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Genome-Wide Transcriptional Analysis of the Phosphate Starvation Stimulon of *Bacillus subtilis*†

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Bacillus subtilis **responds to phosphate starvation stress by inducing the PhoP and SigB regulons. While the PhoP regulon provides a specific response to phosphate starvation stress, maximizing the acquisition of phosphate (P_i) from the environment and reducing the cellular requirement for this essential nutrient, the SigB regulon provides nonspecific resistance to stress by protecting essential cellular components, such as DNA and membranes. We have characterized the phosphate starvation stress response of *B. subtilis* at a genome-wide level using DNA macroarrays. A combination of outlier and cluster analyses identified putative new members of the PhoP regulon, namely, *yfkN* (2′,3′ cyclic nucleotide 2′-phosphodiesterase), *yurI* (RNase), *yjdB* (unknown), and *vpr* (extracellular serine protease). YurI is thought to be responsible for the nonspecific degradation of RNA, while the activity of YfkN on various nucleotide phosphates suggests that it could act on substrates liberated by YurI, which produces 3′ or 5′ phosphoribonucleotides. The putative new PhoP regulon members are either known or predicted to be secreted and are likely to be important for the recovery of inorganic phosphate from a variety of organic sources of phosphate in the environment.**

When *Bacillus subtilis* encounters phosphate starvation stress, it responds by inducing groups of genes that function to restrict the metabolic consequences of the limited supply of this essential nutrient. These groups of genes are collectively referred to as the phosphate (Pho) stimulon. The phosphate stimulon includes at least two well-described regulons, namely, the sigma B (σ^B) general stress regulon and the phosphate starvation-specific PhoP regulon. When *B. subtilis* encounters phosphate starvation, genes of the SigB regulon are induced by the alternative sigma factor, σ^B, and genes of the PhoP regulon are either induced or repressed by activated PhoP (namely, PhoP~P).

The σ^B general stress regulon contains >100 genes (58, 64). These genes provide a nonspecific response to stress by encoding proteins that protect the DNA, membranes, and proteins from the damaging effects of stress. Proteins induced by σ^B help the cell to survive potentially harmful environmental conditions, such as heat, osmotic, acid, or alkaline shock (6, 21, 23, 26). This protective function is thought to be particularly important in maintaining the viability of nongrowing cells.

The PhoP regulon currently consists of 34 members. Six operons (*phoPR* [56, 60], *phoB-ydhF* [7, 14], *pstSAC-pstBA-pstBB* [3, 67], *phoD-tatAD* [7, 19], *resABCDE* [10], and *tuaABC DEFGH* [40, 72]) and five monocistronic genes (*glpQ* [7], *phoA* [30, 31], *tatCD* [34], *ykoL* [60], and *yttP* [62]) are induced and two operons (*tagAB* and *tagDEF* (39) are repressed in response to phosphate starvation. *phoA* and *phoB* encode alkaline phosphatases (APases) which facilitate the recovery of inorganic phosphate (P_i) from organic sources (11, 30); *phoD* encodes a phosphodiesterase/APase, putatively involved in cell wall teichoic acid turnover, and is secreted exclusively by the twin arginine transporter (*tatCD*) pathway (34); the *pstSAC-pstBA-pstBB* operon encodes a high-affinity phosphate transporter for the uptake of P_i at low P_i concentrations (3, 67); *glpQ* encodes a glycerophosphoryl diester phosphodiesterase involved in the hydrolysis of deacylated phospholipids (7); the *tuaABCDEFGH* operon encodes teichuronic acid biosynthesis; the *tagAB* and *tagDEF* operons encode polyglycerolteichoic acid biosynthesis (7, 39, 49); and the *phoPR* and *resABCDE* operons encode two-component signal transduction systems PhoP-PhoR and ResD-ResE (29, 30, 37, 40, 49, 51). The functions of three putative Pho regulon genes (*ydhF*, *ykoL*, and *yttP*) are currently unknown (7, 62, 69).

The induction or repression of PhoP regulon genes is mediated by the binding of PhoP~P to Pho box sequences: direct repeats of TT(A/T/C)ACA with a 5-bp ± 2-bp spacer (18). For efficient binding at promoters where PhoP~P is essential and sufficient for promoter function, four TT(A/T/C)ACA-like sequences with an 11-bp periodicity on the coding strand between nucleotides −60 and −20 relative to the transcription start site are required. Deletion of a single repeat from the core binding region severely reduces PhoP binding and transcriptional activation in vivo and in vitro (40). In the case of genes induced by PhoP~P, the PhoP-binding sites are located on the coding strand of the promoter region. Repressed genes usually have consensus sequences on the noncoding strand (39), although the *resD* promoter is an exception (75).

Recently, we and others have proposed the inclusion of additional genes in the PhoP regulon. Ogura and coworkers (54) analyzed the composition of the PhoP regulon by DNA

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)	Reference or source
B. subtilis strains		
168	*trpC2*	4
ML6	*trpC2 sigB*ΔHindIII-EcoRV::Cm^r	32
168-PR	*trpC2 phoR*ΔBalI::Tc^r	61
168-MLPR	*trpC2 sigB*ΔHindIII-EcoRV::Cm^r *phoR*ΔBalI::Tc^r	61
BFS1243	*trpC2 yurI*::Em^r	MICADO[a]
YFKNd	*trpC2 yfkN*::Em^r	MICADO
YBCOdd	*trpC2 ybcO*::Em^r	MICADO
BFS436	*trpC2 yjdB*::Em^r	MICADO
BFA1234-PR	*trpC2 phoR*ΔBalI::Tc^r *yurI*::Em^r	This study
YFKNd-PR	*trpC2 phoR*ΔBalI::Tc^r *yfkN*::Em^r	This study
YBCOdd-PR	*trpC2 phoR*ΔBalI::Tc^r *ybcO*::Em^r	This study
BFA1243-ML	*trpC2 sigB*ΔHindIII-EcoRV::Cm^r *yurI*::Em^r	This study
YFKNd-ML	*trpC2 sigB*ΔHindIII-EcoRV::Cm^r *yfkN*::Em^r	This study
YBCOdd-ML	*trpC2 sigB*ΔHindIII-EcoRV::Cm^r *ybcO*::Em^r	This study
YBCOdd-MLPR	*trpC2 sigB*ΔHindIII-EcoRV::Cm^r *phoR*ΔBalI::Tc^r *ybcO*::Em^r	This study
E. coli strain XL1-Blue	*supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1 lac* [F′ *proAB*$^+$*lacI*^{q} *lacZ*Δ*M15* Tn*10* (Tc^r)]	Stratagene Europe
Plasmids		
pMUTIN4	Ap^r Em^r *spoVG-lacZ* P_{spac} (8.61 kb)	79
pBluescript II KS(+)	Cloning vector, Ap^r	Stratagene Europe
p*phoA*	pBluescript II KS(+) containing a 978-bp insert of *phoA* Ap^r	This study
p*yheK*	pBluescript II KS(+) containing a 485-bp insert of *yheK* Ap^r	This study

[a] MICADO, Microbial Advanced Database Organization.

microarray analysis, after overproduction of PhoP. They identified *yycP* and *yjdB* as potential members of the Pho regulon, although they were unable to confirm this observation by *lacZ* reporter gene studies. Prágai and Harwood (62) putatively identified two additional members of the PhoP regulon, namely, *yhbH* and *yhaX*. These genes had the same expression characteristics, since their induction in response to phosphate starvation was dependent on PhoPR and the sporulation-specific sigma factor, SigE. However, more recent studies (60) failed to demonstrate binding of PhoP to the control region of *yhaX*. This indicates either that *yhaX* is activated by PhoP indirectly via another regulatory pathway or that binding of PhoP~P to the *yhaX* promoter region requires an additional factor(s) (60). Consequently, we have not included these genes as members of the PhoP regulon. PhoP~P is known to function with $E\sigma^E$ holoenzyme, since it enhances transcription at the SigE-dependent P_{E2} promoter of *phoPR*. Paul and colleagues (56) have shown that autoregulation of *phoPR* involves the up-regulation of this lowly expressed promoter as well as the more highly expressed SigA promoters.

To gain a global perspective on the transcriptional responses of *B. subtilis* to phosphate starvation, we monitored genome-wide changes in gene expression during phosphate starvation using DNA macroarrays. By comparing the response of the wild-type strain to those of the *sigB* and *phoR* mutants, potential new members of the PhoP regulon were identified and subsequently analyzed using a combination of Northern hybridization and reporter gene analyses. The data represent the most comprehensive analysis of the response of *B. subtilis* to P_i starvation.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Bacterial strains and plasmids are listed in Table 1. Strains were grown in Luria-Bertani (LB) medium, low-phosphate medium (LPM) or high-phosphate medium (HPM) (63). The concentration of phosphate was 0.42 mM in LPM and 5.0 mM in HPM. When required, the concentrations of antibiotics were, per milliliter, 0.3 μg of erythromycin, 25 μg of lincomycin, 12.5 μg of tetracycline, and 5 μg of chloramphenicol.

DNA manipulations and general methods. Extraction of plasmid and chromosomal DNA, restriction endonuclease digestion, agarose gel electrophoresis, transformation of *Escherichia coli* cells, and PCR and bioinformatical analyses were carried out as described previously (61, 63). Enzymes, molecular size markers, and deoxynucleotides were purchased from Roche Diagnostics, Ltd. (Lewes, United Kingdom) and Amersham Pharmacia Biotech, Ltd. (Little Chalfont, United Kingdom).

Transcriptome analysis by DNA macroarray hybridization. Total RNA was extracted from the wild-type strain and *phoR* and *sigB* mutants before, during (T_0), and after entry into the stationary growth phase, which was provoked by phosphate starvation. Cell harvesting, preparation of RNA, synthesis of radioactively labeled cDNA, and hybridization of *B. subtilis* macroarrays (Sigma-Genosys, The Woodlands, Tex.) were performed as described by Eymann and coworkers (22). Each analysis was carried out twice, using two independently isolated RNA preparations and two different array batches. The arrays were exposed to a phosphorescent screen which was subsequently scanned with a Storm 860 PhosphorImager (Molecular Dynamics, Sunnyvale, CA) at a resolution of 50 μm and a 16-bit color depth. For quantification of the hybridization signals and background subtraction, ArrayVision software (version 5.1; Imaging Research, Ontario, Canada) was used. Calculation of normalized intensity values of the individual spots was performed using the overall-spot-normalization function of ArrayVision. To avoid extreme expression ratios for genes close to or below the detection limit, genes with signal intensity values corresponding to less than twice the background were not counted in the analysis. Subsequently, the average of the normalized intensity values of the duplicate spots of each gene was used to calculate the expression level ratios. Data analysis (statistical analysis, visualization, and generation of lists) was performed using the GeneSpring software (version 4.2.12; Silicon Genetics, Redwood City, CA).

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE2667.

Determination of outliers. Genes whose expression differed significantly in response to phosphate starvation were determined by two independent methods. The data were analyzed by a method similar to the previously described iterative outlier analysis (12, 41). In cases where multiple hybridization reactions were carried out using the same RNA sample, the data were averaged and treated as a single value. The ratios of the values obtained before (control) and after phosphate starvation were transformed to $\log_2$. The geometric mean and standard deviation of the entire population were then calculated: any gene which had

a ratio of more than 2.5 standard deviations away from the mean was considered an outlier, i.e., significantly induced or repressed. These outlier genes were subsequently removed from the population, and the means and standard deviations were recalculated. Again, any gene more than 2.5 standard deviations away from the mean was considered an outlier. This process was repeated until few or no outliers were detected. In these experiments generally three to four iterations were needed to identify all outliers in the population. The lists of outliers from the two independently grown and prepared samples were compared, and only those genes that were considered to have changed significantly in both data sets were considered further.

Northern blotting. Total RNA was extracted from *B. subtilis* strains (168, 168-PR, and ML6) with phenol (42). Northern blot analysis was performed according to the manufacturer's instructions, using 5 μg of total RNA per lane (DIG Northern Starter kit; Roche Diagnostics GmbH, Mannheim, Germany). Digoxigenin (DIG)-labeled probes for *yfkN*, *yjdB*, *yurI*, and *vpr* were obtained by in vitro transcription from T7 RNA promoter-containing PCR products of the respective genes by T7 RNA polymerase. Synthesis of the templates by PCR was performed using the following pairs of oligonucleotides: for *vpr* forward (FOR), 5′-CAGCTATTCTCAGGCTTC-3′; for *vpr* reverse (REV), 5′-CTAATACGA CTCACTATAGGG AGAGCTTAATCGTTGGGAC-3′; for *yfkN* FOR, 5′-AGGTGCAGGATATCGTAG-3′; for *yfkN* REV, 5′-CTAATACGACTCACT ATAGGGAGCCTGATATGTGACACCG-3′); for *yjdB* FOR, 5′-CTTTAT CGATTTCTGCGT-3′; for *yjdB* REV, 5′-CTAATACGACTCACTATAGGG AGAACAAAGTAATCGTGGCT-3′; for *yurI* FOR, 5′-CGTATTATCAGC GGACAC-3′; and for *yurI* REV, 5′-CTAATACGACTCACTATAGGGAGC ATTCGAGCAGGACAGA-3′.

Hybridization probes specific for *phoA* and *yheK* were DIG labeled by in vitro transcription from HindIII-linearized plasmid pPhoA and pYheK, respectively, with T7 RNA polymerase. The plasmids contained sequences internal to the *phoA* and *yheK* genes amplified by PCR with primers *phoA* FOR (5′-CGCGAA GCTTGCCTTACATAAGAGCCT-3′) and *phoA* REV (5′-CGCGGATCCTTG ATTTCTTCAGATGTG-3′) for pPhoA and primers *yheK* FOR (5′-CGCGAA GCTTTTCGGGTAGTAGTGTTG-3′) and *yheK* REV (5′-CGCGGATCCTCC GA ATATTAGCTTTTT-3′) for pYheK. The primers included either BamHI or HindIII restriction sites at their 3′ ends (underlined). The PCR products were cleaved with BamHI and HindIII and inserted into pBluescript KSII linearized with the same enzymes.

