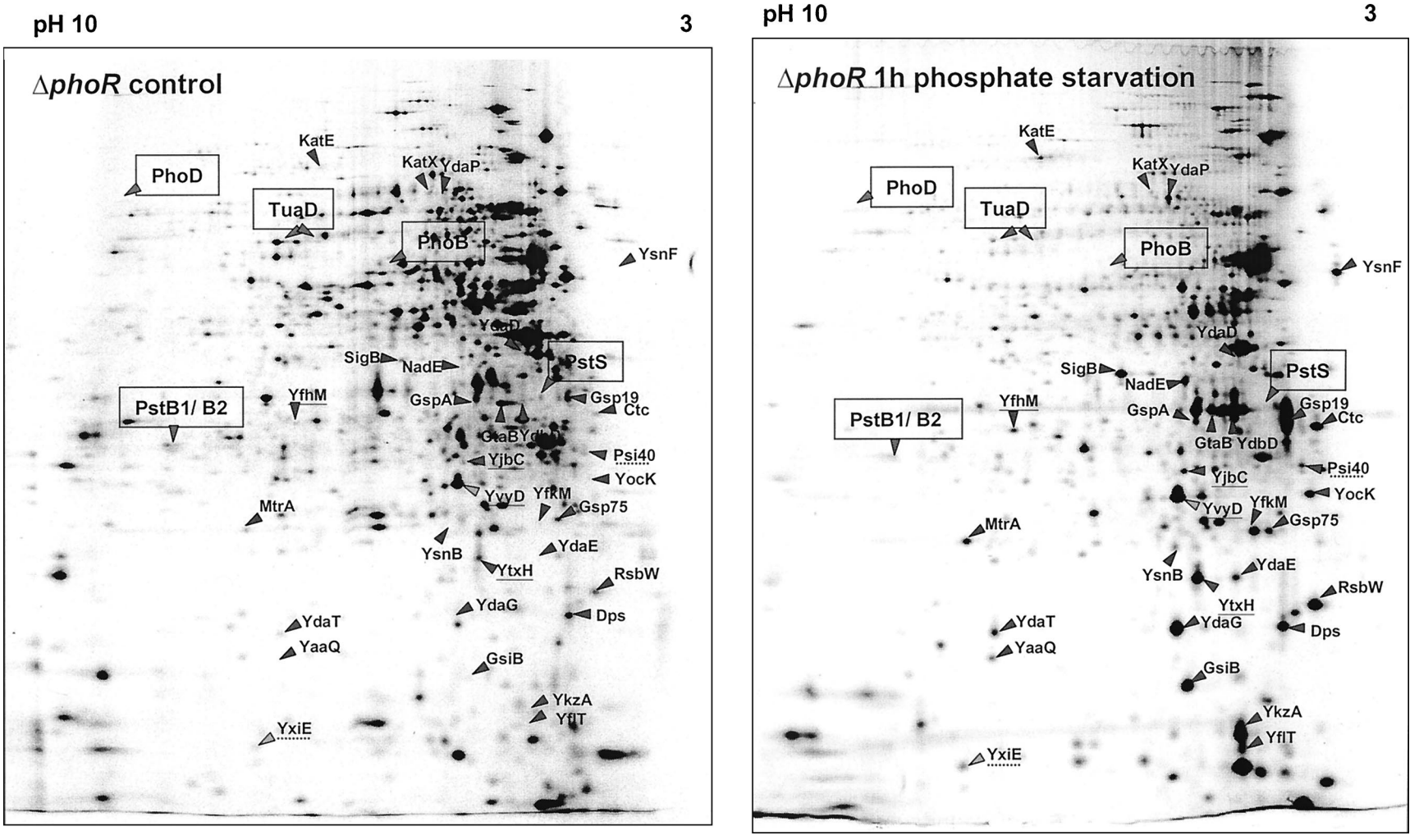

FIG. 2. Synthesis of cytoplasmic phosphate starvation-inducible proteins in the *B. subtilis phoR* mutant labeled with L-[^{35}S]methionine before (Δ*phoR* control) and 1 h after entry into the stationary phase provoked by phosphate starvation (Δ*phoR* 1h phosphate starvation). For details, see the legend to Fig. 1.

