

# Analysis of *Bacillus subtilis* *tagAB* and *tagDEF* Expression during Phosphate Starvation Identifies a Repressor Role for PhoP~P

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**The *tagAB* and *tagDEF* operons, which are adjacent and divergently transcribed, encode genes responsible for cell wall teichoic acid synthesis in *Bacillus subtilis*. The *Bacillus* data presented here suggest that PhoP and PhoR are required for direct repression of transcription of the two operons under phosphate starvation conditions but have no regulatory role under phosphate-replete conditions. These data identify for the first time that PhoP~P has a negative role in Pho regulon gene regulation.**

Teichoic acid, an essential anionic polymer containing polyglycerol or polyribitol phosphate, is the major cell wall component of *Bacillus subtilis* grown in phosphate-replete media (2, 17). Approximately 15% of cellular phosphorus is stored in the form of teichoic acid under these conditions (1). During phosphate starvation, however, the cell ceases to produce teichoic acid and replaces it with an anionic phosphate-free polymer, teichuronic acid (5, 13). As a result, the cell saves phosphorus for cellular metabolism and DNA synthesis. When phosphate becomes available again, the cell will restore synthesis of teichoic acid and stop production of teichuronic acid (reference 5; for reviews, see references 1 and 29).

The genes responsible for synthesis of teichoic acid and teichuronic acid have been cloned and analyzed (16, 27, 28). The genes which are involved in teichuronic acid synthesis, the *tuaABCDEF* operon, are repressed when phosphate is in excess and turned on under phosphate starvation conditions (14a, 28). Expression of this operon under phosphate-limiting conditions is directly activated by PhoP and PhoR, a pair of bacterial two-component regulatory proteins (24, 25). PhoP, the response regulator, binds to the *tuaA* promoter at *B. subtilis* Pho boxes, as it does to other Pho regulon promoters which are induced during phosphate starvation (14a). Divergently transcribed operons *tagAB* and *tagDEF* encode products which are directly involved in teichoic acid synthesis. The gene products of *tagAB* are poorly characterized, although assumptions about their functions have been made based on the similarity of the sequences of their products to those of other proteins (15, 16). The *tagDEF* operon, on the other hand, encodes CDP-glycerol pyrophosphorylase (20), polyglycerol-phosphate glucosyltransferase (22), and polyglycerol-phosphate glycerol phosphate transferase (21). Expression of the *tagA* and *tagD* operons is reduced when the cell is grown in low-phosphate medium (18).

It has recently been suggested that not all proteins whose synthesis is regulated in response to changing phosphate levels are members of the Pho regulon (6, 19). To understand if the *tagA* and *tagD* operons are repressed by PhoP and PhoR, we studied expression of the divergently transcribed operons in a *phoP* mutant and a *phoR* mutant. We also used gel shift and DNase I footprinting assays to determine if PhoP directly regulates expression of these genes. We conclude that PhoP~P

directly binds to and is essential for transcriptional repression of the *tagAB* and *tagDEF* operons but activates the *tuaABCDEF* operon (14a) and therefore regulates the switch of the two anionic polymers during phosphate starvation.

**Cloning of the promoter region shared by *tagAB* and *tagDEF* operons.** The divergently transcribed operons *tagAB* and *tagDEF* from *B. subtilis* 168 have been cloned and sequenced (16). The transcriptional start sites from *tagA* and *tagD* have been identified by primer extension analysis (15). Two primers, FMH304 (5'-<sup>354</sup>CCCCAATGCAGTAAATCAA<sup>372</sup>-3') and FMH305 (5'-GGATCC<sup>845</sup>AGTTACTGTTAACATAAGGAA<sup>824</sup>-3'), which are located within the *tagA* and *tagD* genes, were used to amplify the promoter region shared by the two operons with *B. subtilis* JH642 (a derivative of *B. subtilis* 168) chromosomal DNA as the template (Fig. 1). The superscript numbers in the primers are the nucleotide numbers assigned by Mauël et al. (16). The 493-bp promoter fragment was cloned into pCR2.1 (Invitrogen) to construct pSE90. The cloned promoter fragment contains nucleotides (nt) -371 to +121 for *tagA* and nt -325 to +167 for *tagD*, relative to the transcriptional start site for each gene. The sequence of the insert was confirmed by DNA sequencing, and it is the same as that for *B. subtilis* 168. Figure 1 shows the sequence of the insert in pSE90 containing the common promoter region of the *tagAB* and *tagDEF* operons.