Enzyme assays. Cultures grown in HPM overnight were diluted 500-fold in fresh LPM or HPM. The cultures were grown at 37°C with agitation at 220 rpm. Samples were collected at hourly intervals for the determination of the optical density at 600 nm (OD_{600}), the APase activity (49), and the β-galactosidase activity (45), as described previously (62). The concentration of P_i in the medium was assayed (61) after removal of the cells by filtration through a filter of 0.45-mm pore size.

Genetic analysis. The whole genome was searched with the sequence pattern $TTHACA_{3-7}TTHACA$ (H = A/C/T) using the Pattern Match function of SubtiList (http://genolist.pasteur.fr/SubtiList/) (47). Targets were reported only if they were located upstream of gene boundaries within 150 bp of the start codon. A deviation of 1 bp was allowed in each repeat of the above sequence.

Final evaluation of the macroarray data included the consideration of putative operon structures derived from the genome sequence as well as previously known operons. Genes induced significantly were analyzed for their transcriptional organization using the SubtiList database (http://genolist.pasteur.fr/SubtiList/) and Artemis software (www.sanger.ac.uk/).

RESULTS

A combination of enzymatic, proteomic, and reporter gene studies have shown that when *B. subtilis* encounters phosphate starvation, genes of the phosphate stimulon are either induced or repressed. To characterize the changes in gene expression with respect to the onset of phosphate starvation, global transcriptome analyses were performed using DNA macroarrays containing 4,107 protein-encoding genes of the *B. subtilis* genome (36). In addition to the wild-type *B. subtilis* strain 168, *sigB*- and *phoR*-null mutants were also analyzed to facilitate the tentative assignment of genes to the SigB and PhoR regulons.

Genome-wide analysis of the response of *B. subtilis* to phosphate starvation. *B. subtilis* strain 168 (wild type) was grown in LPM, which facilitates phosphate starvation-induced entry into stationary phase. The concentration of inorganic phosphate in the culture medium and APase activity of the culture were monitored throughout growth, confirming the exhaustion of P_i from the medium and the concomitant induction of the PhoP regulon (Fig. 1). Samples of culture were removed before, during, and after the onset of phosphate starvation and used for the extraction of total cell RNA.

The quality of the extracted RNA was confirmed by Northern blot analyses using gene-specific riboprobes specific for *phoA*, which belongs to the PhoP regulon (Fig. 1), and *yheK*, which belongs to the *sigB* regulon (data not shown). The RNA was ^{33}P labeled in a copy DNA reaction using reverse transcriptase and gene-specific primers (Sigma Genosys). The resulting labeled target molecules were hybridized to probes spotted in duplicate on DNA macroarrays (Panorama *B. subtilis* gene array; Sigma Genosys). Data from replicate arrays showed good reproducibility with a typical Pearson correlation coefficient of ~0.94. Each experiment was performed twice to generate two biologically independent sets of time course data; one set of representative growth curves are shown in Fig. 1. Genes listed in Table 2 were considered significant if they met the following criteria: (i) included by the outlier analysis (see Material and Methods) between pre- and postphosphate starvation in a single time series; (ii) included from a separate outlier analysis in an independent time series; and (iii) at least twofold above the background: if both time points have values less than twice the background, the gene was excluded from the analysis. This analysis was designed to eliminate false-positive results, although this rigorous procedure may lead to false-negative results.

Analysis of the DNA macroarray data identified 24 genes in the wild type that were induced in response to phosphate starvation, several of which were members of the PhoP and σ^B regulons (Table 2, previously identified PhoP regulon members and remaining wild-type genes). *phoB*, a member of the PhoP regulon that encodes a secreted APase, had the highest induction factor (183-fold). Other well-established members of the PhoP regulon, such as *phoA* (APase) and *phoD* (phosphodiesterase/APase) were also significantly induced (Table 2, previously identified PhoP regulon members). However, some members of the PhoP regulon did not show a significant level of induction. These included the *resABCDE* operon, which has been shown previously (5) to be induced in response to phosphate starvation in a PhoP~P-dependent manner. Another omission is *phoPR*, which we have recently confirmed to be a member of the PhoP regulon but which is only weakly induced (approximately twofold) in response to phosphate starvation (60). It is likely that this level of induction is not sufficient to be included in the stringent outlier analysis.

The PhoP regulon also includes the divergent operons *tagAB* and *tagDEF*, encoding proteins responsible for the synthesis of the anionic polymer, teichoic acid. These genes have been shown previously to be repressed about twofold in response to phosphate starvation (39). Together with the concomitant induction of the *tua* operon, these genes bring about a change in the composition of the cell wall from one predominately consisting of the phosphate-containing teichoic acid to one composed mainly of the non-phosphate-containing teichuronic acid (37). The array analysis was not sensitive enough to detect repression of the *tag* genes, since their expression was not

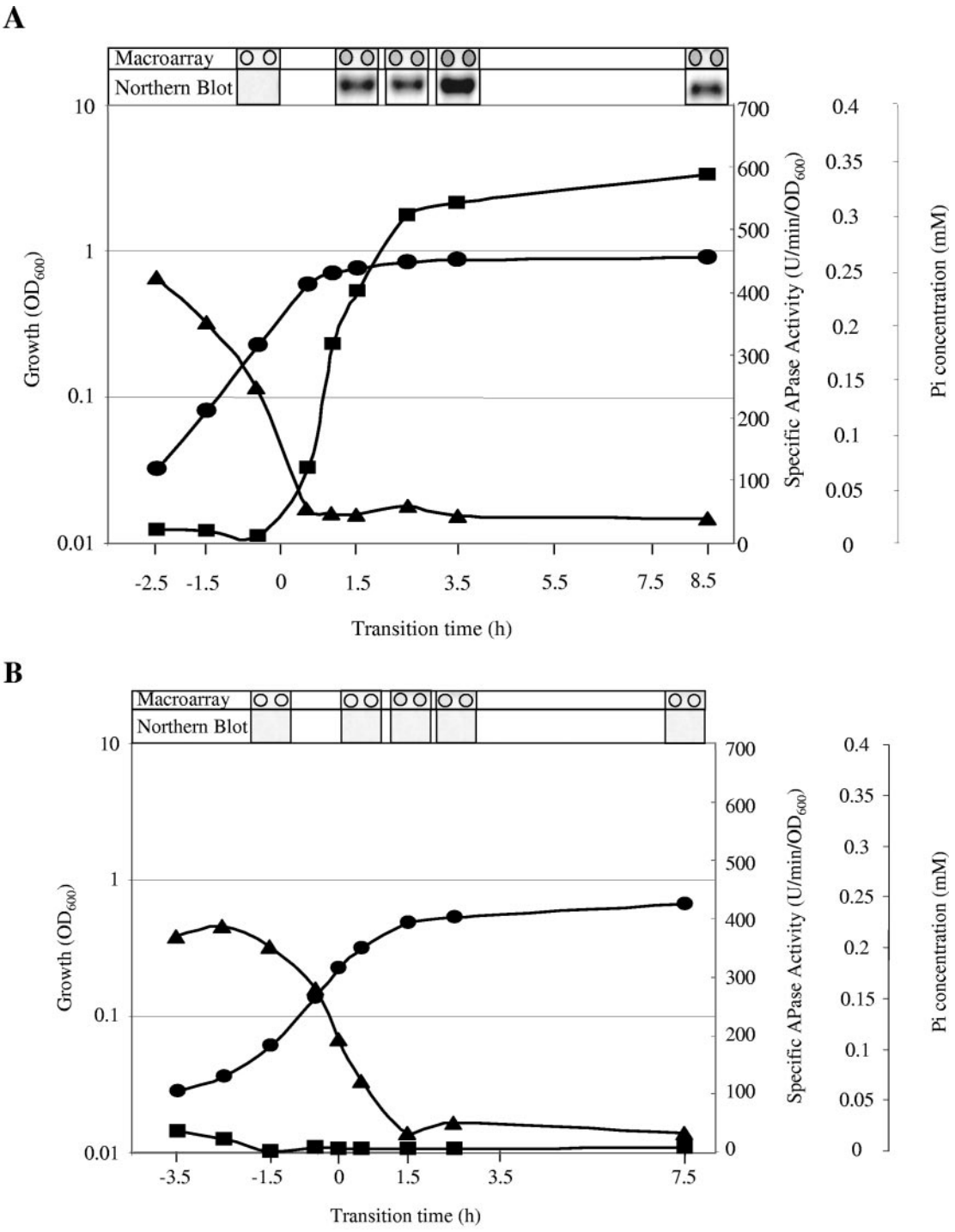

FIG. 1. Representative growth curves and sampling points of various *B. subtilis* strains grown in LPM. OD_{600} values (●), APase production (■), concentration of P_i in the medium (▲), and RNA isolation time points are shown for the wild-type strain 168 (A) and *phoR*-null (B) and *sigB*-null (C) mutant strains. Isolated RNA was used for the Northern blots and macroarray experiments. The hybridization for the Northern blot was carried out with DIG-labeled RNA probes specific for the *phoA* gene. Five micrograms of RNA was applied per lane; after the filters were capillary blotted, they were hybridized to *phoA*-specific riboprobes. For the DNA array analysis, total RNA was isolated and used as the template for reverse transcriptase incorporating radiolabeled [^{33}P]dATP, which was hybridized to a whole-genome macroarray (Sigma-Genosys, The Woodlands, Tex.). Normalization and quantification were performed as described in Materials and Methods.

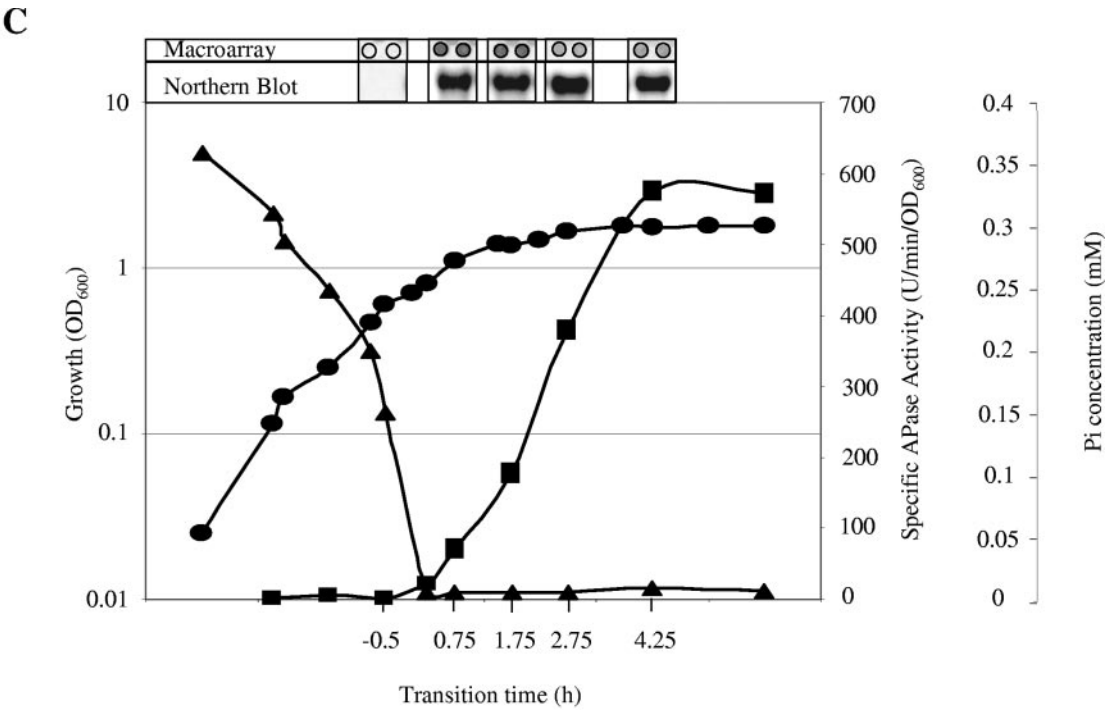

FIG. 1—*Continued.*

significantly down-regulated in response to phosphate starvation (44). However, several, but not all of the genes in the *tua* operon were included in the outlier analysis as being induced in response to phosphate starvation.

The changes in induction ratios of PhoP-regulated genes range from 2 to >100. The differences are often accounted for by the basal levels of expression. For example, the *phoPR* operon exhibits a low change in induction value because of a significant level of expression during phosphate-replete growth. This basal expression ensures that phosphate-replete cells synthesize the signal transduction pathway required to detect and respond to declining phosphate concentrations.

The SigB regulon currently contains approximately 120 genes, identified using DNA array analysis and promoter prediction (58, 64). However, only one σ^B-regulated gene, *ydbD*, was significantly up-regulated in the wild type in response to phosphate starvation. YdbD is a manganese-containing catalase that is thought to prevent oxidative damage by destroying peroxides and related oxidizing compounds (64). We have shown that several members of the SigB regulon are induced in response to phosphate starvation using reporter genes (62) and Northern blotting (Fig. 1) and confirmed that phosphate starvation activates the SigB regulon exclusively via the RsbP phosphatase (data not shown). The failure of the DNA arrays to detect significant levels of induction of other members of the SigB regulon in the wild type may reflect the relatively low sensitivity and incremental response of the RsbP energy-sensing pathway (discussed below).

Eight additional genes, namely, *yfkN*, *bpr*, *glcU* (previously *ycxE*), *cotP* (previously *ydfT*), *lytB*, *rapA*, *yukJ*, and *yxnB*, which are not currently members of the PhoP and SigB regulons were significantly induced in the wild type in response to phosphate starvation (Table 2, remaining wild-type genes). These are discussed in detail later. In addition, *yhaX* and *yhbH* are included by the outlier analysis if a later time point is used (T_5).

Sixty-nine genes are significantly repressed in the wild type in response to phosphate starvation. Many of these genes are involved in nucleic acid and protein synthesis, including 11 *rps* genes, 11 *rpl* and 5 *rpm* genes (encoding small and large ribosome subunit proteins), 4 *rpo* genes (encoding components of RNA polymerase), and 5 genes involved in purine synthesis. The data confirm previous studies showing that genes associated with the information processing machinery are down-regulated in response to growth arrest (27).

