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REVIEW PAPER

The Pho Regulons of Bacteria

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Abstract—Bacterial Pho regulons contain genes whose products are involved in the transport and assimilation of inorganic phosphate. The expression of these genes is regulated by a specific two-component signal transduction system. The paper summarizes data on the organization and function of Pho regulons in gram-negative and gram-positive bacteria, with particular emphasis on the Pho regulons of the best studied bacteria *Escherichia coli* and *Bacillus subtilis.*

Key words: phosphate regulation, signal transduction, *B. subtilis, E. coli.*

Phosphorus is an essential component of bacterial nutrition, which plays an important part in cell metabolism. This chemical element is a constituent of amino and nucleic acids, phospholipids, lipopolysaccharides, important nucleotide cofactors (which are involved in energy transport and many catalytic processes), and some proteins, into which phosphorus is incorporated through posttranslational modification. Bacteria meet their requirements for phosphorus through the assimilation of various phosphorus-containing compounds. The assimilation consists of two primary stages, the transport of such compounds into cells and the incorporation of phosphorus into ATP [1]. The preferable source of phosphorus in bacteria is inorganic phosphate (Pi), which, depending on its concentration in the medium, enters a cell with the aid of various transport systems. When extracellular Pi is in excess, it is transported into the cell by the so-called Pit (phosphate inorganic transport) system. But under phosphate-limiting conditions, Pi is transported by the Pst (phosphate-specific transport) system [2, 3].

Phosphate starvation conditions induce the so-called *psi* (phosphate starvation–inducible) genes [4, 5], which code for proteins involved in the transport of Pi and other phosphorus-containing compounds, the pore proteins of the outer membrane, phosphomonoesterases (acidic and alkaline phosphatases and 5'-nucleotidase), phosphodiesterases, proteins involved in the biosynthesis of cell-wall constituents and in the utilization of polyphosphates and phosphonates, etc. All *psi* genes, which are part of the Pho (phosphate starvation) regulon, occur under the same physiological and genetic control. The *pho* genes are regulated by two-component signal transduction systems, which consist of the protein pairs PhoB–PhoR in gram-negative bacteria and PhoP–PhoR in gram-positive bacteria. The PhoB and PhoP proteins are transcriptional regulators, which directly bind to the *pho* gene promoters. PhoR is a transmembrane sensory histidine protein kinase, which responds to Pi in the medium and is involved in the specific phosphorylation and dephosphorylation of transcriptional regulators. The transcription of the *pho* genes is induced by the phosphorylated form of transcriptional regulators [5–8].

The regulatory systems responding to phosphorus deficiency in the medium are found in gram-negative and gram-positive bacteria, yeasts [9], cyanobacteria [10], and archaebacteria [11]. Hence, the Pho regulon can be considered a universal system responding to a deficiency of phosphate in the medium.

THE Pho REGULON OF *ESCHERICHIA COLI*

ThE Functional Proteins of the E. coli

Pho regulon. Using two-dimensional electrophoresis, Van Bogelen *et al.* [12] showed that the PhoB–PhoR system of *E. coli* regulates by 137 genes, 118 of which are induced and the other 19 are suppressed by phosphorus starvation conditions. More than 30 of these genes have been cloned and sequenced.

The products of the Pho regulon genes are found in all of the cell compartments (Table 1). The PhoE pore protein of the outer membrane facilitates the diffusion of phosphorus-containing compounds through this membrane under phosphorus starvation conditions [13]. When these compounds occur in the periplasm, most of them cannot penetrate the cytoplasmic membrane because of their large size and are hydrolyzed by alkaline (PhoA) or acidic (AppA) phosphatase and 5'-nucleotidase with the formation of Pi [14]. The periplasmic Pi binds to the PstS protein and is transported through the cytoplasmic membrane by the highly specific Pst system [15]. The PstC and PstA proteins of this system form a channel in the membrane, through which Pi is transported to the cytoplasm. The membrane-bound PstB protein provides the transport process with energy [16]. The cytoplasmic protein PhoU, which is

†Deceased.

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Table 1. The Pho regulon genes of *E. coli*

Gene or operon	Encoded product or function	Cellular location	References
phoE	Pore protein	Outer membrane	[13]
phoA	Alkaline phosphatas	Periplasm	[14]
appA	Acidic phosphatase		
nuc	5'-Nucleotidase		
pstS	Pi-binding protein		[15]
ugpB	G3P-binding protein		[18, 19]
phnC			[21, 23]
phcD	Phosphonate carrier		
phnE			
pstA	Channel for Pi	Cytoplasmic membrane	[15]
pstC			
ugpA	Channel for G3P		[18, 19]
ugpE			
phoR	Sensor protein		[1, 30]
pstB	Component of transport system for Pi	Membrane-bound proteins	[16]
phoU	Modulator of Pi transduction		[17]
ugpC	Transport of G3P		[18, 19]
ugpQ	Glycerophosphodiesterase		[18, 19]
ppX	Synthesis of polyphosphates		[20]
ppK	Utilization of polyphosphates		[20]
phoB	Inducer of Pho regulon	Cytoplasm	[34, 1]
phnF	Regulator of phosphonate operon		[21, 23]
phnO			
phnG	Biodegradation of phosphonates		[21, 23]
phnH			
phnI			
phnJ			
phnK			
phnL			
phnM			
phnN			
phnP			
phnH	ATPase		[44]

encoded by one of the *pstSCAB* operon genes, is not directly involved in phosphate transport, but serves as a signal transduction mediator in the Pho regulon [17].

Along with inorganic phosphate, *E. coli* cells can utilize organic phosphates as phosphorus sources. Glycerol-3-phosphate (G3P) and glycerophosphodiesters is transported into cells by the PhoB-dependent Ugp system, which consists of the periplasmic G3P-binding UgpB protein, the integral membrane proteins UgpE and UgpA, and the UgpC permease [18, 19]. The *ugp* operon is induced under the conditions of orthophosphate deficiency. In addition to the aforementioned proteins, this operon also encodes the UgpQ phosphodiesterase, which hydrolyzes the phosphodiesters that are transported by the Ugp system. The PpX and PpK proteins are involved in the metabolism of polyphosphates [20].

