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

Susanne Gebhard and Gregory M. Cook


Updated information and services can be found at: http://jb.asm.org/content/190/4/1335

These include:

REFERENCES

This article cites 38 articles, 25 of which can be accessed free at: http://jb.asm.org/content/190/4/1335#ref-list-1

CONTENT ALERTS

Receive: RSS Feeds, eTOCs, free email alerts (when new articles cite this article), more»

Information about commercial reprint orders: http://journals.asm.org/site/misc/reprints.xhtml
To subscribe to another ASM Journal go to: http://journals.asm.org/sitesubscriptions/
Differential Regulation of High-Affinity Phosphate Transport Systems of *Mycobacterium smegmatis*: Identification of PhnF, a Repressor of the *phnDCE* Operon

Susanne Gebhard and Gregory M. Cook*

Department of Microbiology and Immunology, Otago School of Medical Sciences, University of Otago, P.O. Box 56, Dunedin, New Zealand

Received 6 November 2007/Accepted 6 December 2007

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 phosphorus sources, such as phosphite (e.g., as in the Ptx system of *Pseudomonas stutzeri*) (22) 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 multiple 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 meliloti* (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-
phonate/phosphite transporters of several gram-negative bacteria such as *E. coli* (21), *P. stutzeri* (40), and *S. meliloti* (3) but appears to be specific for phosphate and not phosphonate or phosphate in *M. smegmatis* (5). A gene adjacent to but divergently transcribed from the *M. smegmatis* *phnDCE* operon has been identified as a putative transcriptional regulator of the *M. smegmatis* *phnF* (36). The *phn* operon of *E. coli* also contains a *phnF* gene that is proposed to have a role in the regulation of gene expression, but no definite function has been assigned to its gene product (21).

In the present study, we investigated the mechanisms of regulation of the *phnDCE* and the *pstSCAB* operons of *M. smegmatis*. We used allelic exchange mutagenesis and RNA analysis to investigate the role of PhnF in transcriptional regulation of the *phnDCE* and *pstSCAB* operons. We also used a *pstS* deletion mutant to determine whether the *phnDCE* operon is part of the phosphate regulon in *M. smegmatis*. Site-directed mutagenesis of the region between *phnD* and *phnF* revealed two putative binding sites for PhnF in the promoters of *phnDCE* and *phnF*.

### MATERIALS AND METHODS

**Bacterial strains and growth conditions.** All strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown in Luria-Bertani (LB) medium at 37°C with agitation (200 rpm). The *M. smegmatis* 

### TABLE 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strains</th>
<th>Description</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacterial strain or plasmid</strong></td>
<td><strong>Description</strong></td>
<td><strong>Source or reference</strong></td>
</tr>
<tr>
<td><em>E. coli</em> DH10B</td>
<td>F’ mcrA Δ(mrr-hsdRMS-mcrBC) Δ80lacZ ΔM15 ΔlacX74 deoR recA1 araD139</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Δ(ara leu)7697 galU galK rpsL endA1 napG</td>
<td></td>
</tr>
<tr>
<td><em>M. smegmatis</em> mc155</td>
<td>Electrocompetent wild-type strain of <em>M. smegmatis</em></td>
<td>32</td>
</tr>
<tr>
<td>SG62</td>
<td>mc155 ΔphnF::aphA-3; Km’</td>
<td>This study</td>
</tr>
<tr>
<td>SG95</td>
<td>mc155 ΔpstS::aacC-1; Gm’</td>
<td>5</td>
</tr>
<tr>
<td>SG111</td>
<td>SG62 with pSG41 integrated in attB; Km’ Hyg’</td>
<td>This study</td>
</tr>
<tr>
<td>SG120</td>
<td>SG95 with pSG43 integrated in attB; Gm’ Hyg’</td>
<td>5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Description</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBluescript II KS</td>
<td>Cloning vector; Ap’</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pUC18K</td>
<td><em>E. coli</em> plasmid containing an excisable, nonpolar kanamycin resistance cassette; Km’ Ap’</td>
<td>19</td>
</tr>
<tr>
<td>pX33</td>
<td><em>E. coli</em>-mycobacterium shuttle vector for allelic exchange mutagenesis in mycobacteria, pPR23, a constitutive <em>cylE</em> marker; <em>Gm’</em> Sac’ ts</td>
<td>5, 26</td>
</tr>
<tr>
<td>pJEM15</td>
<td><em>E. coli</em>-mycobacterium shuttle vector for the creation of transcriptional promoter fusions to lacZ; Km’</td>
<td>34</td>
</tr>
<tr>
<td>pUHA267</td>
<td><em>E. coli</em> vector with mycobacteriophage L5 integrase and attP for integration into attB of mycobacteria; Hyg’</td>
<td>AgResearch, Wallaceville, New Zealand</td>
</tr>
<tr>
<td>pSG10</td>
<td>pJEM15 harboring a 500-bp <em>phnD-lacZ</em> fusion; Km’</td>
<td>5</td>
</tr>
<tr>
<td>pSG16</td>
<td>pX33 harboring ΔphnF::aphA-3; Km’ Gm’ Sac’ ts</td>
<td>This study</td>
</tr>
<tr>
<td>pSG18</td>
<td>pJEM15 harboring a 500-bp <em>phnF-lacZ</em> fusion</td>
<td>This study</td>
</tr>
<tr>
<td>pSG41</td>
<td>pUHA267 harboring <em>phnF</em> with its native promoter; Hyg’</td>
<td>5</td>
</tr>
<tr>
<td>pSG42</td>
<td>pJEM15 harboring a 500-bp <em>pstS-lacZ</em> fusion; Km’</td>
<td>This study</td>
</tr>
<tr>
<td>pSG43</td>
<td>pUHA267 harboring <em>pstS</em> with its native promoter; Hyg’</td>
<td>5</td>
</tr>
</tbody>
</table>

