The Phn system of *Mycobacterium smegmatis*: a second high-affinity ABC-transporter for phosphate

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Uptake of inorganic phosphate, an essential but often limiting nutrient, in bacteria is usually accomplished by the high-affinity ABC-transport system Pst. Pathogenic species of mycobacteria contain several copies of the genes encoding the Pst system (*pstSCAB*), and two of the encoded proteins, PstS1 and PstS2, have been shown to be virulence factors in *Mycobacterium tuberculosis*. The fast-growing *Mycobacterium smegmatis* contains only a single copy of the *pst* operon. This study reports the biochemical and molecular characterization of a second high-affinity phosphate transport system, designated Phn. The Phn system is encoded by a three-gene operon that constitutes the components of a putative ABC-type phosphonate/phosphate transport system. Expression studies using *phnD*– and *pstS–lacZ* transcriptional fusions showed that both operons were induced when the culture entered phosphate limitation, indicating a role for both systems in phosphate uptake at low extracellular concentrations. Deletion mutants in either *phnD* or *pstS* failed to grow in minimal medium with a 10 mM phosphate concentration, while the isogenic wild-type strain mcH155 grew at micromolar phosphate concentrations. Analysis of the kinetics of phosphate transport in the wild-type and mutant strains led to the proposal that the Phn and Pst systems are both high-affinity phosphate transporters with similar affinities for phosphate (i.e. apparent *Kₐ* values between 40 and 90 μM P). The Phn system of *M. smegmatis* appears to be unique in that, unlike previously identified Phn systems, it does not recognize phosphonates or phosphite as substrates.

**INTRODUCTION**

The assimilation of Pᵢ or other phosphorus compounds from the environment has been studied in several microorganisms. Detailed studies of Pᵢ transport systems have focussed on *Escherichia coli* (van Veen, 1997; Wanner, 1996), *Acinetobacter johnsonii* (van Veen, 1997), *Sinorhizobium meliloti* (Bardin et al., 1998; Voegele et al., 1997; Yuan et al., 2006) and *Bacillus subtilis* (Allenby et al., 2004; Qi et al., 1997). Pᵢ transporters have also been identified in a number of other bacteria, and there are noticeable parallels between the organizations of transport systems in different species.

Under conditions of excess Pᵢ in the environment, bacteria utilize a low-affinity transporter, the Pit system (phosphate inorganic transport). This transporter is constitutively expressed, recognizes metal phosphate as its substrate and is driven by the proton-motive force (ΔμH⁺). The Pit system has been studied in the most detail in *E. coli* (Hoffer et al., 2001; van Veen et al., 1994b), *A. johnsonii* (van Veen et al., 1993, 1994a) and *S. meliloti* (Bardin et al., 1998; Voegele et al., 1997).

Bacteria also contain an inducible, high-affinity Pᵢ transporter, which operates under Pᵢ limitation. This is usually the ATP-binding cassette (ABC)-type transport system Pst (phosphate specific transport). It consists of four components, PstSCAB, where PstS is the substrate-binding protein, PstC and PstA are transmembrane proteins, and PstB is the ATPase. Pst recognizes Pᵢ, rather than metal phosphate, as its substrate. Well characterized Pst systems include those of *E. coli*, *S. meliloti* and *B. subtilis* (Allenby et al., 2004; Qi et al., 1997; Wanner, 1996; Yuan et al., 2006).

Additionally, many bacteria are able to utilize phosphonates or phosphite as alternative sources of phosphorus. Phosphonates are compounds that contain a direct carbon–phosphorus link, and they can be degraded by *E. coli* (Metcalfe & Wanner, 1991), *Klebsiella aerogenes* (Imazu et al., 1998), *Pseudomonas stutzeri* (White & Metcalf, 2004b), *S. meliloti* (Bardin et al., 1996; Voegele et al., 1997), some streptomycete isolates (Obojska et al., 1999), and other microorganisms (reviewed by Kononova & Nesmeyanova, 2002). In *E. coli* and *K. aerogenes*, phosphonates and...
phosphite are taken up into the cells by the ABC-type Phn system, consisting of the three components PhnCDE, where PhnC is the ATPase, PhnD the substrate-binding protein, and PhnE the permease. Phn is a non-specific substrate for this system (McTevet & Wanner, 1991). S. melliloti utilizes a high-affinity ABC-type transport system, PhoCDET, for the uptake of both P_i and phosphonates. PhoC and PhoD are homologous to PhnC and PhnD, respectively, while PhoE and PhoT both encode transmembrane proteins homologous to PhnE (Bardin et al., 1996; Voegel et al., 1997).

The genes encoding high-affinity P_i transport systems, such as the pst genes, or for transport systems for alternative phosphorus sources, such as the phn genes, are generally induced when the organism encounters P_i-limited conditions (Bardin & Finan, 1998; Qi et al., 1997; Sola-Landa et al., 2005; Wanner, 1996; White & Metcalf, 2004a). This induction is mediated by the P_i-responsive two-component regulatory system, PhoBR in Gram-negative micro-organisms (Bardin & Finan, 1998; von Kruger et al., 2001; Peirs et al., 2003; Wanner, Chang, 1987), or PhoPR in Gram-positives (Hulett et al., 1994; Perez et al., 2001; Sola-Landa et al., 2003). In both cases, PhoR is a membrane-bound sensor and PhoP or PhoB are the response regulators. In E. coli and Mycobacterium smegmatis, repression of the Pho response requires PstS, and mutations in pstS lead to constitutive expression of Pho regulon genes (Kriakov et al., 2003; Wanner, 1996).

*Mycobacterium tuberculosis* contains several copies of genes for components of a Pst system, encoding three different PstS, two PstC and PstA each, and one PstB protein (Braibant et al., 1996a). Other slow-growing species of mycobacteria also contain several copies of *pst* genes (Braibant et al., 1996a; Lefevre et al., 1997). Mutants in some of these genes have been created and they were defective in P_i transport and had reduced virulence *in vivo* (Collins et al., 2003; Peirs et al., 2005). The reason for the presence of several high-affinity *P* _i_ transport systems in pathogenic mycobacteria is not understood. The fast-growing *M. smegmatis* contains only a single copy of the *pst* genes, organized as a *pstSCAB* operon, and mutants in these genes display reduced P_i uptake and an increased requirement for P_i for growth (Bhatt et al., 2000; Kriakov et al., 2003). P_i transport by mycobacteria is poorly characterized, and no detailed studies are available on the requirements for P_i for the growth of these bacteria. In a previous study by our group, a three-gene operon (phnDCE) of *M. smegmatis* with homology to the Psn phosphonate/phosphate transport systems of other bacteria was identified, but no function was assigned to these genes (Tran et al., 2005).