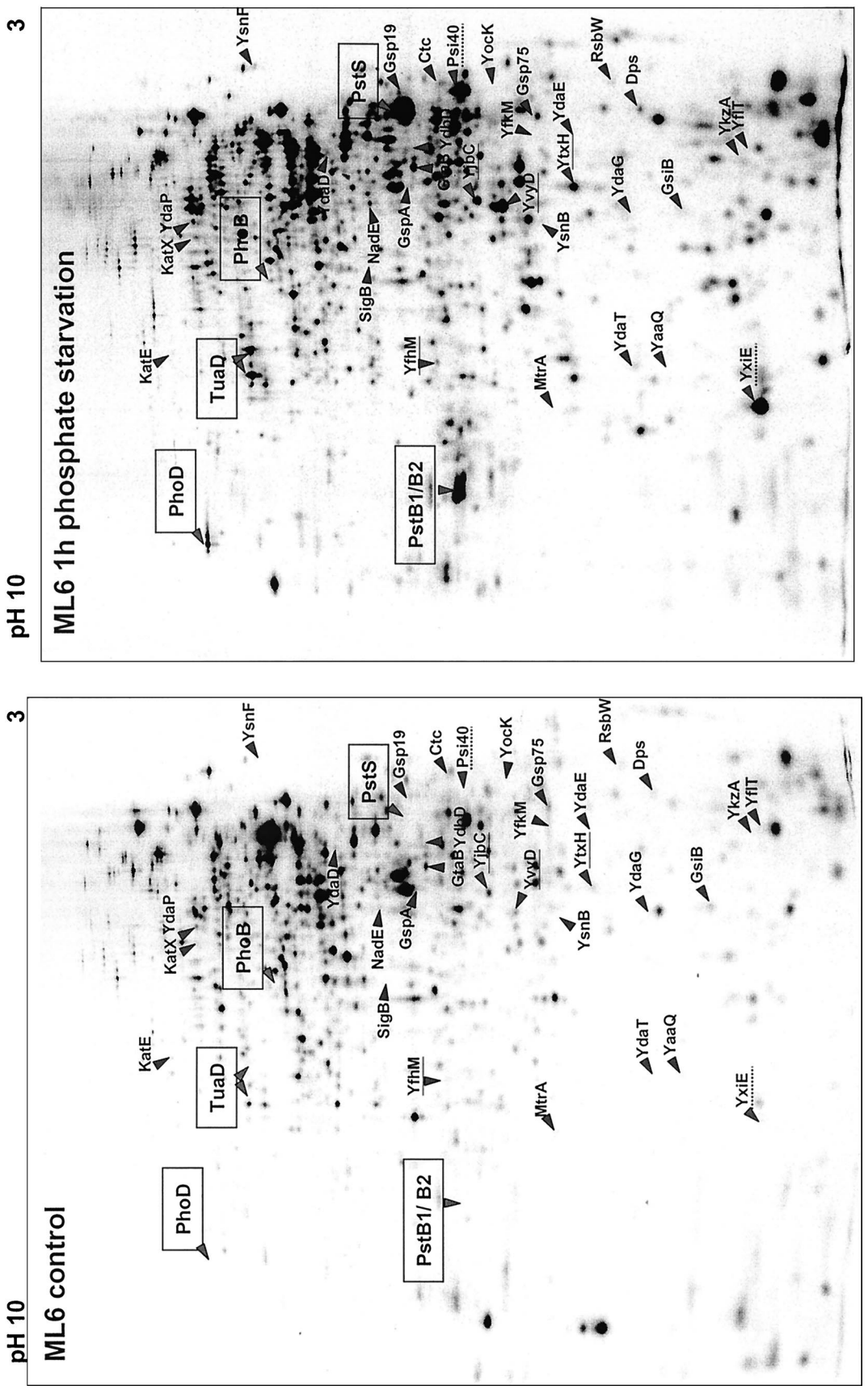

FIG. 3. Synthesis of cytoplasmic phosphate starvation-inducible proteins in the *B. subtilis sigB* mutant labeled with L[^{35}S]methionine before (ML6 control) and 1 h after entry into the stationary phase provoked by phosphate starvation (ML6 1h phosphate starvation). For details, see the legend to Fig. 1.

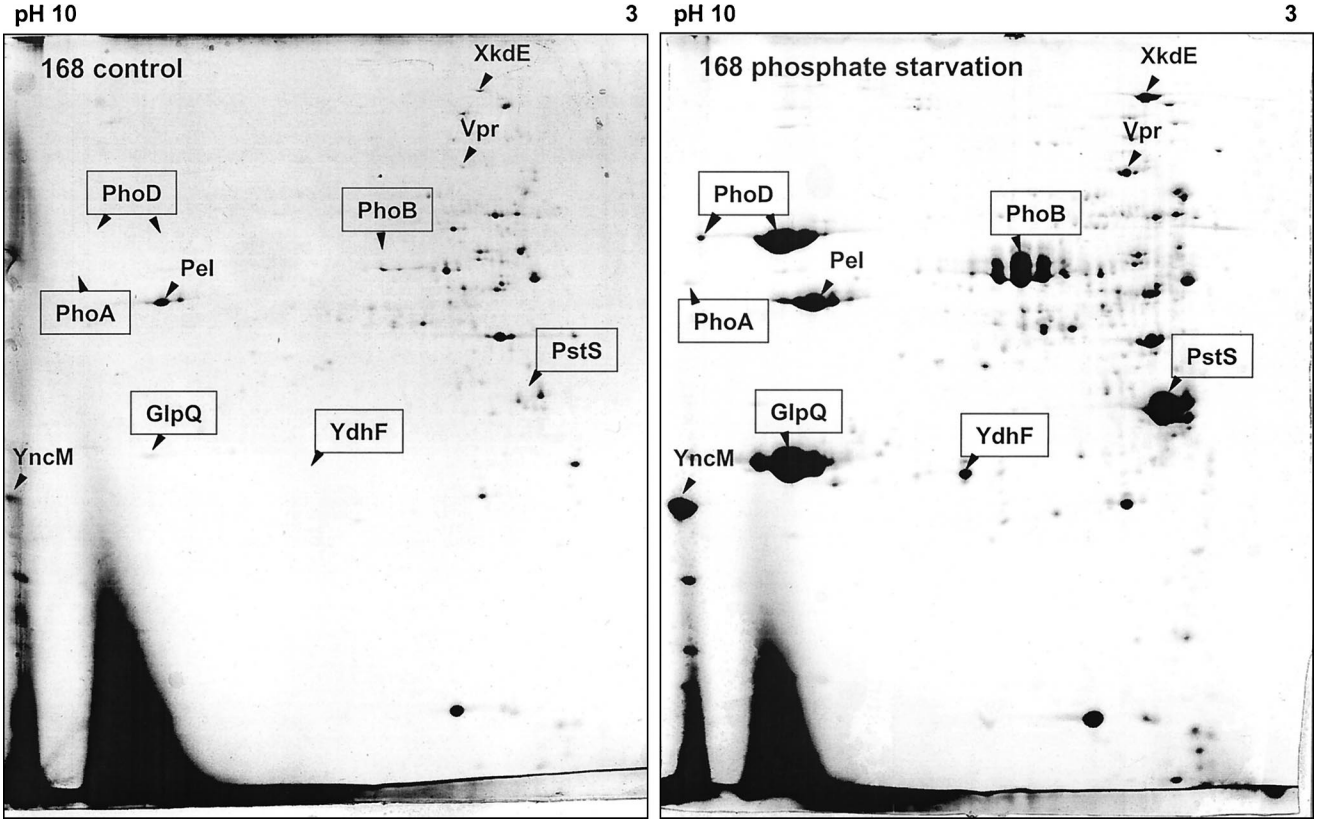

FIG. 4. Extracellular phosphate starvation-inducible proteins in the *B. subtilis* wild type. PhoPR-dependent proteins are indicated by boxes, and the other labeled proteins are PhoPR-independent phosphate starvation-inducible proteins. The 2D gels were stained with Coomassie blue R-250.

unpublished data). The GTP cyclohydrolase MtrA was identified as a new member of the σ^B regulon (Fig. 1). Furthermore, the proteins with currently unknown functions—YdaT, YaaQ, and YsnB—which were identified as σ^B-dependent proteins by the last two approaches (31; Petersohn et al., unpublished), could be localized on the proteome map.