---

mrr-hsdRMS-mcrBC (32) and derived strains were routinely grown at 37°C with agitation at 200 rpm in LB medium containing 0.05% (wt/vol) Tween 80 (LBt) or in Middlebrook 7H9 medium (Difco) supplemented with 10% albumin-dextrose-catalase enrichment (ADC; Becton Dickinson) and 0.05% (wt/vol) Tween 80. *M. smegmatis* transfor-

mants were grown at 28°C for the propagation of temperature-sensitive vectors and at 40°C for allelic exchange mutagenesis. For *P. stutzeri*, *S. meliloti*, and *M. smegmatis* cultures, cells were grown in high-phosphate medium to an optical density of 600 nm (OD600) of 0.7 to 1.2, washed twice in sterile 0.85% (wt/vol) saline with 0.05% (wt/vol) Tween 80, and resuspended to an OD600 of 0.7 in phosphate-free Sauton’s medium, and incubated at 37°C with agitation at 200 rpm for 2 h. Selective media contained kanamycin (50 μg mL⁻¹ for *E. coli*; 20 μg mL⁻¹ for *M. smegmatis*), gentamicin (20 μg mL⁻¹ for *E. coli*; 5 μg mL⁻¹ for *M. smegmatis*), or hygromycin (200 μg mL⁻¹ for *E. coli*; 50 μg mL⁻¹ for *M. smegmatis*). Solid medium contained 1.5% agar.

**DNA manipulation and cloning of constructs.** All molecular biology techniques were carried out according to standard procedures (31). Restriction or DNA-modifying enzymes and other molecular biology reagents were obtained from Roche Diagnostics or New England Biolabs.

Genomic DNA of *M. smegmatis* was isolated by a method modified from that described by Gonzalez-y-Merchand et al. (7). In brief, cells grown on LBt agar were resuspended in 200 μL of lysis buffer (4 M guanidine thiocyanate, 1 mM β-mercaptoethanol, 10 mM EDTA, 0.1% (wt/vol) Tween 80, snap-frozen in dry ice, and heated to 65°C for 10 min. Snap-freezing/heat was repeated, and the cells were cooled on ice for 5 min. The aqueous phase was extracted twice with chloroform, and DNA was precipitated by isopropanol. The pellet was washed once in 70% ethanol, air dried, and dissolved in deionized water.