The aim of this study was to determine whether the putative PhnDCE transport system of *M. smegmatis* has a role in the uptake of P_i and phosphonates, and to compare the characteristics of the putative Phn system to those of the Pst system. The expression of the phn and pst operons in response to P_i limitation was investigated using transcriptional fusions to *lacZ*. Mutants of both systems were created and characterized regarding their growth at different P_i concentrations, as well as kinetics of P_i uptake into whole cells.

**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 r.p.m.). *M. smegmatis* strain mc²155 (Snapper et al., 1990) and derived strains were routinely grown at 37 C, 200 r.p.m. in LB containing 0.05% (w/v) Tween 80 (LBT) or in Middlebrook 7H9 medium (Difco) supplemented with 10% albumin–glucose [dextrose]-catalse enrichment (ADC; Becton Dickinson) and 0.05% (w/v) Tween 80. *M. smegmatis* transformants were grown at 28 C for propagation of temperature-sensitive vectors and at 40 C for allelic-exchange mutagenesis. For P_i limitation studies, *M. smegmatis* was grown in modified Sauton’s medium (ST). The composition of this medium per litre was as follows: 0.5 g MgSO_4_, 2 g citric acid, 1 g l-asparagine, 0.3 g KCl, 1 g glycerol, 0.5 g Tween 80, 320 µl of 0.5 M FeCl_3, and 100 µl of 1 M NaHCO_3_. High-phosphate medium contained 10 mM or 100 mM K_2HPO_4_. For low P_i concentrations (1 mM and less), P_i was added to P_i-free medium from a 0-2 M stock of KH_2PO_4. As alternative sources of phosphorus, methylphosphonate or phosphite were added to P_i-free medium to final concentrations of 1 mM or 5 mM, and phosphonoacetate was added to final concentrations of 1 mM or 2 mM. Cells used as inocula for low-phosphate media were washed in 2 vols saline/Tween 80 (0.85% NaCl, 0.05% (w/v) Tween 80). 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 media contained 1.5% agar.

Optical density was measured at 600 nm (OD₆₀₀) using culture samples diluted in saline to bring OD₆₀₀ to below 0.5 when measured in cuvettes of 1 cm light path length in a Jenway 6300 spectrophotometer. Media were inoculated to an initial OD₆₀₀ of 0.005. Growth rates were determined by plotting the log₁₀ of the OD₆₀₀ versus time.

**DNA manipulation and cloning of constructs.** All molecular biology techniques were carried out according to standard procedures (Sambrook et al., 1989). 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 modification of the method of Gonzalez-γ-Merchand et al. (1996). In brief, cells grown on LBT agar were resuspended in 200 µl lysis buffer (4 M guanidine thiocyanate, 1 mM β-mercaptoethanol, 10 mM EDTA, 0.1%, w/v; Tween 80), snap-frozen in solid CO₂/ethanol and heated to 65 C for 10 min. Snap-freezing/heating was repeated and the cells were then cooled on ice for 5 min. The aqueous phase was extracted twice with chloroform and DNA precipitated by 2-propanol. The pellet was washed once in 70% ethanol, air-dried and dissolved in deionized water.

To create a transcriptional fusion of the phn operon, a 580 bp PCR product, encompassing approximately 500 bp of DNA upstream of phnD plus 85 bp of its coding region, was amplified using primers PPhnF (5’-AAATTTGGGCCCGACATCAATCCCTGGGCCTT-3’) and PPhnR (5’-AAATTTGGTACCGCTTGTCGGAGCCCGAACAG-3’). The product was cloned into the ApaI and Asp718 sites of pJEM15 (Timm et al., 1994), creating plasmid pS910. To create a transcriptional fusion of the pst operon, an 800 bp PCR product, encompassing approximately 560 bp of DNA upstream of pstS plus 200 bp of its coding region, was amplified using primers PpstF (5’-AATTTGTAACCGCTCAAAACCCCGCTTTCTCTCTG-3’) and PpstR
The Phn system of *Mycobacterium smegmatis*

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description*</th>
<th>Source or reference</th>
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<tr>
<td><strong>E. coli</strong></td>
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<tr>
<td>DH10B</td>
<td>F&lt;sup&gt;−&lt;/sup&gt; mcrA Δmrr–lisdRMS–mrcBCΔ pho88ΔlacZ ΔM15 ΔlacX74 deoR recA1 araD139 Δ(ara leu)7697 galU galK rpsL endA1 mupG</td>
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<td><strong>M. smegmatis</strong></td>
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<td>mc&lt;sup&gt;2&lt;/sup&gt;155</td>
<td>Electrocompetent wild-type strain of <em>M. smegmatis</em></td>
<td>Snapper et al. (1990)</td>
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<td>SG34</td>
<td>mc&lt;sup&gt;1&lt;/sup&gt;155 ΔphnD::aphA-3; Km&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
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<td>SG95</td>
<td>mc&lt;sup&gt;1&lt;/sup&gt;155 ΔpstS::aacC-1; Gm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
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<tr>
<td>SG106</td>
<td>SG34 with pSG38 integrated in attB; Km&lt;sup&gt;+&lt;/sup&gt;, Hyg&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
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<tr>
<td>SG118</td>
<td>mc&lt;sup&gt;1&lt;/sup&gt;155 ΔphnD::aphA-3 ΔpstS::aacC-1; Km&lt;sup&gt;+&lt;/sup&gt;, Gm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
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<td>SG120</td>
<td>SG95 with pSG43 integrated in attB; Gm&lt;sup&gt;+&lt;/sup&gt;, Hyg&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
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<td><strong>Plasmids</strong></td>
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<tr>
<td>pBluescript II KS</td>
<td>Cloning vector; Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Stratagene</td>
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<td>pUC18K</td>
<td><em>E. coli</em> plasmid containing an excisable, non-polar kanamycin resistance cassette; Km&lt;sup&gt;+&lt;/sup&gt; Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Menard et al. (1993)</td>
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<td>pBSL141</td>
<td><em>E. coli</em> plasmid containing an excisable aacC-1 gene; Gm&lt;sup&gt;+&lt;/sup&gt; Ap&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>pPR23</td>
<td><em>E. coli</em>–mycobacteria shuttle vector for allelic-exchange mutagenesis in mycobacteria; Gm&lt;sup&gt;+&lt;/sup&gt; Sac&lt;sup&gt;+&lt;/sup&gt;, ts</td>
<td>Pelicic et al. (1997)</td>
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<td>pX33</td>
<td>pPR23 carrying a constitutive xylE marker; Gm&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>pJEM15</td>
<td><em>E. coli</em>–mycobacteria shuttle vector for the creation of transcriptional promoter fusions to lacZ; Km&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Timm et al. (1994)</td>
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<td>pUHA267</td>
<td><em>E. coli</em> vector with mycobacteriophage L5 integrase and attP for integration into attB of mycobacteria; Hyg&lt;sup&gt;+&lt;/sup&gt;</td>
<td>AgResearch, Wallaceville, NZ</td>
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<td>pSG3</td>
<td>pX33 harbouring ΔphnD::aphA-3; Km&lt;sup&gt;+&lt;/sup&gt; Gm&lt;sup&gt;+&lt;/sup&gt; Sac&lt;sup&gt;+&lt;/sup&gt;, ts</td>
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<td>pSG30</td>
<td>pPR23 harbouring ΔpstS::aacC-1; Gm&lt;sup&gt;+&lt;/sup&gt; Sac&lt;sup&gt;+&lt;/sup&gt;, ts</td>
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<td>pSG38</td>
<td>pUHA267 harbouring phnD&lt;sup&gt;+&lt;/sup&gt; with its native promoter; Hyg&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>pJEM15 harbouring a 560 bp pstS-lacZ fusion; Km&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>pSG43</td>
<td>pUHA267 harbouring pstS&lt;sup&gt;+&lt;/sup&gt; with its native promoter; Hyg&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
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*Km<sup>+</sup>, kanamycin resistance; Gm<sup>+</sup>, gentamicin resistance; Hyg<sup>+</sup>, hygromycin resistance; Ap<sup>+</sup>, ampicillin resistance; Sac<sup>+</sup>, sucrose sensitivity; ts, temperature sensitivity.

