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Differential Regulation of High-Affinity Phosphate Transport Systems of *Mycobacterium smegmatis*: Identification of PhnF, a Repressor of the *phnDCE* Operon

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The uptake of phosphate into the cell via high-affinity, phosphate-specific transport systems has been studied with several species of mycobacteria. All of these species have been shown to contain several copies of such transport systems, which are synthesized in response to phosphate limitation. However, the mechanisms leading to the expression of the genes encoding these transporters have not been studied. This study reports on the investigation of the regulation of the *pstSCAB* and the *phnDCE* operons of *Mycobacterium smegmatis*. The *phn* locus contains an additional gene, *phnF*, encoding a GntR-like transcriptional regulator. Expression analyses of a *phnF* deletion mutant demonstrated that PhnF acts as a repressor of the *phnDCE* operon but does not affect the expression of *pstSCAB*. The deletion of *pstS*, which is thought to cause the constitutive expression of genes regulated by the two-component system SenX3-RegX3, led to the constitutive expression of the transcriptional fusions *pstS-lacZ*, *phnD-lacZ*, and *phnF-lacZ*, suggesting that *phnDCE* and *phnF* are conceivably new members of the SenX3-RegX3 regulon of *M. smegmatis*. Two presumptive binding sites for PhnF in the intergenic region between *phnD* and *phnF* were identified and shown to be required for the repression of *phnD* and *phnF*, respectively. We propose a model in which the transcription of *pstSCAB* is controlled by the two-component SenX3-RegX3 system, while *phnDCE* and *phnF* are subject to dual control by SenX3-RegX3 and PhnF.

Phosphorus is an essential nutrient for all cells and is required for energy metabolism and for the synthesis of important biological molecules such as phospholipids and nucleic acids. The main source of phosphorus for bacteria is inorganic phosphate. To ensure the supply of phosphorus under conditions of phosphate limitation, bacteria possess a high-affinity phosphate-specific ABC transport system (Pst), and some species contain additional systems for the utilization of alternative phosphorous sources, such as phosphite (e.g., as in the Ptx system of *Pseudomonas stutzeri*) or phosphonates (e.g., as in the Phn system of *Escherichia coli*) (20, 37). In the slow-growing pathogenic species of mycobacteria, multiple copies of the genes encoding the Pst system have been identified (17), and two of these genes, *pstS1* and *pstS2*, were shown to be important for the virulence of *Mycobacterium tuberculosis* (25). We recently showed that the fast-growing *M. smegmatis* also requires several high-affinity phosphate-specific transport systems for growth (5), suggesting that this is a general characteristic of mycobacteria. The reasons for the presence of multiples of such transporters are not well understood, but this characteristic has been proposed to constitute an adaptation of the bacteria to grow and survive in a variety of phosphate-limited environments (17). If this is the case, it appears likely that the expression of multiple high-affinity phosphate transport systems in mycobacteria should be regulated differentially.

Transcription of the genes for bacterial high-affinity phosphate transport systems is usually regulated by a two-component regulatory system, PhoBR in gram-negative bacteria (37) and PhoPR in gram-positive bacteria (13, 33), where PhoR acts as the sensor kinase and PhoB or PhoP acts as the cognate response regulator. Additionally, the Pst system and the repressor PhoU are required for signal transduction and, together with PhoR, are thought to form a membrane-bound repressor complex under phosphate-replete conditions (37). Mutations in Pst have been shown to lead to constitutive activation of the Pho regulon genes in a number of bacteria such as *E. coli* (39), *Sinorhizobium melloti* (41), and *M. smegmatis* (15).

Recently, the sensor kinase SenX3 and the response regulator RegX3 were identified as composing the phosphate-responsive two-component regulatory system of *M. smegmatis* (6). RegX3 was shown to bind to the promoters of several genes, including *pstS*, the first gene of the operon that encodes the Pst transport system (6). The authors proposed that SenX3 is unlikely to sense the phosphate availability in the medium directly but probably relies on the Pst transporter to relay this information and thus regulate the activity of SenX3, similar to the situation in *E. coli* (6). While putative RegX3 binding sites were identified in the promoter regions of SenX3, *phoA* (encoding alkaline phosphatase), and *pstS*, the sequence conservation between these regions was too weak to predict which other genes might be controlled by SenX3-RegX3 (6).