Genome-wide analysis of a *phoR*-null mutant. The global response of a *phoR*-null mutant to phosphate starvation (Fig. 1B) was determined to identify PhoPR-dependent genes and to monitor the cell's response to the additional stress imposed by the lack of a phosphate-specific stress response. Twenty-nine genes were significantly induced in the *phoR*-null mutant in response to phosphate starvation according to the outlier analysis (Table 2, genome-wide analysis of a *phoR*-null mutant). As expected, none were current members of the PhoP regulon; however, in contrast to the response of the wild-type strain, 24 of the phosphate starvation-induced genes were members of the SigB regulon (7, 58, 64). These data indicate that, in the absence of a functional PhoP regulon, the extent of the energy stress detected via RsbP is enhanced, leading to the significant induction of about one quarter of the genes in the SigB regulon. This response was reflected in a markedly higher level of expression of the genes in the SigB regulon in the *phoR*-null strain compared to the wild type (Table 3).

TABLE 2. List of *B. subtilis* genes exhibiting significantly increased mRNA levels during phosphate starvation conditions as determined by DNA macroarray analysis

Gene	Potential Pho box sequences upstream of the start codon	Function[a]	Fold induction ratio[b]			Transcriptional organization[a] [reference(s)]	Secreted/nonsecreted protein[c]
			WT	*phoR*	*sigB*		
Previously identified PhoP regulon members							
glpQ		Glycerophosphoryl diester phosphodiesterase	**10.0**	3.9	**38.2**	***glpQ*** (7)	Secreted
phoA	ATGATC**AACA**GCCGCAT**TTAACA**AAG-T_{37} ATG	Alkaline phosphatase A	**36.9**	1.2	**132.1**	***phoA*** (65)	Secreted
phoB	ATCTTT**AAAA**TCGA**TTAATA**CTAG-N_{65} TTG	Alkaline phosphatase III	**183.3**	1.7	**166.2**	***phoB***-*ydhF* (7)	Secreted
phoD	TCAG**TTCACA**CTTC**TTCACA**GTCG-N_{55} ATG	Phosphodiesterase/alkaline phosphatase	**59.0**	2.8	**51.9**	***phoD***-*tatAD-tatCD* (34)	Secreted
phoP		Two-component response regulator	1.7	1.7	**4.8**	***phoP***-*phoR* (56, 60)	Nonsecreted
phoR		Two-component sensor histidine kinase	1.9	0.5	**3.6**	*phoP*-***phoR*** (56, 60)	Nonsecreted
pstA		Phosphate ABC transporter (permease)	**8.8**	1.5	**5.4**	*pstS-pstC*-***pstA***-*pstBA-pstBB* (3, 67)	Nonsecreted
pstBA		Phosphate ABC transporter (ATP-binding protein)	**85.1**	1.8	**48.5**	*pstS-pstC-pstA*-***pstBA***-*pstBB* (3, 67)	Nonsecreted
pstBB		Phosphate ABC transporter (ATP-binding protein)	8.6	1.8	**6.9**	*pstS-pstC-pstA-pstBA*-***pstBB*** (3, 67)	Nonsecreted
pstC		Phosphate ABC transporter (permease)	**31.0**	N/A	**18.8**	*pstS*-***pstC***-*pstA-pstBA-pstBB* (3, 67)	Nonsecreted
pstS	CCTT**TTTACA**TAGAACC**TTTACT**CTAT-N_{45} ATG	Phosphate ABC transporter (binding protein)	**32.1**	1.5	**18.1**	***pstS***-*pstC-pstA-pstBA-pstBB* (3, 67)	Secreted
resA	AAA**TTTCACA**TAACC**TTCAAA**AAGT-N_{64} GTG	Essential protein similar to cytochrome *c* biogenesis protein	2.2	0.9	1.0	***resA***-*resB-resC-resD-resE* (10)	Nonsecreted
resB		Required for cytochrome *c* synthesis	0.8	0.6	0.7	*resA*-***resB***-*resC-resD-resE* (10)	Nonsecreted
resC		Required for cytochrome *c* synthesis	1.1	0.8	N/A	*resA-resB*-***resC***-*resD-resE* (10)	Nonsecreted
resD	ACAG**TTCTCA**AACT**TTCTCA**CGAT-N_{41} ATG	Two-component response regulator	1.1	0.9	0.9	*resA-resB-resC-resD-resE* ***resD***-*resE* (10)	Nonsecreted
resE		Two-component sensor histidine kinase	1.3	0.9	0.6	*resA-resB-resC-resD-resE resD*-***resE*** (10)	Nonsecreted
tagA		Teichoic acid biosynthesis	0.5	0.64	N/A	***tagA***-*tagB* (44, 66)	Nonsecreted
tagB		Involved in polyglycerol phosphate teichoic acid biosynthesis	1.6	0.71	N/A	*tagA*-***tagB*** (44, 66)	Nonsecreted
tagD		Glycerol-3-phosphate cytidylyltransferase	0.4	0.47	1.7	***tagD***-*tagE-tagF* (44, 66)	Nonsecreted
tagE		UDP-glucose:polyglycerol phosphate glucosyltransferase	0.4	N/A	N/A	*tagD*-***tagE***-*tagF* (44, 66)	Nonsecreted
tagF			N/A	N/A	N/A	*tagD-tagE*-***tagF*** (44, 66)	Nonsecreted
tuaA	AGTA**TTAACA**ACAT**TTATCA**GAAA-N_{11} GTG	Biosynthesis of teichuronic acid	**19.8**	0.6	5.5	***tuaA***-*tuaB-tuaC-tuaD-tuaE-tuaF-tuaG-tuaH* (37)	Nonsecreted
tuaB		Biosynthesis of teichuronic acid	3.1	0.6	5.1	*tuaA*-***tuaB***-*tuaC-tuaD-tuaE-tuaF-tuaG-tuaH* (37)	Nonsecreted

tuaC		Biosynthesis of teichuronic acid	**23.1**	1.8	**7.6**	*tuaA-tuaB-**tuaC**-tuaD-tuaE-tuaF-tuaG-tuaH* (37)	Nonsecreted
tuaD		Biosynthesis of teichuronic acid	**14.4**	1.3	**10.0**	*tuaA-tuaB-tuaC-**tuaD**-tuaE-tuaF-tuaG-tuaH* (37)	Nonsecreted
tuaE		Biosynthesis of teichuronic acid	**10.4**	0.8	N/A	*tuaA-tuaB-tuaC-tuaD-**tuaE**-tuaF-tuaG-tuaH* (37)	Nonsecreted
tuaF		Biosynthesis of teichuronic acid	**24.9**	1.2	**16.4**	*tuaA-tuaB-tuaC-tuaD-tuaE-**tuaF**-tuaG-tuaH* (37)	Nonsecreted
tuaG		Biosynthesis of teichuronic acid	**15.3**	2.6	**18.1**	*tuaA-tuaB-tuaC-tuaD-tuaE-tuaF-**tuaG**-tuaH* (37)	Nonsecreted
tuaH		Biosynthesis of teichuronic acid	3.8	0.9	13.7	*tuaA-tuaB-tuaC-tuaD-tuaE-tuaF-tuaG-**tuaH*** (37)	Nonsecreted
tatAd		Similar to hypothetical proteins	2.7	1.5	1.5	*phoD-**tatAD**-tatCD* (34)	Nonsecreted
ydhF		Similar to unknown proteins from *B. subtilis*	**76.9**	0.72	**43.9**	*phoB-**ydhF*** (7)	Secreted
ykoL	ATTC**TTTACA**TTAGA**TTCATA**CCAC-N_{56} ATG	Unknown	3.6	N/A	3.2	*ykzB-**ykoL ykoL*** (60)	Nonsecreted
yttP		Similar to unknown proteins	1.2	0.8	1.3	***yttP***	Nonsecreted
Remaining wild-type genes							
bpr	GTAA**TTCAGA**TTG**TCTACA**GTTA-N_{88} ATG	Bacillopeptidase F	**9.7**	2.4	**14.2**	***bpr***	Secreted
lytB		Modifier protein of major autolysin LytC	**52.8**	N/A	N/A	*lytA-**lytB**-lytC* (38)	Secreted
rapA		Response regulator aspartate phosphatase	**8.7**	6.0	**7.6**	***rapA**-phrA* (57)	Nonsecreted
glcU		Glucose uptake	**5.3**	1.3	N/A	***glcU**-gdh* (52)	Nonsecreted
ydbD		Similar to manganese-containing catalase	**9.2**	3.8	1.1	*ydbE-**ydbD*** (8)	Nonsecreted
cotP		Spore coat protein	**5.4**	0.6	N/A	***cotP*** (68)	Nonsecreted
yfkN	AATA**GTTACA**AAATATT**CTTACA**ATAG-N_3 GTG	Similar to 2′,3′-cyclic-nucleotide 2′-phosphodiesterase	**6.3**	1.1	**17.3**	***yfkN***	Secreted
yukJ		Unknown	**5.5**	2.7	**4.7**	***yuxI-yukJ***	Nonsecreted
yxnB		Unknown	**9.8**	3.5	**18.5**	*yxbB-yxbA-**yxnB**-asnH-yxaM* (83)	Nonsecreted
Genome-wide analysis of a *phoR*-null mutant							
csbD		σ^B-transcribed gene	1.7	**29.7**	N/A	***csbD*** (2)	Nonsecretory protein
csbX		σ^B-transcribed gene	1.1	**6.0**	N/A	***csbX**-bofC (24)*	Nonsecreted
dps		Stress- and starvation-induced gene controlled by σ^B	1.6	**6.0**	N/A	***dps*** (6)	Nonsecreted
gsiB		General stress protein	6.0	**63.5**	1.4	***gsiB*** (35)	Nonsecreted
gspA		General stress protein	2.6	**19.8**	N/A	***gspA*** (5)	Nonsecreted
rsbW		Negative regulation of σ^B-dependent gene expression	2.3	**10.7**	0.7	*rsbR-rsbS-rsbT-rsbU-rsbV-**rsbW**-sigB-rsbX rsbV-**rsbW**-sigB-rsbX* (82)	Nonsecreted

Continued on following page

TABLE 2—*Continued*

Gene	Potential Pho box sequences upstream of the start codon	Function[a]	Fold induction ratio[b]			Transcriptional organization[a] [reference(s)]	Secreted/nonsecreted protein[c]
			WT	*phoR*	*sigB*		
rsbX		Indirect negative regulation of σ^B-dependent gene expression	1.8	**5.8**	N/A	*rsbR-rsbS-rsbT-rsbU-rsbV-rsbW-sigB-**rsbX** rsbV-rsbW-sigB-**rsbX*** (82)	Nonsecreted
skfA	GGG**TTAAAA**TATTCTA**TTTATA**CTAA-N_{124} ATG	Unknown	9.9	**5.1**	**32.8**	***skfA**-skfB-skfC-skfD-skfE-skfF-skfG-skfH* (25)	Nonsecreted
ybyB		Unknown	8.5	**29.0**	N/A	***ybyB***	Nonsecreted
ycsD		Similar to hydroxymyristoyl-(acyl carrier protein) dehydratase	6.0	**8.6**	N/A	***ycsD**-ycsE*	Nonsecreted
ydaD		Similar to alcohol dehydrogenase	2.0	**10.2**	N/A	***ydaD**-ydaE* (59)	Nonsecreted
ydaE		Unknown	3.3	**97.6**	N/A	*ydaD-**ydaE*** (59)	Nonsecreted
ydaP		Unknown	2.8	**11.2**	N/A	*mutT-**ydaP***	Nonsecreted
yfkJ		Similar to protein-tyrosine phosphatase	1.9	**6.9**	1.5	***yfkJ**-yfkI-yfkH*	Nonsecreted
yfkM		Similar to hypothetical proteins	1.2	**29.6**	N/A	*yfkK-yfkL-**yfkM*** (59)	Nonsecreted
yflT		Unknown	8.1	**22.7**	0.5	*yfmA-**yflT***	Nonsecreted
ygxB		Similar to hypothetical proteins from *B. subtilis*	2.2	**16.7**	N/A	***ygxB***	Nonsecreted
yheK		Similar to hypothetical proteins	4.1	**27.3**	N/A	***yheK*** (61)	Nonsecreted
yjbC		Induced by phosphate starvation in a σ^B-dependent and PhoPR-independent manner	1.3	**4.8**	2.1	***yjbC**-spx* (*yjbD*) (7)	Nonsecreted
ykzA	TTA**TTTTCCA**TTTTG**TTCACC**AACT-N_{124} ATG	Similar to general stress protein	10.7	**13.7**	N/A	***ykzA*** (81)	Nonsecreted
yocB		Unknown	2.3	**7.4**	N/A	***yocB***	Nonsecreted
yqgZ		Similar to hypothetical proteins	5.1	**9.1**	N/A	***yqgZ***	Nonsecreted
yrzI		Unknown	1.6	**4.6**	N/A	***yrzI***	Nonsecreted
ysnF	TAT**ATTCACA**CAT**TTTTCA**CCTT-N_{68} ATG	Unknown	1.6	**27.0**	N/A	***ysnF***	Nonsecreted
ytxG	TATG**TATACA**GCCCAG**TACACA**TGTT-N_{55} ATG	Similar to general stress protein	1.1	**21.7**	1.7	***ytxG**-ytxH-ytxJ*	Nonsecreted
ytzE		Similar to transcriptional regulator	4.7	**12.9**	7.7	***ytzE***	Nonsecreted
yvgO		Unknown	1.5	**5.0**	0.6	***yvgO***	Secreted
yvyD	AAAG**TTCACT**GAA**TTTTCA**CAAA-N_{79} ATG	Similar to ribosomal protein S30AE family	1.4	**22.2**	1.1	***yvyD*** (17)	Nonsecreted
ywzA		Similar to hypothetical proteins from *B. subtilis*	4.9	**36.6**	N/A	***ywzA***	Nonsecreted
Genome-wide analysis of a *sigB*-null mutant							
asnH		Asparagine synthetase	N/A	N/A	**259.4**	*yxbB-yxbA-**yxnB**-asnH-yxaM* (83)	Nonsecreted
bpr	GTAA**TTCAGA**TTG**TCTACA**GTTA-N_{88} ATG	Bacillopeptidase F	**9.7**	2.4	**14.4**	***Bpr***	Secreted
metC		Methionine biosynthesis	1.8	0.6	**13.1**	*metI-**metC*** (9)	Nonsecreted
pel		Pectate lyase	4.0	2.2	**4.0**	***pel*** (53)	Secreted

