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 mc²155 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 K_m values between 40 and 90 $\mu\text{M P}_i$). 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.

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INTRODUCTION

The assimilation of P_i or other phosphorus compounds from the environment has been studied in several micro-organisms. Detailed studies of P_i transport systems have focussed on *Escherichia coli* (van Veen, 1997; Wanner, 1996), *Acinetobacter johnsonii* (van Veen, 1997), *Sinorhizobium meliloti* (Bardin *et al.*, 1998; Voegelé *et al.*, 1997; Yuan *et al.*, 2006) and *Bacillus subtilis* (Allenby *et al.*, 2004; Qi *et al.*, 1997). P_i 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 P_i 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 ($\Delta\mu\text{H}^+$). 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; Voegelé *et al.*, 1997).

Bacteria also contain an inducible, high-affinity P_i transporter, which operates under P_i 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 P_i , 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* (Metcalf & Wanner, 1991), *Klebsiella aerogenes* (Imazu *et al.*, 1998), *Pseudomonas stutzeri* (White & Metcalf, 2004b), *S. meliloti* (Bardin *et al.*, 1996; Voegelé *et al.*, 1997), some streptomycete isolates (Obojska *et al.*, 1999), and other micro-organisms (reviewed by Kononova & Nesmeyanova, 2002). In *E. coli* and *K. aerogenes*, phosphonates and

Abbreviations: ABC, ATP-binding cassette; MU, Miller units; Pit, phosphate inorganic transport; Pst, phosphate-specific transport.

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. P_i is a non-specific substrate for this system (Metcalf & Wanner, 1991). *S. meliloti* 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; Voegelé *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.*, 1999; 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; Lefèvre *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 Phn 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]-catalase 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 MgSO₄, 2 g citric acid, 1 g L-asparagine, 0.3 g KCl, 1 g glycerol, 0.5 g Tween 80, 320 µl 0.5 M FeCl₃ and 100 µl 1 M NH₄Cl. High-phosphate medium contained 10 mM or 100 mM K₂HPO₄. 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 KH₂PO₄. 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-y-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'-AAATTTGGGCCCGCACTCAATCCCTGGGCCTT-3') and PphnR (5'-AAATTTGGTACCGCTTGTCTGAGCCCGAACAG-3'). The product was cloned into the *Apal* and *Asp718* sites of pJEM15 (Timm *et al.*, 1994), creating plasmid pSG10. 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'-AAATTTGGTACCGTCAACAACGCCGCTTCTCGTG-3') and PpstR