As stated above, we recently identified a second high-affinity phosphate ABC transport system of *M. smegmatis*, the PhnDCE system, which has a sequence similarity to the phos-
In the present study, we investigated the mechanisms of regulation of the \( \text{phnDCE} \) operon of \( M. \text{smegmatis} \). We used allelic exchange mutagenesis and RNA analysis to investigate the role of PhnF in transcriptional regulation of the \( \text{phnDCE} \) operon. We also used a \( \text{pstS} \) deletion mutant to determine whether the \( \text{phnDCE} \) operon is part of the phosphate regulon in \( M. \text{smegmatis} \). Site-directed mutagenesis of the region between \( \text{phnDCE} \) promoted transcription from the \( \text{phnDCE} \) promoter in the presence of phosphate.

**Materials and Methods**

**Bacterial strains and growth conditions.** All strains and plasmids used in this study are listed in Table 1. \( E. \text{coli} \) strains were grown in Luria-Bertani (LB) medium at 37°C with agitation (200 rpm). The \( M. \text{smegmatis} \) electrocompetent wild-type strain of \( M. \text{smegmatis} \) was used in this study. The study are listed in Table 1.

**Plasmids**

- **pBluescript II KS**
  - Cloning vector; Ap<sup>r</sup>
  - Strategene

- **pUC18K**
  - \( E. \text{coli} \) plasmid containing an excisable, nonpolar kanamycin resistance cassette; Km<sup>r</sup>
  - 19

- **pX33**
  - \( E. \text{coli} \)-mycobacterium shuttle vector for allelic exchange mutagenesis in \( M. \text{smegmatis} \), \( \text{pPR23} \), carrying a constitutive \( \text{yE} \) marker; Gm<sup>r</sup> Sac<sup>c</sup>
  - 5, 26

- **pJEM15**
  - \( E. \text{coli} \)-mycobacterium shuttle vector for the creation of transcriptional promoter fusions to lacZ; Km<sup>r</sup>
  - 34

- **pUHA267**
  - \( E. \text{coli} \) vector with mycobacteriophage L5 integrase and attP for integration into \( \text{attB} \); Myc<sup>r</sup> Hg<sup>r</sup>
  - AgResearch, Wallaceville, New Zealand

- **pSG16**
  - pJEM15 harboring a 500-bp \( \text{phnD-lacZ} \) fusion; Km<sup>r</sup>
  - 5

- **pSG12**
  - pJEM15 harboring a 560-bp \( \text{pstS-lacZ} \) fusion; Hg<sup>r</sup>
  - 5

- **pSG14**
  - pUHA267 harboring \( \text{phnF} \) with its native promoter; Hg<sup>r</sup>
  - 5

- **pSG42**
  - pJEM15 harboring a 560-bp \( \text{pstS-lacZ} \) fusion; Km<sup>r</sup> Sac<sup>c</sup>
  - This study

- **pSG41**
  - pJEM15 harboring a 560-bp \( \text{phnF-lacZ} \) fusion; Km<sup>r</sup> Hg<sup>r</sup>
  - This study

**Strains**

- **E. coli** DH10B
  - F<sup>−</sup> \( \text{mcrA} \) \( \Delta(\text{mrr-hsdRMS-mcrBC}) \) \( \delta80\text{lacZ} \) \( \Delta\text{M15} \) \( \Delta\text{lacX74 deoR recA1 ada139} \)

- **M. smegmatis**
  - \( \text{mc}^{\text{155}} \)
  - Electrocompetent wild-type strain of \( M. \text{smegmatis} \)
  - This study
  - 32