**Expression of *tagAB* and *tagDEF* is repressed by PhoP and PhoR during phosphate starvation.** Mauël et al. (18) used a 399-bp intergenic region between *tagA* and *tagD* to make *lacZ* fusions of the two gene promoters and found that expression of *B. subtilis* 168 *tagA* and *tagD* is decreased in phosphate-limited culture. To examine if the lowered expression of the two promoters is dependent on the *phoP* and *phoR* genes, both *tagA* and *tagD* promoter-*lacZ* fusions were introduced into a *phoP* nonpolar deletion mutant (MH5600 [11]), a *phoR* deletion mutant (MH5124 [11]), and the parent strain (JH642) as follows. The promoter region shared by *tagA* and *tagD* was subcloned from pSE90 into pDH32 (26) in two different orientations to make *tagA-lacZ* (pSE91) and *tagD-lacZ* (pSE92) fusions. Single-copy promoter-*lacZ* fusion strains were obtained by integrating the promoter-*lacZ* fusions into the *amy* locus of each strain. The resultant strains containing the *tagA-lacZ* fusion in the parent, *phoP*, or *phoR* background were named MH5630, MH5634, and MH5632, respectively. The strains containing *tagD-lacZ* fusion in the parent, *phoP*, or *phoR* background were named MH5631, MH5635, and MH5633, respectively.

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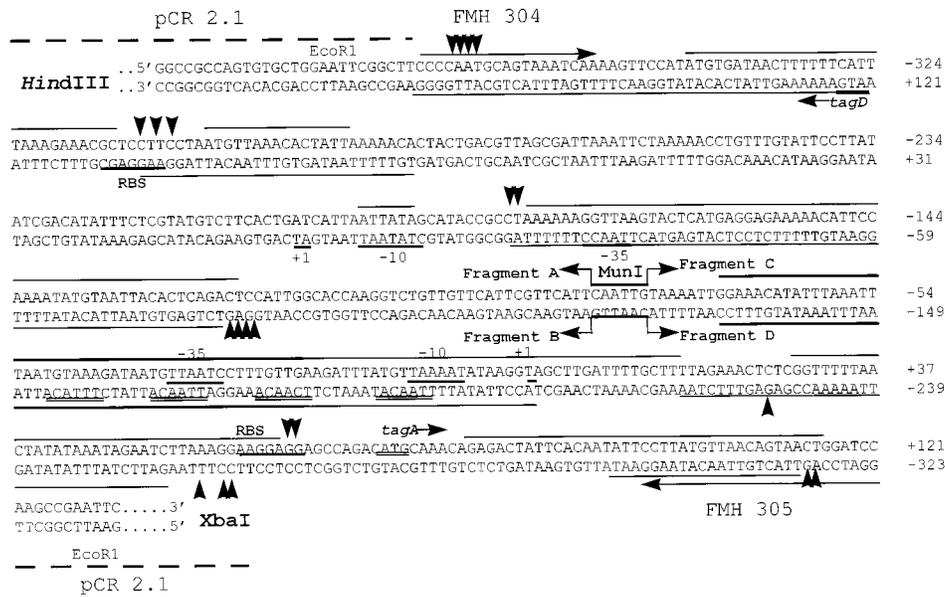