pksE		Involved in polyketide synthesis	2.3	0.6	**3.4**	*pksB-pksC-pksD-**pksE**-acpK-pksF*	Nonsecreted
ppsA		Plipastatin synthetase	N/A	1.6	**4.7**	***ppsA**-ppsB-ppsC-ppsD-ppsE* (77)	Nonsecreted
rapA		Response regulator aspartate phosphatase	**8.7**	6.2	**7.6**	***rapA**-phrA* (57)	Nonsecreted
skfA	GGG**TTAAAA**TATTCTA**TTTATA**CTAA-N_{124}ATG	Unknown	9.9	**5.1**	**32.8**	***skfA**-skfB-skfC-skfD-skfE-skfF-skfG-skfH*	Nonsecreted
spoIIAA		Stage II sporulation	N/A	N/A	**5.7**	***spoIIAA**-spoIIAB-sigF* (70)	Nonsecreted
spoIIB		Stage II sporulation	4.3	N/A	**9.9**	***spoIIB***	Nonsecreted
srfAA	TTAG**TTCATA**AGAA**TTAAAA**GCTG-N_{70} ATG	Surfactin production and competence	2.2	1.0	**3.3**	***srfAA**-srfAB-srfAC-srfAD*	Nonsecreted
thrC		Threonine biosynthesis	0.7	0.6	**7.7**	*hom-**thrC**-thrB* (55)	Nonsecreted
yfkN	ATA**GTTACA**AAATATT**CTTACA**ATAG-N_{38}GTG	Similar to 2′,3′-cyclic-nucleotide 2′-phosphodiesterase	**6.3**	1.1	**17.4**	***yfkN***	Secreted
yhcR		Similar to 5′-nucleotidase	1.7	1.5	**4.3**	***yhcR**-yhcS*	Nonsecreted
yhzC	CCGG**TTTACG**GCAT**TTTGCA**GGAT-N_{105} ATG	Unknown	2.0	1.2	**6.5**	***yhzC***	Nonsecreted
yjdB	ATTA**TTAACA**TTTA**TTTACA**AGGA-N_{63} ATG	Unknown	2.6	0.8	**5.1**	***yjdB***	Secreted
ykzF		Unknown	1.5	2.9	**3.9**	*ykuJ-ykuK-**ykzF**-ykuL-ykuM*	Nonsecreted
yoeB	TATT**GTAACA**TTT**GTAACA**TAAG-N_{29} ATG	Unknown	0.7	N/A	**4.0**	***yoeB***	Nonsecreted
ytzE		Similar to transcriptional regulator (DeoR family)	4.7	**12.9**	**7.6**	***ytzE***	Nonsecreted
yuiB		Similar to hypothetical proteins from *B. subtilis*	2.7	2.3	**11.6**	*yuiA-**yuiB***	Nonsecreted
yukJ		Unknown	**5.5**	2.7	**4.7**	*yuxI-**yukJ***	Nonsecreted
yuxI		Unknown	N/A	1.8	**8.4**	***yuxI**-yukJ*	Nonsecreted
ywkC		Unknown	3.0	1.7	**4.4**	*ywkD-**ywkC***	Nonsecreted
yxbB		Similar to hypothetical proteins	4.9	6.2	**26.9**	***yxbB**-yxbA-yxnB-asnH-yxaM* (83)	Nonsecreted
yxnB		Unknown	**9.8**	3.5	**18.2**	*yxbB-yxbA-**yxnB**-asnH-yxaM* (83)	Nonsecreted
yycO		Unknown	3.6	N/A	**15.0**	***yycO**-yycP* (20)	Secreted

[a] Function and putative transcriptional organization are according to the associated reference. When no reference has been detected in the literature, the organization is according to the SubtiList database (http://genolist.pasteur.fr/SubtiList) (48). Gene names in parentheses are alternative names.

[b] Calculated fold induction ratios (between pre- and postphosphate starvation) for genes averaged from two biologically independent experiments. Genes that fulfill the criteria for the outlier analysis are given in bold type and underlined. Genes that did not fulfill the outlier analysis criteria are shown as N/A. WT, wild type.

[c] Whether the protein is predicted to be secreted or nonsecreted according to Tjalsma and coworkers (76).

Most of the members of the SigB regulon that are induced in response to phosphate starvation are of unknown or experimentally unconfirmed function. *dps* (6) encodes a homologue of the Dps/PexB protein of *E. coli* which protects the chromosome from acid, heat, and oxidative stress (43); a *dps*-null mutant exhibits severely reduced resistance to oxidative stress (6). *yjbC* is involved in salt tolerance, and a *yjbC*-null mutant is almost as sensitive to salt as a *sigB*-null mutant (58). Other SigB-induced genes appear to have a detoxification role; *ykzA* (80) encodes a protein that is similar to Ohr, an organic hydrogen peroxide resistance protein of *Xanthomonas campestris* (46), while *yqgZ* encodes a putative arsenate reductase (64). *gsiB*, encoding a general stress protein of unknown function, showed the highest level of induction (>200-fold) in response to phosphate starvation. The exceptional stability (half-life of ~20 min) of *gsiB* mRNA has been attributed to the presence of a strong ribosome binding site (35).

Four additional genes were induced significantly in the *phoR*-null mutant in response to phosphate starvation but are not currently members of the PhoP and SigB regulons: *skfA* (previously *ybcO*), *yrzI*, *ytzE*, and *ycsD*.

Genome-wide analysis of a *sigB*-null mutant. The global response of a *sigB*-null mutant to phosphate starvation (Fig. 1C) was determined to facilitate the identification of SigB-dependent genes and to monitor the cell's response to the additional stress imposed by the lack of the general stress response. Forty-two genes were significantly induced in the *sigB*-null mutant in response to phosphate starvation (Table 2, genome-wide analysis of a *sigB*-null mutant). As expected, none were members of the SigB regulon but 16 of the genes were members of the PhoP regulon (Table 2, previously identified PhoP regulon members). In confirmation of the findings of Prágai and Harwood (62), members of the PhoP regulon were generally upregulated in the *sigB*-null mutant compared to the wild type (Fig. 2A). A number of genes that are induced in the wild type in response to phosphate starvation are also induced in the *sigB*-null mutant (*yfkN*, *bpr*, *yjdB*, *yukJ*, *ynxB*, and *rapA*). These are good candidates for inclusion in the Pho regulon and are discussed in detail below.

A number of genes that were induced in the *sigB* mutant during the transition to phosphate limitation were not induced in the wild type. Four such genes are involved in the synthesis of antimicrobial compounds: *srfAA* and *ppsA* are involved in the synthesis of the bioactive lipopeptides surfactin (16) and plipastatin (78), respectively; *skfA* encodes an antimicrobial peptide (25); and *pksE* is involved in the synthesis of an antimicrobial polyketide. *bpr*, *pel*, and *yhcR*, encoding bacillopeptidase F, pectate lyase, and a putative 5′ nucleotidase, respectively, are macromolecular hydrolases. *asnH*, *metC*, and *thrC* are involved in the biosynthesis of amino acids. *rapA*, *spoIIAA*, and *spoIIB* are involved in sporulation or sporulation-associated events. *yjdB*, a gene of unknown function, was previously identified by Ogura and coworkers (54) as being a potential member of the PhoP regulon. Our data tend to confirm this assignment, since the *yjdB* promoter region contains putative Pho box-like sequences and this gene is induced in the *sigB*-null mutant but not in the *phoR*-null mutant. The remaining 11 genes (Table 2) are of unknown function.

Clustering of genes based on expression patterns. The analysis of the macroarray data was extended using K-means clustering (33). K-means clustering partitions the data into a predetermined number of clusters on the basis of the similarity of their expression profiles. The analysis involves the iterative reallocation of the cluster members to minimize intracluster scattering. Using the normalized data, genes with little or no expression were excluded by using the "filter on expression" script in the GeneSpring software package, arbitrarily setting the minimum expression to 1 for at least three out of the six time points. Genes not conforming to this requirement were excluded, leaving 2,162 genes in the analyzed data set. We selected the number of clusters to be 15 with "standard correlation" as the similarity measure. The analysis was performed on the *sigB*-null data set, since members of the PhoP regulon generally show enhanced levels of expression in this background (see Fig. S1 in the supplemental material). Cluster 13, containing 139 genes, included 20 genes currently identified as members of the PhoP regulon (see Fig. S2 in the supplemental material).

Comparison of the cluster analyses for the wild-type and *phoR*-null mutant data sets (see Fig. S3 in the supplemental material) show, as expected, little or no expression of the PhoP regulon genes in the *phoR*-null mutants. This method also identified *vpr*, *yurI*, *yfkN*, and *bpr* as potential additional members of the PhoP regulon.

Analysis of promoter regions for PhoP-like consensus sequences. To identify potential PhoP binding sequences of Pho member candidates, the *B. subtilis* genome was interrogated using the Pattern Match function of SubtiList (http://genolist.pasteur.fr/SubtiList/) (47). The PhoP consensus $TTHACA_{3-7}$ TTHACA (H = A/C/T) (11) can be repeated as few as twice, as in the case of the *resA* promoter that additionally requires ResD for induction, or as many as eight times in the case of the *tuaA* promoter. In all cases there is always an even numbers of repeats. Only targets which were upstream of gene boundaries and within 150 bp of the start codon were reported, and in addition, genes were reported only if they were transcribed monocistronically or were the first gene in a polycistronic operon. A deviation of 1 bp from the consensus was allowed in each part of the search sequence. The pattern match gave good results with known PhoP promoters, correctly predicting 8 out of 11 PhoP-regulated promoters (Table 2). PhoP-repressed promoters, such as *tagAB* and *tagDEF*, were not considered, as their consensus sequence usually comprise two repeats on the noncoding strand. Of the promoters not identified by this method, *phoP* has a very weak consensus sequence (60), and *yttP* and *glpQ* would be included if a 2-bp deviation from the consensus sequence were used.

TABLE 3. Total expression of genes in the PhoP and SigB regulons

Genes	Total expression of genes in strain[a]:		
	Wild-type	*sigB*-null mutant	*phoR*-null mutant
PhoP regulon members	3.9	4.0	1.1
SigB regulon members	3.4	1.9	9.4

[a] Total expression of genes in the PhoP (28) and SigB regulons (58). The data are shown as a percentage of the total normalized expression levels for that experiment. Time points were chosen at or close to $T_{1.5}$. Data for each strain are an average of two biologically independent experiments.

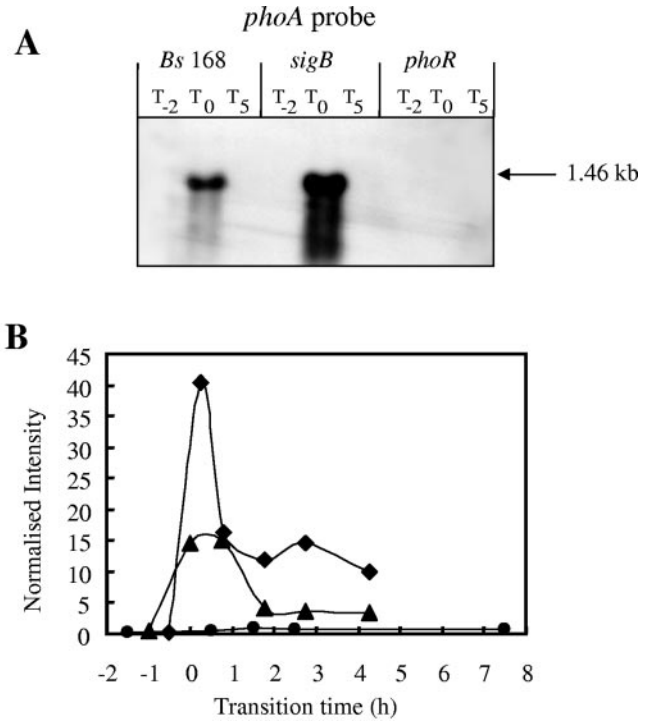

FIG. 2. Northern blot analyses and transcriptional profiles for the *phoA* gene. (A) RNA was isolated from wild-type *B. subtilis* strain 168 (*Bs* 168) and *sigB*-null and *phoR*-null mutants. Bacteria were grown in LPM, and samples were taken 2 h before (T_{-2}) and 0 h (T_0) and 5 h (T_5) after entry into the stationary growth phase, which was provoked by phosphate starvation. Five micrograms of RNA was applied per lane; after the filters were capillary blotted, they were hybridized to a *phoA*-specific riboprobe. Transcript size was determined by comparison with DIG-labeled RNA size markers (Roche Diagnostics, Mannheim, Germany). (B) RNA was isolated from wild-type *B. subtilis* (168) (◆) and *sigB*-null (▲) and *phoR*-null (●) mutants grown in LPM. Samples were taken before, during, and after entry into the stationary growth phase, which was provoked by phosphate starvation. Total RNA was isolated and used as the template for reverse transcriptase incorporating radiolabeled [^{33}P]dATP, which was hybridized to a whole-genome macroarray (Sigma-Genosys, The Woodlands, Tex.). Normalization and quantification were performed as described in Materials and Methods.

Analyses of new potential members of the PhoP regulon. The outlier analysis identified 29 genes that were induced significantly in the wild type or *sigB*-null mutant in response to phosphate starvation but crucially not in the *phoR*-null mutant. Five of these genes, namely, *bpr*, *rapA*, *yfkN*, *yukJ*, and *yxnB*, were induced in the wild type and *sigB*-null mutant and had not previously been identified as members of the PhoP regulon; they were therefore considered to be good candidates for inclusion in the regulon. Additionally, *yjdB* was chosen for further analysis, as Ogura and coworkers (54) identified it as a potential member of the PhoP regulon member in experiments in which *phoP* was overexpressed in the absence of the *phoPR* operon. Our analysis showed that *yjdB* was induced in the *sigB*-null mutant and had Pho box-like repeats in its promoter region. *rapA* was not analyzed further, since its expression in response to growth arrest has been the subject for previous studies (73).