E. coli cells are able to utilize naturally occurring or synthetic phosphonates (Phn), which have a very stable carbon–phosphorus (C–P) bond [21, 22]. Phosphonates are utilized as phosphorus sources with the involvement of phosphonatase or C–P lyase, the enzymes that differ

in the substrate specificity and the mechanism of the catalytic cleavage of the C–P bond. *E. coli* utilizes phosphonates via the C–P lyase pathway [23], whereas *Pseudomonas aeruginosa* and *Salmonella typhimurium* via the phosphonatase pathway [24, 25]. The bacterium *Enterobacter aerogenes* was found to implement both mechanisms of the C–P bond cleavage [26]. All 14 genes of *E. coli* involved in the uptake and utilization of phosphonates are organized in the *phnCDEFGHIJKLMNOP* operon with the single *phnC* promoter. The three products of these genes (the periplasmic binding protein PhnD, the integral membrane protein PhnE, and the permease PhnC) are components of the Phn transport system; the seven products (PhnG, PhnH, PhnI, PhnJ, PhnK, PhnL, and PhnM) are organized in the membrane-bound multienzyme complex of C–P lyase; PhnN and PhnP are auxiliary proteins; and the remaining two proteins, PhnF and PhnO, likely perform a regulatory function. The genes of all the aforementioned proteins are components of the Pho regulon. The recently described new genes of this regulon, *iciA* [27] and *psiE* [28], code for proteins, whose function is still unknown.

The Regulatory Proteins of the E. coli Pho Regulon

As described above, the Pho regulon genes of *E. coli* are expressed coordinately due to the functioning of the two-component PhoB–PhoR signal transduction system. The genes of the PhoB and PhoR proteins are organized in the autoregulatory *phoB–phoR* operon [29].

The sensory histidine protein kinase PhoR of *E. coli* has a molecular mass of 49.6 kDa and consists of 431 amino acid residues [30]. The two N-terminal transmembrane segments of this protein anchor it in the cytoplasmic membrane. PhoR spans the membrane, the major portion of this protein being exposed to the cytoplasm and the minor portion, to the periplasm. One of the 13 histidine residues of PhoR, His-213 at the C-terminus, is responsible for the autophosphorylation of this protein [31]. The bifunctional role of PhoR is to mediate signal transduction and to interact with PhoU. The transmembrane segments of PhoR orient this protein in such a manner as to provide for its most efficient interaction with the PhoU protein, which is a component of the Pst system. PhoR possesses not only protein kinase activity but also phosphatase activity against the PhoB response regulator [32, 33]. Both activities are associated with the same protein domain, whose elements interact with each other to provide for the functional rearrangement of the protein.

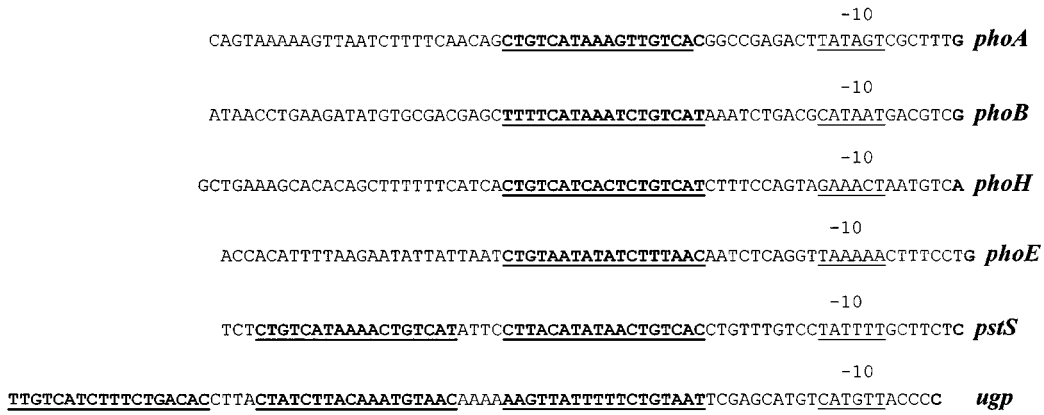
The PhoB regulator protein has a molecular mass of 26.3 kDa and contains 229 amino acid residues [34]. The N-terminal functional domain of this protein is responsible for its phosphorylation, and the C-terminal domain is responsible for its interaction with RNA polymerase [4]. The active center of PhoB is made up of three acidic amino acid residues, Asp-53, Asp-10, and Glu-9. Biochemical and mutation analyses showed that Asp-53 is responsible for autophosphorylation and that Thr-83 is essential for the transfer of the phosphoryl group. The substitution of any of the three Arg residues, two Thr residues, and two Gly residues in the C-terminal domain of PhoB results in the loss of its DNA-binding capacity, indicating the important role of these residues in the interaction of PhoB with the target DNA.

The residue Glu-177 is likely involved in the interaction of PhoB with RNA polymerase, since its substitution for Lys eliminates the ability of the protein to induce the transcription of the *pho* genes, without altering the DNA-binding and the phosphate-receptive abilities of PhoB [4, 35]. Sola *et al.* [36] determined the three-dimensional crystalline structure of the N-terminal receptive domain of PhoB, and Okamura *et al.* [37] determined the structure of the C-terminal regulatory domain. There is evidence that the N-terminal domain can block the C-terminal domain, thereby inhibiting the oligomerization and the DNA-binding ability of PhoB. The reception of a signal from the PhoR sensory protein gives rise to the following intramolecular events: the phosphorylation of Asp-53 eliminates the blockade of the C-terminal domain and thereby allows the dimerization of the N-terminal domain and restores the ability of the C-terminal domain to interact with the promoter region of DNA [38, 39].