In the next step, we investigated whether any genes are induced by phosphate starvation independently of either PhoPR or σ^B. Only five proteins induced by phosphate starvation in a σ^B- or PhoPR-independent manner could be identified; they were YvyD, YtxH, YjbC, YfhM, and YxiE. Among these, a few σ^B-controlled and PhoPR-independent proteins remained phosphate starvation inducible even in a *sigB* mutant background, indicating complex regulation by other sigma factors in addition to σ^B. YvyD and YtxH were presumed to be induced in the wild type via σ^B and in the *sigB* mutant in a σ^H-dependent manner after phosphate starvation because both proteins were phosphate starvation inducible in the *sigB* mutant (Fig. 3). The Psi protein YjbC, with still-unknown function, and YfhM, which is similar to epoxide hydrolases, appeared to be regulated by σ^B and extracytoplasmic function sigma factors (16, 31; Petersohn et al., unpublished). All these proteins belong to the σ^B regulon, forming a link to other Psi regulons. Only YxiE and Psi40 were induced by phosphate starvation independently of σ^B and PhoPR. The identification of Psi40 by MALDI-TOF mass spectrometry failed.

Analysis of the secretome from phosphate-starved *B. subtilis* cells. The Pho regulon includes the APases PhoA and PhoB and the APase-APDase PhoD, which were used as reporter enzymes for monitoring the phosphate starvation response. Because these enzymes were expected to be secreted into the medium, we also analyzed the extracellular protein fraction from phosphate-starved cells using 2D gels (secretome analysis). The supernatant was collected when APase activity was at the maximum. As shown in Fig. 4, the PhoPR-dependent APase PhoB and the APase-APDase PhoD could be identified as very strongly induced and most prominent proteins on the secretome map. Surprisingly, we detected only a low level of the major APase PhoA, which should account for 75% of the total APase activity. Furthermore, the phosphate binding protein PstS was also found in the extracellular fraction. Besides these known Pho regulon proteins, we also identified a second, very strongly induced phosphodiesterase, the glycerophosporyl diester phosphodiesterase GlpQ, involved in the hydrolysis of deacylated phospholipids. Furthermore, the lipoprotein YdhF, with still-unknown function, was identified as a new member of the Pho regulon secreted into the medium. Besides these PhoPR-dependent proteins, a few extracellular proteins induced by phosphate starvation in a PhoR mutant could be identified (data not shown). Among these were the serine protease Vpr, YncM, the pectate lyase Pel, and XkdE, encoded by the prophage PBSX genome.

Transcriptional studies of *glpQ* and *ydhF* as new members of the Pho regulon. To verify the protein data and explain the regulatory mechanisms of gene expression after phosphate starvation, we used Northern hybridization and primer extension analyses.

By secretome analysis, GlpQ was identified as a very strongly phosphate starvation-induced protein. The *glpQ* gene is part of the *glpQT* operon, encoding a glycerol 3-phosphate (G3P) per-

mease and a glycerophosphoryl diester phosphodiesterase which is involved in the hydrolysis of deacylated phospholipids (10, 28, 37). Northern hybridization analyses using a *glpQ*-specific mRNA probe showed very strong induction of the 1.1-kb *glpQ*-specific transcript after phosphate starvation in the wild type; the transcript failed to be induced in the *phoR* mutant (Fig. 5B). This result indicates that *glpQ* is transcribed monocistronically after phosphate starvation from a promoter which is located upstream of *glpQ* and which requires the PhoPR system for transcriptional activation. We were not able to detect the 2.4-kb bicistronic *glpTQ* transcript shown by Nilsson et al. (28). In primer extension experiments, the 5′ end of the *glpQ*-specific message was preceded by the sequence 5′ ACACGC-N_{17}-TATTAT 3′ (Fig. 5C and D). The promoter has a −10 region that is similar to the consensus sequence for a σ^A promoter; however, there is no sequence similarity at the −35 region. Because induction depends on the PhoPR system, we examined the promoter region for putative PhoP binding sites. The putative "Pho boxes," consisting of the four TT(A/T) ACA-like direct repeats, are located from −22 to −60 in the promoter region of *glpQ* and are underlined in Fig. 5.

The second new PhoPR-dependent gene identified was *ydhF*. The *ydhF* gene is located downstream of the APase gene *phoB* (Fig. 6A). Transcriptional studies using a *ydhF*-specific mRNA probe showed the strong induction after phosphate starvation in the wild type of a 2.2-kb transcript which failed to be induced in the *phoR* mutant (Fig. 6B). This result indicates that *ydhF* might be cotranscribed with *phoB* initiating from the *phoB* promoter, which depends on the PhoPR system.

Transcriptional studies of *yjbC*, *yfhM*, and *yxiE* as non-PhoPR-dependently phosphate starvation-induced genes. Besides the specific Pho regulon, there is the large σ^B-dependent general stress regulon which is induced after entry into the stationary phase provoked by starvation for phosphate. However, a small group of proteins is induced independently of either σ^B or PhoPR, indicating the presence of other groups of Psi proteins. *yjbC*, *yfhM*, and *yxiE* might belong to these groups. Transcriptional studies were performed to identify the regulatory mechanisms responsible for σ^B- and PhoPR-independent induction.

The *yjbC*-specific transcriptional start points were mapped in previous studies by Petersohn et al. (31), suggesting double control of *yjbC* by σ^B and probably σ^W. Our transcriptional data showed strong induction of a promoter located upstream from putative P_W after phosphate starvation (P_1) (Fig. 7C and D). Transcription initiating from P_1 was induced in a σ^B-independent manner. The transcriptional start point was preceded by the sequence 5′ GAGCAG-N_{17}-AAAAAA 3′, which shares no similarities with consensus sequences from known promoters. The putative σ^W-dependent transcript initiating from P_2 also was induced weakly after phosphate starvation. However, we did not find any induction at the σ^B promoter (P_B) after phosphate starvation (data not shown), indicating that σ^B does not contribute in a substantial way to the induction of *yjbC* in response to phosphate starvation. This result could be explained by competition between the other two sigma factors and σ^B with the RNA polymerase core enzyme. Northern blot data showed the induction of a 1.2-kb mRNA, indicating that *yjbC* may be cotranscribed with the downstream *yjbD* gene (Fig. 7B).