Analysis of the transcriptional profiles by K-means clustering identified four putative new members of the PhoP regulon, namely, *bpr*, *yfkN*, *vpr*, and *yurI*.

We selectively analyzed expression of seven of these putative new members of the PhoP regulon using strains carrying transcriptional reporter gene fusions generated during the *Bacillus* Functional Analyses (BFA) project. In BFA mutants, the target gene was inactivated using the pMUTIN integration vector that simultaneously fuses its transcription to a *lacZ* reporter gene (79). Mutants were grown in LPM and HPM, and the production of β-galactosidase and APase was monitored throughout growth. β-Galactosidase assays were performed on a minimum of three independent cultures. Additionally, selective Northern blot analyses were performed on RNA extracted from the wild type and *phoR*- and *sigB*-null mutants during exponential phase and phosphate starvation-induced stationary phase. The hybridization of a riboprobe against a well-established member of the PhoP regulon, namely, *phoA*, was used as a control. These analyses confirmed that little or no *phoA* mRNA transcripts were detected at T_{-2}, confirming the lack of induction of this gene prior to phosphate starvation. As expected, a single prominent band of ~1.45 kb was detected at T_0 (Fig. 2A), disappearing at T_5, confirming the transient expression of the PhoP regulon (28) in response to phosphate starvation. A more intense band was seen at T_0 in the *sigB* mutant than in the wild type, while, as expected, no transcript was seen in the *phoR* mutant. The quantitative transcription profiles for *phoA* in the wild type and *phoR*- and *sigB*-null mutants obtained from the DNA macroarrays are shown in Fig. 2B.

yfkN, encoding an extracellular 2′,3′ cyclic nucleotide 2′-phosphodiesterase (13), is a good candidate for inclusion in the PhoP regulon, since it is induced in response to phosphate starvation in a PhoR-dependent manner and has a Pho box-like sequence (Table 2) in the vicinity of its putative σ^A promoter 38 bases upstream of the GTG start codon. Growth of a BFA *yfkN* mutant (YFKNd-PR) in LPM showed the concomitant induction of β-galactosidase and APase activities at T_0 (Fig. 3). The introduction of a *sigB*-null mutation into this strain had very little influence on the induction of *yfkN* (~100 nmol *o*-nitrophenyl [ONP]/min/OD unit at T_0), while the introduction of the *phoP*-null mutation reduced it 100-fold (~1 nmol ONP/min/OD unit at T_0). No β-galactosidase activity was detected in HPM (data not shown), indicating that transcription of *yfkN* was induced only in response to phosphate starvation. Northern blot analyses confirmed the induction of the *yfkN* transcript in the wild type and *sigB* mutant and the absence of this transcript in phosphate-replete conditions (P_i >0.1 mM) (Fig. 4A). The size of the primary transcript (4.45 kb) was consistent with the predicted length of *yfkN*, indicating that this gene comprises a monocistronic operon. Discrete smaller bands are likely to be processed products (3). The Northern blot analyses confirmed the data obtained in the reporter gene experiments and transcriptional profiling (Fig. 4B), namely, that *yfkN* is induced at T_0 in the wild type and *sigB* mutant but not in the *phoR* mutant. *yfkN* showed fourfold hyperinduction at T_0 in the *sigB*-null mutant using DNA macroarray, and twofold hyperinduction was detected using β-galactosidase reporter fusion (Fig. 3 and 4) at T_1. On the basis of this evidence, we have putatively assigned *yfkN* as a new member of the PhoP regulon.

bpr, encoding bacillopeptidase (71), was also a good candidate for inclusion in the PhoP regulon, since it is induced in response to phosphate starvation in a PhoR-dependent manner and has a Pho box-like sequence (Table 2) in the vicinity of a SigA promoter and 88 bases upstream of its ATG start codon. Northern blot analyses, using a *bpr*-specific riboprobe, confirmed the induction of the *bpr* transcript at T_0, but this induction was seen in all strains. Since its expression is both PhoR and SigB independent, we conclude that *bpr* is induced in response to growth arrest by an as-yet identified regulator (data not shown).

yjdB was previously identified by Ogura and coworkers (54) as being a potential member of the PhoP regulon. The data shown here support its inclusion in the PhoP regulon, as it is induced in response to phosphate starvation in a PhoR-dependent manner, and the promoter region contains Pho box-like sequences (Table 2) 63 bases upstream of the ATG start codon. Northern blot analyses using a riboprobe specific to the monocistronic *yjdB* gene showed induction of a single strong band of ~0.4 kb at T_0 which was much reduced at a later time point (T_5). The band intensity was highest in the *sigB* mutant and lowest in the *phoR* mutant (Fig. 5A), and this was confirmed by the DNA array data (Table 2). The strength of the transcriptional profiles of *yjdB* in the wild type and *sigB* mutant were similar, whereas little induction was seen with the *phoR* mutant (Fig. 5B). On the basis of this evidence and that of supporting reporter gene data (data not shown), we have putatively assigned *yjdB* as a new member of the PhoP regulon.

yurI was identified as a candidate for inclusion in the PhoP regulon by K-means clustering. *yurI* encodes an RNase which shows 78% amino acid identity to the RNase Bsn. Bsn has no apparent sequence specificity and can hydrolyze RNA endonucleolytically to yield 5′-phosphorylated oligonucleotides (50). Growth of a BFA *yurI* mutant, BFA1234, in LPM showed the concomitant induction of β-galactosidase and APase at T_0 (Fig. 6). The introduction of a *sigB*-null mutation into this strain had little influence on the induction of *yurI* (~300 nmol ONP/min/OD unit at T_0), while the introduction of the *phoR*-null mutation reduced it 300-fold (~1 nmol ONP/min/OD unit at T_0). No transcription activity was detected in HPM (data not shown), confirming the phosphate starvation-specific induction of *yurI*. Northern blot analyses confirmed the induction of the *yurI* transcript in the wild type and *sigB*-null mutant, with a much lower level of transcript present in phosphate-replete conditions (P_i concentration of >0.1 mM) (Fig. 7A). The size of the primary transcript (~0.9 kb) was consistent with the predicted length of *yurI*. A second band of ~0.5 kb that was observed at T_5 in the wild type and the *sigB*-null mutant and to a lesser extent in the *phoR*-null mutant may indicate posttranscriptional processing or multiple promoters. The Northern blot analyses confirmed the data obtained in the reporter gene experiments and transcriptional profiling (Fig. 6 and 7), namely, that *yurI* was induced at T_0 in the wild type and *sigB* mutant but not in the *phoR* mutant. On the basis of this evidence, we have putatively assigned *yurI* as a new member of the PhoP regulon. In order to determine whether the product of *yurI* was responsible for the nonspecific degradation of RNA in the environment as a source of P_i, the wild type and a *yurI*-null mutant were grown on LPM agar supplemented with RNA. Plate tests confirmed the absence of a secreted RNase in the *yurI* mutant (data not shown).

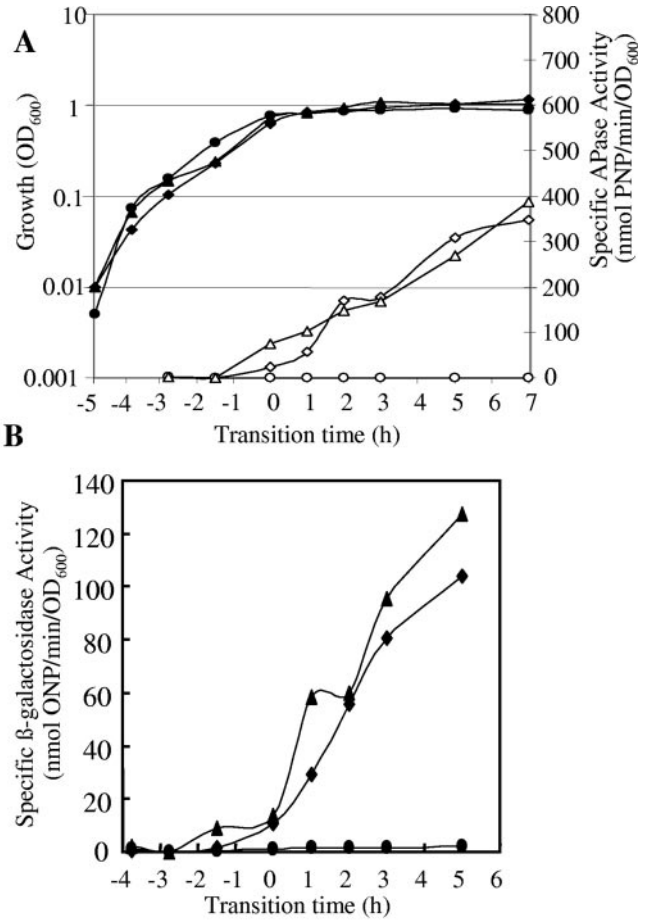

FIG. 3. Growth and reporter activity of *B. subtilis yfkN-lacZ* fusion mutants grown in LPM. (A) OD_{600} values of *lacZ* fusion *yfkN-lacZ* (◆), Δ*sigB yfkN-lacZ* (▲), and Δ*phoR yfkN-lacZ* (●) mutants are shown with closed symbols. APase activities of *yfkN-lacZ* (◇), Δ*sigB yfkN-lacZ* (△), and Δ*phoR yfkN-lacZ* (○) strains are shown with open symbols. PNP, *p*-nitrophenyl. (B) Specific β-galactosidase activities of *yfkN-lacZ* (◆), Δ*sigB yfkN-lacZ* (▲), and Δ*phoR yfkN-lacZ* (●) strains are shown with closed symbols.

vpr was identified as a candidate for inclusion in the PhoP regulon by K-means clustering. Since no pMUTIN constructs were available, we used Northern blot analysis and *vpr*-specific riboprobes to analyze the transcription of *vpr*. This confirmed the transient induction of the *vpr* transcript in response to phosphate starvation in the wild type and *sigB*-null mutant but not in the *phoR* mutant (Fig. 8). The size of the primary transcript (~2.5 kb) was consistent with the predicted length of *vpr*, indicating that this gene is transcribed as part of a monocistronic operon. The DNA macroarray data showed that the transcription levels of *vpr* were induced threefold in the wild type and eightfold in the *sigB* mutant at T_0 compared to the transcription levels in the *phoR* mutant. On the basis of this evidence and the fact that the promoter has PhoP box-like consensus sequences, we have putatively assigned *vpr* as a new member of the PhoP regulon.

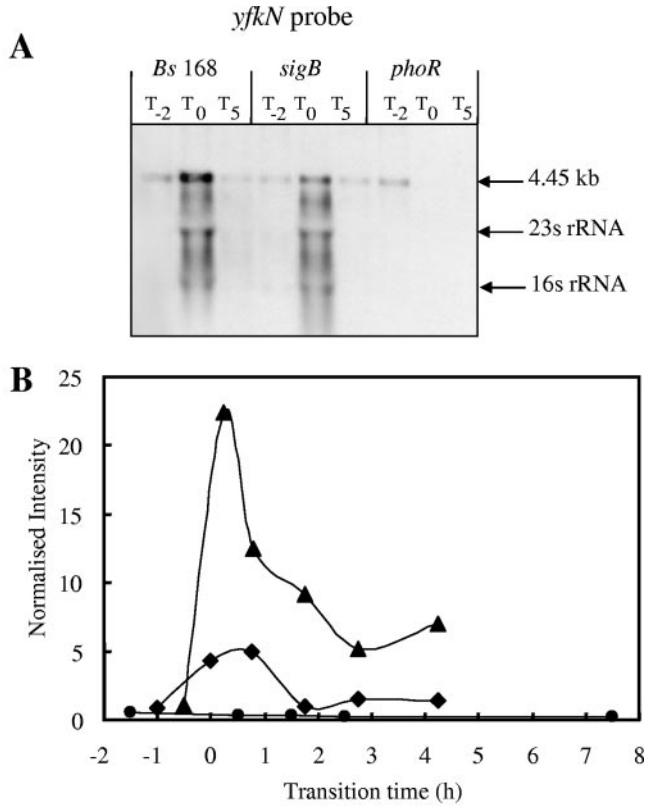

FIG. 4. Northern blot analyses and transcriptional profiles for the *yfkN* gene. (A) RNA was isolated from wild-type *B. subtilis* strain 168 (*Bs* 168) and *sigB*- and *phoR*-null mutants. Bacteria were grown in LPM, and samples were taken 2 h before (T_{-2}) and 0 h (T_0) and 5 h (T_5) after entry into the stationary growth phase, which was provoked by phosphate starvation. Five micrograms of RNA was applied per lane; after the filters were capillary blotted, they were hybridized to *yfkN*-specific riboprobes. Transcript size was determined by comparison with DIG-labeled RNA size markers (Roche Diagnostics, Mannheim, Germany). The arrows labeled 16S rRNA and 23S rRNA indicate the locations of these rRNA species that are known to trap smaller RNA species (1). (B) RNA was isolated from wild-type *B. subtilis* (168) (◆) and *sigB*-null (▲), and *phoR*-null (●) mutants grown in LPM. Samples were taken before, during, and after entry into the stationary growth phase, which was provoked by phosphate starvation. Total RNA was isolated and used as the template for reverse transcriptase incorporating radiolabeled [^{33}P]dATP, which was hybridized to a whole-genome macroarray (Sigma-Genosys, The Woodlands, Tex). Normalization and quantification were performed as described in Materials and Methods.