- **SG62**
  - mc<sup>155</sup> \( \text{DphnF::aphA-3} \); Km<sup>r</sup>
  - 5

- **SG95**
  - mc<sup>155</sup> \( \Delta\text{pstS::aacC-1} \); Gm<sup>r</sup>
  - 5

- **SG111**
  - SG62 with pSG41 integrated in \( \text{attB} \); Km<sup>r</sup> Hg<sup>r</sup>
  - This study
  - 5

- **SG120**
  - SG95 with pSG43 integrated in \( \text{attB} \); Gm<sup>r</sup> Hg<sup>r</sup>
  - 5

**Transduction**

- **pSG62**
  - gmr, gentamicin resistance; hyg<sup>r</sup>, hygromycin resistance; ap<sup>r</sup>, ampicillin resistance; sac<sup>s</sup>, sucrose sensitivity; ts, temperature sensitivity.

**Site-directed mutagenesis of the region between \( \text{phnDCE} \) and \( \text{phnF} \).** In the present study, we investigated the mechanisms of regulation of the \( \text{phnDCE} \) operon of \( M. \text{smegmatis} \). We used allelic exchange mutagenesis and RNA analysis to investigate the role of PhnF in transcriptional regulation of the \( \text{phnDCE} \) operon. We also used a \( \text{pstS} \) deletion mutant to determine whether the \( \text{phnDCE} \) operon is part of the phosphate regulon in \( M. \text{smegmatis} \). Site-directed mutagenesis of the region between \( \text{phnD} \) and \( \text{phnF} \) revealed two putative binding sites for PhnF in the promoters of \( \text{phnDCE} \) and \( \text{phnF} \).
CACCACAGGAAACAGCTATGA-3'). The resulting 850-bp product was digested with EcoRI and BamHI. PCR products of approximately 850 bp flanking the phnF gene of M. smegmatis were amplified by using the primers phnFK01 (5'-AAATTTAATGCGGGCCGAGCGCAGCATCAGG-3') with primer phnFK02 (5'-AAATTTATTAGCAGGATCGCCGAGCTGAAACTG-3'). PCR products were gel purified and used as templates for the mutagenesis of IRU-2. A fragment containing the wild-type IRU-2 was amplified by using the primers SDMIRU2-1 (5'-H11032 GTGGGTGCTATACCA-3') and SDMIRU2-2 (5'-H11032 ACCGGTTGCATCCGGC-3') with primer phnFK03 (5'-AAATTTTGGATACCGCCTGCTCGACCTGA-3'). PCR products of approximately 850 bp flanking the phnF locus, eliminating 80% of the phnF coding sequence in exchange for the kanamycin resistance marker. Allelic replacement of phnF was carried out essentially as described by Pelicic et al. (26) and was achieved by growing a culture of M. smegmatis carrying pSG16 in Middlebrook 7H9-ADC medium at 28°C with agitation (200 rpm) to an OD600 of approximately 0.6 to 0.8, followed by plating onto low-salt LBT plates (2 g NaCl liter⁻¹) containing gentamicin and 10% sucrose at 40°C, selecting for double-crossover events. Replacement of phnF with the kanamycin marker created strain SG62 (ΔphenF::aph-3). For Southern hybridization analysis, Small-digested genomic DNA of the putative mutants was separated on a 1% agarose-Tris-acetate-EDTA gel and transferred onto a nylon membrane (Hybond-N; Amersham) by vacuum blotting. Blots were labeled by random priming using [α-32P]dCTP (Amersham) and Ready-To-Go DNA-labeling beads (Amersham).