FIG. 1. Sequence of the promoter region shared by *tagAB* and *tagDEF* operons. The 493-bp fragment containing promoters for both *tagAB* and *tagDEF* operons were cloned into pCR2.1. The junction of pCR2.1 and the promoter fragment is shown with the relevant restriction sites indicated above the sequence. The primers FMH304 and FMH305 within the coding region of *tagD* and *tagA*, respectively, are also indicated by arrows. The  $-10$  regions, ribosomal binding sites, transcriptional start sites for  $\sigma^A$ , and translational start sites for both *tagA* and *tagD* are labeled and underlined (14). Thin lines above or below the sequences, binding sites for PhoP~P only; thick lines above or below the sequences, binding sites for both PhoP and PhoP~P; arrowheads, hypersensitive sites. To number the nucleotides, the top strand (the coding strand for *tagA*) and the bottom strand (the coding strand for the *tagD*) are numbered separately, with transcriptional start sites of each gene indicated by +1. To facilitate the location of the protection area by PhoP (Fig. 4), the fragments A to D and the *MunI* restriction site are indicated by bent arrows and labeled. The four repeated TTAACA-like sequences located on the *tagA* noncoding strand upstream of  $-10$  are also doubly underlined.

All six strains described above were grown in both high-phosphate defined medium (HPDM) containing 5 mM phosphate (23) and low-phosphate defined medium (LPDM) containing 0.42 mM phosphate (10). The promoter activities were detected under these conditions as described previously (10), and alkaline phosphatase (APase) activity was measured as a reporter for Pho regulon gene expression (10). Under high-phosphate conditions, transcription from *tagA* in the three strains was basically the same (Fig. 2D), as was that of the *tagD* promoter (Fig. 3D). There was no APase produced under these conditions, indicating no induction of the Pho response during phosphate-replete growth (Fig. 2C and 3C). These results strongly argue that neither PhoP nor PhoR plays a role in regulation of either of the two *tag* promoters under high-phosphate conditions. Phosphate is the limiting nutrient in LPDM, and cultures enter into stationary phase due to a phosphate deficiency. APases were induced in the parent strain but were not induced in either the *phoP* or the *phoR* strain (Fig. 2A and 3A). In LPDM, transcription from both the *tagA* and *tagD* promoters was dramatically decreased in the parent strain (Fig. 2B and Fig. 3B) at the same time that APase was induced in the same culture. In contrast, transcription from the *tagA* and *tagD* promoters continued during the whole growth period in either the *phoP* or the *phoR* strain (Fig. 2B and 3B). These results strongly suggest that both PhoP and PhoR are required for repression of transcription of these two promoters under phosphate starvation conditions. Transcription of *tagD* was two- to threefold higher than that of *tagA*, as reported by Mäuel et al. (18). However, under our culture conditions, the decrease in the level of either *tagA* or *tagD* transcription in the parent strain during phosphate starvation was more dramatic (threefold) compared to the results from Mäuel et al. (less than twofold) (18).

The level of *tagA* and *tagD* transcription changed little in

either the *phoP* or the *phoR* mutant during phosphate starvation, corroborating the cell wall biochemical quantitation data (19). Under the same conditions, *tuaA* transcription is not turned on (14a), and no teichuronic acid synthesis is induced in these mutant strains (19). In order to survive, these mutants continue to synthesize teichoic acid for cell wall assembly, although the limiting phosphate reserve has to be used for this purpose. However, in the parent strain, the cell represses teichoic acid synthesis and switches to teichuronic acid synthesis during phosphate starvation to conserve the limited phosphorus sources (5). The activation of *tuaA* transcription (14a) and repression of *tagA* and *tagD* transcription require *phoP* and *phoR*.

Since the *tagAB* and *tagDEF* operons were regulated by PhoP and PhoR during phosphate starvation, these operons should be considered part of the Pho regulon. The definition of the Pho regulon genes, therefore, is broadened to include not only the genes which are activated by PhoP~P, but genes which are repressed by PhoP~P as well.