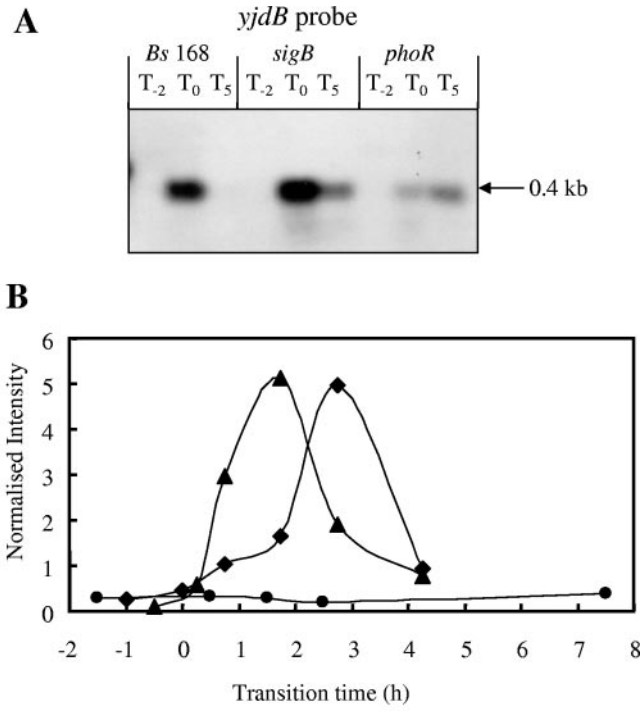

FIG. 5. Northern blot analyses and transcriptional profiles for the *yjdB* gene. (A) RNA was isolated from wild-type *B. subtilis* strain 168 (*Bs* 168) and *sigB*-null and *phoR*-null mutants. Bacteria were grown in LPM, and samples were taken 2 h before (T_{-2}) and 0 h (T_0) and 5 h (T_5) after entry into the stationary growth phase, which was provoked by phosphate starvation. Five micrograms of RNA was applied per lane; after the filters were capillary blotted, they were hybridized to *yjdB*-specific riboprobes. Transcript size was determined by comparison with DIG-labeled RNA size markers (Roche Diagnostics, Mannheim, Germany). (B) RNA was isolated from wild-type *B. subtilis* (168) (◆) and *sigB*-null (▲) and *phoR*-null (●) mutants grown in LPM. Samples were taken before, during, and after entry into the stationary growth phase, which was provoked by phosphate starvation. Total RNA was isolated and used as the template for reverse transcriptase incorporating radiolabeled [^{33}P]dATP, which was hybridized to a whole-genome macroarray (Sigma-Genosys, The Woodlands, Tex.). Normalization and quantification were performed as described in Materials and Methods.

yxnB and *yukJ* were identified as potential PhoR- and SigB-independent members of the Pho stimulon, and Northern blot analyses confirmed their induction at T_0 in the wild type and *sigB*- and *phoR*-null mutants (data not shown). To determine whether *yxnB* was induced in response to phosphate starvation, strains containing a reporter gene fusion to *yxnB* were grown in LPM and HPM. The data indicated that *yxnB* was induced at T_0 in both high- and low-phosphate media, indicating that this gene is induced in response to growth arrest, independently of phosphate concentration (data not shown).

DISCUSSION

Soil-dwelling bacteria, such as *B. subtilis*, have evolved physiological responses to overcome environmental stress. One of the most commonly encountered stresses in soil is phosphate starvation (28). *B. subtilis* responds to phosphate starvation via the transient induction of a variety of genes with diverse functions. In this study we have characterized the phosphate starvation stimulon of *B. subtilis* at the whole-genome level using DNA macroarrays. Although the main aim was to obtain a global perspective of the *B. subtilis* cell to the phosphate starvation response, comparison of the data with existing information obtained using complementary technologies has provided information on the relative advantages and disadvantages of DNA array data. To this end, we have characterized the phosphate starvation response in wild-type *B. subtilis* and *sigB*- and *phoR*-null mutants in which the general and specific responses to phosphate starvation are nonfunctional, respectively. The data shown here and previously (7, 22, 60) indicate that the phosphate starvation stimulon consists, at least, of the phosphate starvation-specific PhoP regulon and the σ^B general stress regulons. The SigB-dependent stress proteins provide a nonspecific, prospective stress resistance (26), while the PhoP

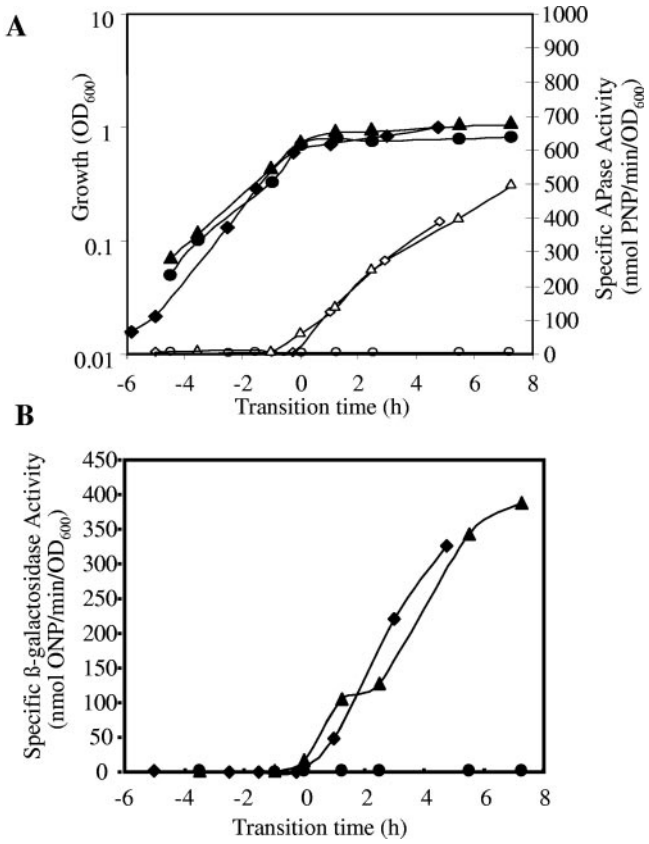

FIG. 6. Growth and reporter activity of *B. subtilis yurI-lacZ* fusion mutants grown in LPM. (A) OD_{600} values of *lacZ* fusion mutant *yurI-lacZ* (◆), Δ*sigB yurL-lacZ* (▲), and Δ*phoR yurL-lacZ* (●) strains are shown with closed symbols. APase activities of *yurI-lacZ* (◇), Δ*sigB yurL-lacZ* (△), and Δ*phoR yurL-lacZ* (○) strains are shown with open symbols. PNP, *p*-nitrophenyl. (B) Specific β-galactosidase activities of *yurI-lacZ* (◆), Δ*sigB yurL-lacZ* (▲), and Δ*phoR yurL-lacZ* (●) strains are shown.

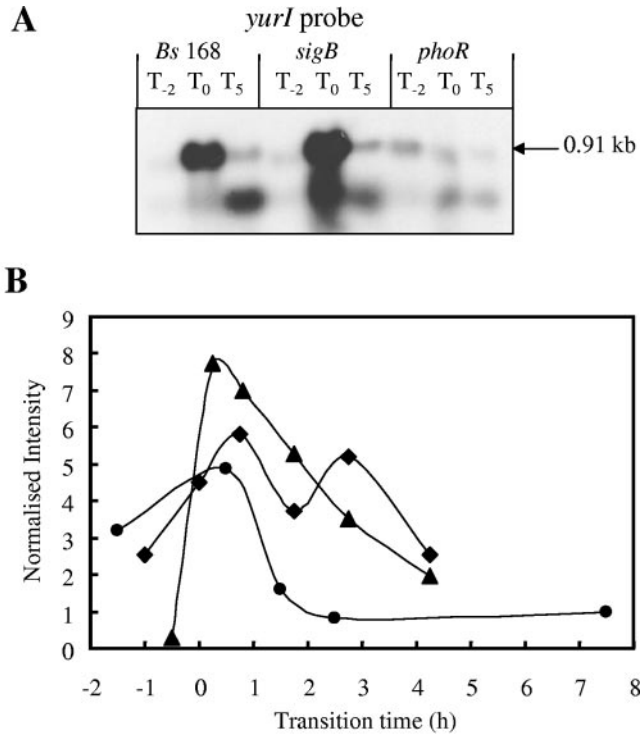

FIG. 7. Northern blot analyses and transcriptional profiles for the *yurI* gene. (A) RNA was isolated from wild-type *B. subtilis* strain 168 (*Bs* 168) and *sigB*- and *phoR*-null mutants. Bacteria were grown in LPM, and samples were taken 2 h before (T_{-2}) and 0 h (T_0) and 5 h (T_5) after entry into the stationary growth phase, which was provoked by phosphate starvation. Five micrograms of RNA was applied per lane; after the filters were capillary blotted, they were hybridized to *yurI*-specific riboprobes. Transcript size was determined by comparison with DIG-labeled RNA size markers (Roche Diagnostics, Mannheim, Germany). (B) RNA was isolated from wild-type *B. subtilis* (168) (◆) and *sigB*-null (▲) and *phoR*-null (●) mutants grown in LPM. Samples were taken before, during, and after entry into the stationary growth phase, which was provoked by phosphate starvation. Total RNA was isolated and used as the template for reverse transcriptase incorporating radiolabeled [^{33}P]dATP, which was hybridized to a whole-genome macroarray (Sigma-Genosys, The Woodlands, Tex.). Normalization and quantification were performed as described in Materials and Methods.

regulon provides proteins with functions specific for making alternative sources of phosphate available.

The response of *B. subtilis* to phosphate starvation has been extensively studied during the last 15 years. Prominent among the cell's response is the induction of APases and a high-affinity phosphate-specific transporter which cooperatively recover phosphate from organic sources and transport it into the cell. Important marker genes involved, namely, *glpQ*, *phoB*, *phoD*, and *pstS* (7), were all identified as significantly induced, confirming the validity of the methodology. However, it should be borne in mind that different transcriptomic approaches (e.g., DNA arrays, Northern blotting, and reporter gene technology) do not detect and measure the same elements: although they usually show the same trends, factors such as mRNA stability and processing can influence their congruence.

The outlier analysis identified 24 genes in the wild type which were significantly induced in response to phosphate starvation, 15 of which were previously known members of the PhoP regulon. In contrast, only one of these genes (*ydbD*) was a previously known member of the SigB regulon (58). This method provided a stringent method of analysis which reduced the identification of false-positive results, but probably at the expense of excluding genes that are only weakly induced in response to phosphate starvation.

Hulett and coworkers showed that the *resABCDE* operon was induced under phosphate starvation conditions (10) and that the ResDE two-component system modulates this activity, as mutations in these genes lead to decreased PhoP regulon expression (74). However, our experiments provided no evidence for the induction of the *res* operon in response to phosphate starvation. Discrepancies between our results and those of Hulett and colleagues may be due to differences between in the growth media or strain genotypes.

The majority of PhoP-regulated genes are expressed at T_0, the most notable exceptions being *yhaX* and *yhbH*, which are induced at T_3 (62). The timing of their expression is compatible with the observation that they are expressed from SigE-dependent promoters. However, we have shown previously that PhoP does not bind in the region to the *yhaX* promoter (60), indi-

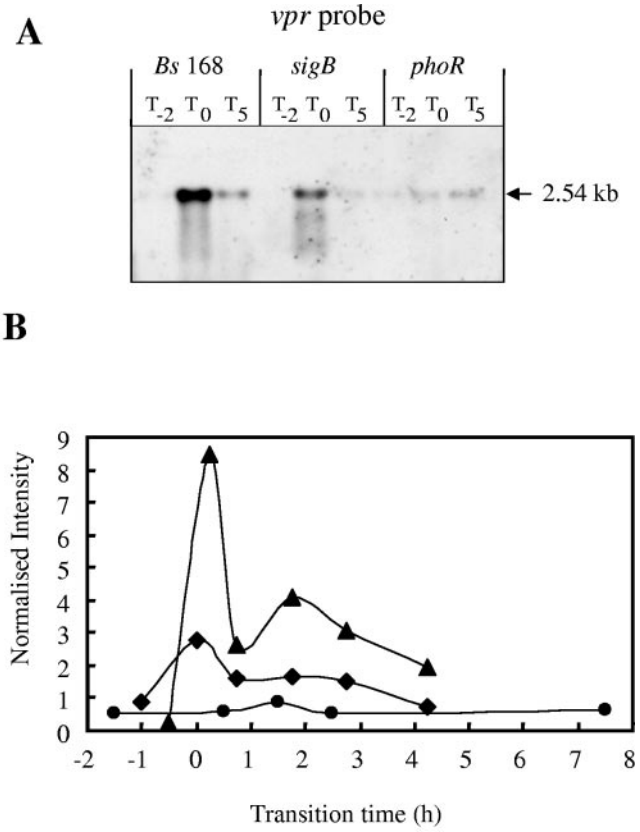

FIG. 8. Northern blot analyses and transcriptional profiles for the *vpr* gene. (A) RNA was isolated from wild-type *B. subtilis* strain 168 (*Bs* 168) and *sigB*-null and *phoR*-null mutants. Bacteria were grown in LPM, and samples were taken 2 h before (T_{-2}) and 0 h (T_0) and 5 h (T_5) after entry into the stationary growth phase, which was provoked by phosphate starvation. Five micrograms of RNA was applied per lane; after the filters were capillary blotted, they were hybridized to *vpr*-specific riboprobes. Transcript size was determined by comparison with DIG-labeled RNA size markers (Roche Diagnostics, Mannheim, Germany). (B) RNA was isolated from wild-type *B. subtilis* (168) (◆) and *sigB*-null (▲) and *phoR*-null (●) mutants grown in LPM. Samples were taken before, during, and after entry into the stationary growth phase, which was provoked by phosphate starvation. Total RNA was isolated and used as the template for reverse transcriptase incorporating radiolabeled [^{33}P]dATP, which was hybridized to a whole-genome macroarray (Sigma-Genosys, The Woodlands, Tex.). Normalization and quantification were performed as described in Materials and Methods.

cating either that PhoP regulates *yhaX* indirectly or that a product of the Pho regulon is required for its transcription. On the basis of this evidence, we do not currently include *yhaX* as a member of the PhoP regulon. Additionally, because of the similarity of its induction kinetics, we currently also exclude *yhbH*.

Twenty-nine genes were induced significantly in the *phoR*-null mutant in response to phosphate starvation. As expected, none of the currently recognized PhoP regulon genes are included, but a large number of these genes belong to the SigB regulon. They include SigB-dependent genes, such as *gsiB* and *dps*, that were shown previously to be induced in response to phosphate starvation (7). Dps is required for oxidative stress resistance and is thought to bind DNA to prevent damage (6). Six other SigB-dependent genes were significantly induced in response to phosphate starvation: *yheK*, *ykzA*, *ysnF*, and *csbD* were identified by screening BFA mutants (62), and *yjbC* and *ytxH* were identified in a two-dimensional gel electrophoresis study of the phosphate starvation proteome (7).