The PCR products were sequenced to confirm the allelic exchange mutation was cloned into the integrative vector pUHA267 (16). A 1.2-kb PCR product encompassing the phnF gene plus 428 bp of upstream DNA was amplified by PCR using the primers pphnFR (5'-AAATTTAGCTT CATAGTCGGAGGCCTGGACG-3') and pphnDR (5'-AAATTTAAGCTTCA CATAGTCGGAGGCCTGGACG-3') and cloned into the HindIII site of pUHA267, creating the plasmid pSG41. Site-directed mutagenesis of the putative PhnF binding sites (inverted repeat unit [IRU-1] and IRU-2) was carried out using PCR over-lap extension (11). For the mutagenesis of IRU-1, a 158-bp PCR product was amplified with the primers PphnFR and SMIRU1-1 (5'-ACGTCTGTGTCTATCACAAGCGGACC CGCCGTGCAGC-3') and a 247-bp PCR product was amplified with the primers SMIRU1-2 (5'-GTCCCGGTGTATAGACCAAGACAGTGTCCGTT CGTCCT-3') and PphnDR (5'-AAATTTGTACCGTGTCAGGACAGCAGGCA sequences amplified using primers SMIRU2-1 (5'-ACGTCTGTGTCTATCACAAGCGG ACCGTGCAGC-3') and SMIRU2-2 (5'-AGGTCATGGAGGTCGACG-3') and an 189-bp PCR product was amplified by using the primers SMIRU2-3 (5'-ACGTCTGTGTCTATCACAAGCGG ACCGTGCAGC-3') and PphnDR. These fragment pairs were then used in over-lap extension PCR, using the primers PphnFR and SDMIRU1-1 (5'-H11032 GTCCGCTGTGATAGACACAGACGTATTCGCTGGCCGTCTGACGAG-3') or the phnF-specific primer phnF-IRU1 (5'-AAATTTGATACCGCCTGCTCGACCTGA-3'). The resulting DNAs were purified, and a deoxyribosyladenine tail was added by following the kit instructions. Purified deoxyribosyladenine-tailed DNAs were then used as a template for PCR using the oligo(dt) anchor primer and the phnF-specific primer phnD-IRU2 (5'-ACGGAGCACCTTCTTGG-3') or the phnF-specific primer phnF-IRU2 (5'-GGTGAAGGCATCCGAGC-3'). The resulting PCR products were gel purified and used as templates for a second PCR using the PCR anchor primer and the nested phnD-specific primer phnD-IRU3 (5'-CGACCCGAGGACTGCTAGGC-3') or the phnF-specific primer phnF- IRU3 (5'-GTGAAGGCATCCGAGC-3'). These PCR products were cloned into pGEM-T Easy (Promega) according to the manufacturer's instructions. Three clones containing the correctly sized insert for phnD (ca. 400 bp) or phnF (ca. 550 bp) were sequenced using the primer phnD-IRU3 or phnF-IRU3, respectively, giving consistent results for each gene.

β-Galactosidase and inorganic phosphate assays. To determine the threshold pH for induction leading to the induction of phnF, cells carrying pMSG81 were grown in medium containing 200 μM phosphate. The medium was modified from the standard Sauton's medium used in this study to contain higher concentrations of the carbon source (5 g of glycerol liter⁻¹) and the nitrogen source (4 g 1-aspargine liter⁻¹), providing both nutrients in excess. At various time points, 2 ml to 4 ml of the culture was removed to determine the OD600. Cells were then pelleted, and cell pellets and supernatants were stored at −20°C. β-Galactosidase activity was determined for the 0- to 1-h time point was determined with cells of the inoculum culture. β-Galactosidase activities were determined as described previously (5) and were expressed as Miller units (MU) (23), calculated as the increase in A420 per min per 1 ml of cell suspension used (normalized to an OD600 of 1.0) and multiplied by a factor of 1,000. Statistical analyses were performed using a two-tailed, unequal t test. The F1 concentration in the supernatant was determined in inorganic phosphate assay as described previously (24).

DNA and protein sequence analysis. The provisional genome sequence of M. smegmatis strain mc²155 was accessed via The Institute for Genomic Research (TIGR) website (http://www.tigr.org; GenBank accession number CP000480). Sequence data for M. tuberculosis were obtained from the Institut Pasteur website (http://genolist.pasteur.fr/Tuberculist). Protein sequence alignments were carried out using a ClustalW function and the BLOSUM62 matrix of BioEdit (9). Secondary-structure predictions were performed using JPred (4). Promoter areas were searched for motifs, using motif discovery and search tool MEME software, available at the San Diego Supercomputer Center website (http://meme.sdsc.edu/meme/).