**Both PhoP and PhoP~P bind to the promoter region shared by *tagA* and *tagD*.** PhoP regulates the Pho regulon genes by binding to the target promoters (13a, 14, 14a). To determine if regulation of the *tagA* and *tagD* promoters by PhoP is through direct binding of PhoP to these promoters, we performed gel shift assays (data not shown) and DNase I footprinting assays using the promoter region shared by both *tagA* and *tagD* in pSE90. PhoP bound to multiple regions within these two promoters (data not shown). To obtain clearer footprinting data, the promoter region was dissected into two subregions by DNA digestion at the unique *MunI* restriction site, which is located between the  $-35$  regions of the two promoters (Fig. 1). Figure 4 shows that PhoP~P bound to multiple regions whereas unphosphorylated PhoP bound only to a region in which multiple TTAACA-like sequences were found (Fig. 1). It has

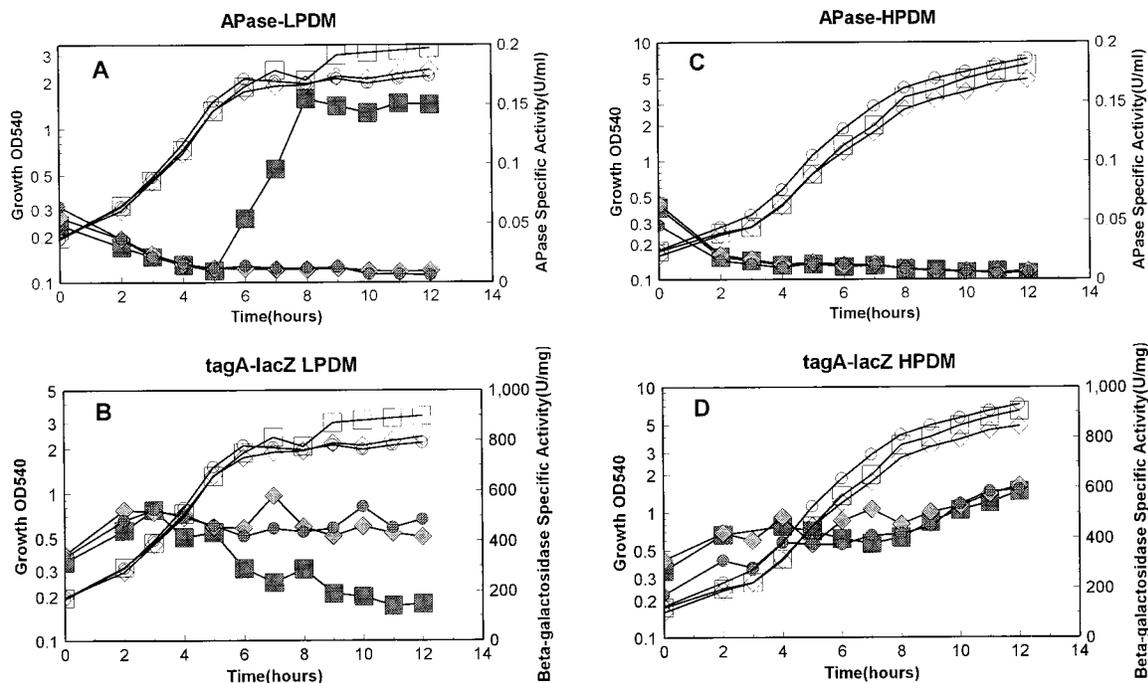


FIG. 2. Promoter activity of *tagA* under phosphate starvation and phosphate-replete conditions. Strains containing the *tagA-lacZ* fusion in the parent strain or the *phoP* or *phoR* mutant strain were grown in LPDM (0.42 mM) or HPDM (5 mM) as described previously (10). A 12-h growth was monitored with respect to optical density at 540 nm, (OD<sub>540</sub>), APase activity, and β-galactosidase, which represents promoter activity. (A and B) APase and β-galactosidase activities, respectively, in LPDM; (C and D) APase and β-galactosidase activities, respectively, in HPDM. Open symbols, growth; filled symbols, APase or β-galactosidase activity. Strains included MH5630 (squares [parent]), MH5632 (diamonds [*phoR*]), and MH5634 (circles [*phoP*]).

