Chapter 17

Molecular Mechanisms of Phosphate Homeostasis in *Escherichia coli*

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Additional information is available at the end of the chapter

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Abstract

Life's processes absolutely require inorganic phosphate for structural and energetic purposes. *Escherichia coli* has developed sophisticated mechanisms to acquire phosphate and to maintain intracellular amounts at optimal levels. The processes by which these simple cells maintain stable intracellular concentrations of phosphate are termed phosphate homeostasis, which involves mechanisms to balance the import, assimilation, sequestration, and export of phosphate. This chapter introduces the proteins involved in phosphate homeostasis and reviews information concerning the multiple phosphate transporters and the mechanisms by which they are regulated. It also introduces new concepts of how this bacterium responds to elevated extracellular levels of phosphate and presents a model for the integration of all of these processes to achieve homeostasis. The predominant importers are PitA, PitB, and the PstSCAB complex. Assimilation, or the incorporation of Pi into organic molecules, occurs primarily through the formation of ATP. Gene regulation relies on the PhoB/PhoR two-component system and the formation of a signaling complex at the membrane. The amount of intracellular phosphate can be fine-tuned through the formation or degradation of polyphosphate. Polyphosphate formation requires adequate supplies of ATP. In addition, when intracellular phosphate levels become too high, phosphate can be exported through PitA, PitB, or the YjbB transporters.

Keywords: phosphate homeostasis, ABC transporter, phosphate transporter, polyphosphate, two-component signal transduction

1. Introduction

Inorganic phosphate (Pi) is essential for life. For example, it is found in the hydrophilic ends of the amphipathic lipids in the cellular membranes that define the boundaries of a cell. Together with the sugars ribose or deoxyribose, it makes up the structural backbone of DNA or RNA through its phosphodiester bonds. The cell's energy currency is based upon the energy released from the hydrolysis of the phosphoanhydride bonds between the phosphates of ATP or of the other nucleotides. Moreover, the biochemical activities of many proteins are regulated by the phosphorylation of specific amino acids—histidine and aspartate in bacteria, as well as serine, threonine, and tyrosine.

Because of its essential roles, cells must maintain intracellular Pi pools at optimal levels. In bacteria, such as *Escherichia coli*, this is believed to be between 1 and 10 mM [1–3]. Pi is assimilated into biological molecules through the synthesis of ATP from ADP and Pi. The mechanisms to control intracellular Pi levels include multiple transport proteins with characteristic patterns of expression, different affinities for Pi, and rates of Pi transport [4]. *E. coli* cells also employ a well-studied sensory transduction system that monitors extracellular Pi levels to control the expression of genes for scavenging Pi under limiting conditions and to utilize alternate phosphorous sources. Additionally, there are also metabolic reactions that control the amount of polyphosphate, a Pi storage compound.

The primary Pi importers in *E. coli* are PitA, PitB, and PstSCAB [5]. PitA and PitB are secondary transporters that bring neutral metal-Pi complexes into the cell at the expense of a proton [6, 7]. PstSCAB is a Pi-specific ABC transporter that imports Pi at the expense of ATP hydrolysis [8, 9]. Proteins that export Pi include PitA, PitB, and GlpT, which is a glycerol-3-phosphate:Pi antiporter [10], UhpT, which is a hexose-6-phosphate:Pi antiporter [11], and potentially YjbB, which has been suggested to be a Pi exporter [12]. The signal transduction system that controls gene expression in response to limiting extracellular Pi levels has at its heart the histidine kinase PhoR and the response regulator PhoB [4, 13]. When PhoB receives a phosphoryl group from PhoR, it binds to DNA and activates the transcription of a number of genes for the high-affinity acquisition of Pi (including the PstSCAB transporter) and the utilization of alternate sources of phosphorous [14–17]. At least 31 genes have been shown to be directly controlled and positively regulated by PhoB. They are called the Pho regulon and include *phoA*, which encodes the periplasmic enzyme alkaline phosphatase, *pstSCAB*, *phoB*, and *phoR* [4]. Alkaline phosphatase removes phosphoryl groups from organophosphate molecules. The members of the Pho regulon that are involved in utilizing alternate phosphorous sources are *ugpBAECQ*, which encodes a glycerol-3-phosphate ABC transporter and a phosphodiesterase and *phnCDEFGHIJKLMNOP*, which encodes a phosphonate transporter and enzymes of a C-P lyase complex that produces a phosphoribosyl product from imported phosphonate. Phosphonates are compounds that contain a carbon-phosphorous bond. In addition to the 31 genes that have been demonstrated to be directly controlled by PhoB [4, 18], 2D-polyacrylamide gels and computational methods suggest that possibly 400 proteins may be controlled directly or indirectly by PhoB [19, 20]. These include genes that are both up- and down-regulated.

The presence of the PhoBR signal transduction system underscores the need for maintaining a minimal intracellular level of Pi when extracellular Pi is limiting. That too much intracellular Pi can also be a problem is underscored by the phenotype of a *phoU* mutant [21]. *phoU* is the fifth gene in the *pstSCAB-phoU* operon and its function is to control the activity and the amount of the PstSCAB transporter [22]. It has been shown that *phoU* mutations cause a severe growth defect, probably because these cells become poisoned by too much intracellular Pi [21, 23, 24]. Taken together, these observations suggest that *E. coli* cells possess homeostatic mechanisms that maintain intracellular Pi levels within an optimal range. It is the purpose of this chapter to introduce the reader to the principle players involved in Pi homeostasis and to highlight advances in our understanding of the mechanisms involved.

2. The multiple Pi importers

E. coli is capable of using multiple transporters to bring Pi into cells. Three of them, PitA, PitB, and PstSCAB, are individually capable of supporting growth with Pi as the only source of phosphorous [6]. The others, GlpT, UhpT, and PhnCDE, are capable of secondarily importing Pi but are not able to support growth when the sole source of phosphorous is Pi [5]. GlpT primarily transports glycerol-3-phosphate, UhpT transports hexose-6-phosphates and PhnCDE brings phosphonates into the cell. Complicating many of the early studies on Pi transport was the use of the K10 strain of *E. coli*, which harbored a G220D mutation in the *pitA* gene [25]. The interpretations of some of the genetic and biochemical studies of Pi transport in these strains are therefore difficult because many early strains contained compensatory mutations in other genes that restored growth on Pi [21].

2.1. PitA and PitB—the low-affinity Pi importers

The low-affinity PitA and PitB transporters utilize the energy stored in the proton-motive force to bring neutral metal-Pi complexes into the cell [6, 25, 26]. These homologous proteins each contain 499 amino acid residues and show 80.8 and 89.8% sequence identity and similarity, respectively (see **Figure 1A**). Amino acid identities between the two proteins are indicated by vertical lines and similarities are indicated with two dots. A membrane topology model for these two proteins was created using the SCAMPI2 web server [27] and is shown in **Figure 1B**. This model predicts that PitA and PitB have 10 transmembrane helices with the N- and C-termini facing the periplasm. The sequences of the predicted transmembrane helices are surrounded by green boxes in **Figure 1A**. Support for this N_{out}-C_{out} topology model comes from a recent paper in which the authors tagged the C-termini of 601 inner membrane proteins from *E. coli* with alkaline phosphatase and green fluorescent proteins (GFPs) [28]. Because alkaline phosphatase is only active in the periplasm and GFP is only fluorescent in the cytoplasm, they concluded that PitA and PitB have a C_{out} topology [28]. These two proteins show very high levels of amino acid identity and similarity within the predicted 10 transmembrane segments (91.4 and 96.7%, respectively). The greatest degree of divergence is found in a putative 127-amino acid cytoplasmic loop domain (L7) located between helices 7 and 8. This loop shows 59.1% identity and 75.6% similarity between the two proteins suggesting that it may contribute to differences in protein stability, potential binding partners, or means of regulation.