RESULTS AND DISCUSSION

Identification of a putative regulator in the phn locus of M. smegmatis. The PhnDCE phosphite transport system of M. smegmatis is encoded by a three-gene operon (Fig. 1A) (5). A gene adjacent to and divergently transcribed from this operon, MMSMEG_0650, has been annotated as a GntR family transcriptional regulator encoding a 244-amino-acid protein. According to the results of a conserved-domain search (18), the protein was called PhnF (36) (Fig. 1A). A search for TIGRFAM and Pfam matches for PhnF, using the search tools available via the TIGR website (http://cmr.tigr.org), revealed that the N terminus (amino acids 5 to 65) of the protein contained a helix-turn-helix motif of a GntR-like transcriptional regulator. The C-terminal part of the protein (amino acids 85 to 224) was a Ubic trancriptional regulator-associated (UTRA) domain, which is common to members of the HutC subfamily of GntR-like regulators and has been pro-
posed to function in ligand binding (2). The predicted secondary structure of this region of *M. smegmatis* PhnF (Fig. 1B) showed a fold similar to the typical tandem arrangement of two \( \alpha_2\beta\alpha_2\beta\) repeats, further confirming that like the PhnF protein of *E. coli* (27), the *M. smegmatis* PhnF protein also belongs to the HutC subfamily. While few residues of the ligand binding pocket are conserved among members of this subfamily, two large residues in sheet 2 and one polar residue in sheet 3 appear to be universally conserved (2). An alignment between the *E. coli* and the *M. smegmatis* PhnF proteins shows that with the exception of Leu135 (*E. coli* numbering), these residues are conserved in the *M. smegmatis* protein (Fig. 1B). The crystal structure of *E. coli* PhnF showed that five residues conserved among PhnF orthologues delineate the proposed binding cavity (8). Only two of these residues (R181 and S228 [*E. coli* numbering]) are also conserved in the *M. smegmatis* protein (Fig. 1B). *E. coli* PhnF is thought to respond to the presence of alkylphosphonates (2, 8), but this seems unlikely to be the signal recognized by *M. smegmatis* PhnF, because the PhnDCE system does not appear to transport such compounds and because *M. smegmatis* cannot utilize phosphonates as phosphorous sources for growth (5). Differences in the signaling molecule recognized by the proteins from *E. coli* and *M. smegmatis* could explain the poor conservation of binding site residues between them. No *phnF* homologues have been identified in any of the other sequenced mycobacterial genomes, but a locus containing homologues to all four of the *phn* genes of *M. smegmatis* has been annotated in the genome of another actinomycete, *Nocardia farcinica* IFM 10152 (14).

The expression of *phnF* is induced by phosphate limitation. Both of the operons for the high-affinity phosphate transport systems identified in *M. smegmatis* to date, *phnDCE* and *pstSCAB*, are induced when the culture enters phosphate limitation at a threshold phosphate concentration of 40 \( \mu \text{M} \) (5). If PhnF had a role in the regulation of one or both of these operons, then it was conceivable that the expression of the *phnF* gene also responded to the concentration of phosphate in the growth medium. To test this hypothesis, we created a transcriptional fusion of the putative promoter area (ca. 500 bp) of *phnF* to that of *lacZ* (*pSG18*). An *M. smegmatis* strain carrying this construct was grown in a phosphate-limited minimal medium as described previously (5), and the phosphate concentration, the \( \beta\)-galactosidase activity, and the OD_{600} were monitored throughout growth. Activities of the *phnF-lacZ* fusion increased rapidly from between 25 and 40 MU under phosphate-replete conditions to approximately 150 MU when the phosphate concentration in the medium dropped to below 40 \( \mu \text{M} \) (Fig. 2). The expression of *phnF-lacZ* therefore followed the same expression pattern as that of *phnD-lacZ* and *pstS-lacZ*, even though the magnitude of induction (ca. 4-fold) was much smaller than those observed for *phnD* and *pstS* (26-fold and 17-fold, respectively) (5). These data strongly suggest that PhnF is involved in the adaptation of *M. smegmatis* to phosphate-limited conditions.