To create a deletion construct for *pstS*, the gentamicin resistance cassette (Gm<sup>+</sup>), aacC-1, from pBSL141 (Alexeeyev et al., 1995) was excised from the plasmid using *PstI* and *NotI*. PCR products flanking the *pstS* gene of *M. smegmatis* for approximately 780 bp (left flank) and 1 kb (right flank) were amplified using the primers pSGK01 (5′-AAATTTGTCAGTCAACAAAGCGCGTTCTTGTTG-3′) with pSGK02 (5′-AAATTTCTCGAGCTCGCCATTCTTGCTG-3′) and pSGK03 (5′-AAATTTGTCAGTCAACAAAGCGCGTTCTTGTTG-3′) and pSGK04 (5′-AAATTTGTCAGTCAACAAAGCGCGTTCTTGCTG-3′) with pSGK05 (5′-AAATTTCTCGAGCTCGCCATTCTTGCTG-3′), respectively. The left flank PCR product was digested with *BamHI* and *PstI*, the right flank PCR product with *NotI* and *XbaI*. Both flanks and the gentamicin cassette were ligated into the *BamHI* and *XbaI* sites of pBluescript II KS (Stratagene). The resulting assembly, left flank/Gm<sup>+</sup>/right flank, was subcloned as a *BamHI/XbaI* fragment into pPR23, resulting in plasmid pSG30. The expected double-crossover event would result in a deletion-insertion at the *pstS* locus, eliminating 76% of the *pstS* coding sequence in exchange for the gentamicin resistance marker.

Allelic replacement of *phnD* and *pstS* was essentially carried out as described by Pelicic et al. (1997). Deletion of *pstS* was achieved by growing a culture of *M. smegmatis* carrying pSG30 at 28 °C with agitation (200 r.p.m.) to an OD<sub>600</sub> of approximately 0.6 to 0.8, followed by plating onto low-salt LBT plates (2 g NaCl<sup>−1</sup>) containing gentamicin and 10% sucrose at 40 °C, selecting for double-crossover.

(5′-AAATTTTGATACCCCTCGGCAGTGTTCTTGTG-3′). The product was cloned into the *Asp718* site of pJEM15, creating plasmid pSG42.

To create a construct for the deletion of *phnD*, the kanamycin resistance cassette (Km<sup>+</sup>), encoded by *aphA-3*, was amplified from pUC18K (Menard et al., 1993) by PCR using the primers 3′ mcsPUC (5′-GTTTTTACCCAGCAACTCTT-3′) and 5′ mcsPUC (5′-CACACAGAGAAACAGCTATG-3′). The resulting 850 bp product was digested with *SacI* and *HindIII*. PCR products of approximately 1–2 kb flanking the *phnD* gene of *M. smegmatis* on either side were amplified using the primers phnDK01 (5′-AAATTTTACAGTGGGCCGCTGTAACAGATTG-3′) with phnDK02 (5′-AAATTGGCTGCTTTCGGAGACCGAACACG-3′) (left flank) and phnDK03 (5′-AAATTTTACAGTGGGCCGCTGTAACAGATTG-3′) with phnDK04 (5′-AAATTGGCTGCTTTCGGAGACCGAACACG-3′) (right flank). The left flank PCR product was digested with *SpeI* and *SacI*, the right flank PCR product with *HindIII* and *SpeI*. Both flanks and the kanamycin cassette were ligated into the *SpeI* site of pBluescript II KS (Stratagene). The resulting assembly, left flank/Km<sup>+</sup>/right flank, was subcloned as a *SpeI* fragment into pPR23 (Pelicic et al., 1997) carrying a constitutive xylE marker, generating plasmid pSG3. The expected double-crossover event would result in a non-polar deletion-insertion at the *phnD* locus, eliminating 85% of the *phnD* coding sequence in exchange for the kanamycin resistance marker.
events. Replacement of pstS with the gentamicin marker created strain SG95 (ΔpstS::aacC-1). For the deletion of phnD, a two-step protocol was used. A culture of M. smegmatis carrying pSG3 was grown at 28 °C as described above and then plated onto LBT solid medium containing kanamycin. Incubation at 40 °C in the presence of kanamycin selected for integration of the whole deletion construct into the chromosome of M. smegmatis via a single-crossover event at either the left or right flank. Colonies that formed a yellow product when exposed to 250 mM catechol due to the presence of the phnD marker in the background of the phnD deletion strain were screened by Southern hybridization analysis for correct integration of the construct. One integrant was chosen and grown in 2 ml TH9/ADC containing kanamycin at 37 °C, 200 r.p.m. to an OD₆₀₀ of 0-7. Aliquots of this culture were then plated onto low-salt LBT plates containing kanamycin and 10 % sucrose and incubated at 40 °C, to select for a second crossover event resulting in loss of the plasmid and replacement of phnD with the kanamycin marker. Colonies that did not form the yellow product after exposure to catechol were picked onto LBT plates containing kanamycin, and counter-picked onto LBT plates containing gentamicin, to confirm loss of the plasmid backbone. Candidate clones (Km', Gm') were screened by Southern hybridization analysis for correct deletion of phnD. Replacement of phnD with the kanamycin marker created strain SG34 (ΔphnD::aph-3).