The *yfhM* gene was also found during the promoter search for σ^B-dependent genes (16); additionally, it was found as a σ^B-dependent gene by DNA array technology (Petersohn et al., unpublished). Our Northern blot data showed that a transcript of 1.8 kb was weakly induced after more than 1 h of phosphate starvation in the wild type but was completely absent in the *sigB* mutant (Fig. 8B). This transcript might initiate from the σ^B promoter located in front of *yfhK*, which was also found as a σ^B-dependent gene by DNA array technology (Petersohn et al., unpublished). This result indicates that *yfhK*, *yfhL*, and *yfhM* may be cotranscribed (Fig. 8A). Interestingly, the σ^B-dependent transcript was induced severalfold more strongly in the *phoR* mutant than in the wild type. The second transcript, corresponding to the 1.2-kb mRNA seen on the Northern blot, was not induced after phosphate starvation. The third, 0.9-kb transcript seemed to be induced in all strains tested, possibly because of transcription from another as-yet-unidentified promoter located in the region upstream of *yfhM*. This phosphate starvation induction seems to be independent of either σ^B or PhoPR.

The *yxiE* gene was induced after phosphate starvation in a σ^B-independent as well as a PhoPR-independent manner. Northern blot analyses revealed the induction of a 0.5-kb *yxiE*-specific transcript in the wild type, the *sigB* mutant, and the *phoR* mutant, indicating that *yxiE* is transcribed monocistronically (Fig. 9B). The mechanism leading to the induction of *yxiE* after phosphate starvation is unknown.

DISCUSSION

The phosphate starvation response of *B. subtilis* was analyzed using the 2D protein gel electrophoresis technique for comparison of the cytoplasmic and secreted proteins from wild-type *B. subtilis*, the *phoR* mutant, and the *sigB* mutant. These analyses showed that the phosphate stimulon consists of two main regulons, the phosphate starvation-specific Pho regulon and the σ^B general stress regulon. However, there appeared to be a small group of phosphate starvation-induced proteins (e.g., YjbC, YfhM, and YxiE) which might be controlled by currently unknown mechanisms.

The Pho regulon genes are well characterized (5, 9, 18, 22, 23, 33, 35, 36), and most of these were identified on the cytoplasmic as well as the extracellular proteome map. On the cytoplasmic proteome map, three components of the high-affinity phosphate transport system could be identified. These included, in addition to the previously identified phosphate binding protein PstS (12), the ATP binding proteins PstB1 and PstB2. PstS was the most strongly induced protein. The low-level induction of PstS observed in the *phoR* mutant is consistent with the transcriptional data for the *pstS* promoter (33). Because the activated *pstS* promoter could be activated only by PhoP-P, it was proposed that PhoP might be phosphorylated by other histidine kinases in the *phoR* mutant (32). The stronger induction of *pstS* than of other Pho regulon genes was found to be due to the secondary PhoP binding region in addition to the core binding region, which is located within the coding region of *pstS* (24). The integral inner membrane proteins PstA and PstC, which are the other components of the phosphate transport system, could not be identified, probably because the separation of hydrophobic membrane proteins by isoelectric focusing is currently very difficult.

The very alkaline isoelectric point as well as the hydrophobicity may be also the reasons why only the UDP-glucose 6-dehydrogenase TuaD was localized on the proteome map and the detection of the remaining seven proteins encoded by the *tuaABCDEFGH* operon failed. The *tua* operon encodes proteins involved in the biosynthesis of teichuronic acids, which replace phosphate-containing teichoic acids (23). The Pho regulon further includes the major APase structural genes, *phoA* and *phoB*, which account for 98% of total APase activity (5, 17, 18, 19). APase specific activity increases 500-fold after transition into the stationary phase under low-phosphate conditions. A third APase structural gene, *phoD*, encodes an en-