been found that the DNA regions containing multiple TTAA CA-like sequences (separated by about five nonconserved nucleotides) were bound by both unphosphorylated PhoP and PhoP~P in all of the tested Pho promoters (13a, 14, 14a)

and have been proposed to compose the *B. subtilis* PhoP core binding region (13a, 14a). Four TTAACA-like sequences in the *tagA* and *tagD* promoters were located in the region between -10 and -50 of the *tagA* promoter but on the noncod-

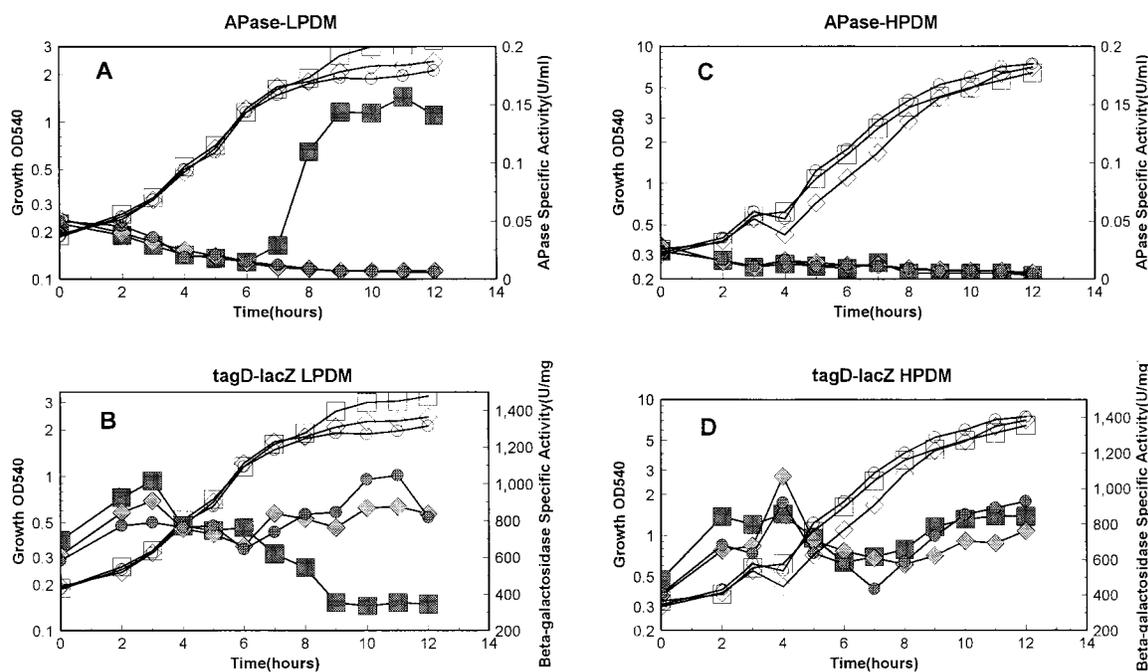


FIG. 3. Promoter activity of *tagD* under phosphate starvation and phosphate-replete conditions. Strains containing the *tagD-lacZ* fusion in the parent strain or the *phoP* or *phoR* mutant strain were grown in LPDM and HPDM as described in the legend to Fig. 2. Strains included MH5631 (squares [parent]), MH5633 (diamonds [*phoR*]), and MH5635 (circles [*phoP*]). OD<sub>540</sub>, optical density at 540 nm.