Thus, signal transduction from the membrane to the genetic apparatus of cells occurs through the autophosphorylation of the C-terminal domain of the sensor protein, which then transfers its phosphoryl group to the N-terminal domain of the regulator protein. This results in an altered ability of the latter protein to influence gene transcription. The other known sensory–regulatory systems of bacteria respond to external signals in a similar way. These are the NtrB/NtrC and EnvZ/OmpR systems of *E. coli*, which regulate the nitrogen metabolism and the osmoadaptation of this bacterium [40, 41], and the VirA/VirG system of *Agrobacterium tumefaciens*, which regulates the transfer of bacterial DNA into plant cells [42].

The Mechanism of the Interaction of the PhoB Response Regulator of E. coli with Promoters

The transcription of the Pho regulon is catalyzed by the major form of RNA polymerase, containing the σ^{70} subunit. To be initiated, the transcription requires the PhoB regulator protein, which serves as the inducer and the repressor at a time [1, 7]. Correspondingly, the promoters of the Pho regulon genes contain elements which are recognizable both by the σ^{70} subunit of RNA polymerase and by the PhoB protein. The *pho* gene promoters have a conservative sequence in the –10 region, which is necessary for interaction with the σ^{70}-containing RNA polymerase and lack the conservative sequence in the –35 region (Fig. 1) [45, 46]. On the other hand, the latter region of all of the Pho regulon



The consensus sequence of the *pho* box in *E. coli*

CTGTCATA(A/T)A(T/A)CTGTCA(C/T)

Fig. 1. Promoters of the Pho regulon genes in *E. coli*. The sites of initiation of mRNA synthesis are given in bold letters; the *pho* boxes are additionally underlined. The consensus sequence of the *pho* box is reproduced from [4].

genes contains a specific sequence, the so-called *pho* box, which is responsible for interaction with the PhoB regulator protein. The *pho* box is located 10 nucleotides upstream of the –10 region and represents a sequence of 18 nucleotides with two identical 7-nucleotide repeats separated by the AT-linker (Fig. 1) [4]. The phosphorylated PhoB (PhoB~P) recognizes this sequence and interacts with it, thereby initiating gene transcription [7]. The promoters of some Pho regulon genes, such as the *pst* and *ugp* operon promoters, have several *pho* boxes. For the expression of these genes to be maximal, the response regulator must interact with all of these *pho* boxes. The response regulator interacts with DNA in the form of a dimer, whose subunits interact with different nucleotide repeats, thereby enhancing the DNA-binding capacity of PhoB~P [38, 45].

The binding of the PhoB response regulator to the *pho* boxes was confirmed experimentally, both in vitro and in vivo, for some genes of the *E. coli* Pho regulon, namely, *phoA*, *phoB*, *pstS* [7, 43], *phoE* [13], *phnC* [46], *ugpB* [18], and *phoH* [44].

The fact that RNA polymerase fails to bind to the Pho regulon gene promoters in the absence of the functionally active (phosphorylated) PhoB response regulator suggests that PhoB~P enhances the interaction of RNA polymerase with the promoter lacking the –35 region, which is necessary for interaction with the σ^{70} subunit of RNA polymerase. To determine the subunit of RNA polymerase that is responsible for interaction with PhoB, Makino *et al.* [47] obtained the *rpo* mutants defective in the expression of *pho* genes. The *rpoD* mutants had substituted amino acids at the C-terminus of the σ^{70} subunit of RNA polymerase. The respective mutant holoenzymes could efficiently transcribe the PhoB-independent genes *tac, trp, mel*, and *lac*, but none of the studied genes of the Pho regulon (*phoE, pstS, phoA, ugpB*, and *phoK*). Based on these data, Makino *et al.* suggested that PhoB~P, when bound to the *pho* box, directly interacts with the C-terminus of the σ^{70} subunit of RNA polymerase. The interaction likely involves the residues Glu-575 and Asp-570, since point mutations at these amino acid residues (the substitutions $Glu^{575} \longrightarrow Lys^{575}$ and $Asp^{570} \longrightarrow Gly^{570}$) did not influence the initiation of transcription from the PhoB-independent promoters but blocked transcription from the *pho* gene promoters. The interaction of PhoB with the σ^{70} subunit of RNA polymerase compensates for the absence of the interaction of this subunit with the –35 regions of the *pho* promoters. The stretch of 10 nucleotides between the –10 region and the *pho* box is necessary for the interaction of the PhoB protein with the first helix turn of the helix–loop–helix structure of the 4.2 domain of the σ^{70} subunit of RNA polymerase [47]. These inferences were derived from experiments with mutant RNA polymerases, whose σ^{70} subunits contained various deletions in their C-termini [48]. When bound to a promoter, the PhoB protein alters the DNA conformation, thereby enhancing the interaction of the σ^{70} subunit of RNA polymerase with the promoter [49].

Regulation of the Expression of the E. coli Pho Regulon Genes

The expression of the *E. coli* Pho regulon genes is subject to both positive and negative controls [1]. The sensory protein PhoR is involved in both types of control, either in the form of the repressor $PhoR^R$ or in the form of the activator $PhoR^A$ of the PhoB response regulator. $PhoR^R$ is formed when Pi is in excess. This process requires the presence of the PhoU mediator and the intact Pst system. $PhoR^A$ is formed under phosphorus