To create a transcriptional fusion of *phnF*, a PCR product encompassing 420 bp of DNA upstream of *phnF* plus 110 bp of its coding region was amplified using the primers PhnFF (5’-AAAAATTGGCGGCCCAGATGTCGGAGCTGGAC-3’) and PhnFR (5’-AAATTTGGATACGGACCCGATCCGCAATC-3’). The product was cloned into the ApaI and Apa1 sites of pEIM15 (34), creating the plasmid pSG18.

To create a construct for the deletion of *phnF*, the kanamycin resistance (Kmr) cassette, encoded by *apha-3*, was amplified from pUC18 (19) by PCR, using the primers 3’mcspUC (5’-GTTTTCTCCAGTCGAAGCGGT-3’) and 5’mcspUC (5’-
Phosphate transport system regulation of M. smegmatis

RESULTS AND DISCUSSION

Identification of a putative regulator in the phn locus of M. smegmatis.

The PhnDCE phosphate 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, MSMEG_0650, has been annotated as a GntR family transcriptional regulator encoding a 244-amino-acid protein. Analysis of the gene sequence revealed a movement into the PhnDCE operon via the TIGR website (http://cmr.tigr.org), which highlighted the presence of a 320-bp fragment containing a helix-turn-helix motif. Similarity searches were performed using the Clustal W program, and the resulting sequence was compared to other proteins using the Swiss-Prot database. The sequence of the PhnF protein was called PhnF (36) (Fig. 1A).

The transcriptional start sites (TSSs) of phnD and phnF were determined using primer extension analysis. The transcriptional start site of phnD was determined using a cDNA library generated using a 5'-rapid amplification of cDNA ends (RACE) kit and a specific primer phnF-RACE1 (5'-ACGAAGCACACCTTCTTG-3'). The transcriptional start sites of phnF were determined using a cDNA library generated using a 5'-rapid amplification of cDNA ends (RACE) kit and a specific primer phnF-RACE2 (5'-ACTGCGGCTGCATGACGTAGAG-3'). The transcriptional start sites were determined using a gel-based method, and the resulting sequences were confirmed using DNA sequencing.

DNA and protein sequence analysis.

The provisional genome sequence of M. smegmatis strain mc^155 was accessed via The Institute for Genomic Research (TIGR) website (http://www.tigr.org; GenBank accession number CP000480). Sequence data for M. tuberculosis was obtained from the Institut Pasteur website (http://genolist.pasteur.fr/TuberulList). Protein sequence alignments were carried out using a Clustal W program, and the resulting sequences were compared to other proteins using the Swiss-Prot database. The resulting sequences were compared to other proteins using the Clustal W program, and the resulting sequences were compared to other proteins using the Swiss-Prot database.

RESULTS AND DISCUSSION

Identification of a putative regulator in the phn locus of M. smegmatis.

The PhnDCE phosphate 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, MSMEG_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 UbC transcriptional regulator-associated (UTRA) domain, which is common to members of the HUc 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 \( \alpha \)-\( \beta \)-\( \alpha \)-\( \beta \) 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 \( \mu \)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 rapidly increased 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 \( \mu \)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
and pstSCAB, phospho-DCE, phospho-F.

In the wild-type strain, both of the Medium (5 g glycerol liter\(^{-1}\), 4 g \(\text{L-asparagine}\) liter\(^{-1}\), 200 \(\mu\text{M}\) P\(_{i}\)) and monitored for growth, expressed as OD\(_{600}\) (■), phosphate concentration in the medium (\(P_i\)) (△), and \(\beta\)-galactosidase activity (β-Gal), expressed as MU (□). Representative results of two independent experiments are shown.

cloned into pX33, as described in Materials and Methods, and \(M.\) \(\text{sme}gmatis\) mc\(^{2}\)155 was transformed with the resulting plasmid. Knockout mutants were selected as described previously (26). The replacement of \(\text{phnF}\) with the antibiotic marker introduced two additional SmaI restriction sites, resulting in a band shift from 6.7 kb in the wild type to 3.2 kb in the deletion mutant (strain SG62) in Southern hybridization analysis of SmaI-digested genomic DNA probed with a radiolabeled PCR product of the left flank of the deletion construct (Fig. 3). For complementation, an integrative plasmid containing \(\text{phnF}\) plus ca. 500 bp of the region upstream of the predicted translational start site was introduced into strain SG62, creating strain SG111.