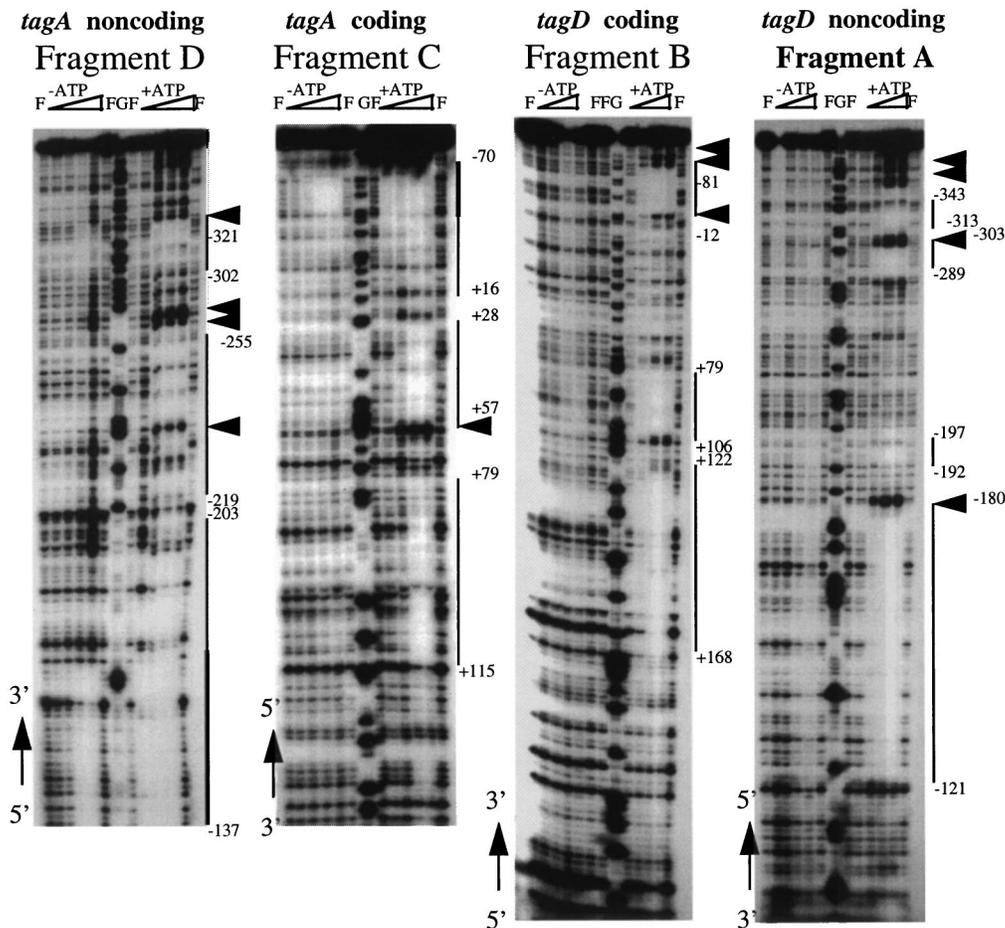


FIG. 4. DNase I footprinting assays of the *tagA* and *tagD* promoters by PhoP and PhoP~P. Plasmid pSE90 was digested with *MunI* (Fig. 1), end labeled with Klenow fragment, and digested with *HindIII* to obtain the fragment A showing nt -371 to -79 of the *tagD* noncoding strand. To get fragment B showing nt +168 to -124 of the *tagD* coding strand, pSE90 was digested with *HindIII*, end labeled with Klenow fragment, and digested with *MunI*. Fragment C showing nt -82 to +121 of the *tagA* coding strand was made by digesting pSE90 with *XbaI*, end labeling with Klenow fragment, and further digesting with *MunI*. Fragment D showing nt -121 to -323 of the *tagA* noncoding strand was made by digesting pSE90 with *MunI*, end labeling with Klenow fragment, and further digesting with *XbaI*. The ends were labeled in the presence of [ $\alpha$ - $^{32}$ P]dCTP. The footprinting experiments were done as described previously (14). The amounts of PhoP added to each reaction mixture were 40 ng (55 nM), 200 ng (275 nM), 1  $\mu$ g (1.4  $\mu$ M), and 5  $\mu$ g (6.9  $\mu$ M) from left to right. To each reaction mixture, 0.7  $\mu$ g of \*PhoR, the cytoplasmic domain of PhoR (14), was added. +ATP, reaction mixtures containing ATP which were used for producing PhoP~P; -ATP, reaction mixtures for unphosphorylated PhoP; F, lanes without PhoP; G, the G sequencing lane; thick lines, binding sites for both PhoP and PhoP~P; thin lines, binding sites for PhoP~P alone; arrowheads, hypersensitive sites. The numbers of the arrowheads do not reflect the numbers of hypersensitive sites shown in Fig. 1 because of space constraints due to clustering. To correlate the locations of the footprinting results with the sequences, the 5' and 3' ends of each sequence are labeled along with the sequencing gels.