Table 1. Bacterial strains and plasmids

Strain or plasmid	Description*	Source or reference
<i>E. coli</i>		
DH10B	F ⁻ <i>mcrA</i> Δ (<i>mrr</i> – <i>hsdRMS</i> – <i>mcrBC</i>) ϕ 80d <i>lacZ</i> Δ M15 Δ <i>lacX74</i> <i>deoR</i> <i>recA1</i> <i>araD139</i> Δ (<i>ara</i> <i>leu</i>)7697 <i>galU</i> <i>galK</i> <i>rpsL</i> <i>endA1</i> <i>nupG</i>	Hanahan <i>et al.</i> (1991)
<i>M. smegmatis</i>		
mc ² 155	Electrocompetent wild-type strain of <i>M. smegmatis</i>	Snapper <i>et al.</i> (1990)
SG34	mc ² 155 Δ <i>phnD</i> :: <i>aphA</i> -3; Km ^r	This study
SG95	mc ² 155 Δ <i>pstS</i> :: <i>aacC</i> -1; Gm ^r	This study
SG106	SG34 with pSG38 integrated in <i>attB</i> ; Km ^r , Hyg ^r	This study
SG118	mc ² 155 Δ <i>phnD</i> :: <i>aphA</i> -3 Δ <i>pstS</i> :: <i>aacC</i> -1; Km ^r , Gm ^r	This study
SG120	SG95 with pSG43 integrated in <i>attB</i> ; Gm ^r , Hyg ^r	This study
Plasmids		
pBluescript II KS	Cloning vector; Ap ^r	Stratagene
pUC18K	<i>E. coli</i> plasmid containing an excisable, non-polar kanamycin resistance cassette; Km ^r Ap ^r	Menard <i>et al.</i> (1993)
pBSL141	<i>E. coli</i> plasmid containing an excisable <i>aacC</i> -1 gene; Gm ^r Ap ^r	Alexeyev <i>et al.</i> (1995)
pPR23	<i>E. coli</i> –mycobacteria shuttle vector for allelic-exchange mutagenesis in mycobacteria; Gm ^r Sac ^s , ts	Pellicic <i>et al.</i> (1997)
pX33	pPR23 carrying a constitutive <i>xylE</i> marker; Gm ^r	This study
pJEM15	<i>E. coli</i> –mycobacteria shuttle vector for the creation of transcriptional promoter fusions to <i>lacZ</i> ; Km ^r	Timm <i>et al.</i> (1994)
pUHA267	<i>E. coli</i> vector with mycobacteriophage L5 integrase and <i>attP</i> for integration into <i>attB</i> of mycobacteria; Hyg ^r	AgResearch, Wallaceville, NZ
pSG3	pX33 harbouring Δ <i>phnD</i> :: <i>aphA</i> -3; Km ^r Gm ^r Sac ^s , ts	This study
pSG10	pJEM15 harbouring a 500 bp <i>phnD</i> – <i>lacZ</i> fusion; Km ^r	This study
pSG30	pPR23 harbouring Δ <i>pstS</i> :: <i>aacC</i> -1; Gm ^r Sac ^s , ts	This study
pSG38	pUHA267 harbouring <i>phnD</i> ⁺ with its native promoter; Hyg ^r	This study
pSG42	pJEM15 harbouring a 560 bp <i>pstS</i> – <i>lacZ</i> fusion; Km ^r	This study
pSG43	pUHA267 harbouring <i>pstS</i> ⁺ with its native promoter; Hyg ^r	This study

*Km^r, kanamycin resistance; Gm^r, gentamicin resistance; Hyg^r, hygromycin resistance; Ap^r, ampicillin resistance; Sac^s, sucrose sensitivity; ts, temperature sensitivity.

(5'-AAATTTGGTACCCTCGGCGATCGCGTTCTGTTGC-3'). The product was cloned into the *Asp*718 site of pJEM15, creating plasmid pSG42.

To create a construct for the deletion of *phnD*, the kanamycin resistance cassette (Km^r), encoded by *aphA*-3, was amplified from pUC18K (Menard *et al.*, 1993) by PCR using the primers 3'mcspUC (5'-GTTTCCAGTCACGACGTT-3') and 5'mcspUC (5'-CACAC-AGGAAACAGCTATGA-3'). The resulting 850 bp product was digested with *Sac*I and *Hind*III. PCR products of approximately 1.2 kb flanking the *phnD* gene of *M. smegmatis* on either side were amplified using the primers *phnD*KO1 (5'-AAATTTACTAGT-GGGGCTGGCTAACGAATGT-3') with *phnD*KO2 (5'-AAATTGAGCTCGCTTGTGCGAGCCCCGAACAG-3') (left flank) and *phnD*KO3 (5'-AAATTTAAGCTTACGGGCTCCGAGAGAGTGCAA-3') with *phnD*KO4 (5'-AAATTTACTAGTGAGAACACCTGGGAGC-ACA-3') (right flank). The left flank PCR product was digested with *Spe*I and *Sac*I, the right flank PCR product with *Hind*III and *Spe*I. Both flanks and the kanamycin cassette were ligated into the *Spe*I site of pBluescript II KS (Stratagene). The resulting assembly, left flank/Km^r/right flank, was subcloned as a *Spe*I fragment into pX33 [pPR23 (Pellicic *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.