**Deletion of \(\text{phnF}\) leads to the overexpression of \(\text{phnDCE}\) but not \(\text{pstSCAB}\).** In order to determine any of the effects that the deletion of \(\text{phnF}\) would have on the expression of the \(\text{phnDCE}\) and \(\text{pstSCAB}\) operons, we analyzed the relative amounts of mRNA synthesized by both of the operons in the wild type and the \(\text{phnF}\) deletion strain (SG62) under high- and low-phosphate conditions. In the wild-type strain, both of the \(\text{phnDCE}\) and \(\text{pstSCAB}\) operons were expressed at low levels in the high-phosphate samples, and expression was increased strongly in phosphate-starved cells (Fig. 4A), as was expected from previous results obtained from transcriptional \(\text{lacZ}\) fusion analyses (5). The deletion of \(\text{phnF}\) had no effect on the expression of the \(\text{pstSCAB}\) operon, which showed the same expression patterns in the SG62 \(\triangle\text{phnF}\) strain, the SG111 complemented strain, and the wild-type strain (Fig. 4A, top panel). In contrast, the expression of \(\text{phnDCE}\) was strongly increased in the phosphate-starved cells of the \(\text{phnF}\) deletion mutant strain SG62 compared to that in the cells of the wild-type strain (Fig. 4A, bottom panel). The expression level of \(\text{phnDCE}\) under high-phosphate conditions was not changed in the SG62 strain. Complementation of the \(\text{phnF}\) 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 \(\text{phnF}\). These data clearly show that PhnF acts as a repressor of \(\text{phnDCE}\). PhnF appears to have no role in the regulation of \(\text{pstSCAB}\). The fact that the deletion of \(\text{phnF}\) does not lead to full constitutive expression of \(\text{phnDCE}\) 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 \(\text{pstS}\) leads to the constitutive expression of \(\text{pstSCAB}, \text{phnDCE}, \) and \(\text{phnF}\).** As mentioned above, the expression of the \(\text{phnDCE}\) 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 \(\text{pstSCAB}\) operon (6). SenX3, like its functional counterpart PhoR of \(E.\) \(\text{coli}\), lacks any significant extracellular loops, which led to the proposal that the Pst system of \(M.\) \(\text{sme}gmatis\) functions as the actual sensor of extracellular phosphate concentration (6). Accordingly, mutations in either \(\text{pstS}, \text{pstC},\) or \(\text{pstA}\) are sufficient for the derepression of \(\text{phoA}\) expression (15), which is known to be under the control of SenX3-RegX3 (6). We therefore utilized the \(M.\) \(\text{sme}gmatis\) \(\text{pstS}\) deletion mutant (strain SG95) and the \(\text{pstS}\)-complemented strain (SG120) (5) to determine whether the expression of \(\text{phnDCE}\) was affected by \(\text{pstS}\) 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 \(\text{phnDCE}\) was
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 (ΔpstS), 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

FIG. 4. Dot blot analysis of RNA from wild-type and mutant strains. Fourfold dilutions of total RNA isolated from cells grown in high-phosphate medium (100 mM; +) or subjected to phosphate starvation for 2 h (−) were spotted onto nylon membranes. Membranes were probed with radiolabeled PCR products from internal fragments of pstC (A, top panel) or phnC (A, bottom panel, and B). Amounts of total RNA per spot are shown in ng. Strains are indicated above the autoradiographs. (A) WT, wild-type; ΔphnF, phnF deletion strain SG62; cphnF, phnF-complemented strain SG111. (B) WT, wild-type; ΔpstS, pstS deletion strain SG95; c pstS, pstS-complemented strain SG120. Representative results of two to three independent experiments are shown. Below each panel, 16S and 23S rRNA bands from 300 ng total RNA per sample on an agarose gel stained with ethidium bromide are shown as controls. Lanes correspond to the samples in the rows above.
regulation of phnDCE and phnF and whether there is direct interaction between RegX3 and these promoters.