ing strand. In contrast, in other Pho promoters which are activated by PhoP~P, the TTAACA-like sequences were located at and upstream of the -22 region of the coding strand (12, 14a). The appearance of the PhoP core binding region on different strands may be important for the different role of PhoP~P in the regulation of these promoters. PhoP~P binding sites within the *tagA* and *tagD* promoters covered the -10 region, partially covered the ribosomal binding sites, and extended to the 5' coding regions for each of the two genes. However, binding of PhoP~P on *tuaA*, which is activated by PhoP~P, is only located upstream of -10 (14a). PhoP~P binding in the 5' coding region may also be critical for the negative role of PhoP~P in *tagA* and *tagD* regulation.

Mäuel et al. (16) found a sequence in the *tagA* and *tagD* promoter region which shows similarity with the *Escherichia coli* Pho box. A similar sequence has also been found in the *phoA* and *phoB* promoters. However, these sequences, at least for transcription of the *phoA* and *phoB* promoters, are not necessary (3, 13a).

**A model for PhoP~P regulation of cell wall synthesis.** We have shown that PhoP is the substrate for PhoR, its cognate kinase, with respect to phosphorylation and dephosphorylation (14). It has been proposed that PhoP~P is the active form for stimulation of Pho regulon gene transcription during phosphate starvation (7-9, 14a). In control of *B. subtilis* cell wall synthesis, we have shown that under phosphate starvation conditions PhoP is required for activation of the *tuaABCDEF* operon (14a) and simultaneous repression of the *tagAB* and *tagDEF* operons. Therefore, teichuronic acid is synthesized and teichoic acid synthesis is inhibited under these conditions (5, 13). In this switch process, we propose that PhoP~P plays a key role in activation of *tuaA* and repression of *tagA* and *tagD* by binding to different regions of these promoters (14a). In the *phoP* or *phoR* mutant, transcription of genes for teichuronic acid synthesis is not initiated due to the lack of the activator, PhoP~P, during phosphate starvation; However, the cell continues to transcribe the genes for teichoic acid synthesis to compensate for cell wall anionic polymers because repression