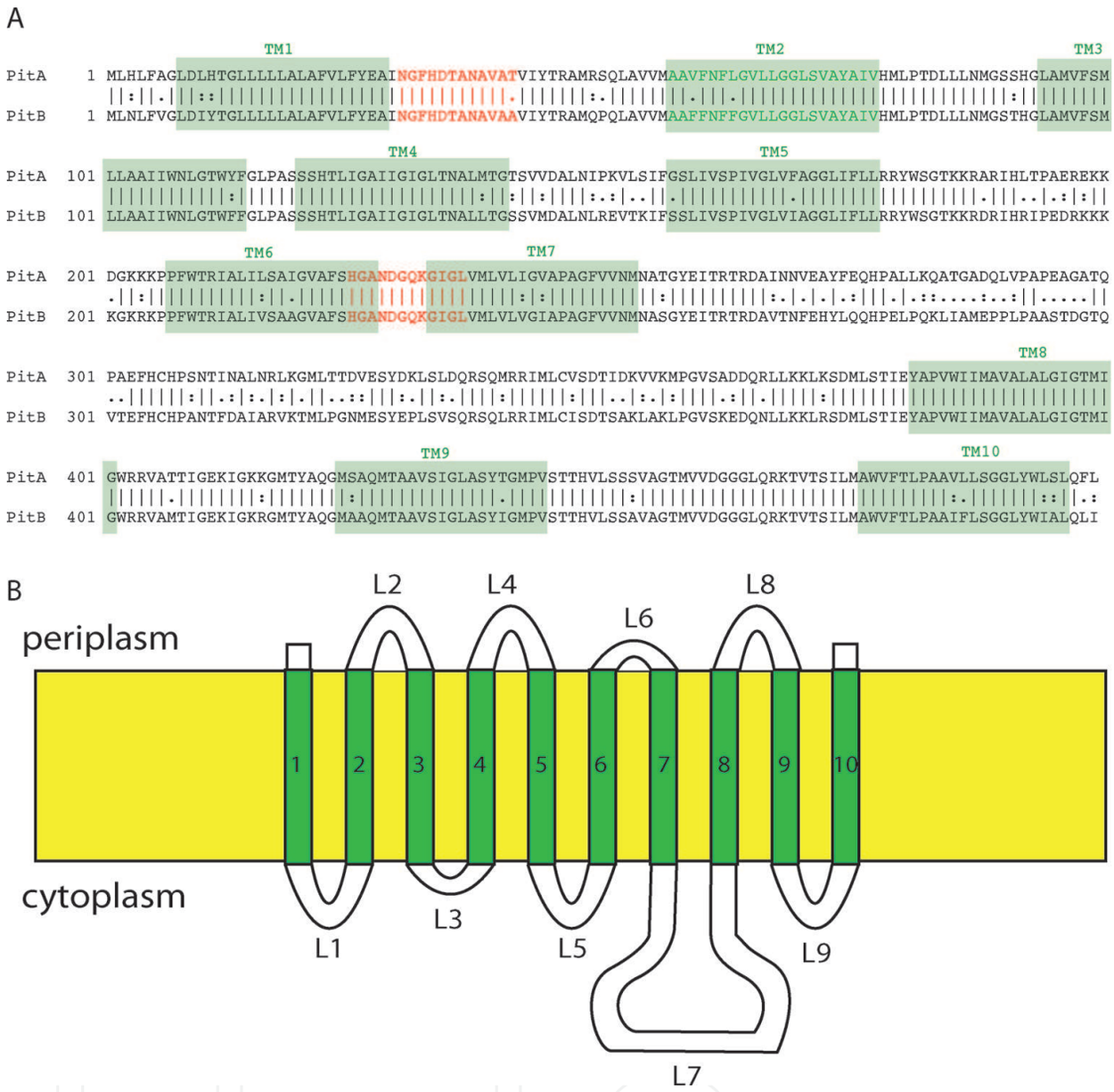

Figure 1. Sequence alignments of PitA and PitB with an accompanying topology model. (A) PitA and PitB amino acid sequences are given in one-letter code and are aligned. The alignment was made using the European Molecular Biology Open Software Suite (EMBOSS) [29]. Predicted transmembrane helices are boxed in green and the conserved signature motifs are marked with a red font. (B) Topology model of PitA and PitB. The model includes an N_{out}-C_{out} topology. The predicted transmembrane helices are labeled TM1–TM10 and the connecting loops are labeled L1–L9.

Analysis of the kinetic properties of Pi uptake in whole cells where *pitA* and *pitB* were expressed from the pBR322 plasmid showed that the PitA protein has a K_m^{app} of 1.9 μM and a V_{max}^{app} of 58 nmol of Pi minute^{-1} milligram (dry weight)$^{-1}$, whereas the values for PitB are 6 μM and 67 nanomoles of Pi minute^{-1} milligram (dry weight)$^{-1}$ [6].

It was originally thought that *pitA* expression was constitutive, but it has recently been shown that it is positively regulated by the availability of Zn(II) and also by limiting Pi [7]. *pitB* expression appears to be repressed when cells are grown in limiting Pi conditions [25], so its

function may be more important during growth in Pi-replete environments. The mechanisms for the regulation of these genes are not known.

PitA and PitB are members of the PiT family of Pi transporters within the transporter classification database (TC #2.A.20) [30] and the PHO4 family within the Pfam database [31]. These families include bacterial, archaeal, and eukaryotic members, suggesting that these Pi transporters developed early in evolution and that they continue to play important functions in all domains of life. A conserved signature sequence has been identified in both the N- and C-terminal ends of these transporters that has the common core sequence of **G**(**A**FGST)(**N**H)(**D**N)(**V**ATIG)(**A**QSG)(**N**KA)(**A**STG)(IMVF)(GAS)(TPIL), with the bolded amino acids representing the most common amino acids at that position. This signature sequence is highlighted with red letters in **Figure 1A**. The human proteins from this family are thought to be involved in housekeeping functions and are called PiT1 and PiT2, whereas the *Neurospora crassa* and *Saccharomyces cerevisiae* members are called Pho-4 and Pho89, respectively [32, 33]. Mutations in the signature sequence of the PiT2 protein block Pi transport [34]. In addition to their role in Pi transport, the PiT1 and PiT2 proteins are also receptors for the gamma-retroviruses [32]. This protein family includes both Na^+-dependent and H^+-dependent Pi symporters. PiT1, PiT2, Pho-4, and Pho89 are sodium-dependent transporters, whereas PitA, PitB, and the Pht2_1 proteins from *Arabidopsis thaliana* are proton-dependent Pi symporter [35].