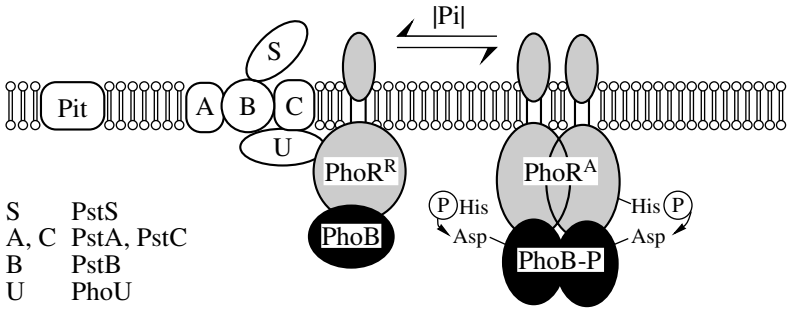


Fig. 2. Diagram schematically representing the regulation of the expression of the Pho regulon genes in *E. coli*. When phosphate in the medium is in excess, the expression of the Pho regulon genes is repressed due to formation of the repressor complex, which includes the regulatory proteins PhoR and PhoU and the Pst proteins. Under phosphorus starvation conditions, when the PstS protein is no longer saturated with Pi, conformational alterations in the Pst system eliminate $PhoR^R$ from the repressor complex, which leads to the spontaneous formation of the activator $PhoR^A$. $PhoR^A$ is autophosphorylated and then transfers its phosphoryl group to PhoB, forming PhoB~P. The phosphorylated PhoB binds to the target sequences of promoters and induces the transcription of the Pho regulon genes [52].

starvation conditions. In spite of the fact that the repression of the *pho* genes requires the functional Pst system, it does not depend on phosphate transport. For instance, Cox *et al*. showed that the missense mutation in the PstA protein impairs phosphate transport but does not influence *pho* gene repression [50]. The Pho regulon genes are derepressed, with the involvement of $PhoR^A$ and PhoB, when the concentration of Pi in the medium falls below 4 mm. The expression of the Pho regulon genes does not depend on the intracellular concentration of Pi, which comprises 9–13 mM under an excess of Pi in the medium and remains at a sufficiently high level (~7 mM) even under a deficiency of Pi [51].

Figure 2 schematically represents the Wanner model of the expression of the Pho regulon genes at different concentrations of phosphate in the medium [52]. The model is based on the results of the genetic and biochemical studies of the PhoB and PhoR proteins. When phosphate in the medium is in excess, the expression of the Pho regulon genes is repressed due to formation of the repressor complex, which includes the PhoR and PhoU proteins and all four proteins of the Pst system (PstA, PstB, PstC, and PstS). The complex is formed only when the Pi-binding protein PstS is completely saturated with phosphate. The binding of Pi to the PstS protein provokes conformational alterations in the Pst system. These alterations are transmitted, via the PhoU protein, to PhoR, which transforms to the repressor form $PhoR^R$. The interactions between the Pst carrier and PhoR or between PhoU and PhoR are essential for the formation of the repressor ($PhoR^R$). $PhoR^R$ can inactivate PhoB~P by means of its dephosphorylation. Under phosphorus starvation conditions, when the PstS protein is no longer saturated with Pi, conformational alterations in the Pst system eliminate $PhoR^R$ from the repressor complex, which leads to the spontaneous formation of the activator $PhoR^A$. $PhoR^A$ is autophosphorylated at the histidine residue. Then the phosphoryl group is transferred from the histidine residue of $PhoR^A$ to the aspartate residue of PhoB, forming PhoB~P. The phosphorylated protein PhoB~P binds to DNA, inducing the transcription of the Pho regulon genes [52].

Cross Regulation of the Pho Regulon Genes

This type of regulation of the *pho* genes is independent of extracellular phosphate. Cross regulation implies that the regulator protein of a two-component regulatory system is controlled by another regulatory system. The hypothesis of cross regulation was advanced by Wanner to explain the finding that acetyl phosphate (an intermediate of phosphorus metabolism that serves as a donor of a phosphate group for ATP) is an inducer of the Pho regulon [53]. Acetyl phosphate controls the Pho regulon due to changes in the proportion between the concentrations of ATP (or other nucleotide phosphate) and acetyl phosphate. The expression of the Pho regulon genes is induced when this proportion decreases. There are two phosphate-independent regulatory systems which activate the PhoB protein in the absence of its native kinase, PhoR. Both systems are associated with the central metabolism of bacterial cells and both are regulated by carbon sources, albeit differently. One system functions during bacterial growth on glucose and requires the sensor protein CreC (PhoM) [54]. The other system functions during bacterial growth on pyruvate and is associated with the synthesis of acetyl phosphate, an intermediate of the phosphotransacetylase/acetate kinase metabolic pathway. PhoB is activated by acetyl phosphate either directly [55] or via one of the sensor proteins [56, 57].

In cells grown on glucose or acetate, i.e., when the intracellular concentration of acetyl phosphate is low, PhoB is activated with the involvement of histidine protein kinase, EnvZ, by a mechanism which is still unclear [57]. Presumably, EnvZ is autophosphorylated with acetyl phosphate supplying a phosphate group, after which the phosphate group is transferred to PhoB. Alternatively, EnvZ may catalyze the phosphorylation of PhoB with acetyl phosphate as the donor of a phos-

Table 2. The Pho regulon genes of *B. subtilis*

Gene or operon	Encoded product or function	References
phoP–phoR	Signal transduction system proteins	[84, 85]
phoA	Alkaline phosphomonoesterase	[86]
phoB	Alkaline phosphomonoesterase	[87, 88]
phoD	Alkaline phosphomonoesterase/phosphodiesterase	[89]
pstSACB$_1$B$_2$	Proteins of Pi transport system	[90]
tuaABCDEFGH	Biosynthesis of teichuronic acids	[91]
tagAB/tagDEF	Biosynthesis of teichoic acids	[92]
glpQT	Proteins of organic phosphate transport system	[78]
ydhF	Unknown	[78]

phate group. The possibility cannot also be excluded that acetyl phosphate acts as an effector molecule, which enhances either the autophosphorylation of EnvZ with ATP supplying a phosphate group or the transfer of the phosphate group from EnvZ~P to PhoB.

In the absence of PhoR, CreC, or acetyl phosphate, the response regulator PhoB can be activated in vitro by the foreign sensory kinase VanS, which is the transcriptional gene regulator responsible for the vancomycin resistance of *Enterococcus faecium* [56]. The cytoplasmic domain (including residues Met^{95} to Ser^{384}) of the sensory kinase VanS interacts with PhoB, thereby activating the *phoA* gene of the *E. coli* alkaline phosphatase. VanS~P efficiently transfers its phosphoryl group to PhoB (the degree of transfer reaches 90% in 5 min when the VanS/PhoB ratio is 1 : 4). Such an efficiency of the phosphoryl group transfer is comparable with that observed in the case of the PhoR–PhoB system [58]. Thus, cross regulation is a form of global control, which links various two-components regulatory systems to each other and to cell metabolism.