To create a phnD pstS double deletion mutant, strain SG34 was transformed with pSG30 and the mutant was created using a two-step protocol as described above. Gentamicin was used for selection in all events. Replacement of pstS with the gentamicin marker in the background of the phnD deletion strain created strain SG118 (ΔphnD::aph-3 ΔpstS::aacC-1).

For Southern hybridization analysis, Smal- or EcoRI-digested genomic DNA of putative mutants was separated on a 1 % agarose/TAE gel and transferred onto a nylon membrane (Hybond-N+, Amersham) by vacuum blotting. Probes were labelled by random priming using [α-³²P]dCTP (Amersham) and Ready-To-Go DNA-labelling beads (Amersham).

Constructs used for complementation of allelic-exchange mutants were cloned into the integrative E. coli/mycobacteria shuttle vector pUHA267 (Lee et al., 1991). For complementation of the phnD mutation, a 1-7 kb PCR product encompassing the phnD gene plus 500 bp of upstream DNA was amplified by PCR using primers cphnDF (5'-AAATTTGTTACCGGACTCATATTCTGGGGCCTT-3') and cphnDR (5'-AAATTTGTTACCGGACTCATATTCTGGGGCCTT-3') and cloned into the Asp718 site of pUHA267, creating plasmid pSG38. For complementation of the pstS mutation, a 1-5 kb PCR product encompassing the pstS gene plus 500 bp upstream DNA was amplified by PCR using primers PpstF and cptsR (5'-AAATTTGTTACCGGACTCATATTCTGGGGCCTT-3') and cloned into the Asp718 site of pUHA267, creating plasmid pSG43.

β-Galactosidase and inorganic phosphate assays. To determine the threshold Pₙ concentration leading to induction of the phn and pst operon genes, strains carrying pSG10 or pSG42 were grown in medium containing an intermediate Pₙ concentration (200 μM). The medium was modified from the standard ST medium used in this study to contain higher concentrations of the carbon source (5 g glycerol l⁻¹) and nitrogen source (4 g L-asparagine l⁻¹), providing both nutrients in excess. At various time points, 2 ml to 4 ml of the culture where removed to determine OD₆₀₀. Cells were then pelleted and cell pellets and supernatants were stored at −20 °C. β-Galactosidase activity for the 0 h time point (t₀) was determined in cells of the inoculum culture. To measure β-galactosidase activities, cell pellets were resuspended in 1 ml Z-buffer (61 mM Na₂HPO₄, 39 mM NaH₂PO₄, 10 mM KCl, 1mM MgSO₄3 containing 0-05 % (w/v) Tween 80, and OD₆₀₀ was adjusted to 0.2 in 1.5 ml Z-buffer. Of this suspension, 200, 400 and 800 μl were used in each assay and the volumes increased to 1 ml with Z-buffer. Cells were then permeabilized by adding 40 μl chloroform and 20 μl 0-1 % (w/v) SDS and vortexing for 5 s. Tubes were rested at room temperature for 5 to 10 min and then the reaction was started by the addition of 200 μl 4 mg ml⁻¹ stock-solution of ONPG. Reactions were incubated at room temperature until the assay with the highest activity of β-galactosidase showed a pale yellow colour (approx. 8 to 20 min). The reaction was stopped by the addition of 500 μl 1 M sodium carbonate. Cells were removed by centrifugation at 16 100 g for 3 min and the assay quantified spectrophotometrically at a wavelength of 420 nm (A420). A blank control consisted of 1 ml Z-buffer in the assay. Activity of β-galactosidase was expressed as Miller units (MU) and was calculated as the increase in A₄₂₀ per minute, per 1 ml cell suspension (normalized to an OD₆₀₀ of 1-0) used, multiplied by a factor of 1000.

The Pₙ concentration in the supernatant was determined by inorganic phosphate assay as described previously (Monk et al., 1991).

Phosphate transport assays. M. smegmatis was grown in high-phosphate ST medium to an OD₆₀₀ of approximately 1-0, harvested by centrifugation (7700 g, 20 min), washed in 4 vols buffer (50 mM MOPS pH 7-5, 5 mM MgCl₂) containing 0-05 % (w/v) Tween 80, and resuspended to an OD₆₀₀ of 0-7 in Pₙ-free ST medium. Cells were then incubated at 37 °C, 200 r.p.m. for 2 h to deplete intracellular stores of Pₙ and to induce expression of high-affinity Pₙ transport systems as described previously (Bhatt et al., 2000). The presence of an appropriate energy source in the medium ensured that the cells were energized at the time of the assay. As a control, cells were resuspended to an OD₆₀₀ of 1-0 and starved of Pₙ in the presence of 200 μg chloramphenicol ml⁻¹. The final OD₆₀₀ of each culture was measured prior to the uptake assay. Each assay contained 200 μl cell suspension, 2 μl radioactive Pₙ stock ([³²P]orthophosphate; >92-5 TBq mmol⁻¹; Amersham), and 5 μl unlabelled Pₙ stock to give a final concentration of 5 μM to 200 μM Pₙ in the assay mix. Pₙ was added to give approximately 150,000 to 300,000 c.p.m., resulting in a final concentration of 165 μM to 1-65 nM of radioactive Pₙ in the assay, depending on the age of the radioactive stock. The cell suspension aliquots (200 μl) were pre-incubated at 37 °C in 5 ml polystyrene test-tubes. Transport was started by addition of 7 μl of the appropriate mix of [³²P] and unlabelled Pₙ, and the tubes were incubated again at 37 °C for 0 to 30 seconds. The reactions were stopped by the addition of 2 ml cold 0-1 M LiCl and rapid filtration through 0-45 μm HA MF membrane filters (Millipore), using a vacuum manifold (Millipore) with an applied vacuum of approximately 80 psi. Filters were washed again with 2 ml 0-1 M LiCl, air-dried in 4 ml scintillation vials and covered in 2 ml scintillation fluid (Amersham). The amount of radioactivity taken up by the cells was determined with a 1214 Rackbeta liquid scintillation counter (LKB Wallac) using the ³¹C-window and counting each vial for 1 min. The amount of Pₙ taken up by the cells was calculated from the counts on the filters of each time-point relative to a t₀ control (i.e. simultaneous addition of [³²P] and LiCl), the total counts initially added to the assay, and the excess of unlabelled Pₙ over [³²P]. Rates of Pₙ uptake were expressed as nmol Pₙ min⁻¹ (mg protein)⁻¹. Controls for non-specific binding of [³²P] were carried out by pre-incubating cells with nigericin and valinomycin (15 μM each) for 30 min as previously described (Rao et al., 2001).

The Pₙ concentration in suspensions of whole cells of M. smegmatis was determined as described previously (Meyers et al., 1998). A cell suspension of OD₆₀₀ 1-0 had a protein content of 64 μg ml⁻¹, and this value was the same for the wild-type and 152
mutants. $V_{\text{max}}$ values of $P_i$ transport were determined from Michaelis–Menten plots; $K_m$ values were determined from double-reciprocal plots of the same data.