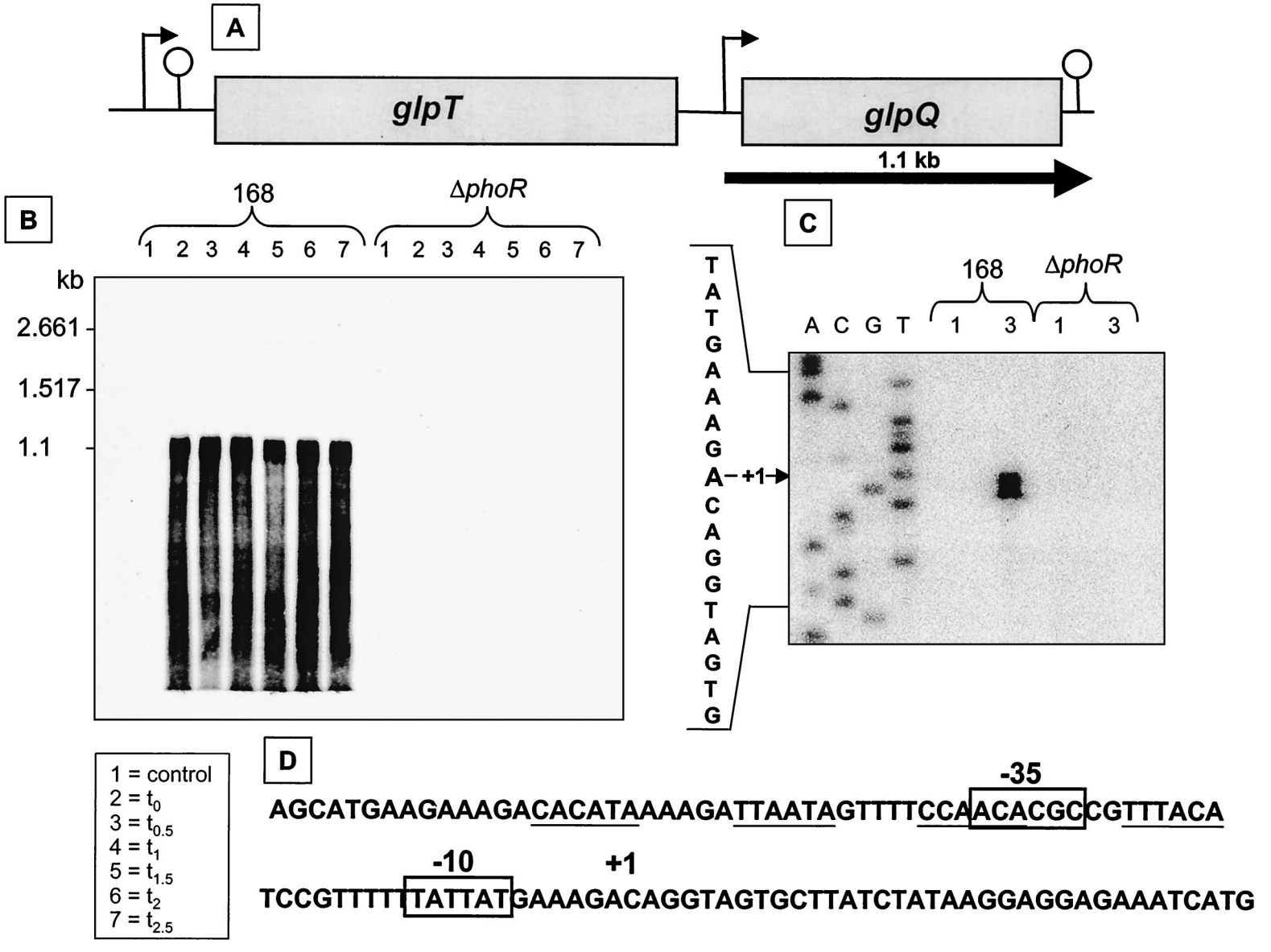

FIG. 5. Physical organization of *glpTQ* (A), transcript analyses of *glpQ* (B and C), and sequence of the *glpQ* promoter region (D). For Northern blot (B) and primer extension (C) experiments, 10 μg of RNA each was isolated from wild-type *B. subtilis* 168 and the *phoR* mutant before (control) and at different times after (30, 60, 90, 120, and 150 min) entry into the transient phase (t_0) provoked by phosphate starvation. The probable 5′ end of the σ^A-dependent *glpQ* message is marked by +1 (C and D). The −10 and −35 promoter sequences of the σ^A-dependent transcript are indicated by boxes, and the putative PhoP binding sites are underlined (D). The dideoxy sequencing ladder (ACGT) (C) extends from the same primer as that used for the primer extension experiments and is complementary to that determined by DNA sequencing.