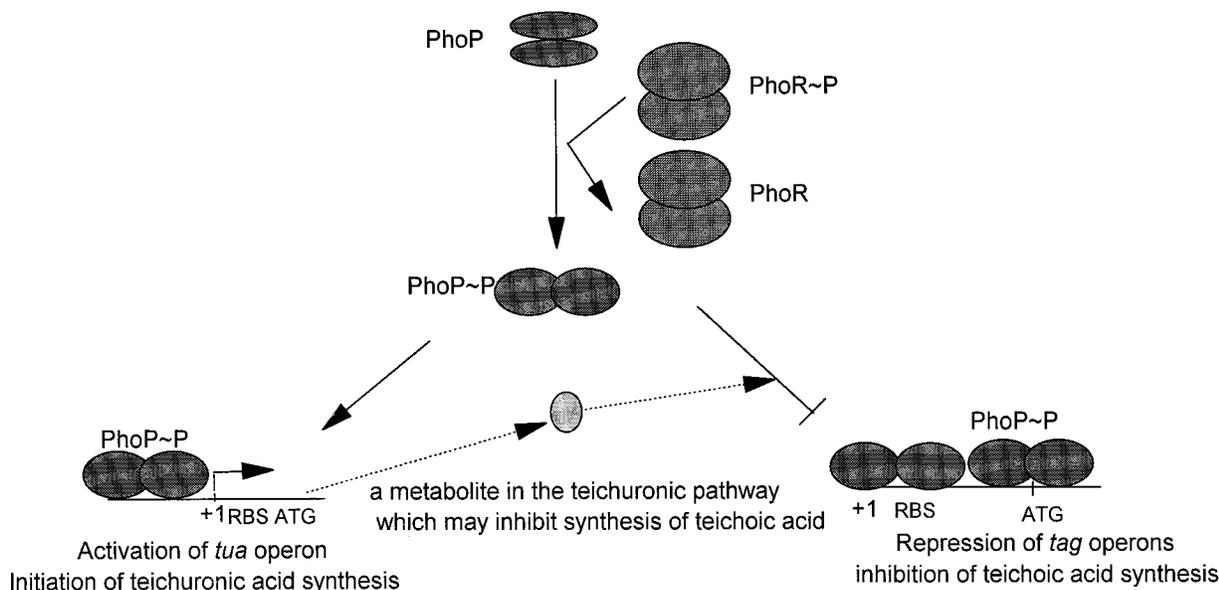


FIG. 5. A model for teichoic acid and teichuronic acid synthesis regulated by PhoP~P. Under phosphate starvation conditions, PhoP~P phosphorylated by PhoR binds to both the *tag* operon promoters and the *tua* operon promoter. As a result, PhoP~P binds to the  $-10$  region and/or the ribosomal binding site and to the 5' end of the coding region to repress transcription from the *tag* operons and simultaneously binds upstream of the  $-10$  region of the *tua* operon to activate transcription. A component involved in teichuronic acid synthesis has also been suggested to repress teichoic acid synthesis during phosphate limitation (19), as indicated by the arrows with dashed lines. The number of the PhoP dimers in this model is not meant to reflect the physiological state. The number of PhoP dimers binding to any promoter is unknown.

from PhoP~P does not exist. Continued synthesis of teichoic acid has also been observed in the mutants unable to synthesize teichuronic acid (*gtab*) during phosphate limitation (19). The proposed explanation for this phenomenon was that a component of teichuronic acid synthesis is involved in repression of teichoic acid synthesis (Fig. 5). Any repression by a metabolite dependent on teichuronic synthesis must require PhoP and PhoR (14a), and this idea is supported by the observation that no repression of teichoic acid was detected in a *phoP* or *phoR* mutant under phosphate-limited conditions.

When excess phosphate is provided, PhoP~P is dephosphorylated by PhoR. In the absence of PhoP~P, teichuronic acid synthesis is not induced and teichoic acid synthesis is relieved from repression. Thus, PhoP and PhoR do not play a role during phosphate-replete growth.

This is the first time that the negative role of PhoP~P has been observed for Pho regulation. That the PhoP-PhoR two-component regulatory system participates in the regulation of *B. subtilis* cell wall anionic polymer synthesis distinguishes the *B. subtilis* Pho regulon from the well-studied *E. coli* Pho regulon. These data together with *tuaA* activation establish the importance of the Pho regulon in *B. subtilis* cell wall synthesis and perhaps in other gram-positive bacteria whose cell walls contain teichoic acid and teichuronic acid anionic polymers.

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