**DNA and protein sequence analysis.** Preliminary sequence data for *M. smegmatis* mc²155 were obtained from the Institute for Genomic Research website at http://www.tigr.org. *phnDCE* correspond to the loci MSMEG0640 to MSMEG0638, and *pstSCAB* correspond to MSMEG5750 to MSMEG5753.

## RESULTS

### Phosphate-limited growth of *M. smegmatis*

To determine the $P_i$ requirements of *M. smegmatis* for growth, mc²155 was grown in ST medium containing between 2-5 µM and 100 µM $P_i$. Under these conditions, both the specific growth rate ($\mu$) and the cell yield (expressed as final OD$_{600}$) of the cultures increased with increasing $P_i$ concentration (Fig. 1a). OD$_{600}$ was a reliable indicator of cell yield of cultures of *M. smegmatis* as determined by viable cell counts. Increasing $P_i$ concentrations to above 100 µM led to no further increase in growth rate or cell yield (data not shown). Because the substrate concentration in the growth medium can be expected to fall substantially during growth of *M. smegmatis*, growth rates were determined in the initial phase of exponential growth, i.e. between 14 h and 20 h of incubation. In Fig. 1(b), $\mu$ is plotted as a function of $P_i$ concentration, according to the Monod equation. The same data plotted as $\mu$ versus $\mu [P_i]^{-1}$, in analogy to an Eadie–Hofstee plot of enzyme kinetics, showed biphasic growth kinetics, indicating a change in the kinetics of $P_i$ utilization of *M. smegmatis* when $P_i$ concentrations drop below approximately 30 µM. At concentrations above 30 µM, the apparent saturation constant, which is the $P_i$ concentration that supports half-maximal growth rate (Pirt, 1975), was 17 µM, and the maximal growth rate was 0.3 h$^{-1}$ (2.3 h doubling time). At lower $P_i$ concentrations, the apparent saturation constant was 3.4 µM, and the maximal growth rate was 0.19 h$^{-1}$ (3.7 h doubling time) (Fig. 1c).

To investigate the ability of *M. smegmatis* to utilize phosphonates or phosphate as the sole phosphorus source, mc²155 was inoculated into $P_i$-free ST medium supplemented with methylphosphonate (1 mM or 5 mM), phosphonoacetate (1 mM or 2 mM) or phosphite (1 mM or 5 mM). None of these alternative phosphorus compounds supported growth of *M. smegmatis* (data not shown).

### Induction of *phnD–lacZ* and *pstS–lacZ* occurs under $P_i$-limited conditions

In order to investigate how expression of the genes encoding the putative phosphonate/phosphate transport system Phn (*phnDCE*) and the high-affinity $P_i$ transport system Pst (*pstSCAB*) responds to the $P_i$ concentration in the growth medium, strains of *M. smegmatis* carrying transcriptional lacZ-fusion constructs of the *phn* operon (pSG10) and of the *pst* operon (pSG42) were grown in modified ST medium as described in Methods. The $P_i$ concentration, $\beta$-galactosidase activities and OD$_{600}$ were monitored throughout growth (Fig. 2). The $P_i$ concentration in the medium decreased rapidly as the strains entered exponential growth, and $P_i$ was depleted from the medium completely as the cultures reached an OD$_{600}$ between 1.5 and 2.5. Stationary-phase cultures had an OD$_{600}$ of 5, suggesting that the bacteria had accumulated considerable intracellular stores of $P_i$ to maintain growth after $P_i$ had been depleted from the medium. For both promoter fusions, $\beta$-galactosidase activities increased significantly once the $P_i$ concentration in the medium dropped below values of approximately 40 µM. Activities of the *phnD–lacZ* fusion in pSG10 increased rapidly from values of 4 to 7 MU to reach a maximum value of 185 MU (26-fold induction over $h_0$) in late exponential phase, with a final value of approximately 100 MU in stationary phase (Fig. 2a). Activities of the *pstS–lacZ* fusion in pSG42 increased from 8 to 16 MU under

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**Fig. 1.** Phosphate-limited growth of *M. smegmatis*. (a) Growth, expressed as OD$_{600}$, in ST medium with 100 µM (●), 60 µM (▲), 40 µM (●), 10 µM (◆), 2.5 µM (■) $P_i$. (b) Growth rates ($\mu$) plotted as a function of $P_i$ concentration. (c) Data from (b) plotted as $\mu$ versus $\mu [P_i]^{-1}$ divided by $P_i$ concentration in µM. Results were reproducible in two independent experiments. Panels (b) and (c) show the data from both experiments.
phosphate-replete conditions to a maximum of 136 MU (17-fold induction over $t_0$) in late exponential phase, with approximately 110 MU in stationary phase (Fig. 2b). These data strongly suggest that both the Phn and Pst systems have a role in $P_i$ transport under $P_i$-limited growth conditions.

Construction of phnD and pstS deletion mutants

To further investigate the role of the Phn system in *M. smegmatis*, a deletion mutant in the gene encoding the substrate-binding protein, i.e. phnD, was created by allelic-exchange mutagenesis. A construct for the replacement of phnD with a kanamycin cassette was cloned into pX33 as described in Methods, transformed into *M. smegmatis* mc^{155}. Attempts at creating a phnD deletion mutant by selection for a double-crossover event as described by Pelicic *et al.* (1997) were unsuccessful, and we therefore decided to take a two-step approach of plasmid integration and excision (Fig. 3a). Colonies displaying the expected SmaI band pattern for integration of the plasmid via the left flank were readily obtained (Fig. 3b, lane 2) and one such colony was chosen to select for a second crossover event, resulting in the deletion of phnD and creating strain SG34 (Fig. 3b, lane 4). No colonies were obtained that displayed the correct band pattern for integration of the plasmid via the right flank, and the remaining colonies contained a single 8-3 kb band (Fig. 3b, lane 1), suggesting the loss of one copy of the left flank.

To be able to distinguish between the Phn system and the previously identified Pst system of *M. smegmatis* (Bhatt *et al.*, 2000; Kriakov *et al.*, 2003), a deletion mutant in pstS, encoding the substrate-binding protein of the Pst system, was created. The construct for the replacement of pstS with a gentamicin marker was cloned into pPR23 (Pelicic *et al.*, 1997) as described in Methods, transformed into *M. smegmatis* mc^{155}, and knockout mutants were selected as described previously (Pelicic *et al.*, 1997). Replacement of pstS with the gentamicin marker led to the introduction of an additional EcoRI restriction site, resulting in a band shift from 3-5 kb in the wild-type to 1-7 kb in the deletion mutant (strain SG95) in Southern hybridization analysis of EcoRI-digested genomic DNA probed with a radiolabelled PCR product of the left flank of the deletion construct (Fig. 3c). This hybridization result further confirmed that the genome of *M. smegmatis* contains only a single copy of the pst operon.