The Pho regulons have been revealed and investigated, to different degrees, not only in *E. coli* but also in other gram-negative bacteria, such as *Salmonella typhimurium* [25, 59, 60], *Shigella flexneri* [61], *Shigella dysenteriae*, *Klebsiella pneumoniae* [62], *Klebsiella aerogenes* [63], *Pseudomonas aeruginosa* [64, 65], *Agrobacterium tumefaciens* [66, 67], *Haemophilus influenza*, *Branhamella catarrhalis* [68], *Sinorhizobium meliloti* [69–71], *Bradyrhizobium japonicum* [72], *Acinetobacter* spp. [73, 74], *Vibrio parahaemolyticus* [75], *Vibrio cholerae* [76], and *Caulobacter crescentus* [77].

THE Pho REGULON OF *BACILLUS SUBTILIS*

The investigation of the *psi* genes of *B. subtilis* by two-dimensional electrophoresis showed that the cell response to phosphate starvation is determined by the genes of two regulons, the specific phosphate starvation–inducible Pho regulon and the general σ^B-dependent stress regulon [78]. The Pho regulon genes comprise a smaller portion of the *psi* genes of *B. subtilis.*

The Pho regulon of *B. subtilis* is organized in a way similar to that of *E. coli*, with differences related to specific physiological features of bacilli, in particular, the production of endospores, which involves more than 100 proteins, whose synthesis is coordinately regulated by a cascade of reactions with the involvement of σ-factors [79, 80]. In addition to the PhoP–PhoR signal transduction system of *B. subtilis*, which is homologous to the PhoB–PhoR system of *E. coli*, the former bacterium has two other regulatory systems: the multicomponent system of Spo proteins, which regulates spore formation [81], and the two-component ResD–ResE system, which regulates the aerobic and anaerobic respiration of bacilli [82, 83].

The Functional Proteins of the Pho Regulon of B. subtilis

The Pho regulon genes of *B. subtilis* encode more than 20 proteins (Table 2). The *phoA* gene codes for the major alkaline phosphatase (phosphatase A), which is synthesized by vegetative bacillar cells in response to phosphate starvation. The *phoB* gene codes for phosphomonoesterase, which is synthesized from two promoters, Pv (under phosphorus starvation conditions) and Ps (under the conditions of spore formation). The *phoD* gene, which is also expressed in response to phosphorus starvation, codes for the enzyme that possesses both phosphodiesterase and phosphomonoesterase activities. Furthermore, the Pho regulon contains genes of the *tuaABCDEFGH* operon, which are involved in the synthesis of teichuronic acids, the *tagAB* and *tagDEF* operons, which are involved in the synthesis of teichoic acids, the *pstSCAB$_1$B$_2$* operon, which codes for the phosphate transport system proteins (the Pi-binding protein PstS, the integral membrane proteins PstA and PstC, and the PstB$_1$ and PstB$_2$ ATPases), and the *phoP–phoR* operon, which codes for the regulator proteins

PhoP and PhoR. Recently, Antelman *et al.* have also described two new genes of the *B. subtilis* Pho regulon, *glpQ* and *ydhF* [78]. The *glpQ* gene is a component of the *glpQT* operon, encoding glycerol-3-phosphate permease and glycerophosphodiester phosphodiesterase, which are involved in the hydrolysis of deacylated phospholipids. The *ydhF* gene, located downstream the alkaline phosphatase gene *phoB*, is cotranscribed from the promoter of the latter gene. The function of the product of the *ydhF* gene is unknown.

The products of the Pho regulon of *B. subtilis* provide for all the necessary reactions of the adaptive response of this bacterium. Under phosphorus starvation conditions, *B. subtilis* cells enhance the uptake of extracellular Pi via the Pst system, secreting into the medium a set of hydrolytic enzymes (phosphomonoesterase and phosphodiesterase among them), which allow a wider range of phosphorus-containing substrates to be utilized. In response to phosphorus starvation, *B. subtilis* cells stop the synthesis of the cell-wall teichoic acids, which are rich in phosphate groups necessary for cell metabolism, and activate the synthesis of teichuronic acids [93, 94].

The Regulatory Proteins of the Pho Regulon of B. subtilis

The two-component PhoP–PhoR system of *B. subtilis* is the major regulatory system controlling the expression of the *pho* genes of this bacterium. The response regulator PhoP binds to gene promoters, either inducing or repressing gene transcription. Under an excess of Pi in the medium, the *phoP–phoR* operon is expressed at a low constitutive level. But when the concentration of extracellular Pi is below 0.1 mM, the transcription of the operon increases 3–4 times [90, 95]. The phosphate starvation–inducible expression of this operon depends on the concentration of its products, PhoP and PhoR.

The response regulator PhoP consists of 241 amino acid residues and has a molecular mass of 27.7 kDa [84]. The amino acid sequences of the PhoP regulator of *B. subtilis* and the PhoB regulator of *B. subtilis* are identical by 60%. The sensory protein PhoR of *B. subtilis* contains 579 amino acid residues, has a molecular mass of 50.3 kDa, and is identical to the PhoR sensor of *E. coli* by 47% [85]. The N-terminal domain of the *B. subtilis* PhoR is associated with the cell membrane and has a structure typical of most sensor kinases. Two hydrophobic transmembrane segments of PhoR flank a large periplasmic domain of this protein, which also contains a large cytoplasmic domain linking the N-terminal domain to the C-terminal highly conservative kinase domain. This domain was found only in the PhoR proteins of *E. coli* and *B. subtilis*. The large periplasmic and extended cytoplasmic domains of PhoR likely sense the external signal, thereby determining cell response to changes in the concentration of Pi in the medium [96].