The questions arise as to why the analysis did not include all members of the PhoP regulon and why no members of the SigB regulon were significantly induced. One explanation of the weak response of the SigB regulon may be that its activation by the RsbP phosphatase on the energy side of the signal transduction pathway is not as strong as the induction by environmental stress that is mediated via RsbU (Fig. 9). Hyperinduction of the SigB regulon can occur if a mild physical stress is applied during energy depletion (80).

The overexpression of genes belonging to the SigB regulon in a *phoR*-null mutant has been described previously by Prágai and Harwood (62), who observed a two- to fourfold increase in expression of SigB-dependent genes. The data obtained from the DNA arrays show that expression of 124 SigB genes (64) in response to phosphate starvation was higher in the *phoR* mutant than in the wild-type strain (Table 3). This increased expression seems to indicate that the SigB response is proportional to the level of the stress. This might occur if an active PhoP regulon reduces the extent to which the cellular concentration of ATP falls in response to phosphate starvation. In the absence of an active PhoP regulon, the cellular ATP concentration may be lower, triggering a greater response from the energy-sensing pathway of the SigB regulon.

Putative new members of the PhoP regulon. Two methods of analysis were used to identify new candidate members of the PhoP regulon: outlier analysis and K-means cluster analysis. Genes *bpr*, *skfA*, *yfkN*, *yjdB*, *yukJ*, *yurI*, *yxnB*, and *vpr* were identified as being induced in response to phosphate starvation and as potential members of the PhoP regulon. Subsequent Northern blot analysis of these candidate genes identified that *bpr*, *yxnB*, and *yukJ* were induced at T_0 in a PhoR- and SigB-independent manner and are therefore not members of either the PhoP or SigB regulon. These data confirm the importance of secondary analysis of candidate genes identified solely by DNA array data, and a combination of Northern blotting and reporter gene analyses was used to confirm genes *yfkN*, *yurI*, *yjdB*, and *vpr* as putative new members of the PhoP regulon.

Northern blot analysis showed that the *yfkN*, *yurI*, *yjdB*, and *vpr* genes were induced transiently at T_0 and that this signal was absent from RNA isolated from a *phoR*-null strain. Northern blot analysis confirmed that each of these genes was transcribed from monocistronic operons: *yfkN* is transcribed as an 4.45-kb transcript; *yurI* as transcripts of 0.9 kb and 0.5 kb, indicating that it may be posttranscriptionally processed; *yjdB* as a 0.4-kb transcript and *vpr* as a 2.5-kb transcript. In the case of *yfkN* and *yurI*, the introduction of a *phoR* mutation into their respective BFA strains resulted in the loss of phosphate starvation-induced β-galactosidase activity.

Interestingly, many of the previously identified PhoP regulon members and all of the putative new members described here are known or predicted to be secreted. These include alkaline phosphatases, phosphodiesterases, glycerophosphoryl diester phosphodiesterases, and now, a protease, RNase, and 2′,3′ cyclic nucleotide phosphodiesterase, 2′ (or 3′) nucleotidase, and 5′ nucleotidase. These proteins all have the potential

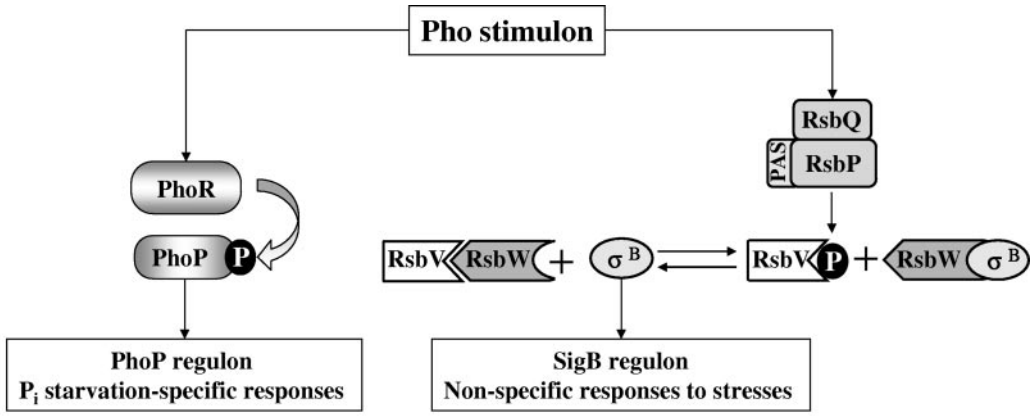

FIG. 9. Pho stimulon of *B. subtilis*. Model for the activation of the PhoP and SigB (σ^B) regulons in response to phosphate starvation (energy) stress. The activation of PhoP via phosphorylation by PhoR leads to the induction of genes involved in the recovery and acquisition of P_i (28). The energy-sensing pathway of the SigB regulon is mediated via the Per-Arnt-Sim (PAS) domain of the RsbP phosphatase and RsbQ. Activated RsbP removes the serine phosphate (P) from RsbV~P, which in turn sequestrates anti-σ^B factor RsbW. Released from its anti-σ factor, SigB is now free to interact with the core RNA polymerase to induce the nonspecific general stress genes.

for salvaging P_i from organic sources in the environment. Ultimately, the recovered P_i is transported into the cell using the *pit* (low-affinity) and the *pst* (high-affinity) phosphate-specific-transporters.

Vpr is an extracellular serine protease (71) and may help recover phosphate from phosphoproteins and has been identified as a protease capable of processing subtilin (15).

YurI is predicted to be a secreted protein (76) with nonspecific hydrolytic activity on extracellular RNA to generate 3′ or 5′ phosphonucleotides. These phosphonucleotides provide substrates for APases (e.g., PhoA) and YfkN, which exhibits 2′,3′ cyclic nucleotide phosphodiesterase, 2′ (or 3′) nucleotidase, and 5′ nucleotidase activities (13) to provide a complete pathway for the recovery of P_i from extracellular RNA.

In conclusion, our data show the value of the global transcriptomic analysis that is possible using DNA macroarrays, and this has enabled us to identify five putative new members of the PhoP regulon. However, despite using the rigorous outlier technique, the technology still identified a number of putative PhoP regulon genes that were rejected by subsequent analysis by Northern blotting or reporter gene fusion technology. The global transcriptome analysis revealed the relatively weak induction of the SigB regulon during phosphate starvation and the hyperinduction of this regulon in the absence of the specific, PhoPR-mediated response.

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REFERENCES

1. **Abdel-Fattah, W. R., Y. Chen, A. Eldakak, and F. M. Hulett.** 2005. *Bacillus subtilis* phosphorylated PhoP: direct activation of the Eσ^A- and repression of the Eσ^E-responsive *phoB*-P_{S+V} promoters during Pho response. J. Bacteriol. **187:**5166–5178.
2. **Akbar, S., S. Y. Lee, S. A. Boylan, and C. W. Price.** 1999. Two genes from *Bacillus subtilis* under the sole control of the general stress transcription factor σ^B. Microbiology **145:**1069–1078.
3. **Allenby, N. E., N. O'Connor, Z. Pragai, N. M. Carter, M. Miethke, S. Engelmann, M. Hecker, A. Wipat, A. C. Ward, and C. R. Harwood.** 2004. Post-transcriptional regulation of the *Bacillus subtilis pst* operon encoding a phosphate-specific ABC transporter. Microbiology **150:**2619–2628.
4. **Anagnastopoulos, C., and J. Spizizen.** 1961. Requirements for transformation in *Bacillus subtilis*. J. Bacteriol. **81:**741–746.
5. **Antelmann, H., J. Bernhardt, R. Schmid, and M. Hecker.** 1995. A gene at 333 degrees on the *Bacillus subtilis* chromosome encodes the newly identified σ^B-dependent general stress protein GspA. J. Bacteriol. **177:**3540–3545.
6. **Antelmann, H., S. Engelmann, R. Schmid, A. Sorokin, A. Lapidus, and M. Hecker.** 1997. Expression of a stress- and starvation-induced *dps/pexB*-homologous gene is controlled by the alternative sigma factor σ^B in *Bacillus subtilis*. J. Bacteriol. **179:**7251–7256.
7. **Antelmann, H., C. Scharf, and M. Hecker.** 2000. Phosphate starvation-inducible proteins of *Bacillus subtilis*: proteomics and transcriptional analysis. J. Bacteriol. **182:**4478–4490.
8. **Asai, K., S. H. Baik, Y. Kasahara, S. Moriya, and N. Ogasawara.** 2000. Regulation of the transport system for C4-dicarboxylic acids in *Bacillus subtilis*. Microbiology **146:**263–271.
9. **Auger, S., W. H. Yuen, A. Danchin, and I. Martin-Verstraete.** 2002. The *metIC* operon involved in methionine biosynthesis in *Bacillus subtilis* is controlled by transcription antitermination. Microbiology **148:**507–518.
10. **Birkey, S. M., W. Liu, X. Zhang, M. F. Duggan, and F. M. Hulett.** 1998. Pho signal transduction network reveals direct transcriptional regulation of one two-component system by another two-component regulator: *Bacillus subtilis* PhoP directly regulates production of ResD. Mol. Microbiol. **30:**943–953.
11. **Bookstein, C., C. W. Edwards, N. V. Kapp, and F. M. Hulett.** 1990. The *Bacillus subtilis* 168 alkaline phosphatase III gene: impact of a *phoAIII* mutation on total alkaline phosphatase synthesis. J. Bacteriol. **172:**3730–3737.
12. **Britton, R. A., P. Eichenberger, J. E. Gonzalez-Pastor, P. Fawcett, R. Monson, R. Losick, and A. D. Grossman.** 2002. Genome-wide analysis of the stationary-phase sigma factor (sigma-H) regulon of *Bacillus subtilis*. J. Bacteriol. **184:**4881–4890.
13. **Chambert, R., Y. Pereira, and M. F. Petit-Glatron.** 2003. Purification and characterization of YfkN, a trifunctional nucleotide phosphoesterase secreted by *Bacillus subtilis*. J. Biochem. (Tokyo) **134:**655–660.
14. **Chesnut, R. S., C. Bookstein, and F. M. Hulett.** 1991. Separate promoters direct expression of *phoAIII*, a member of the *Bacillus subtilis* alkaline phosphatase multigene family, during phosphate starvation and sporulation. Mol. Microbiol. **5:**2181–2190.
15. **Corvey, C., T. Stein, S. Dusterhus, M. Karas, and K. D. Entian.** 2003. Activation of subtilin precursors by *Bacillus subtilis* extracellular serine proteases subtilisin (AprE), WprA, and Vpr. Biochem. Biophys. Res. Commun. **304:**48–54.
16. **Cosmina, P., F. Rodriguez, F. de Ferra, G. Grandi, M. Perego, G. Venema, and D. van Sinderen.** 1993. Sequence and analysis of the genetic locus responsible for surfactin synthesis in *Bacillus subtilis*. Mol. Microbiol. **8:**821–831.
17. **Drzewiecki, K., C. Eymann, G. Mittenhuber, and M. Hecker.** 1998. The *yvyD* gene of *Bacillus subtilis* is under dual control of σ^B and σ^H. J. Bacteriol. **180:**6674–6680.