Additionally, a phnD pstS double deletion mutant was created to determine whether *M. smegmatis* harboured other genes that encode high-affinity $P_i$ transport systems. Strain SG34 was transformed with the pstS deletion construct (pSG30), and selection for the desired mutation was carried out using the two-step approach as described above. Colonies displaying the expected EcoRI band pattern for integration of the plasmid via the left flank (4-3 kb and 1-7 kb) or via the right flank (3-5 kb and 2-3 kb) when probed with a radiolabelled left flank PCR product, were both readily obtained (Fig. 3d, lanes 1 and 2). One such colony was chosen to select for a second crossover event, resulting in strain SG118, which carried deletions of both phnD and pstS (Fig. 3d, lane 4).

Mutants in the Phn and Pst systems require high concentrations of phosphate for growth in minimal medium

Transposon mutants of *M. smegmatis* in pstS, pstA and pstC have been described previously, and all of these mutants failed to grow on 10 mM $P_i$ in minimal medium (Kriakov *et al.*, 2003). We therefore conducted growth experiments in ST medium to confirm that the non-polar deletion mutant in pstS created in the present study, strain SG95, was also unable to grow on 10 mM $P_i$ in a minimal medium (Fig. 4a). Furthermore, strains SG34 (Fig. 4a) and SG118 (data not shown) also failed to grow under these conditions. In contrast, all mutants showed the same growth characteristics as the wild-type when grown in more complex media such as LBT or ADC-enriched 7H9 medium (data not shown), which is also consistent with previous results obtained for $P_i$ transporter mutants of mycobacteria (Kriakov *et al.*, 2003; Peirs *et al.*, 2005). Transformation

![Fig. 2. Transcriptional activities of the phn and pst operons during phosphate-limited growth. Cells were grown in modified ST medium (5 g glycerol $l^{-1}$, 4 g L-asparagine $l^{-1}$, 200 μM $P_i$) and monitored for growth, expressed as OD$_{600}$ ( ), phosphate concentration in the medium (×), and β-galactosidase activity (β-Gal), expressed as Miller units (MU) (□). (a) Cells carrying pSG10. (b) Cells carrying pSG42. Experiments were carried out in duplicate; representative results are shown.](image-url)
of SG34 with pSG38, which supplied a single copy of \textit{phnD} to the cell, resulted in strain SG106. Transformation of strain SG95 with pSG43, supplying a single copy of \textit{pstS}, resulted in strain SG120. Both complemented strains, SG106 and SG120, displayed a fully restored wild-type phenotype and were able to grow in ST medium containing as little as

\begin{figure}[h]
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\includegraphics[width=\textwidth]{fig3}
\caption{Construction of \textit{pstS} and \textit{phnD} deletion mutants of \textit{M. smegmatis} mc\textsuperscript{2}155. (a) Schematic diagram of the two-step approach for allelic replacement of \textit{phnD}. The delivery vector pSG3 was constructed as described in Methods. Integration of the vector via the left flank (Int A) or right flank (Int B) and replacement of \textit{phnD} with the kanamycin cassette (KO) are shown. Restriction sites of \textit{SmaI} and fragment sizes as detected in Southern hybridization analysis (b) are indicated. LF, left flank; RF, right flank; WT, wild-type. (b) Southern hybridization analysis (see a) of integration and excision of the \textit{phnD} deletion construct. \textit{SmaI} digests of genomic DNA from candidate colonies of the wild-type mc\textsuperscript{2}155 carrying the integrated deletion construct for \textit{phnD} in the right or left flank (lanes 1 and 2 respectively), from the wild-type (lane 3) and from the \textit{phnD} deletion mutant SG34 (lane 4) were probed with radiolabelled left flank PCR product of the deletion construct. (c) Southern hybridization analysis of replacement of \textit{pstS} with the gentamicin marker. \textit{EcoRI}-digests of genomic DNA from the wild-type (lane 1) and \textit{pstS} deletion mutant SG95 (lane 2) were probed with radiolabelled left flank PCR product of the deletion construct. (d) Southern hybridization analysis of replacement of \textit{pstS} in strain SG34. \textit{EcoRI}-digested genomic DNA from candidate colonies of SG34 carrying the integrated deletion construct for \textit{phnD} in the left or right flank (lanes 1 and 2 respectively), from strain SG95 (lane 3), and from the \textit{phnD \textit{pstS} double deletion mutant SG118 (lane 4) were probed with radiolabelled left flank PCR product of the \textit{pstS} deletion construct. Molecular masses of detected bands are indicated in kb.}
\end{figure}
0.1 mM Pi (Fig. 4b), proving the role of *phnD* and *pstS*, respectively, in the observed growth characteristics of the mutants.

**Determination of initial rates of Pi transport in whole cells of *M. smegmatis***

For assays of Pi uptake, cells were grown in ST medium, harvested, washed and resuspended in Pi-free medium. This was followed by incubation at 37°C for 2 h to deplete endogenous Pi levels as described previously (Bhatt *et al.*, 2000) and to induce the Phn and Pst transport systems (Fig. 2). Pi transport was measured by following the accumulation of [33P]orthophosphate ([33P]) inside whole cells of *M. smegmatis* over time. As shown for strain mc^2^155 in Fig. 5(a), [33P] accumulation was linear over a period of approximately 30 s, and no more than 15% of the total radioactivity added to the reaction was taken up into the cells in that time. In all further experiments, initial rates of transport were therefore determined between 0 and 30 s.

Cells that were grown in high-phosphate medium (100 mM Pi) and subjected to Pi starvation in the presence of 200 μg chloramphenicol ml^−1^ to prevent synthesis of inducible transport systems, showed no measurable uptake of Pi (Fig. 5a), indicating that the rates determined for induced cells were indeed due to the induction and activity of high-affinity transport systems. This further confirms the data obtained for the transcription of the *phn* and *pst* operons shown in Fig. 2.

To confirm that the accumulation of radioactivity in the cells was due to active transport and not caused by diffusion or non-specific binding of [33P] to the cells, we performed transport assays in the presence of metabolic inhibitors. Addition of nigericin and valinomycin (15 μM each), which dissipate the proton motive force in *M. smegmatis* (Rao *et al.*, 2001), inhibited transport by approximately 86% compared to an untreated control, demonstrating that accumulation of [33P] in *M. smegmatis* was via an energy-dependent mechanism (Fig. 5b).