It has recently been suggested that neither PitA nor PitB play primary roles in Pi transport, but function instead for the purpose of metal ion transport [4]. However, considering the homologies between PitA and PitB with other Pi transporters from other organisms, it seems unlikely that they are retained in this genome primarily to function as transporters of divalent metal cations, which have their own primary transporters, as well [36]. Clearly, further work is needed to better understand the roles of PitA and PitB in Pi homeostasis.

2.2. PstSCAB—the high-affinity Pi importer

The PstSCAB protein is a high-affinity Pi transporter that has a K_m of 0.4 μM Pi and a V_{max} of 16 nmol Pi mg (dry weight)$^{-1}$ min^{-1} [37]. It is a member of the ATP-binding cassette (ABC) superfamily from the transporter classification and Pfam databases [30, 31]. This protein superfamily employs the hydrolysis of ATP to bring a variety of substrates across biological membranes, both as importers and as exporters [38]. Members of this protein superfamily are found among the bacteria, archaea, and eukaryotes. Prokaryotic importers, such as the PstSCAB protein, utilize an extra-cytoplasmic substrate-binding protein that binds substrates and presents them to their membrane-spanning proteins [39]. PstS is the periplasmic substrate-binding protein. PstC and PstA compose the membrane-spanning components of the transporter [40, 41]. The most highly conserved feature within the superfamily is the nucleotide-binding domain, also called the ATP-binding cassette, which binds ATP, hydrolyzes it, and then releases it in order to provide the energy for transport [42]. PstB contains the nucleotide-binding domain for this transporter [43]. The crystal structures of several ABC importers have been solved, which has shed some light onto the mechanisms of transport [44]. Of particular note is the structure of the putative molybdate transporter, called ModABC, from the archaeon *Archaeoglobus fulgidus* [45]. Like the PstSCAB transporter, this protein also imports an

oxyanion. A clue to understanding the mechanisms of Pi transport through the PstSCAB protein comes from sequence similarities between the molybdate, sulfate, and Pi transporters. The most highly conserved sequences within this group are found in a region of the protein that creates a cavity within the membrane-spanning region and a gate that most likely represents the pathway through which the substrate must pass. The published ModABC structure is of the protein in a nucleotide-free conformation and shows 12 transmembrane helices situated in an inward-facing conformation with the gate at the periplasmic surface of the membrane. It has been proposed that PstSCAB, like other transporters in this superfamily, utilizes an alternating access mechanism to transport their substrates in which they alternate between inward- and outward-facing states that are driven by substrate binding, ATP hydrolysis, ADP release, and subsequent ATP binding (see **Figure 2**) [44]. ATP binding across the PstB dimer interface would be predicted to close the cavity and lead to an outward-facing structure that can receive Pi from the substrate-loaded, periplasmic PstS protein. This event would trigger ATP hydrolysis that would flip the outward-facing transmembrane components to an inward-facing conformation, thereby opening the gate and allowing Pi to gain access to the cytoplasm. The cycle would be continued as ADP is released and ATP is rebound.

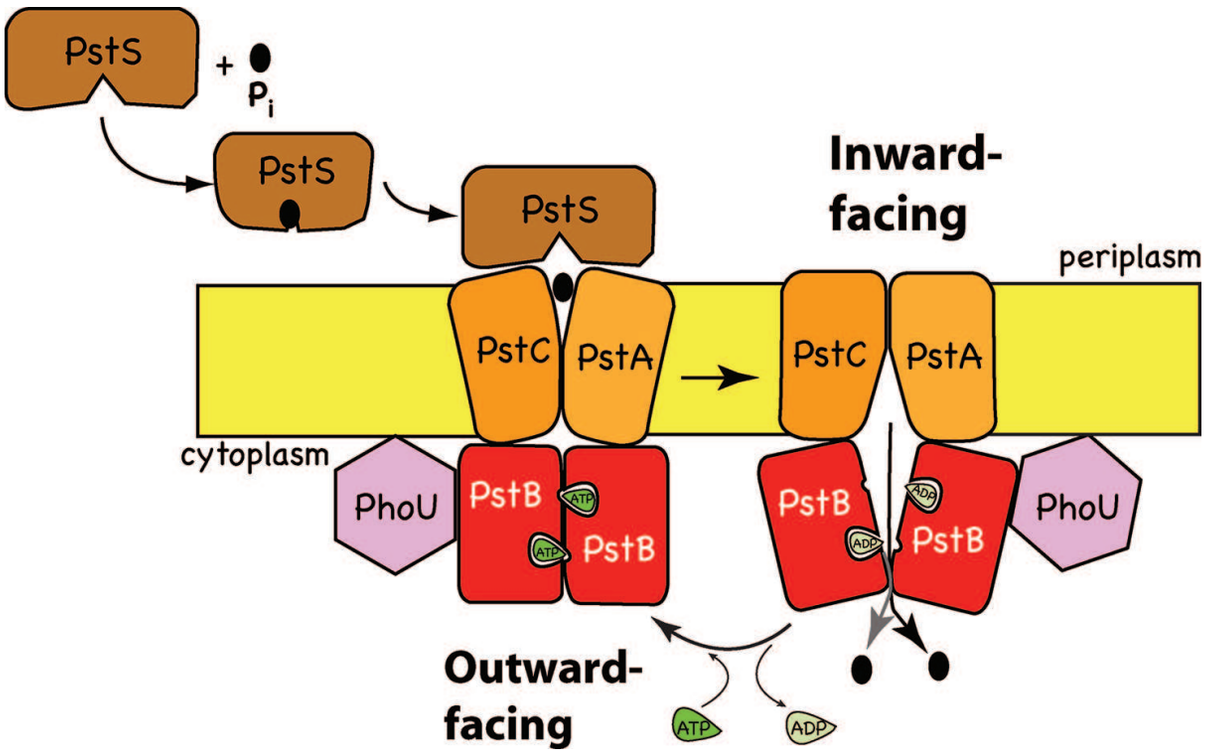

Figure 2. Model of the mechanism of Pi import through the PstSCAB transporter. Free Pi is bound within the periplasm and presented to the outward-facing PstCAB proteins. This docking triggers ATP hydrolysis, which causes a conformational change that triggers the adoption of an inward-facing conformation. The transported Pi is then released into the cytoplasm, as well as the Pi from the hydrolysis of ATP. The transporter is reset as PstB binds ATP again. The PhoU protein interacts with the PstB protein and slows transport when cytoplasmic Pi concentrations are high.

The Pst transporter is most highly expressed when environmental Pi levels are low. For this reason, it was assumed that it played its most important role in Pi transport under those conditions. More recently, it has been proposed that it plays the primary role in Pi transport

under all conditions [4]. The expression of the *pstSCAB* genes is controlled by the PhoBR two-component system described below. The primary promoter for this operon, and the one which is regulated by Pi levels, is found upstream of the *pstS* gene [46]. Other promoters that are internal to the operon have been identified upstream of the *pstC*, *pstB*, and *phoU* genes and are rather weak; but they may play a role in maintaining a basal level of the PstSCAB transporter under Pi-replete conditions [47].