Construction of a *phnF* deletion mutant. To further elucidate the function of PhnF, we created a *phnF* deletion mutant by allelic exchange mutagenesis. A construct in which *phnF* was replaced with a kanamycin resistance cassette, *aphA*-3, was expressed in *M. smegmatis* mc2155.
The deletion of phnF had no effect on the expression of the pstSCAB operon, which showed the same expression patterns in the SG62 ΔphnF strain, the SG111 complemented strain, and the wild-type strain (Fig. 4A, top panel). In contrast, the expression of phnDCE was strongly increased in the phosphate-starved cells of the phnF deletion mutant strain SG62 compared to that in the cells of the wild-type strain (Fig. 4A, bottom panel). The expression level of phnDCE under high-phosphate conditions was not changed in the SG62 strain. Complementation of the phnF deletion completely restored expression patterns to the wild-type level, confirming that the increase observed for expression under phosphate-starved conditions was indeed due to the deletion of phnF. These data clearly show that PhnF acts as a repressor of phnDCE. PhnF appears to have no role in the regulation of pstSCAB. The fact that the deletion of phnF does not lead to full constitutive expression of phnDCE under high-phosphate conditions suggests that a further regulatory mechanism is required for the induction of the operon. This hypothesis is addressed below.

**Deletion of pstS leads to the constitutive expression of pstSCAB, phnDCE, and phnF.** As mentioned above, the expression of the phnDCE operon appears to be subject to dual regulation: repression by PhnF and activation by a second regulatory mechanism. The most probable candidate for this second regulatory mechanism is the SenX3-RegX3 two-component system, which is reported to regulate the expression of the pstSCAB operon (6). SenX3, like its functional counterpart PhoR of E. coli, lacks any significant extracellular loops, which led to the proposal that the Pst system of *M. smegmatis* functions as the actual sensor of extracellular phosphate concentration (6). Accordingly, mutations in either pstS, pstC, or pstA are sufficient for the derepression of phoA expression (15), which is known to be under the control of SenX3-RegX3 (6). We therefore utilized the *M. smegmatis* pstS deletion mutant (strain SG95) and the pstS-complemented strain (SG120) (5) to determine whether the expression of phnDCE was affected by pstS deletion and thus was likely to be under the control of SenX3-RegX3. Using RNA dot blot analysis, a small but reproducible increase in the expression level of phnDCE was observed from the deletion of *pstS*.
observed with the high-phosphate samples of strain SG95 (ΔpstS) compared to that in the wild-type strain, and no differences in the wild-type expression levels were observed with phosphate-starved cells (Fig. 4B). Complementation of the pstS deletion restored phnDCE expression to the wild-type pattern.

To study this pattern of gene expression in more detail, we introduced the transcriptional phnD-lacZ construct (pSG10) (5) into strain SG95 (ΔpstS) and monitored the expression patterns in both the phosphate-replete and the phosphate-starved cells. As a control, the transcriptional pstS-lacZ construct, pSG42 (5), was introduced into strain SG95, because the pstS promoter is known to be regulated by RegX3 (6) and thus should be constitutively expressed in a pstS deletion background. In cells of the wild-type strain carrying the pstS-lacZ construct pSG42, phosphate starvation led to a five- to sixfold increase in β-galactosidase activity, from ca. 10 MU to 55 MU, compared to that in cells grown under phosphate-replete conditions (Fig. 5A). Cells of the pstS deletion strain SG95 harboring pstS-lacZ had β-galactosidase activities of ca. 80 MU, independent of the phosphate concentration available.