**Determination of Pi transport kinetics in the wild-type and pstS and phnD mutant strains**

In previous studies of Pi transport in mycobacteria, uptake of radioactive Pi isotopes into the cells was measured over 8 to 20 min (Bhatt *et al.*, 2000; Peirs *et al.*, 2005), and most likely reflected steady-state levels of both transport and utilization of Pi. To further characterize Pi transport in *M. smegmatis*, we therefore chose to determine the kinetic parameters of Pi uptake at different external Pi concentrations.
concentrations. Initial rates of transport were determined over a range of P_i concentrations (5 μM to 200 μM), and plots of rates of transport versus P_i concentration showed that uptake followed Michaelis–Menten kinetics (Fig. 6a). The apparent K_m and V_max values of the wild-type were 40 μM P_i and 170 nmol P_i min^{-1} (mg protein)^{-1}, respectively. An Eadie–Hofstee plot of the same data was linear (data not shown). We determined the kinetic parameters of P_i transport in the phnD deletion strain, SG34 (Fig. 6b), the pstS deletion strain, SG95 (Fig. 6c), and the double deletion strain, SG118 (Fig. 6d). No significant differences were observed between these mutant strains and the wild-type, i.e. the K_m values were in the range 40 to 90 μM P_i, and V_max values were between 100 and 200 nmol P_i min^{-1} (mg protein)^{-1}. Moreover, Eadie–Hofstee plots of the same data were linear (data not shown). These results suggest that the Phn and Pst systems have similar affinities for P_i, and that the loss of one of these systems can be compensated for by the remaining system.

Strikingly, strain SG118, which carries deletions in both the Phn and Pst systems, was still able to transport P_i at similar rates to the wild-type. Further study of P_i transport in SG118 revealed that uninduced cells (i.e. P_i-starved in the presence of 200 μg chloramphenicol ml^{-1}) had a basal rate of transport of 10 nmol P_i min^{-1} (mg protein)^{-1} when measured at an external P_i concentration of 200 μM P_i, which is 6% of the rate at the same P_i concentration in induced cells [180 nmol P_i min^{-1} (mg protein)^{-1}]. These findings indicate that P_i transport in SG118 is inducible and therefore most likely due to the presence of a third, as yet unidentified, high-affinity transport system.

The phosphate transport systems of *M. smegmatis* are not competitively inhibited by phosphonates or phosphite

The Phn-like systems characterized to date recognize a variety of phosphonates, as well as phosphite, as substrates.

![Fig. 6](http://mic.sgmjournals.org) Kinetics of phosphate transport in phosphate-starved whole cells of the wild-type and Phn and Pst mutant strains. The initial uptake rates of orthophosphate (^32P, >92.5 TBq mmol^{-1}, Amersham), expressed as nmol P_i min^{-1} (mg mycobacterial protein extract)^{-1}, were measured over 30 s at phosphate concentrations between 5 μM and 200 μM. Data are shown as Michaelis–Menten plots. Insets show Lineweaver–Burk plots of the same data. (a) Wild-type, (b) SG34, (c) SG95, (d) SG118. V, rate of transport in nmol P_i min^{-1} (mg protein)^{-1}; S, P_i concentration in μM.
and are thought to transport P$_i$ non-specifically (Imazu et al., 1998; Metcalf & Wanner, 1991; Metcalf & Wolfe, 1998; Voegel et al., 1997; White & Metcalf, 2004b). The substrate specificity of the high-affinity P$_i$ transport systems of M. smegmatis was determined by testing the ability of alternative substrates (i.e. phosphonates or phosphate) to competitively inhibit P$_i$ uptake at a concentration (1 mM) 10-fold above that of radiolabelled P$_i$ (100 μM) as described previously (Voegel et al., 1997). At a 10-fold excess, any compound recognized as a substrate besides P$_i$ should out-compete radiolabelled P$_i$ for binding by the transport system and therefore strongly reduce the uptake of $^{33}$P into the cell. Excess unlabelled P$_i$ ($^{33}$P) as well as arsenate, a structural analogue of P$_i$, were included in the assay as positive controls for competitive inhibition. In addition to the wild-type, the mutant strains were also tested for their substrate range. We suggested above that the loss of one or two P$_i$ transport systems can be compensated for by increased activity of the remaining systems. It is therefore conceivable that differences in the substrate specificity of the high-affinity transport systems are more easily detected in strains defective in one or more of these systems. The results are summarized in Table 2. Phosphonoacetate, methylphosphonate or phosphate did not inhibit the initial rate of P$_i$ uptake as compared to the control rate in any of the strains tested and are therefore probably not recognized by any of the high-affinity P$_i$ transport systems. Arsenate inhibited the rate of $^{33}$P transport by 86% to 90%, and excess unlabelled P$_i$ inhibited transport by 87% to 92%, in good agreement with the theoretical value of 90% inhibition expected for 10-fold dilution of the radiolabelled substrate with unlabelled substrate.

**DISCUSSION**

We studied P$_i$-limited growth of M. smegmatis and investigated the role of the PhnDCE and PstSCAB transport systems in the physiology of this bacterium, using transscriptionsal analysis, mutagenesis and characterization of P$_i$ transport. Our data show that the phnDCE operon does indeed encode an ABC-transporter for P$_i$, which is induced under P$_i$-limited conditions. In contrast to previously identified Phn-like systems, the Phn system of M. smegmatis does not recognize phosphonates or phosphate as a substrate. Southern hybridization analysis of the phnD and pstS deletion mutants further confirmed that the genome of M. smegmatis harbours only a single copy of the pst and phn operons. Additionally, a third inducible, as yet unidentified P$_i$ transport system is present in M. smegmatis.

Expression analysis of transcriptional lacZ-fusions of the phnDCE and pstSCAB promoter areas revealed that both promoters under investigation were induced once the P$_i$ concentration in the medium dropped to a value below approximately 40 μM. This P$_i$ concentration is consistent with the threshold value (30 μM P$_i$) for P$_i$ limitation, as determined from the growth kinetics of M. smegmatis. While P$_i$ starvation induced expression of genes occurs at a similar concentration in B. subtilis (between 50 μM and 80 μM P$_i$) (Allenby et al., 2005; Hulett & Jensen, 1988), the threshold for induction in Gram-negative bacteria appears to be considerably lower, at about 4 μM P$_i$ in E. coli (Wanner, 1996), and below 10 μM in A. johnsonii (van Veen et al., 1994a).