18. **Eder, S., W. Liu, and F. M. Hulett.** 1999. Mutational analysis of the *phoD* promoter in *Bacillus subtilis*: implications for PhoP binding and promoter activation of Pho regulon promoters. J. Bacteriol. **181:**2017–2025.
19. **Eder, S., L. Shi, K. Jensen, K. Yamane, and F. M. Hulett.** 1996. A *Bacillus subtilis* secreted phosphodiesterase/alkaline phosphatase is the product of a Pho regulon gene, *phoD*. Microbiology **142:**2041–2047.
20. **Eichenberger, P., S. T. Jensen, E. M. Conlon, C. van Ooij, J. Silvaggi, J. E. Gonzalez-Pastor, M. Fujita, S. Ben-Yehuda, P. Stragier, J. S. Liu, and R. Losick.** 2003. The σ^E regulon and the identification of additional sporulation genes in *Bacillus subtilis*. J. Mol. Biol. **327:**945–972.
21. **Engelmann, S., and M. Hecker.** 1996. Impaired oxidative stress resistance of *Bacillus subtilis sigB* mutants and the role of *katA* and *katE*. FEMS Microbiol. Lett. **145:**63–69.
22. **Eymann, C., H. Mach, C. R. Harwood, and M. Hecker.** 1996. Phosphate-starvation-inducible proteins in *Bacillus subtilis*: a two-dimensional gel electrophoresis study. Microbiology **142:**3163–3170.
23. **Gaidenko, T. A., and C. W. Price.** 1998. General stress transcription factor σ^B and sporulation transcription factor σ^H each contribute to survival of *Bacillus subtilis* under extreme growth conditions. J. Bacteriol. **180:**3730–3733.
24. **Gomez, M., and S. M. Cutting.** 1997. Identification of a new σ^B-controlled gene, *csbX*, in *Bacillus subtilis*. Gene **188:**29–33.
25. **Gonzalez-Pastor, J. E., E. C. Hobbs, and R. Losick.** 2003. Cannibalism by sporulating bacteria. Science **301:**510–513.
26. **Hecker, M., and U. Volker.** 1998. Non-specific, general and multiple stress resistance of growth-restricted *Bacillus subtilis* cells by the expression of the σ^B regulon. Mol. Microbiol. **29:**1129–1136.
27. **Huisman, G. W., D. A. Siegele, M. M. Zambrano, and R. Kolter.** 1996. Morphological and physiological changes during stationary phase, p. 1672–1682. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, D.C.
28. **Hulett, F. M.** 2002. The Pho regulon, p. 193–203. *In* A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and its closest relatives: from genes to cells. ASM Press, Washington, D.C.
29. **Hulett, F. M.** 1996. The signal-transduction network for Pho regulation in *Bacillus subtilis*. Mol. Microbiol. **19:**933–939.
30. **Hulett, F. M., C. Bookstein, and K. Jensen.** 1990. Evidence for two structural genes for alkaline phosphatase in *Bacillus subtilis*. J. Bacteriol. **172:**735–740.
31. **Hulett, F. M., J. Lee, L. Shi, G. Sun, R. Chesnut, E. Sharkova, M. F. Duggan, and N. Kapp.** 1994. Sequential action of two-component genetic switches regulates the PHO regulon in *Bacillus subtilis*. J. Bacteriol. **176:**1348–1358.
32. **Igo, M., M. Lampe, C. Ray, W. Schafer, C. P. Moran, Jr., and R. Losick.** 1987. Genetic studies of a secondary RNA polymerase sigma factor in *Bacillus subtilis*. J. Bacteriol. **169:**3464–3469.
33. **Jain, A. K., and R. C. Dubes.** 1988. Algorithms for clustering data. Prentice-Hall, Englewood Cliffs, N.J.
34. **Jongbloed, J. D., U. Martin, H. Antelmann, M. Hecker, H. Tjalsma, G. Venema, S. Bron, J. M. van Dijl, and J. Muller.** 2000. TatC is a specificity determinant for protein secretion via the twin-arginine translocation pathway. J. Biol. Chem. **275:**41350–41357.
35. **Jurgen, B., T. Schweder, and M. Hecker.** 1998. The stability of mRNA from the *gsiB* gene of *Bacillus subtilis* is dependent on the presence of a strong ribosome binding site. Mol. Gen. Genet. **258:**538–545.
36. **Kunst, F., N. Ogasawara, I. Moszer, A. M. Albertini, G. Alloni, V. Azevedo, M. G. Bertero, P. Bessieres, A. Bolotin, S. Borchert, R. Borriss, L. Boursier, A. Brans, M. Braun, S. C. Brignell, S. Bron, S. Brouillet, C. V. Bruschi, B. Caldwell, V. Capuano, N. M. Carter, S. K. Choi, J. J. Codani, I. F. Connerton, A. Danchin, et al.** 1997. The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*. Nature **390:**249–256.
37. **Lahooti, M., and C. R. Harwood.** 1999. Transcriptional analysis of the *Bacillus subtilis* teichuronic acid operon. Microbiology **145:**3409–3417.
38. **Lazarevic, V., P. Margot, B. Soldo, and D. Karamata.** 1992. Sequencing and analysis of the *Bacillus subtilis lytRABC* divergon: a regulatory unit encompassing the structural genes of the N-acetylmuramoyl-L-alanine amidase and its modifier. J. Gen. Microbiol. **138:**1949–1961.
39. **Liu, W., S. Eder, and F. M. Hulett.** 1998. Analysis of *Bacillus subtilis tagAB* and *tagDEF* expression during phosphate starvation identifies a repressor role for PhoP~P. J. Bacteriol. **180:**753–758.
40. **Liu, W., and F. M. Hulett.** 1998. Comparison of PhoP binding to the *tuaA* promoter with PhoP binding to other Pho-regulon promoters establishes a *Bacillus subtilis* Pho core binding site. Microbiology **144:**1443–1450.
41. **Loos, A., C. Glanemann, L. B. Willis, X. M. O'Brien, P. A. Lessard, R. Gerstmeir, S. Guillouet, and A. J. Sinskey.** 2001. Development and validation of *Corynebacterium* DNA microarrays. Appl. Environ. Microbiol. **67:**2310–2318.
42. **Majumdar, D., Y. J. Avissar, and J. H. Wyche.** 1991. Simultaneous and rapid isolation of bacterial and eukaryotic DNA and RNA: a new approach for isolating DNA. BioTechniques **11:**94–101.
43. **Martinez, A., and R. Kolter.** 1997. Protection of DNA during oxidative stress by the nonspecific DNA-binding protein Dps. J. Bacteriol. **179:**5188–5194.
44. **Mauel, C., M. Young, A. Monsutti-Grecescu, S. A. Marriott, and D. Karamata.** 1994. Analysis of *Bacillus subtilis tag* gene expression using transcriptional fusions. Microbiology **140:**2279–2288.
45. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
46. **Mongkolsuk, S., W. Praituan, S. Loprasert, M. Fuangthong, and S. Chamnongpol.** 1998. Identification and characterization of a new organic hydroperoxide resistance (*ohr*) gene with a novel pattern of oxidative stress regulation from *Xanthomonas campestris* pv. phaseoli. J. Bacteriol. **180:**2636–2643.
47. **Moszer, I.** 1998. The complete genome of *Bacillus subtilis*: from sequence annotation to data management and analysis. FEBS Lett. **430:**28–36.
48. **Moszer, I., P. Glaser, and A. Danchin.** 1995. SubtiList: a relational database for the *Bacillus subtilis* genome. Microbiology **141:**261–268.
49. **Muller, J. P., Z. An, T. Merad, I. C. Hancock, and C. R. Harwood.** 1997. Influence of *Bacillus subtilis phoR* on cell wall anionic polymers. Microbiology **143:**947–956.
50. **Nakamura, A., Y. Koide, H. Miyazaki, A. Kitamura, H. Masaki, T. Beppu, and T. Uozumi.** 1992. Gene cloning and characterization of a novel extracellular ribonuclease of *Bacillus subtilis*. Eur. J. Biochem. **209:**121–127.
51. **Nakano, M. M., Y. Zhu, M. Lacelle, X. Zhang, and F. M. Hulett.** 2000. Interaction of ResD with regulatory regions of anaerobically induced genes in *Bacillus subtilis*. Mol. Microbiol. **37:**1198–1207.
52. **Nakatani, Y., W. L. Nicholson, K. D. Neitzke, P. Setlow, and E. Freese.** 1989. Sigma-G RNA polymerase controls forespore-specific expression of the glucose dehydrogenase operon in *Bacillus subtilis*. Nucleic Acids Res. **17:**999–1017.
53. **Nasser, W., A. C. Awade, S. Reverchon, and J. Robert-Baudouy.** 1993. Pectate lyase from *Bacillus subtilis*: molecular characterization of the gene, and properties of the cloned enzyme. FEBS Lett. **335:**319–326.
54. **Ogura, M., H. Yamaguchi, K. Yoshida, Y. Fujita, and T. Tanaka.** 2001. DNA microarray analysis of *Bacillus subtilis* DegU, ComA and PhoP regulons: an approach to comprehensive analysis of *B. subtilis* two-component regulatory systems. Nucleic Acids Res. **29:**3804–3813.
55. **Parsot, C., and G. N. Cohen.** 1988. Cloning and nucleotide sequence of the *Bacillus subtilis hom* gene coding for homoserine dehydrogenase. Structural and evolutionary relationships with *Escherichia coli* aspartokinases-homoserine dehydrogenases I and II. J. Biol. Chem. **263:**14654–14660.
56. **Paul, S., S. Birkey, W. Liu, and F. M. Hulett.** 2004. Autoinduction of *Bacillus subtilis phoPR* operon transcription results from enhanced transcription from $E\sigma^A$- and $E\sigma^E$-responsive promoters by phosphorylated PhoP. J. Bacteriol. **186:**4262–4275.
57. **Perego, M., and J. A. Hoch.** 1996. Cell-cell communication regulates the effects of protein aspartate phosphatases on the phosphorelay controlling development in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **93:**1549–1553.
58. **Petersohn, A., M. Brigulla, S. Haas, J. D. Hoheisel, U. Volker, and M. Hecker.** 2001. Global analysis of the general stress response of *Bacillus subtilis*. J. Bacteriol. **183:**5617–5631.
59. **Petersohn, A., H. Antelmann, U. Gerth, and M. Hecker.** 1999. Identification and transcriptional analysis of new members of the σ^B regulon in *Bacillus subtilis*. Microbiology **145:**869–880.
60. **Pragai, Z., N. E. Allenby, N. O'Connor, S. Dubrac, G. Rapoport, T. Msadek, and C. R. Harwood.** 2004. Transcriptional regulation of the *phoPR* operon in *Bacillus subtilis*. J. Bacteriol. **186:**1182–1190.
61. **Pragai, Z., C. Eschevins, S. Bron, and C. R. Harwood.** 2001. *Bacillus subtilis* NhaC, an Na^+/H^+ antiporter, influences expression of the *phoPR* operon and production of alkaline phosphatases. J. Bacteriol. **183:**2505–2515.
62. **Prágai, Z., and C. R. Harwood.** 2002. Regulatory interactions between the Pho and σ^B-dependent general stress regulons of *Bacillus subtilis*. Microbiology **148:**1593–1602.
63. **Prágai, Z., and C. R. Harwood.** 2000. Screening for mutants affected in their response to phosphate, p.245-249. *In* W. Schumann, S. D. Ehrlich, and N. Ogasawara (ed.), Functional analysis of bacterial genes: a practical manual. John Wiley & Sons, Ltd., Chichester, United Kingdom.
64. **Price, C. W., P. Fawcett, H. Ceremonie, N. Su, C. K. Murphy, and P. Youngman.** 2001. Genome-wide analysis of the general stress response in *Bacillus subtilis*. Mol. Microbiol. **41:**757–774.
65. **Qi, Y., and F. M. Hulett.** 1998. PhoP-P and RNA polymerase σ^A holoenzyme are sufficient for transcription of Pho regulon promoters in *Bacillus subtilis*: PhoP-P activator sites within the coding region stimulate transcription *in vitro*. Mol. Microbiol. **28:**1187–1197.
66. **Qi, Y., and F. M. Hulett.** 1998. Role of Pho~P in transcriptional regulation of genes involved in cell wall anionic polymer biosynthesis in *Bacillus subtilis*. J. Bacteriol. **180:**4007–4010.
67. **Qi, Y., Y. Kobayashi, and F. M. Hulett.** 1997. The *pst* operon of *Bacillus subtilis* has a phosphate-regulated promoter and is involved in phosphate transport but not in regulation of the Pho regulon. J. Bacteriol. **179:**2534–2539.
68. **Reischl, S., S. Thake, G. Homuth, and W. Schumann.** 2001. Transcriptional analysis of three *Bacillus subtilis* genes coding for proteins with the alpha-crystallin domain characteristic of small heat shock proteins. FEMS Microbiol. Lett. **194:**99–103.
69. **Robichon, D., M. Arnaud, R. Gardan, Z. Pragai, M. O'Reilly, G. Rapoport, and M. Debarbouille.** 2000. Expression of a new operon from *Bacillus sub-*

tilis, *ykzB-ykoL*, under the control of the TnrA and PhoP-PhoR global regulators. J. Bacteriol. **182:**1226–1231.
70. **Schmidt, R., P. Margolis, L. Duncan, R. Coppolecchia, C. P. Moran, and R. Losick.** 1990. Control of developmental transcription factor σ^F by sporulation regulatory proteins SpoIIAA and SpoIIAB in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **87:**9221–9225.
71. **Sloma, A., G. A. Rufo, Jr., K. A. Theriault, M. Dwyer, S. W. Wilson, and J. Pero.** 1991. Cloning and characterization of the gene for an additional extracellular serine protease of *Bacillus subtilis*. J. Bacteriol. **173:**6889–6895.
72. **Soldo, B., V. Lazarevic, M. Pagni, and D. Karamata.** 1999. Teichuronic acid operon of *Bacillus subtilis* 168. Mol. Microbiol. **31:**795–805.
73. **Stephenson, S., C. Mueller, M. Jiang, and M. Perego.** 2003. Molecular analysis of Phr peptide processing in *Bacillus subtilis*. J. Bacteriol. **185:**4861–4871.
74. **Sun, G., S. M. Birkey, and F. M. Hulett.** 1996. Three two-component signal-transduction systems interact for Pho regulation in *Bacillus subtilis*. Mol. Microbiol. **19:**941–948.
75. **Sun, G., E. Sharkova, R. Chesnut, S. Birkey, M. F. Duggan, A. Sorokin, P. Pujic, S. D. Ehrlich, and F. M. Hulett.** 1996. Regulators of aerobic and anaerobic respiration in *Bacillus subtilis*. J. Bacteriol. **178:**1374–1385.
76. **Tjalsma, H., A. Bolhuis, J. D. Jongbloed, S. Bron, and J. M. van Dijl.** 2000. Signal peptide-dependent protein transport in *Bacillus subtilis*: a genome-based survey of the secretome. Microbiol. Mol. Biol. Rev. **64:**515–547.
77. **Tosato, V., A. M. Albertini, M. Zotti, S. Sonda, and C. V. Bruschi.** 1997. Sequence completion, identification and definition of the fengycin operon in *Bacillus subtilis* 168. Microbiology **143:**3443–3450.
78. **Tsuge, K., T. Ano, M. Hirai, Y. Nakamura, and M. Shoda.** 1999. The genes *degQ*, *pps*, and *lpa-8* (*sfp*) are responsible for conversion of *Bacillus subtilis* 168 to plipastatin production. Antimicrob. Agents Chemother. **43:**2183–2192.
79. **Vagner, V., E. Dervyn, and S. D. Ehrlich.** 1998. A vector for systematic gene inactivation in *Bacillus subtilis*. Microbiology **144:**3097–3104.
80. **Voelker, U., A. Dufour, and W. G. Haldenwang.** 1995. The *Bacillus subtilis rsbU* gene product is necessary for RsbX-dependent regulation of σ^B. J. Bacteriol. **177:**114–122.
81. **Volker, U., K. K. Andersen, H. Antelmann, K. M. Devine, and M. Hecker.** 1998. One of two *osmC* homologs in *Bacillus subtilis* is part of the σ^B-dependent general stress regulon. J. Bacteriol. **180:**4212–4218.
82. **Wise, A. A., and C. W. Price.** 1995. Four additional genes in the *sigB* operon of *Bacillus subtilis* that control activity of the general stress factor σ^B in response to environmental signals. J. Bacteriol. **177:**123–133.
83. **Yoshida, K., I. Ishio, E. Nagakawa, Y. Yamamoto, M. Yamamoto, and Y. Fujita.** 2000. Systematic study of gene expression and transcription organization in the *gntZ-ywaA* region of the *Bacillus subtilis* genome. Microbiology **146:**573–579.