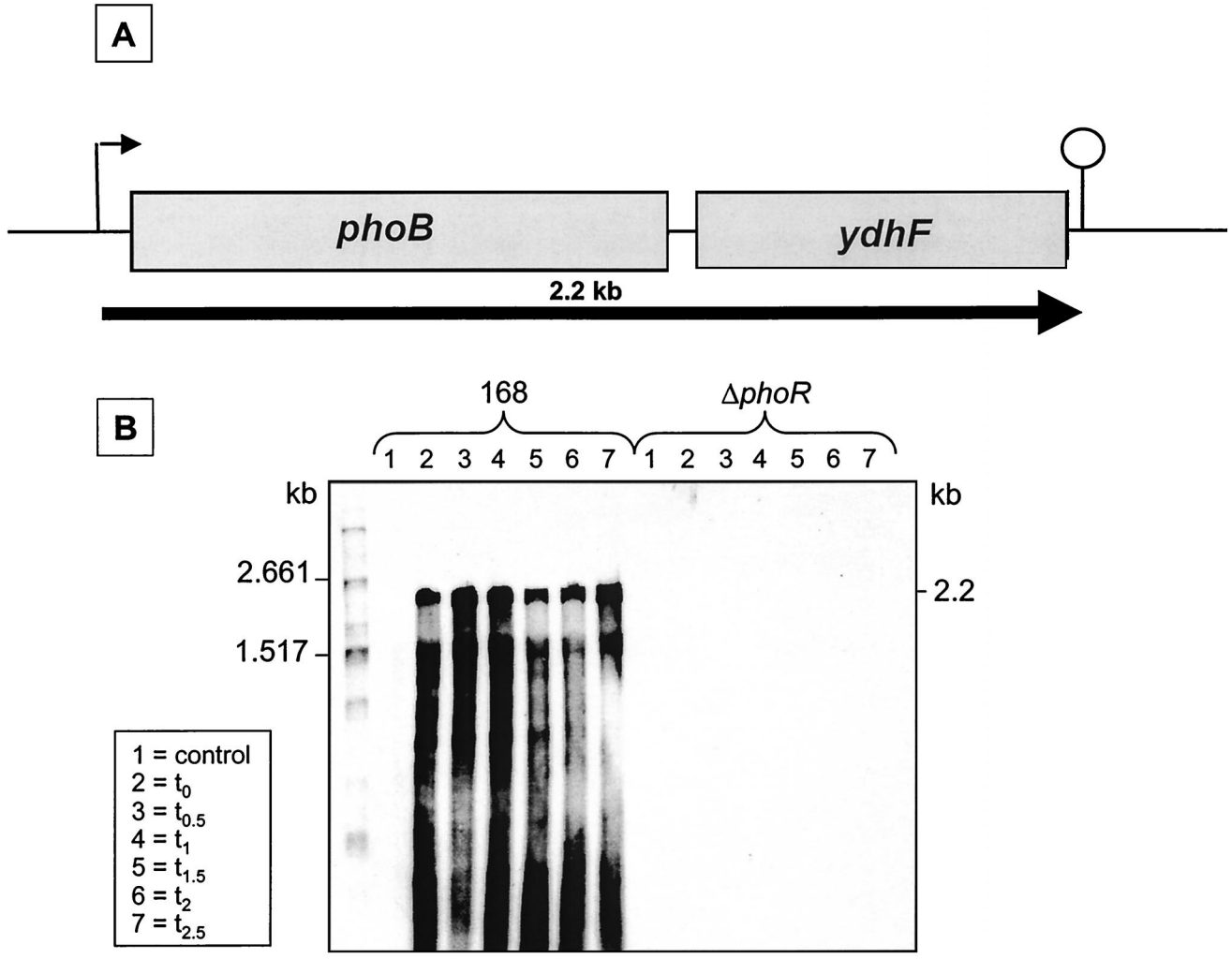

FIG. 6. Physical organization of the *phoB-ydhF* operon (A) and transcript analysis of *ydhF* (B). For Northern blot experiments (B), 10 μg of RNA was isolated from wild-type *B. subtilis* 168 and the *phoR* mutant before (control) and at different times after (30, 60, 90, 120, and 150 min) entry into the transient phase (t_0) provoked by phosphate starvation.

zyme with both APase and phosphodiesterase activities (9) and also belongs to the Pho regulon. However, to a minor extent, the APase PhoB and the APase-APDase PhoD could be also detected in the cytoplasmic fraction. These proteins might represent the unprocessed precursors of the APases still containing the signal peptides. On the secretome map, the APase PhoB and the APase-APDase PhoD belong to the most prominent extracellular proteins induced after phosphate starvation. The major APase, PhoA, was identified only as a minor phosphate starvation-induced protein probably because this enzyme has high specific activity. It is also possible that the main fraction of PhoA is lost because of the very alkaline isoelectric point (pI, 9.9), which is on the border of the IPGs pH range. Furthermore, the lipoprotein YdhF was also secreted. *ydhF* was shown to be cotranscribed with *phoB* initiating from the PhoPR-dependent *phoB* promoter.

Besides these known members of the Pho regulon, we identified GlpQ as the second very strongly induced phosphodiesterase of *B. subtilis* which is regulated by the PhoPR system. The *glpTQ* operon has been described as a member of the glycerol utilization (*glp*) regulon of *B. subtilis* (28). The *glpT* gene encodes a G3P permease that is believed to function as an anion antiporter, where G3P is taken up by the cell in exchange for internal phosphate (26). The *glpQ* gene encodes a glycerophosphoryl diester phosphodiesterase that hydrolyzes deacylated phospholipids to G3P. While the APase-APDase PhoD is believed to cleave the phosphodiester bonds of teichoic acids (9), GlpQ is involved in the degradation of phospholipids. It has been shown previously that the *glpTQ* operon-specific transcript is induced threefold by G3P (28). Therefore, it was concluded that the *B. subtilis glpTQ* operon is homologous with the *Escherichia coli glpTQ* operon, which is also part of the glycerol regulon. However, in *E. coli*, a second transport system recognizing G3P is encoded by the *ugp* operon (6). The *ugp*-dependent transport system is induced after phosphate starvation in a PhoBR-dependent manner (6). In this case, G3P can be used as the sole source of phosphate. The last gene of the *ugp* operon, the *ugpQ* gene, encodes a glycerophosphoryl diester phosphodiesterase which is similar to *B. subtilis* GlpQ. Our studies showed that *B. subtilis glpQ* is part of the Pho regulon and therefore seems to be regulated in a manner similar to *E. coli ugpQ*. However, *glpQ* is transcribed monocistronically after phosphate starvation from a promoter located in the region upstream of *glpQ*. It could be assumed that the G3P