3. The two-component signal transduction system for Pi homeostasis

Two-component-signaling systems constitute the most common signaling pathways in bacteria [48]. These pathways regulate many important cellular processes ranging from cell development and virulence, to motility and metabolism, and most species have over 10–20 different two-component systems [49]. Most frequently, they are composed of receptors on the cell periphery and signal-processing components and targets in the interior of the cell. These pathways rely on a phospho-transfer reaction between the histidine residues of sensor kinases, which generally receive input from the cell surface, and a conserved aspartate residue within the response regulators, which are located in the cytoplasm [50]. Response regulators are most frequently, but not always, transcription factors that interact with RNA polymerase [51].

3.1. PhoB and PhoR—the transcription factor and the histidine kinase

In *E. coli*, gene regulation in response to limiting Pi concentrations depends on the function of seven proteins: the two-component regulatory proteins PhoB and PhoR, as well as the Pst transporter, PstSCAB, and an auxiliary protein PhoU [4]. The hub of this signaling pathway consists of the PhoB and PhoR proteins. PhoB is the response regulator that has an N-terminal receiver domain (Pfam: PF00072, response_reg) and a C-terminal DNA-binding domain (Pfam: PF00486, trans_reg_c). This particular domain architecture represents the largest group of response regulators [31]. The receiver domain has a doubly wound α/β-fold with a central five-stranded beta-sheet [52]. This domain contains the site of aspartyl phosphorylation, which in PhoB is Asp53. The receiver domain of PhoB contains the necessary catalytic residues to transfer a phosphoryl group from the phospho-histidine residue of phospho-PhoR [17]. The C-terminal DNA-binding domain has a winged-helix structure [53]. When PhoB becomes phosphorylated, it forms a dimer that binds to DNA sequences, called pho boxes [17, 53–55]. These short sequences are located upstream of Pho regulon genes to recruit RNA polymerase and initiate transcription by remodeling the RNA polymerase holoenzyme-DNA complex [53, 54, 56].

PhoR is a homodimeric, bifunctional histidine autokinase/phospho-PhoB phosphatase. When environmental Pi is limiting, it autophosphorylates on a conserved histidine residue and subsequently donates this phosphoryl group to PhoB, but when Pi is plentiful, it removes the phosphoryl group from phospho-PhoB [57, 58]. PhoR is an integral membrane protein that is not predicted to contain a significant periplasmic domain but does contain a membrane-spanning region, a cytoplasmic charged region, a Per-ARNT-Sim (PAS) domain (Pfam: PF00989, PAS) [59], and prototypical dimerization/histidine phosphorylation (DHp; Pfam:

PF06580, His_kinase) and catalytic ATP-binding (CA; Pfam: PF02518, HATPase_c) domains at its C-terminus (see **Figure 3**) [57]. PAS domains generally function in signal perception activities [60]. Since PhoR does not contain a significant periplasmic sensory domain, it is assumed that its PAS domain senses a cytoplasmic signal that reflects extracellular Pi concentrations, but the nature of the signal is not completely known. The CA domain harbors the enzymatic activity for transferring a phosphoryl group from ATP to the conserved histidine residue of the DHp domain. The DHp domain consists of a four-helix bundle with the conserved phospho-accepting histidine residue being positioned midway on one face of one of the helices. It has been shown that phosphorylation of PhoR occurs in cis, where the CA domain of one of the monomers phosphorylates the His residue of the same polypeptide chain [61]. The DHp domain also contains all of the residues necessary for phospho-PhoB phosphatase activity [57]. We propose that the control of the opposing kinase and phosphatase activities of PhoR involves the constraint of the CA domains to prevent their access to the DHp domain and simultaneously exposing the residues of the DHp domain that are required for phosphatase function (see **Figure 3**). If this proposal is correct, then how are the interactions between the different PhoR domains controlled?

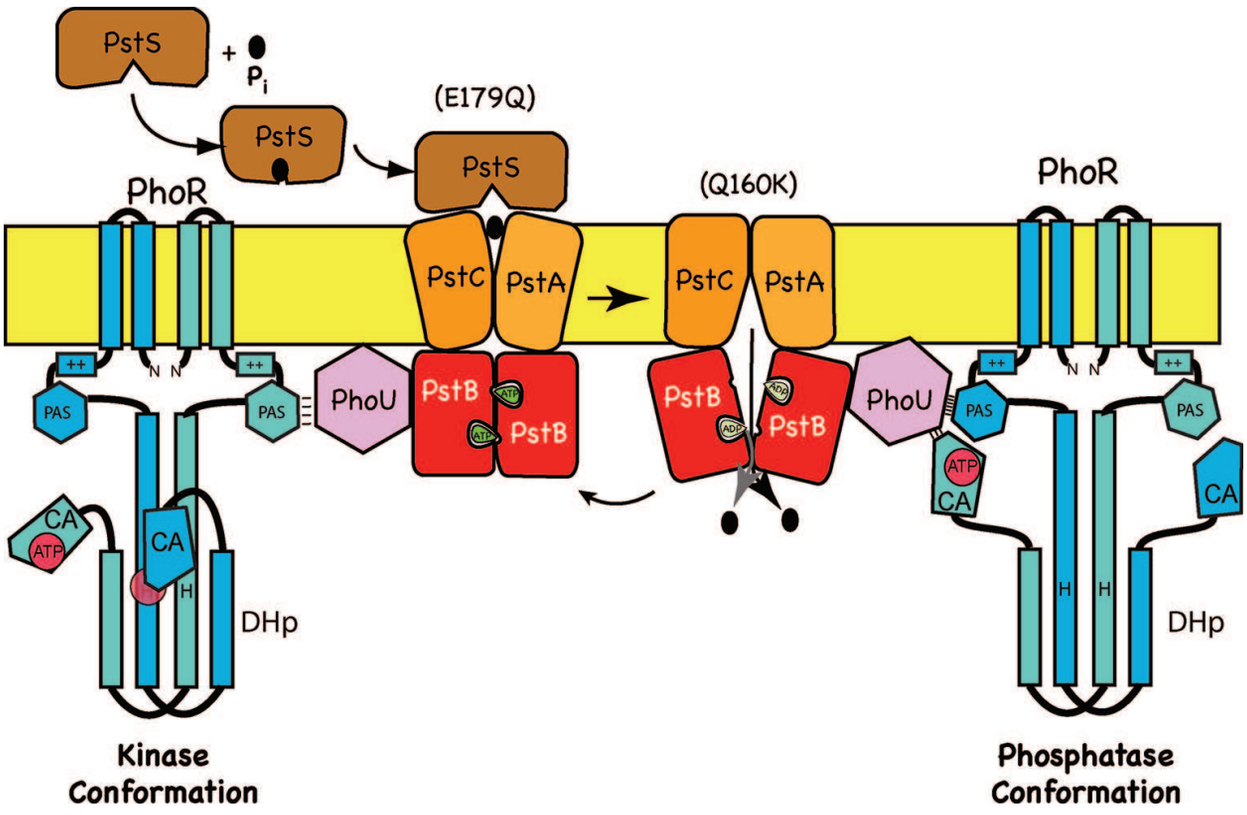

Figure 3. A signaling model involving different conformations of PstSCAB and PhoR. As the Pst transporter switches between its inward- and outward-facing conformations during Pi transport, it interacts differently with PhoR, depending upon its conformation. This interaction is mediated by PhoU. The inward-facing conformation, which is stabilized by the *pstB*Q160K mutation, interacts with PhoR to constrain its CA domain in order to stabilize the phosphatase conformation of PhoR. The outward conformation, which is stabilized by the *pstB*E179Q mutation, does not interact with the CA domain and favors the kinase conformation of PhoR, which allows the CA domain to bind ATP and autophosphorylate its DHp domain.