We created mutants of M. smegmatis, carrying deletions in the genes encoding substrate-binding proteins of both the Phn system (phnD) (Tran et al., 2005), and the Pst system (pstS) (Bhatt et al., 2000; Kriakov et al., 2003), as well as a phnD pstS double mutant. All mutants were unable to grow in ST medium containing 10 mM P$_i$, while they were not impaired in growth in more complex media such as 7H9/ADC or LBT. Kriakov et al. (2003) isolated M. smegmatis transposon mutants in pstS, pstC and pstA, and these also failed to grow in minimal medium (‘home-prepared’ 7H9) with 10 mM P$_i$. Mutants of M. tuberculosis in pstS1 and pstS2 were not tested for growth in minimal medium, but had no growth defect in ADC-enriched 7H9 medium (Peirs et al., 2005). These data are difficult to interpret, as no two groups used the same media for characterization of their mutants. It appears that growth of the mutants depends strongly on the complexity of the medium and that the growth defects are not observed in more complex media. This growth defect in minimal medium cannot be explained by a lack of high-affinity P$_i$ transport, because the mutants were not impaired in P$_i$ uptake, as discussed below. However, mutations in genes of the pst operon have been shown to cause constitutive expression of Pho regulon genes in M. smegmatis (Kriakov et al., 2003). Constitutive activation of the Pho response leads to induction of stationary-phase genes in E. coli (Ruiz & Silhavy, 2003) and to initiation of sporulation in B. subtilis (Hulett, 1995). If a similar mechanism exists in M. smegmatis, it could be speculated that hyper-induction of the Pho response due to deletion of a high-affinity P$_i$ transport system causes a stationary-phase response and thereby prevents growth of the bacterium.

**Table 2. Effect of alternative substrates on the rates of phosphate uptake in M. smegmatis strain mc2155 and derived mutants**

| Cells    | Control* | PnAc | MePn | Pt | As | P$_i$
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<td>mc2155</td>
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<td>252</td>
<td>346</td>
<td>269</td>
<td>27</td>
<td>33</td>
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<tr>
<td>SG34</td>
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<td>188</td>
<td>158</td>
<td>208</td>
<td>25</td>
<td>16</td>
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<td>SG95</td>
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<td>177</td>
<td>174</td>
<td>213</td>
<td>18</td>
<td>15</td>
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<tr>
<td>SG118</td>
<td>170</td>
<td>178</td>
<td>171</td>
<td>186</td>
<td>21</td>
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*Rate of transport at 100 μM $^{33}$P without addition of competitor substrates.
unless such a response is overridden by components of a complex growth medium. Future work will be directed at investigating this hypothesis. Complementation of the pstS and phnD mutants completely restored the wild-type phenotype, confirming that the observed growth defect was indeed due to the deletion of the target gene, and not to the presence of the antibiotic markers used.

Characterization of P_i transport in the wild-type and Phn and Pst system mutant strains, SG35 and SG95, showed no significant differences in the kinetic parameters between these strains. This suggests that the Phn and Pst systems have similar affinities for P_i and that loss of one system can be compensated for by increased activity of the remaining system. The high-affinity P_i transport system of the closely related Streptomyces graminicolor has an apparent K_m of 60 μM P_i (Licha et al., 1997), which is similar to the values obtained here for M. smegmatis. The Pst systems that have been characterized from Gram-negative bacteria and the PhoCDET system of S. meliloti have much lower apparent K_m values between 0·2 and 0·4 μM P_i (Voegele et al., 1997; Willsky & Malamy, 1980; Yuan et al., 2006). This is in good agreement with the lower P_i concentration at which these systems are induced in Gram-negative bacteria as compared to Gram-positives, as discussed above.

A strain with deletions in both the Phn and the Pst systems still exhibited high-affinity P_i uptake. The difficulties encountered when deleting pstS in the phnD mutant background, while deletion of the gene in the wild-type was possible in a single double-crossover attempt, may have suggested that the presence of at least one of these systems was essential for M. smegmatis and that P_i transport in the double mutant might be attributed to a suppressor mutation. Such suppressor mutations, caused by gene amplification or increased expression of the low-affinity, constitutive Pit system, have been isolated for an E. coli pstS pitA double mutant (Hoffer et al., 2001) and an S. meliloti phoC mutant (Voegele et al., 1997). Increased expression of pitA in the phoC mutant of S. meliloti restored the ability of the mutant to grow in the presence of 2 mM P_i and to transport P_i at the same levels as the wild-type (Bardin et al., 1998). However, pitA in S. meliloti is repressed under conditions of P_i limitation (Bardin et al., 1998), whereas P_i transport in the phnD pstS double mutant of M. smegmatis described here was clearly inducible by P_i starvation. It is therefore unlikely that the P_i transport activity of the double mutant strain is due to increased synthesis of a Pit-like low-affinity transport system, but could rather be due to a further, as yet unidentified inducible P_i transport system. The presence of three different, inducible transport systems with K_m values between 40 μM and 90 μM P_i should enable M. smegmatis to easily adapt to changes in the availability of P_i in its environment as has been hypothesized previously for the presence of multiple Pst systems in M. tuberculosis (Lefevre et al., 1997).

Phn-type transport systems are primarily used for the uptake of alternative phosphorus compounds and generally recognize a wide variety of phosphonates, as well as phosphate (Imazu et al., 1998; Metcalf & Wanner, 1991; Metcalf & Wolfe, 1998; Voegele et al., 1997; White & Metcalf, 2004b). They are thought to transport P_i non-specifically. One of the most commonly utilized classes of phosphonates are the alklyphosphonates. These are degraded by the C–P lyase pathway, which is encoded by the phn genes of E. coli and other bacteria (Chen et al., 1990). Phosphonoacetate, degraded by phosphonoacetate lyase (McMullan & Quinn, 1994), can be utilized as a phosphorus source by some micro-organisms, including two streptomycete isolates (Obojska et al., 1999). We therefore tested two phosphonate compounds as well as phosphite for their ability to act as competitive inhibitors for P_i transport in this organism, and none of these compounds inhibited the uptake of ^33P. These data strongly suggest that the PhnDCE transport system of M. smegmatis, in contrast to the previously identified Phn-type systems of Gram-negative bacteria, is specific for P_i. This finding is supported by the inability of the same compounds to support growth of M. smegmatis as the sole phosphorus source.

In summary, we demonstrate that both PhnDCE and PstSCAB of M. smegmatis are high-affinity (K_m values between 40 μM and 90 μM P_i) ABC-type transport systems, which recognize P_i, but not phosphonates or phosphite, as substrates. The Phn system of M. smegmatis is the first Phn-like system described to date that appears to be specific for P_i. The presence of multiple high-affinity P_i transport systems has so far only been shown in pathogenic species of mycobacteria (Braibant et al., 1996a, b; Lefevre et al., 1997; Peirs et al., 2005), while M. smegmatis was thought to contain only a single Pst system (Bhatt et al., 2000; Kriakov et al., 2003). The findings presented here suggest that both slow- and fast-growing mycobacteria require several such transport systems for growth. Furthermore, M. smegmatis contains a third, as yet unidentified system for the uptake of P_i, that is active when the Phn and Pst systems are absent.

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REFERENCES