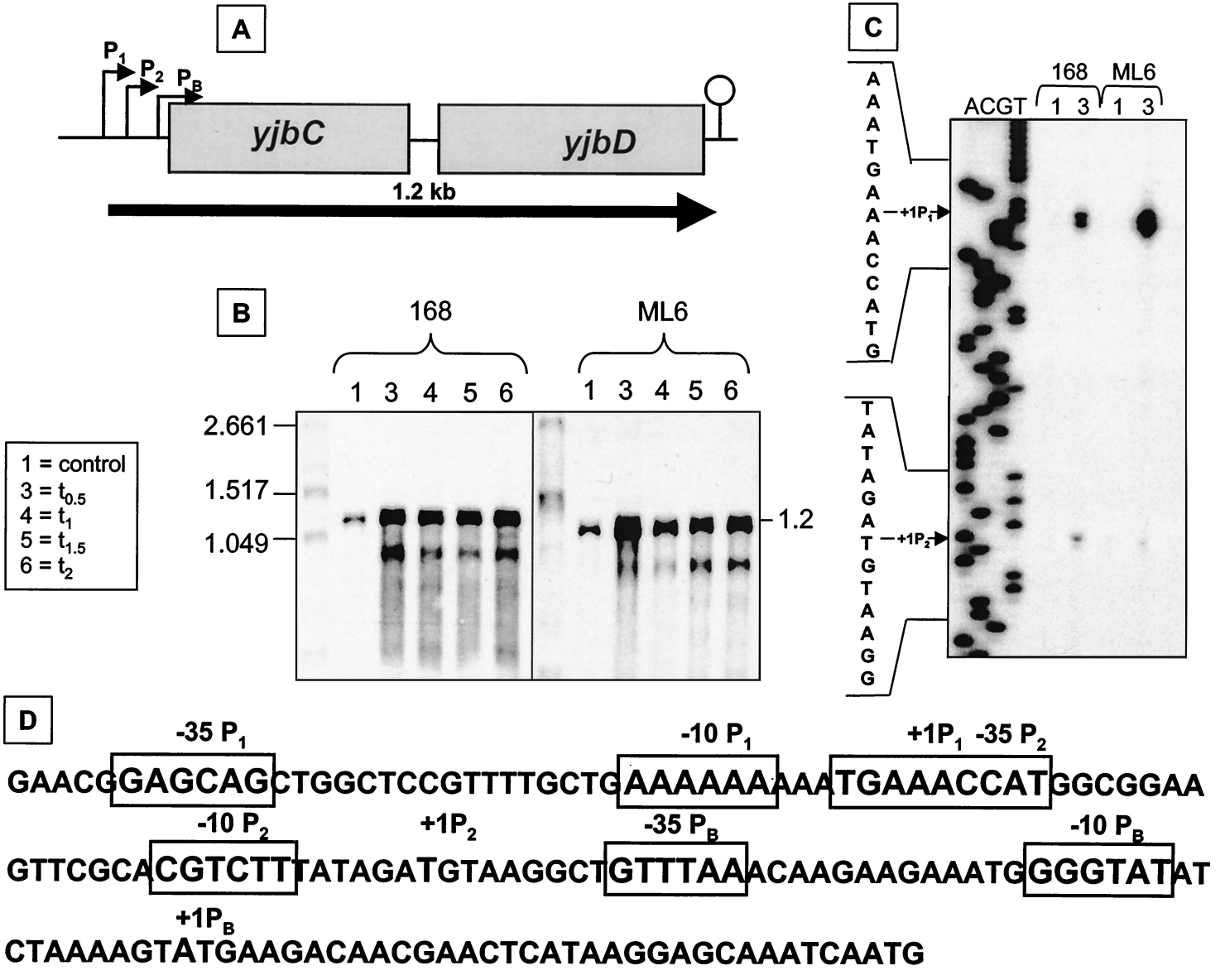

FIG. 7. Physical organization of *yjbCD* (A), transcript analyses of *yjbC* (B and C), and sequence of the *yjbC* promoter region (D). For Northern blot (B) and primer extension (C) experiments, 10 μg of RNA each was isolated from wild-type *B. subtilis* 168 and the *sigB* mutant ML6 before (control) and at different times after (30, 60, 90, and 120 min) entry into the transient phase (t_0) provoked by phosphate starvation. The probable 5′ ends of the *yjbC*-specific transcripts are marked by +1 (C and D). The −10 and −35 promoter sequences are indicated by boxes (D). The dideoxy sequencing ladder (ACGT) (C) extends from the same primer as that used for the primer extension experiments and is complementary to that determined by DNA sequencing.

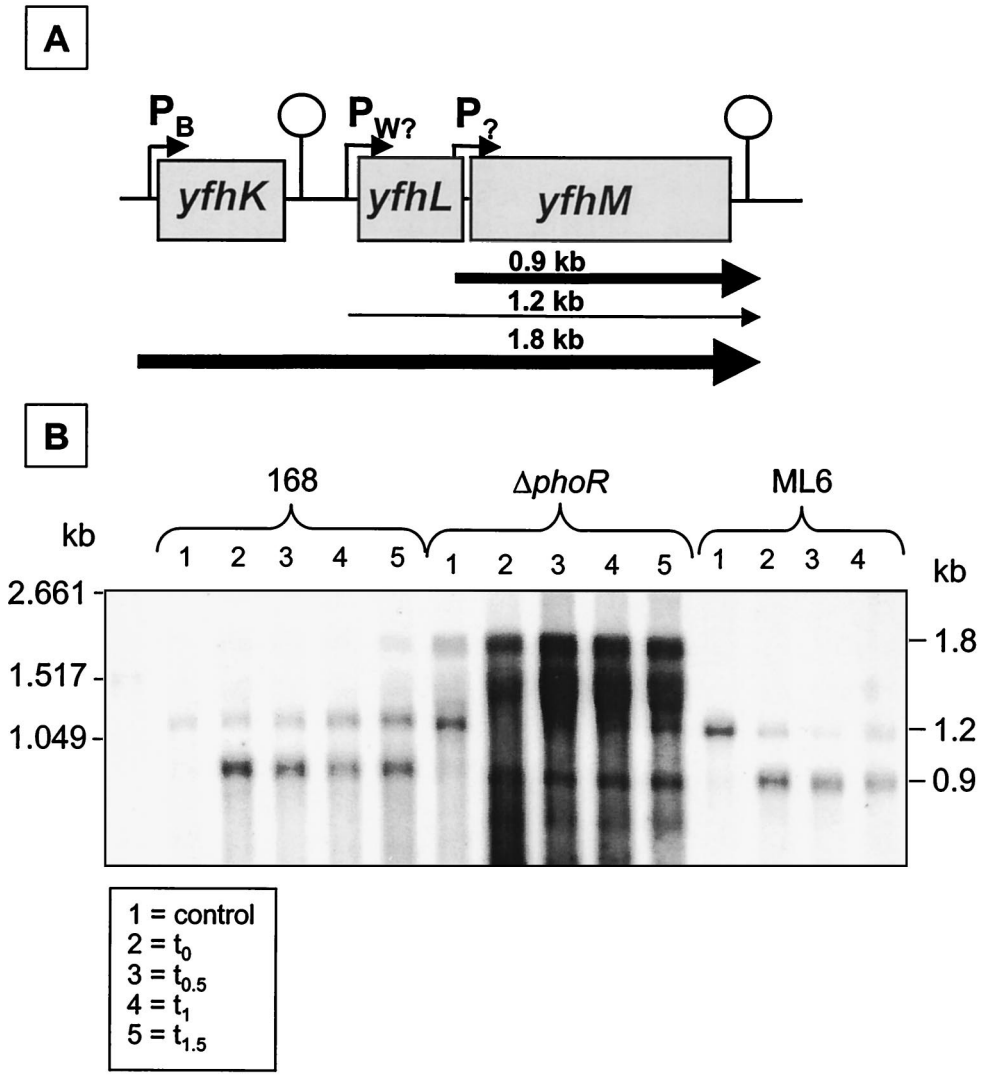

FIG. 8. Physical organization of the *yfhKLM* operon (A) and transcript analysis of *yfhM* (B). For Northern blot experiments (B), 10 μg of RNA was isolated from wild-type *B. subtilis* 168, the *sigB* mutant ML6, and the *phoR* mutant before (control) and at different times after (30, 60, and 90 min) entry into the transient phase (t_0) provoked by phosphate starvation.

permease GlpT is not needed after phosphate starvation because the G3P produced by GlpQ is a substrate for the APases which liberate the inorganic phosphate. *E. coli* seems to possess two systems involved in the transport of G3P, the *glpT*-mediated system (as part of the *glp* regulon) and the *ugp*-dependent transport system (as part of the Pho regulon). In contrast, the *B. subtilis glpTQ* operon is part of the *glp* regulon and, in addition, the *glpQ* gene is part of the Pho regulon controlling its expression independently of *glpT*.