3.2. PstSCAB—the sensor of extracellular Pi

In addition to its role in Pi transport, the Pst transporter is also required for signal transduction. Because PhoR does not have a periplasmic domain, it has been assumed that this transporter is the ultimate sensor of extracellular Pi [5]. In fact, if any of the Pst proteins are absent, the Pho regulon becomes unregulated, leading to the overexpression of Pho regulon genes [5]. Thus, the default biochemical activity of PhoR is an autokinase and the role of the Pst transporter is to negatively regulate this activity and to stimulate its phospho-PhoB phosphatase activity. There are two possibilities for how the Pst protein may function to control the activity of PhoR. The first is by controlling intracellular Pi levels. If PhoR senses intracellular Pi, most likely through its PAS domain, then the Pst system may function by controlling the amount of Pi within the cell. This model seems unlikely for two reasons. Intracellular Pi has been measured by phosphorous nuclear magnetic resonance (^{31}P NMR) and has been shown to be constant under conditions in which the Pho regulon is both repressed and derepressed [2]. Also, there are several mutations in *pstC* and *pstA* that lead to defective transporters, but that retain their signaling capacity, that is, they can still stimulate the phospho-PhoB phosphatase activity of PhoR [40, 41]. The second model for how the Pst transporter functions in signal transduction is that PhoR may somehow sense its transport activity [62]. That is to say, it is not the intracellular level of Pi that is sensed, but how active the transporter is. Support for this model is provided below.

3.3. PhoU—the adaptor protein

In addition to the PstSCAB protein, PhoU is also required for Pi-signal transduction, but not for transport through the complex [21]. When *phoU* is mutated or deleted, PhoR is constitutively active as an autokinase leading to high-level expression of Pho regulon genes. *phoU* mutants show poor growth and frequently accumulate compensatory mutations in *phoR*, *phoB*, or the *pstSCAB* genes [21, 23, 24]. PhoU is a peripheral membrane protein that modulates Pi transport through the PstSCAB complex [22–24]. When Pi is plentiful, PhoU acts like a brake to prevent too much Pi import, with its accompanying ATP hydrolysis [23]. Multiple crystal structures have been reported for PhoU proteins from various organisms [63–65]. PhoU consists of two symmetric, three alpha-helix bundles and metal ions are found associated with two of these structures. The metals are coordinated by highly conserved amino acid residues that are found in each three-helix bundle. PhoU from *Thermotoga maritima* coordinates iron clusters [63], while PhoU from *Streptococcus pneumoniae* shows zinc ions bound [64]. Gardner et al. have recently shown that the soluble form of PhoU from *E. coli* is a dimer that binds manganese or magnesium [66]. Mutagenesis experiments demonstrated that these divalent metals are bound by the same conserved amino acid residues that bind the iron and zinc ions in the two crystal structures. It was also suggested in this study that metal binding may be important for PhoU interactions with the membrane. Alternatively, PhoU may bind Pi through its interactions with these metals.

Two general classes of models have been previously suggested for how PhoU participates in the signaling pathway. It may mediate the formation of a signaling complex between the PstSCAB transporter and PhoR [5, 64] or it may produce a soluble messenger that is

recognized by the cytoplasmic domains of PhoR (consistent with observations reported by Hoffer and Tommassen [67] and by Rao and Torriani [68]). The following section presents new evidence in favor of the Pi-signaling complex model.

3.4. Protein interactions within the Pi-signaling complex

It has recently been demonstrated through bacterial two-hybrid analysis and through co-elution experiments that PhoU interacts with both the PhoR protein and PstB [66]. The two-hybrid experiments used the BACTH system [69]. Adenylate cyclase from *Bordetella pertussis* can be genetically divided into nonfunctional T18 and T25 fragments. The enzyme can be reconstituted in vivo and cAMP can be produced when the two fragments are brought into physical proximity within the cell by fusing interacting proteins with either the T18 or T25 fragments and monitoring cAMP production. Gardner et al. fused various parts of PhoR, or the PstB protein, to the T25 fragment and PhoU to the T18 fragment and indirectly monitored cAMP production by assaying the cAMP-dependent gene β-galactosidase [66]. They found that the interaction between PhoR and PhoU occurred through its PAS domain and that the PstB-PhoU interaction was weaker than the PhoR-PhoU interaction. They then employed a complementary co-elution method by using His-tagged versions of either PstB or PhoR and showed by Western blotting that PhoU was retained on a nickel column in a PstB- or PhoR-dependent manner. In a subsequent paper, Gardner et al. were able to further localize the sites on PhoR and PhoU that are important for the formation of the signaling complex [70]. They knew that the phenotype of a mutant containing the *phoU35* allele was unlike that of a *phoU* deletion mutation. Neither the *phoU35* nor the *phoU* deletion mutants could signal Pi sufficiency and they both constitutively expressed alkaline phosphatase. However, the *phoU35* mutant did not have a severe growth defect [71]. Since the *phoU35* allele encoded a change from alanine at position 147 to glutamic acid (A147E) [72], Gardner et al. hypothesized that the *phoU*35 mutation may disrupt PhoU's interaction with PhoR, preventing the signal for the switch to PhoR phosphatase activity, but that it maintained its interaction with PstB, limiting excess transport of Pi into the cell during Pi-replete conditions. From this assumption, they were able to identify the surface residues Ala147 and Arg148 of PhoU as being important for the interaction with PhoR. Moreover, they employed a scanning mutagenesis approach to identify a surface on the PAS domain of PhoR that is essential for the interaction. Every two amino acids within the PAS domain were sequentially mutated and then tested using the BACTH assay for interactions with PhoU. They identified residues 141–146, 157–162, and 169–176 of PhoR as important for the interaction with PhoU. By using these genetic constraints, they were able to build a plausible three-dimensional model of the docked proteins. This model was then supported by using a bioinformatic method, called direct-coupling analysis that identifies residues from one sequence that tend to co-evolve with residues from another sequence. Proteins that physically interact co-evolve with each other. These analyses supported a model in which PhoU interacts with both the PAS and CA domains of PhoR. Gardner et al. proposed the existence of a Pi-signaling complex in which under high-Pi growth conditions PhoU interacts with PhoR to constrain its CA domain and inhibit its kinase activity and promote its phosphatase activity.

3.5. Conformational signaling model

To answer the question of how PhoR senses the signaling activity of the Pst transporter, we propose that PhoR interacts differently with the alternate outward- and inward-facing conformations of the transporter that are sampled throughout the transport cycle. When Pi is limiting, the transporters are not actively importing Pi and reside primarily in the outward-facing conformation. We propose that this conformation contacts PhoU in such a manner that it does not interact with both the PAS and CA domains of PhoR, which promotes its autokinase activity. It is only under Pi-replete environments when Pi import is occurring that the Pst transporter adopts the inward-facing conformation. We propose that in this conformation, it interacts with PhoU in such a manner to constrain the CA domain of PhoR so that its phosphatase activity is stimulated.