PhoP is subject to the kinase and phosphatase activities of its sensory protein PhoR [96]. In the absence of PhoR, PhoP is only slightly activated. The in vivo study of the transcription of the *tua* operon showed that transcription from the promoter in a strain with a *phoR* deletion depends on the concentration of the phosphorylated form of PhoP. This indicates that PhoP can be phosphorylated by other kinases than PhoR or by low-molecular-weight donors of phosphate groups [94]. It should be noted, however, that acetyl phosphate cannot phosphorylate the PhoP protein in vitro [97].

The response regulators PhoP of *B. subtilis* and PhoB of *E. coli* are considered to be homologous based mainly on the observation that mutations in the genes of these proteins lead to repression of the Pho regulon genes. PhoP and PhoB belong to the OmpR subgroup of regulator proteins, whose DNA-interacting domain has the form of a wing and which require direct repeats in their target DNA [98]. Unlike the N-terminal and C-terminal domains of PhoB, those of PhoP do not mutually inhibit their functional activities. PhoP dimerizes and binds to the target gene promoters, irrespective of whether it is phosphorylated or not. Presumably, the C- and N-terminal domains of PhoP function independently, like the respective domains of the UhpA regulator of hexose phosphate transport in *E. coli* [99] and the BvgA regulator of virulence genes in *Bordetella pertussis* [100].

The second two-component system involved in the regulation of the expression of the Pho regulon genes of *B. subtilis* contains the regulator protein of cell response (ResD) and histidine protein kinase (ResE) [82, 95]. The respective genes *resD* and *resE*, together with the upstream genes of cytochrome *c* biogenesis (*resABC*), constitute the *resABCDE* operon. Initially, the role of the regulatory proteins ResD and ResE was believed to be restricted to their involvement in the aerobic and anaerobic respiration of *B. subtilis* [101]. Then, however, it was found that mutations in the *resD* and *resE* genes are pleiotropic and affect not only respiration but also spore formation, the ability to utilize carbon sources, and the expression of the Pho regulon genes, in particular, the gene encoding alkaline phosphatase [5].

The Pho regulon of *B. subtilis* is regulated not only by PhoP and PhoR but also by the Spo0A protein, a positive and negative transcriptional regulator [81] that is a component of the signal transduction system exerting the positive/negative regulation of some genes expressed in the postexponential growth phase, including genes responsible for cell competence [102], antibiotic synthesis [103], protease synthesis [104], and the initiation of spore formation [105]. The Spo0A response regulator is activated with the involvement of multiple kinases, through the intermediate phosphorylation of the Spo0F and Spo0B proteins [79, 80]. The phosphorylated protein Spo0A~P controls the expression of relevant genes either directly (by binding to

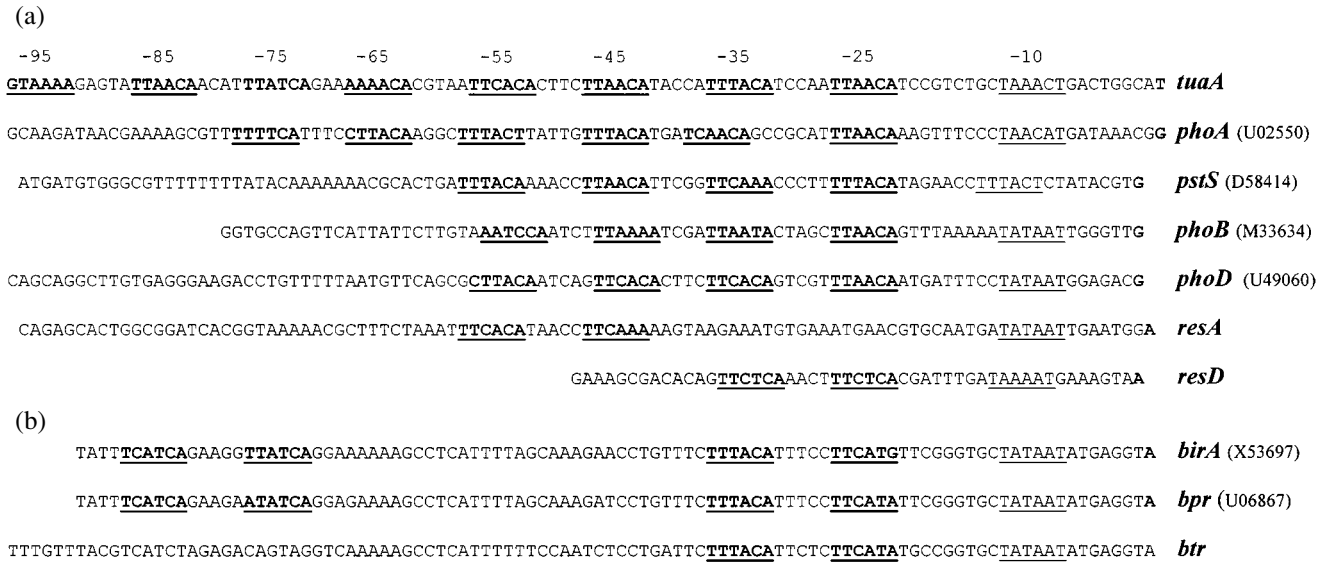


Fig. 3. Promoters of (a) the Pho regulon genes of *B. subtilis* and (b) the guanyl-specific ribonucleases of *B. intermedius* (*birB*), *B. pumilus* (*bpr*), and *B. thuringiensis* (*btr*). The sites of initiation of mRNA synthesis are given in bold letters; the TT(A/T/C)ACA-like hexanucleotides (the *pho* boxes) are additionally underlined. Parenthesizes are the GenBank accession numbers of sequences. The promoters of the *tua* operon, the *resA* and *resD* genes and the *btr* gene are reproduced from [91], [108], and [109], respectively.

their promoters) or indirectly (by regulating the transcription of the respective regulatory proteins). An example of such proteins is AbrB, which is a regulator of many genes expressed in the postexponential growth phase [106].