Besides these phosphate starvation-specific proteins, the σ^B regulon is induced after phosphate starvation. σ^B-dependent general stress proteins are expected to provide nonspecific, multiple, and prospective stress resistance to nongrowing *B. subtilis* cells in anticipation of future stress (15). Our studies have shown that a mutation in the *phoR* gene not only abolishes the transcription of Pho regulon genes but also causes the superinduction of σ^B-dependent proteins (Fig. 2). This finding was also shown on the transcriptional level for the *yfhM* gene, which was much more strongly induced in the *phoR* mutant than in the wild type. Previous studies of Farewell et al. (13) showed competition between σ^{70} and σ^S for limiting amounts of RNA polymerase during the stationary phase in *E. coli*. Thus, we suggest that the superinduction of σ^B-dependent proteins in a *phoR* mutant after entry into the stationary phase provoked by starvation for phosphate is caused by an increased amount of σ^B bound to the RNA polymerase in the absence of the competing PhoP activation of σ^A-dependent genes. Another explanation might be that in the absence of PhoP, the expression of genes that would overcome the phosphate starvation condition is impaired, resulting in increased expression of σ^B-dependent proteins.

Previous studies of Eymann et al. (12) have shown that there is a third group of phosphate starvation-inducible proteins. We also identified a few proteins which remained phosphate starvation inducible in a *phoR* mutant as well in a *sigB* mutant. These proteins can be allocated to two subgroups of phosphate starvation-inducible proteins. The first subgroup includes YtxH, YvyD, YjbC, and YfhM, which are regulated by σ^B and by additional mechanisms leading to σ^B-independent induction by phosphate starvation. *ytxH* and *yvyD* are presumed to be

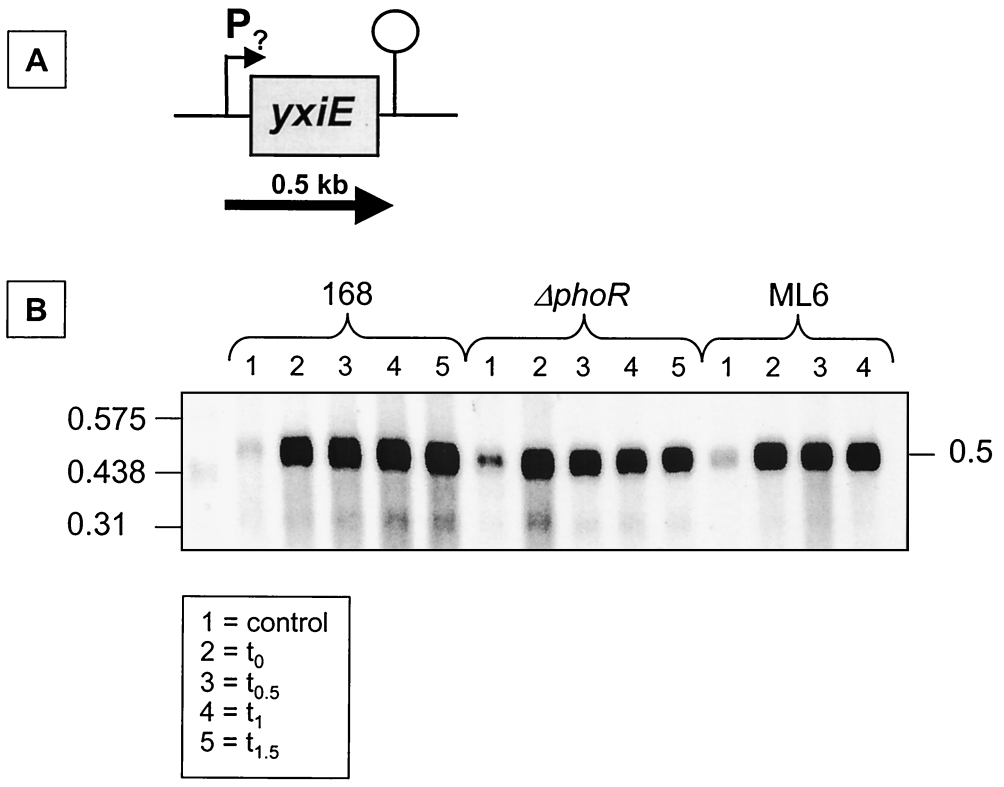

FIG. 9. Physical organization of the *yxiE* gene (A) and transcript analysis of *yxiE* (B). For Northern blot experiments (B), 10 μg of RNA was isolated from wild-type *B. subtilis* 168, the *sigB* mutant ML6, and the *phoR* mutant before (control) and at different times after (30, 60, and 90 min) entry into the transient phase (t_0) provoked by phosphate starvation.

induced at the σ^H promoter in response to phosphate starvation if σ^B is not available, but experimental evidence for this suggestion is still lacking (7, 38). YjbC and YfhM are regulated by σ^B and probably by the ECF sigma factor σ^W, as shown in other studies (16, 31; Petersohn et al., unpublished). The *yjbC* and *yfhM* genes form a link between the σ^B regulon and the σ^H or σ^W regulon (7, 16). The mechanisms causing induction of the σ^B-dependent genes *yjbC* and *yfhM* after phosphate starvation are totally unknown. The second subgroup is regulated completely independently of σ^B. *yxiE* was allocated to this subgroup. Transcriptional analyses raised the question of which mechanisms other than PhoPR or σ^B are involved in the phosphate deficiency response of *B. subtilis*. However, genes such as *yjbC*, *yfhM*, or *yxiE* form only a minor part of the phosphate stimulon. It is obvious that the most important regulons involved in the phosphate starvation response are the specific Pho regulon and the σ^B-dependent general stress regulon. However, this study is not yet a full description of the phosphate stimulon because integral membrane proteins still escaped our proteome approach. The usage of DNA array techniques is a convenient strategy for finding the still-missing genes of the phosphate stimulon.

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