The region between phnF and 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 phnDCE and phnF promoters, we determined the TSSs for both phnD and phnF, using 5' RACE analysis (Fig. 6). The TSS for phnD was determined as the “G” located 65 bp upstream of the translational start. No consensus −10 or −35 sequences could be identified for phnF. The TSS for phnF was determined as the first nucleotide of the GTG start codon, suggesting that phnF is transcribed as a leaderless transcript, a feature which has also been observed for other GntR family transcriptional regulators from actinomycetes (12, 30). Putative −10 (5'-TACGTT-3') and −35 (5'-TCTGAC-3') boxes with some similarities to mycobacterial promoter elements (1, 29) could be identified upstream of the phnF TSS (Fig. 6).

Next, we searched both promoter regions for conserved binding motifs. RegX3 of M. smegmatis has been shown to bind to a loosely conserved inverted repeat (GTGAAC) separated by seven nucleotides in the promoters of phoA, ptsS, and senX3 (6). We analyzed 500-bp regions upstream of phnD and 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 M. tuberculosis 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.

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 Streptomyces coelicolor, has been identified as the 16-bp palindromic sequence ACTGGTCTACACCATT (28). Analysis of the intergenic region between M. smegmatis phnD and phnF revealed the presence of two inverted repeats with a similar 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 M. smegmatis GntR family regulators (38).

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 phnF and phnD plus 93 bp of the start of the phnD coding region and 85 bp of the start of the phnD coding region. IRU-1 was changed to TGGTATAGACCA (mutated nucleotides are underlined), changing the sequence of each half site as well as the palindrome sequence. Start codons are shown in bold; transcriptional start sites are determined as the first nucleotide of the GTG start codon, using 5'-Galactosidase activities are given as reverse traces. The sequence for phnF for HutC-type regulators has been predicted (27), and one of the first three codons of phnF and phnD (the first three amino acids are indicated) and the intergenic region. It should be noted that the sequence for phnF is therefore given as a reverse sequence. Start codons are shown in bold; transcriptional start sites are shown in bold and are indicated as +1. Putative −10 and −35 regions for phnF are underlined. The two IRUs, which constitute presumptive PhnF binding sites, are boxed.
The three mutated fragments as well as a PCR product containing the changes in both regions was amplified.

Changes were introduced into IRU-1 and IRU-2 (see text for details), leading to a mutated sequence of TGGTATACCACA. A third promoter, this part of the IRU-2 was therefore left unchanged, as gray ovals. Flat-headed arrows indicate negative regulation; a pointed arrowhead indicates positive regulation. Dotted lines indicate that the proposed regulation may be direct or indirect. The presumptive PhnF binding sites are shown as black diamonds.

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 the 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

FIG. 7. Effects of site-directed mutagenesis of the IRUs in the intergenic region between phnF and phnD on gene expression. Changes were introduced into IRU-1 and IRU-2 (see text for details), and effects were monitored as the expression of phnD-lacZ (A) and phnF-lacZ (B) transcriptional fusions. Cells were grown in minimal Sauton’s medium containing 100 mM phosphate (open bars) or starved in phosphate-free medium for 2 h (gray bars). The presence of wild-type sequences (+) or site-directed changes (−) in each IRU are shown below the graphs. Results are shown as the means and standard deviations from three independent experiments.

FIG. 8. Model for the regulation of pstSCAB, phnDCE, and phnF in M. smegmatis. Genes are shown as open arrows; proteins are shown as gray ovals. Flat-headed arrows indicate negative regulation; a pointed arrowhead indicates positive regulation. Dotted lines indicate that the proposed regulation may be direct or indirect. The presumptive PhnF binding sites are shown as black diamonds.
the promoters studied here or indirectly via additional regulatory systems and to study how these different regulatory proteins interact with each other or with RNA polymerase. The differences in regulation between the Pst and Phn systems of \textit{M. smegmatis} described here may shed light on the differences between the multiple phosphate transport systems present in other mycobacterial species. It has been proposed previously that the requirement of several such systems by mycobacteria may reflect subtle adaptations of the bacteria to a highly variable environment (17). In this case, the differential regulation of gene expression, as observed here, may be a key feature of mycobacterial phosphate transport systems.

ACKNOWLEDGMENTS

This work was funded by a New Zealand Lottery health grant. S.G. was supported by a University of Otago Prestigious Postgraduate Scholarship.

We thank Vernon Ward for helpful advice regarding the 5’ RACE experiments. We also thank Desmond Collins for supplying the pUHA267 plasmid.

REFERENCES