To test this model, we have recently created two *pstB* mutations that are predicted to lock the transporter into these alternate conformations (Vuppada and McCleary, manuscript in preparation). Based upon work with the homologous maltose transporter [73], an E179Q mutation in *pstB* should lock the transporter into an outward-facing conformation because it cannot hydrolyze ATP and a Q160K mutation should lock it into an inward-facing conformation because it does not bind ATP. The *pstSCAB-phoU* genes were cloned onto a medium-copy number plasmid and were introduced into a Δ*pstSCAB-phoU* strain of *E. coli* to confirm that the plasmid could complement the deletion mutation. Mutations were then introduced into the plasmid by site-directed mutagenesis and confirmed by DNA sequencing. Neither the E179Q nor the Q160K mutants showed high-affinity Pi transport, showing that the transporters were dead. By using alkaline phosphatase expression as a reporter of the Pho regulon, we observed that the E179Q mutant constitutively signaled Pistarvation, whether the cells were grown in Pi-replete or Pi-starvation media. We also observed that the Q160K mutant always signaled Pisufficiency. In other words, these cells always expressed low levels of alkaline phosphatase, presumably resulting from the activation of the phosphatase activity of PhoR. These results support the model in which the inward-facing form of the Pst transporter interacts with PhoU and PhoR in a manner that stimulates the phospho-PhoB phosphatase activity of PhoR. This signaling output of the PhoBR pathway reduces the expression of the PstSCAB transporter when low-level expression is sufficient for maximal growth. It also downregulates other genes whose expression would be wasteful in times of Pi sufficiency.

4. The response to high levels of extracellular Pi

Clues to understanding how *E. coli* and other bacteria cope with high levels of extracellular Pi came from studies on Pi remediation [12, 74, 75]. Excess Pi in natural water sources is a major cause of eutrophication [76]. Toxic cyanobacterial blooms are frequently attributed to Pi accumulation in water sources resulting from agricultural runoff [77]. Normally, Pi is removed from wastewaters by chemical precipitation, which is an expensive process [78]. Biological Pi removal is an alternative to chemical treatments in which bacteria accumulate

excess Pi as polyphosphate (polyP) [79, 80]. The bacteria can then be retained as sludge, which can be separated from the wastewater, which now has a much reduced phosphorous concentration.

PolyP is found in all kingdoms of life [81, 82]. It is a linear chain of variable length of Pi residues that are linked by phosphoanhydride bonds. The cellular amounts of polyP are controlled through its polymerization and depolymerization, presumably to meet cellular needs for free Pi. PolyP can be synthesized from ATP by polyP kinase, encoded by the *ppk* gene [83]. It is degraded by exopolyphosphatase, encoded by the *ppx* gene. The Ppk reaction is fully reversible and cells can also use polyP to synthesize ATP.

To enhance biological removal of Pi from wastewater, Kato et al. cloned the *pstSCAB* and *ppk* genes on plasmids [74]. They found that an *E. coli* strain harboring these plasmids could accumulate up to 16% of their dry weight as phosphorus with over 60% of the cellular phosphorous stored as polyP. They also noted that these strains grew very poorly. Subsequent work from this group showed that *phoU* mutants also accumulated high levels of polyP [75]. It was known that *phoU* mutants expressed the transporter at high levels, even when environmental Pi levels were high. As an additional contribution to understanding the phenotype of a *phoU* mutant, our group showed that PhoU also negatively regulates the activity of the Pst transporter [23]. Those experiments were performed by uncoupling expression of the Pst transporter from its normal PhoB-dependent mechanism through a technique called promoter swapping [84]. We felt that it was important to keep the *pstSCAB-phoU* operon at its normal location in the *E. coli* chromosome, so we developed a technique using Lambda-Red recombineering methodology to swap the P_{tac} promoter for the wild-type P_{pstS} promoter [85]. As we held expression levels constant with an exogenous promoter, we demonstrated that a *phoU* deletion mutant accumulated Pi at a higher rate than cells expressing the *pstSCAB* genes and *phoU*. Other ABC transporters, such as the methionine transporter, have regulatory domains that respond to the cytoplasmic concentrations of transported substrates and function as sites of allosteric inhibition of transport [86–88]. We proposed that PhoU plays a similar role for Pi transport in *E. coli*. We learned from these observations that *E. coli* cells tightly control the amounts of the Pst transporter as well as its activity. When intracellular amounts of Pi become too high,*E. coli* cells store excess Pi as polyP.

In addition to its role as a Pi and energy store, PolyP has many other important functions in *E. coli* [89]. For example, it is involved in metal detoxification and can function as a primitive chaperone to protect against oxidative damage [90–92]. Of importance to our discussion here, Keasling hypothesized that *E. coli* cells could detoxify metals by sequestering them with intracellular polyP. Following hydrolysis of polyP to Pi, the metal/Pi complexes would be exported through the Pit transporters. PolyP is also involved in cell signaling, respiratory chain gene expression, bacterial persistence, and in stress response networks [93–96]. It has recently been shown that when external Pi levels are very high, polyP can even activate PhoB during the stationary phase of growth through the small molecule acetyl phosphate [97]. It is then postulated that phospho-PhoB inhibits the synthesis of c-di-GMP, blocking the production of AI-2, leading to the inhibition of biofilm formation.

4.1. The Tn-seq experiment—identifying the players of the high-Pi response

In order to further investigate cellular processes involved in Pi homeostasis when cells are grown in conditions of high environmental Pi, we performed a Tn-seq experiment. Tn-seq relies on the ability to saturate a bacterial genome by transposon mutagenesis. Cells are grown in a selective environment and individual transposon insertions are mapped using next-generation-sequencing protocols. The frequency of insertions in each gene is used to analyze the importance of each gene under those growth conditions. Those genes that receive few or no insertions are identified as essential (no insertions under any conditions), conditionally essential (no or few insertions under one condition), or conditionally important for fitness (reduced insertion frequency under one particular condition) (see **Figure 4**).