The Mechanism of the Interaction of PhoP with Promoters

The expression of the Pho regulon genes is regulated by means of the direct interaction of the activated regulator protein PhoP with promoters [5]. The –10 region of the *B. subtilis pho* gene promoters contains a consensus sequence recognizable by the σ^A-containing RNA polymerase. Although the –35 region does not contain a strict consensus motif, this region is involved in the recognition and binding of the PhoP regulator protein. The *pho* box necessary for the binding of PhoP~P contains two hexanucleotide fragments TT(A/T/C)ACA separated by a stretch of five nonconservative nucleotides [107]. Such a structure of the binding site implies that the regulator protein interacts with DNA in the form of a dimer. The promoter regions interacting with PhoP contain an even number (from two to eight) TT(A/T/C)ACA-like sequences (Fig. 3). This region is termed the core site of protein binding. DNA footprinting analysis showed that the interaction of PhoP with these regions are absolutely necessary for the activation of the transcription of the following Pho regulon genes: *phoB* [97], *phoA* and *pstS* [110], *tuaA* [91], *tagAB* and *tagDEF* [92], and *phoD* [107].

Thus, the binding sites of the PhoP protein of *B. subtilis* and the PhoB protein of *E. coli* are structurally similar: both sites contain direct repeats separated by nonconservative nucleotide sequences, although the number, nucleotide composition, and distance between these repeats in these two bacteria are different [91, 97].

The regulator protein PhoP is able to interact with the *pho* gene promoters in both the phosphorylated and nonphosphorylated forms, although the phosphorylated PhoP protein is much more active. For instance, PhoP~P binds to the *tua* gene promoter 10 times more efficiently than PhoP [91].

The molecular mechanisms of the interaction of PhoP~P with promoters are studied in detail with reference to the *phoA, pstS*, and *phoD* genes [107, 110]. It was found that, in addition to the core binding sites of the promoters of these genes, Pho~P also interacts with a region lying upstream of these sites in all three genes, as well as with a downstream region of the *phoA* and *pstS* gene promoters and with an internal site located in the coding regions of the genes. It is known that the structural genes of many regulator proteins have internal binding sites. The binding of regulator proteins to these sites usually represses transcription. This is most likely because, when bound to the coding region, the regulator protein blocks the motion of RNA polymerase. Surprisingly, PhoP~P interacting with the coding regions of genes induce their expression. Moreover, Liu and Hulett showed that the internal sites are necessary to provide for the maximum activity of promoters in vivo. Indeed, their deletion reduced promoter activity by 45% (in the case of the *pstS* promoter) and by 75% (in the case of the *phoA* promoter) [110]. To activate promoters, several PhoP~P molecules must coordinately interact with the internal sites.

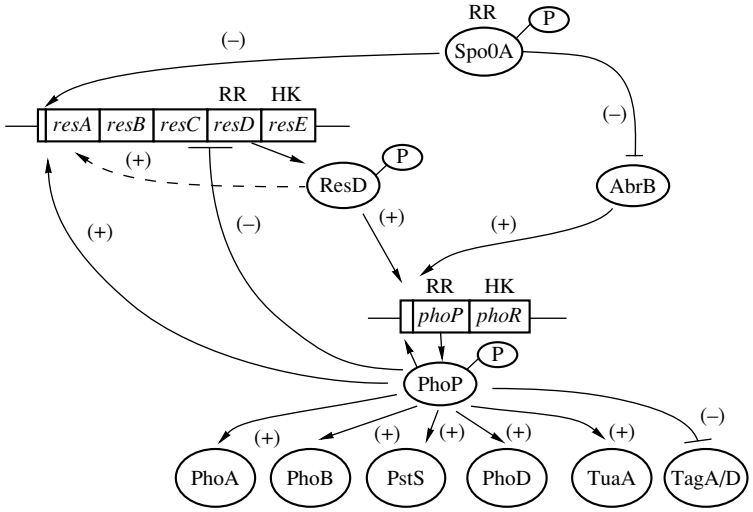


Fig. 4. Putative diagram showing the regulation of the expression of the Pho regulon genes in *B. subtilis.* The Pho regulon is under the control of three regulatory systems, PhoP–PhoR, ResD–ResE, and Spo0A. The PhoP and ResD proteins act as positive regulators. Spo0A functions as a negative regulator of the *pho* genes, repressing the regulator proteins AbrB and ResD. Solid lines show direct interactions. Dashed lines show interactions whose nature is not unambiguously established. Positive regulation is marked by the symbols (↑) and (+), and negative regulation is marked by the symbols (⊥) and (–). HK and RR stand for histidine protein kinase and response regulator, respectively. This diagram is confirmed by the most recent data of Zhang *et al.* [83] and Paul *et al.* [119].

The mechanism of interaction of the regulator protein PhoP with the *pho* genes is similar to that by which the UhpA protein (a regulator of hexose phosphate transport in *E. coli*) interacts with the *uhpT* gene promoter [99]. Two phosphorylated molecules of the regulator protein bound to the high-affinity binding sites of a promoter cause the cooperative binding of the third such molecule to a downstream low-affinity site. The binding of the third PhoP~P molecule to the low-affinity site is likely facilitated by DNA bending between the high- and low-affinity binding sites after the attachment of the first two PhoP~P molecules. In any case, the bound PhoP~P molecules turn out to be close to RNA polymerase, which is necessary for their interaction and the initiation of transcription. DNA bending (or, conversely, straightening) is a general mechanism for activation of prokaryotic gene expression [111]. Makino *et al.* [49] described it with reference to the response regulator PhoB of *E. coli.*

Of particular interest is the mechanism of the interaction of PhoP with the promoters of the *tagAB* and *tagDEF* operons, which code for proteins involved in the biosynthesis of teichoic acids, and the promoter of the *resDE* operon, for which PhoP serves as a repressor [92, 94, 108]. PhoP~P interacts with the core binding site of the promoter and involves in the interaction the site of initiation of mRNA synthesis and the coding region of genes extending to the +115 nucleotide in the case of the *tagA* gene, to the +168 nucleotide in the case of the *tagD* gene, and to the +125 nucleotide in the case of the *resD* gene. The presence of binding sites in the coding regions of genes is likely necessary for the repressor function of PhoP~P. PhoP binds to the *tagD* and *resD* gene promoters only in the phosphorylated form (each of these promoters contains only one PhoP-binding consensus sequence). At the same time, both PhoP and PhoP~P can bind to the *tagA* gene promoter, which has four repeats of consensus sequences. These sequences, unlike those of the other *pho* promoters, are located on the noncoding DNA strand.