In the complemented strain SG120, regulation was restored, and phosphate starvation led to a fivefold induction of β-galactosidase activity, although the absolute levels of activity were lower than in the wild-type strain. These data show that the deletion of pstS causes constitutive expression from the RegX3-dependent pst promoter. Cells of the wild-type strain carrying the phnD-lacZ construct pSG10 displayed a 20-fold induction of β-galactosidase activity in response to phosphate starvation (Fig. 5B). In contrast, the expression of phnD-lacZ in SG95 (ΔpstS) was constitutive at around 100 MU. Complementation of the pstS deletion restored the regulation of expression to that of wild-type levels.

To study the effect of pstS deletion on phnF expression, phnF-lacZ activity was measured in the wild-type strain and in the SG95 strain (ΔpstS). In the wild-type strain carrying the phnF-lacZ construct, phosphate starvation led to a 2.6-fold induction, from 35 MU to 90 MU, while in strain SG95, the expression of phnF-lacZ was constitutive at about 80 MU (Fig. 5C). In the complemented strain SG120, β-galactosidase activity was lower than that in the wild-type strain, but phosphate starvation-dependent induction was restored to a 3-fold level.

These data demonstrate that pstS deletion leads to constitutive expression of phnDCE and phnF under phosphate-replete and phosphate-starved conditions. However, RNA analysis of phnDCE expression in phosphate-replete cells of the pstS deletion mutant indicated that the derepression was only partial. We attribute this discrepancy to the differences in copy numbers of the phnDCE promoter region in the two experiments: for RNA analysis, PhnF is able to exert its repressive effect on the single copy of the phnDCE promoter. In contrast, phnD-lacZ is present as 3 to 10 copies per cell (34), and therefore the effect of PhnF is titrated out. Taken together, these results show that the expression from the phnDCE and phnF promoters, like that from the pstSCAB promoter, is increased in a pstS deletion background. The phnDCE operon and phnF thus appear to be likely new candidates for the SenX3-RegX3 regulon of M. smegmatis, in which phnDCE is under additional control by the repressor PhnF. Further work is required to confirm the involvement of SenX3-RegX3 in the
regulation of \textit{phnDCE} and \textit{phnF} and whether there is direct interaction between RegX3 and these promoters.

The region between \textit{phnF} and \textit{phnD} contains two putative binding sites for PhnF. To gain further understanding of the involvement of the different transcription factors in the regulation of the \textit{phnDCE} and \textit{phnF} promoters, we determined the TSSs for both \textit{phnD} and \textit{phnF}, using 5’ RACE analysis (Fig. 6). The TSS for \textit{phnD} was determined as the “G” located 65 bp upstream of the translational start. No consensus –10 or –35 sequences could be identified for \textit{phnF}. The TSS for \textit{phnF} was determined as the first nucleotide of the GTG start codon, suggesting that \textit{phnF} is transcribed as a leaderless transcript, a sequence for HutC-type regulators has been predicted (27), and sequence of TGGTATAGACCA, which we termed IRU-1 and IRU-2 (Fig. 6). The same sequence has recently been identified as a potential binding site for PhnF by an in silico analysis of the material genes known to respond to phosphate starvation is not yet large enough to identify common motifs in their promoter areas.

As discussed above, PhnF is a member of the HutC subfamily of GntR-like transcriptional regulators. A core recognition sequence for HutC-type regulators has been predicted (27), and more recently, the binding site of a member of this subfamily, DasR from \textit{Streptomyces coelicolor}, has been identified as the 16-bp palindromic sequence ACTGGTCTACACCATT (28). Analysis of the intergenic region between \textit{M. smegmatis phnD} and \textit{phnF} revealed the presence of two inverted repeats with a similar sequence to a loosely conserved inverted repeat (GTGAAC) separated by seven nucleotides in the promoters of \textit{phoh4}, \textit{psitS}, and \textit{senX3} (6). We analyzed 500-bp regions upstream of \textit{phnD} and \textit{phnF} but were unable to identify sequences with obvious similarity to the previously described RegX3 binding sites. Analysis of the same promoter areas, together with 500-bp regions with phosphate-responsive promoter activity for one of the \textit{M. tuberculosis} \textit{pst} operons (35), using a MEME motif discovery and search tool, also did not lead to the identification of potential RegX3 binding sites. It is likely that the number of mycobacterial genes known to respond to phosphate starvation is not yet large enough to identify common motifs in their promoter areas.