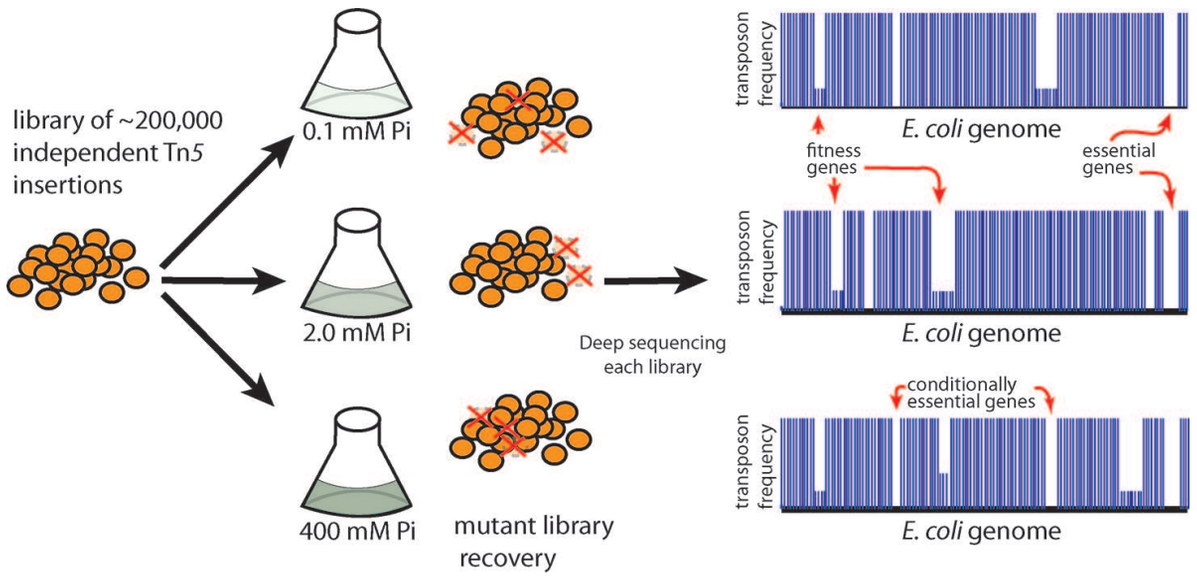

Figure 4. The design of a Tn-seq experiment. A library of transposon insertion mutants was grown in duplicate in liquid cultures containing either 0.1 mM Pi, 2.0 mM Pi, or 400 mM Pi. Chromosomal DNA was prepared from each sample for deep sequencing to identify the sites of insertion and their frequencies. If an insertion does not affect the growth of a strain, then it is assumed that that gene is not required for growth. Genes with no insertions under any conditions were classified as essential genes. Genes with no or very few insertions under one condition were classified as conditionally essential and those with reduced frequency were classified as important for fitness.

Wild-type *E. coli* strain MG1655 that harbors a *rpsL* mutation conferring streptomycin resistance was mutagenized with a mini-Tn*5* transposon delivered from a conjugative plasmid that required the lambda Π protein for replication. The donor strain could be counter-selected because it contained a mutation in the *dapA* gene and required supplementation with diaminopimelic acid. By selecting transconjugants that were kanamycin-resistant and that did not require diaminopimelic acid we were able to obtain a library of about 200,000 independent mutants. Such a library would be predicted to give about 30–50 random insertions per gene. This mutant library was then grown in duplicate in one of three different defined media containing variable Pi concentrations. We used media containing 0.1, 2.0, and 400 mM Pi. Preliminary experiments showed that growth of the wild-type strain in 400 mM Pi was significantly slower than in the other media. This high-Pi medium was also of a significantly

higher osmolarity than the other two media. After growing cells until stationary phase, the cultures were harvested and DNA extractions were performed. The chromosomal DNA was then enzymatically fragmented and a polyC tail was then added to these DNA fragments using terminal transferase. Polymerase chain reactions (PCRs) were then performed using transposon-specific and polyG primers to amply DNA where transposons had inserted. A second round of PCR was then used to add primers for Illumina sequencing. The reads were mapped to the published MG1655 genome and the number of reads was normalized to 4×10^6 reads per sample.

To identify genes that are important for growth in high-Pigrowth conditions, we sorted from low to high each of the genes based upon the quotient of the number of hits in high-Pi media divided by the total number of hits in all three media. We were particularly interested in genes with few hits in the high-Pi medium and were able to identify many genes whose functions are important for fitness under these growth conditions. As mentioned above, the high-Pi growth medium that we employed was also high in osmolarity. As an internal control to identify genes that were important for this growth condition, we were able to identify many top hits as occurring in genes that are known to be important in a high osmolarity response, such as*ompR, envZ, galU, otsB, hupA, cpxR*, and *hupB* [98–100]. OmpR and EnvZ are two-component regulators that respond to changes in osmolarity. GalU and OtsB are involved in the synthesis of trehalose, a compatible solute, that is produced under high osmolarity growth conditions. *hupA* encodes for a component of the HU protein, which is a small DNA-binding protein that helps regulate the expression of the osmoresponsive gene *proU* [101]. CpxR is a response regulator that responds to cell envelope damage and it is known that it participates in the regulation of gene expression in response to osmolarity [99].

We also identified genes that are known to be involved in the control of the Pho regulon, for example, each of the *pstSCAB* genes was found near the top of the list. Mutations in any of these genes lead to elevated expression of the entire Pho regulon, whose genes are involved in the high-affinity acquisition of Pi and the utilization of alternate Pi sources. It is easy to hypothesize why the expression of these genes would be deleterious when Pi levels are very high. With the Pho regulon fully expressed, Pi may be imported through the phosphonate or other transporters without the requisite expression of genes to accommodate the increased Pi. Another common class of genes that had few transposon insertions under high Pi conditions was genes involved in central metabolism of glucose and most importantly in ATP production (*ptsG, pykF, ackA, zwf, pta*, and *sdhBCD*). PstG is the enzyme IIBC component of the phosphotransferase system for glucose uptake [102]. PykF is pyruvate kinase from glycolysis and synthesizes ATP from ADP and phospho*enol*pyruvate. Zwf is glucose-6-phosphate-1-dehydrogenase, which catalyzes the first steps in the Enter Doudoroff or oxidative pentose phosphate pathways [103, 104]. AckA and Pta are acetate kinase and phosphotransacetylase, respectively, and are involved in ATP production, acetyl phosphate synthesis, and acetate secretion [105]. SdhBCD are subunits of succinate dehydrogenase, which is part of the TCA cycle. It is interesting to note that these genes are repressed during growth on glucose [106], so it is unclear why mutations in these genes lower the fitness of *E. coli* grown on glucose high-Pi medium. It is also important to note that there were very few hits under

any condition in the *ppk* gene, suggesting that it was an essential gene under these defined growth conditions, as well as in the genes for ATP synthase. Another intriguing class of genes with low frequency of transposon insertions included genes of unknown function, such as *ydhP, yodD, yniC*, and *glcG*. We conclude from these results that when placed in very high Pi environments cells need to regulate Pi import and continue to synthesize ATP at high rates for the production of polyP. We expect that there are some previously unknown functions that are necessary to deal with high-Pi stresses that are represented by the "y" genes. These may include other transporters, regulators of transporters, or genes for metabolic functions.

4.2. Pi homeostasis model and questions for further research

E. coli inhabits environments with widely ranging Pi concentrations. It is often limiting in environmental conditions and can be quite high in the intestinal lumen of a healthy human [96]. Pi homeostasis is a balancing act of import, export, utilization, and sequestration (see **Figure 5**). Pi can be imported through the secondary transporters PitA and PitB or through the PstSCAB ABC transporter. The multiple transporters that import Pi have various specificities and expression patterns, which allow them to be used primarily under conditions when they are most needed, but which also permits a considerable amount of redundancy in function. Of primary importance in Pi homeostasis is the ability to increase transcription of genes when environmental Pi levels are low for the high-affinity acquisition of Pi and for the utilization of alternate sources of phosphorous. To monitor extracellular Pi, *E. coli* utilizes a Pi-signaling complex consisting of the PstSCAB transporter, PhoU and PhoR. In its two states, it can either activate or deactivate the response regulator PhoB. We propose that the signaling complex does not directly sense extracellular Pi, but senses the activity of the Pst transporter by recognizing its alternate conformational states. It is the inward-facing conformation of the Pst transporter that represents Pi-sufficient environments because it is only formed when Pi is actively transported. Once imported, Pi becomes part of an intracellular pool and can be incorporated into ATP through substrate-level phosphorylation or through oxidative phosphorylation. From ATP or its equivalents, the phosphoryl groups are transferred to all other phosphorylated intermediates of the cell. Cellular growth is inhibited when intracellular Pi levels become too elevated, so cells must have mechanisms to control this parameter also. To maintain its intracellular Pi levels near 10 mM, *E. coli* can either export excess Pi or it can sequester it through the synthesis of PolyP. PitA and PitB are known metal-Pi exporters and rely on high intracellular Pi levels and metals, such as Mg^{2+}, Mn^{2+}, Ca^{2+}, Zn^{2+}, and Co^{2+} for Pi export [7, 107]. Pi export through the Pit proteins contributes to the generation of a proton-motive force. It has also been suggested that YjbB plays a role in Pi export [12]. This protein is very interesting because it consists of two segments, a hydrophobic N-terminal half with sequence similarity to Na^{+}/Pi transporters and a C-terminal half with sequence similarity to PhoU. Motomura et al. showed that overexpression of YjbB resulted in lower intracellular polyP levels and that it released significant amounts of Pi into the medium. These results are consistent with YjbB being a Pi exporter. PolyP serves as a Pi buffer to fine-tune intracellular Pi levels.