Parallel rEgulatory Pathways Controlling the Expression of the B. subtilis Pho Regulon Genes

Signal transduction pathways in *B. subtilis* are not linear and represent networks, which receive external and internal signals to analyze them and to develop an appropriate cell response. Based on numerous experimental data, Hulett *et al.* suggested the existence of a combination of the PhoP–PhoR, ResD–ResE, and Spo0A signal transduction systems in *B. subtilis* cells grown under phosphorus starvation conditions (Fig. 4) [5, 108]. The first two systems serve as the positive regulators of the *pho* genes, and the third system as their negative regulator. Spo0A~P is a very efficient negative regulator, as is evident from the fact that the mutation of one of the genes responsible for the phosphorylation of Spo0A reduces the amount of the Spo0A~P produced but does not eliminate the repression of the *pho* genes.

The *resD* and *abrB* genes, coding for the activator proteins ResD and AbrB, belong to different regulatory pathways repressible by Spo0A~P. The role of these two proteins in the regulation of expression of the *pho* genes was studied using mutants defective in different regulatory loci. For instance, mutations in the *resD* and *abrB* genes reduce the expression of the alkaline phosphatase genes by 80 and 20%, respectively. In the dou-

ble *resD–abrB* mutants, the synthesis of alkaline phosphatase is inhibited by 95%. Therefore, the two positive regulators ResD and AbrB are responsible for almost complete induction of the Pho regulon. Conversely, mutations in *spo0A* and double mutations in *spo0A/resD* and *spo0A/abrB* enhance the synthesis of alkaline phosphatase by, respectively, 500, 200, and 300%. These data indicate that Spo0A~P represses the Pho regulon by decreasing the level of the regulatory proteins AbrB and ResD in bacterial cells.

In the absence of the phosphate deficiency–induced induction, the *phoP–phoR* operon is expressed at a low constitutive level. Phosphorus starvation conditions induce this operon; however, the induction requires the ResD and AbrB proteins, which directly bind to the operon promoter. The induction of the *phoP–phoR* operon by the regulator protein AbrB lasts for a short period of time, since nutrition starvation, including phosphate starvation, activates the Spo system, which produces Spo0A~P and thereby represses the transcription of the *abrB* gene and decreases the content of AbrB in cells.

Under phosphorus starvation conditions, PhoP~P induces the transcription of the *resABCDE* operon, one of the products of which (the regulator protein ResD) provides for an 80% induction of the *phoP–phoR* operon. PhoP~P directly binds to the promoter of this operon, thereby repressing the internal *resDE* promoter, which provides for a low level of synthesis of the ResD protein in the absence of repression by PhoP~P (i.e., under phosphorus sufficient conditions or in mutant strains). Under phosphorus starvation, PhoP~P enhances the expression of the *resABCDE* operon and raises the concentration of the ResD protein to a level at which the internal *resDE* promoter becomes unnecessary.

The complete induction of the Pho regulon genes provides for the functioning of the high-affinity system of phosphate transport encoded by *pstSCAB$_1$B$_2$*, and for the synthesis of degradative enzymes encoded by the *phoA*, *phoB*, and *phoD* genes (these enzymes are necessary to derive phosphate from teichoic acids and other substances) and teichuronic acids encoded by the *tuaA* gene, as well as suppresses the synthesis of teichoic acids encoded by the *tagD* gene and the internal *resDE* promoter. Under phosphorus starvation conditions, the *resABCDE* operon provides for the synthesis of heme *a* and cytochrome *c*, which are necessary for the normal functioning of the electron transport chain [82, 112] and, hence, the incorporation of Pi into ATP. The induction of the Pho regulon continues for several hours until the intracellular phosphate pool is exhausted. Then Spo0A~P represses the expression of the Pho regulon, thereby decreasing the level of the activator proteins AbrB and ResD in cells and initiating spore formation.

The Pho regulons of other bacillar species have not been described. Our investigations showed that the biosynthesis of secretory ribonucleases in most bacilli is induced under the conditions of extracellular phosphate deficiency [113–116] and is regulated coordinately with the biosynthesis of alkaline phosphatases [117]. The promoters of the genes encoding the secretory guanyl-specific ribonucleases of *B. intermedius* (*birA*), *B. pumilus* (*bpr*), and *B. thuringiensis* (*btr*) were found to contain *hexanucleotide tandem sequences similar to the *pho* boxes of the Pho regulon genes in *B. subtilis* (Fig. 3b). Transcriptional analysis with the use of mutant *B. subtilis* strains with deletions in the regulatory genes *phoP* and *phoR* of the Pho regulon showed that the expression of the *birA*, *bpr*, and *btr* genes is regulated by the two-component PhoP–PhoR signal transduction system [117, 118]. In the complex system of reactions with the involvement of high-molecular-weight RNAs, RNases precede phosphatases in the reactions which cleave RNAs to mono- and dinucleoside phosphates. In turn, the mono- and dinucleoside phosphates serve as substrates for phosphomonoesterases. Therefore, RNases are likely involved in the phosphate regulation system of bacilli, which allows them to coordinately regulate the biosyntheses of ribonucleases and phosphohydrolases. Two-component signal transduction systems homologous to the PhoP–PhoR system of *B. subtilis* may also function in *B. intermedius*, *B. pumilus*, and *B. thuringiensis*, regulating the expression of the genes whose products are involved in the metabolism of phosphorus-containing compounds.

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