To further investigate the role of IRU-1 and IRU-2, we introduced site-directed changes on a 377-bp PCR product encompassing the 199-bp intergenic region between \textit{phnF} and \textit{phnD} plus 93 bp of the start of the \textit{phnF} coding region and 85 bp of the start of the \textit{phnD} coding region. IRU-1 was changed to TGGTATAGACCA (mutated nucleotides are underlined), changing the sequence of each half site as well as the palin-

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure6}
\caption{Sequence analysis of the intergenic region between \textit{phnF} and \textit{phnD}. Transcriptional start sites for \textit{phnF} and \textit{phnD} were determined by 5’ RACE analysis as shown in the top and bottom panels, respectively. Start codons in bold; transcriptional start sites are indicated as +1. Putative –10 and –35 regions for \textit{phnF} are underlined. The two IRUs, which constitute presumptive PhnF binding sites, are boxed.}
\end{figure}
The three mutated fragments as well as a PCR product containing the wild-type sequence were then used to construct constructs, in which phosphate starvation led to a ca.

increase in β-galactosidase activity under phosphate-replete conditions compared to that of the wild-type promoter ($P < 0.0001$) (Fig. 7B). Phosphate starvation still led to a twofold induction of expression ($P = 0.0001$). As observed for the phnDCE expression level, the mutation of both IRU-1 and IRU-2 simultaneously further increased the expression of phnF under both conditions tested ($P < 0.05$). These data suggest that IRU-1 is required for the repression of phnDCE transcription, while IRU-2 is required to repress the transcription of phnF. IRU-1 is centered at position −83.5 relative to the phnD TSS. IRU-2 is centered at position −110.5 relative to the phnF TSS. It is likely that IRU-1 and IRU-2 are binding sites for PhnF, and phnF would therefore appear to be subject to autoregulation.

**Conclusions.** In the present study, we investigated the transcriptional regulation of two operons, pstSCAB and phnDCE, encoding high-affinity phosphate transport systems of *M. smegmatis.* We showed that the *phn* locus contains another gene, *phnF,* which encodes a functional transcriptional regulator belonging to the HutC subfamily of GntR-like regulators. Furthermore, we demonstrated that PhnF acts as a repressor of the phnDCE operon but does not affect the expression of pstSCAB. Two presumptive binding sites for PhnF were identified in the region between *phnD* and *phnF,* and these sites are required for the repression of *phnD* and *phnF.* The deletion of *pstS* leads to the constitutive expression of *phnDCE* and *phnF,* suggesting that these genes may be under the control, directly or indirectly, of the SenX3-RegX3 system of *M. smegmatis.*

Based on these findings, we propose a model for the regulation of *phnDCE* and *pstSCAB* operons in *M. smegmatis* (Fig. 8). Both operons are strongly induced by phosphate limitation. The *pstSCAB* operon is under the sole and direct control of SenX3-RegX3. In contrast, *phnDCE* expression at a low-phosphate concentration requires both the derepression by PhnF and the activation by a second system, presumably SenX3-RegX3. Transcription of *phnF* also appears to be under such dual control of activation by SenX3-RegX3 and repression by PhnF itself. Increased expression of PhnF under phosphate-limited conditions may supply the cell with a means to reestablish the repression of the Phn system when phosphate availability improves. Such a phenomenon of induction of repressors for the Pho regulon, *pstSCAB* and *phoU,* has been reported for *E. coli* and seems to be required to terminate the phosphate starvation response (39). Further work is required to determine whether PhnF and RegX3 interact directly with
putative phosphate transport receptors are encoded by the Mycobacterium tuberculosis genome and are present at the surface of Mycobacterium bovis BCG. J. Bacteriol. 179:2900–2906.