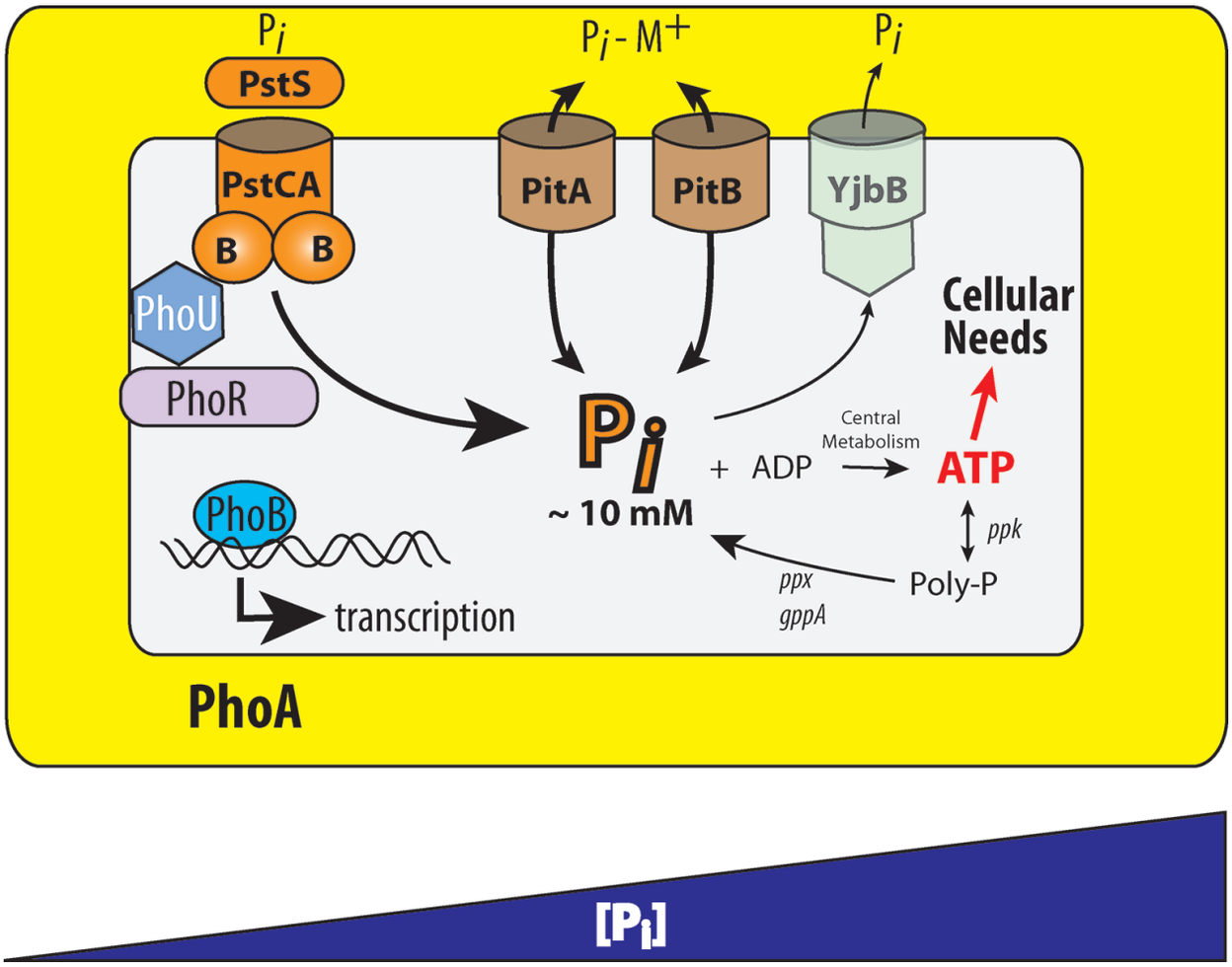

Figure 5. Model for Pi homeostasis in *E. coli*. Intracellular amounts of Pi are maintained within a modest range around 10 mM. The mechanisms for this homeostatic maintenance include the use of multiple Pi importers with variable affinities and rates of Pi transport. Cells also utilize the sophisticated PhoBR two-component-signaling mechanism that directly controls the expression of genes for high-affinity Pi acquisition and for the use of alternate sources of phosphorous. In addition, when Pi levels become too high, the cells sequester Pi by accumulating polyP, which is produced from ATP by the enzyme Ppk or they export it.

While the general outlines of Pi homeostasis have begun to be filled in, there are still important questions that remain. How do cells sense intracellular levels of Pi to control polyP synthesis/degradation and Pi export? What are the roles of the genes that are repressed by the PhoBR system? What are the functions of the unknown genes that were identified by Tn-seq to be important for fitness in very high levels of environmental Pi? What are the control mechanisms for the expression of PitA and PitB? Why does *E. coli* retain both the *pitA* and *pitB* genes? What are their differential functions? What effects does the stoichiometry of PstSCAB, PhoU, PhoR, and PhoB have on signaling, especially at the level of the single cell? Knowledge gained in studying Pi homeostasis will continue to be important in understanding global regulatory mechanisms, as Pi is involved in so many cellular processes. It will also be important in the engineering of organisms for improved Pi bioremediation.

5. Conclusion

Pi homeostasis is essential for life's basic processes. Without the ability to control intracellular levels of Pi within optimal levels, cells would be unable to maintain energy stores, synthesize nucleic acids and phospholipids, or carry out central metabolic pathways. The molecular mechanisms by which *E. coli* cells maintain intracellular Pi levels include utilizing multiple importers with characteristic patterns of expression, affinities for Pi and rates of Pi import [4]. These cells also employ a highly characterized signal transduction system that monitors extracellular Pi levels through the conformational states of the high-affinity Pi importer to control gene expression for scavenging Pi and utilizing alternate phosphorous sources. In addition, polyphosphate plays an important role in fine-tuning the amounts of free intracellular Pi. Understanding these mechanisms is important because this knowledge can be used to design organisms and pathways for the remediation of phosphate pollution. Moreover, the expression of virulence genes in many organisms is controlled by the PhoBR signal transduction system.

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