To create a deletion construct for *pstS*, the gentamicin resistance cassette (Gm^r), *aacC*-1, from pBSL141 (Alexeyev *et al.*, 1995) was excised from the plasmid using *Pst*II and *Not*I. 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 *pst*SKO1 (5'-AAATTTGGATCCGTCACACACGCCGCGTTCTCGTG-3') with *pst*SKO2 (5'-AAATTTCTGCAGCTCGGCGATCGCGTTCTGTTGC-3') and *pst*SKO3 (5'-AAATTTGCGGCCGCTTCAAAGAGCGTTT-GCTCAC-3') with *pst*SKO4 (5'-AAATTTCTAGAACTGGTCG-TTGAATTCGCTTG-3'), respectively. The left flank PCR product was digested with *Bam*HI and *Pst*II, the right flank PCR product with *Not*I and *Xba*I. Both flanks and the gentamicin cassette were ligated into the *Bam*HI and *Xba*I sites of pBluescript II KS (Stratagene). The resulting assembly, left flank/Gm^r/right flank, was subcloned as a *Bam*HI/*Xba*I 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 Pellicic *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 OD₆₀₀ of approximately 0.6 to 0.8, followed by plating onto low-salt LBT plates (2 g NaCl l⁻¹) containing gentamicin and 10% sucrose at 40 °C, selecting for double-crossover

events. Replacement of *pstS* with the gentamicin marker created strain SG95 ($\Delta pstS::aacC-1$). 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 *xylE* marker were screened by Southern hybridization analysis for correct integration of the construct. One integrant was chosen and grown in 2 ml 7H9/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^r, Gm^s) were screened by Southern hybridization analysis for correct deletion of *phnD*. Replacement of *phnD* with the kanamycin marker created strain SG34 ($\Delta phnD::aphA-3$).

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 steps, and no final counter-selection for the loss of the plasmid backbone was carried out. Replacement of *pstS* with the gentamicin marker in the background of the *phnD* deletion strain created strain SG118 ($\Delta phnD::aphA-3 \Delta pstS::aacC-1$).

For Southern hybridization analysis, *Sma*I- or *Eco*RI-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 1.5 kb PCR product encompassing the *phnD* gene plus 500 bp of upstream DNA was amplified by PCR using primers cphnDF (5'-AAATTTGGTACCGCACTCAATCCCTGGGCCTT-3') and cphnDR (5'-AAATTTGGTACCATGACAACGACGTCATC-ACC-3') and cloned into the *Asp*718 site of pUHA267, creating plasmid pSG38. For complementation of the *pstS* mutation, a 1.7 kb PCR product encompassing the *pstS* gene plus 560 bp upstream DNA was amplified by PCR using primers PpstF and cpstSR (5'-AAATTTGGTACCAATCCAGCTGACTAGCTGATCG-3') and cloned into the *Asp*718 site of pUHA267, creating plasmid pSG43.

β -Galactosidase and inorganic phosphate assays. To determine the threshold P_i concentration leading to induction of the *phn* and *pst* operon genes, strains carrying pSG10 or pSG42 were grown in medium containing an intermediate P_i concentration (200 μ M). 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 were 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 (*t*₀) 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,

1 mM MgSO₄) 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 μ l were used in each assay and the volumes increased to 1 ml with Z-buffer. Cells were then permeabilized by adding 40 μ l chloroform and 20 μ l 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 μ l 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 μ l 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 (*A*₄₂₀). 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 *A*₄₂₀ per minute, per 1 ml cell suspension (normalized to an OD₆₀₀ of 1.0) used, multiplied by a factor of 1000.

The P_i 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 P_i-free ST medium. Cells were then incubated at 37 °C, 200 r.p.m. for 2 h to deplete intracellular stores of P_i and to induce expression of high-affinity P_i 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 P_i in the presence of 200 μ g chloramphenicol ml⁻¹. The final OD₆₀₀ of each culture was measured prior to the uptake assay. Each assay contained 200 μ l cell suspension, 2 μ l radioactive P_i stock ([³³P]orthophosphate; >92.5 TBq mmol⁻¹; Amersham), and 5 μ l unlabelled P_i stock to give a final concentration of 5 μ M to 200 μ M P_i in the assay mix. ³³P was added to give approximately 150,000 to 300,000 c.p.m., resulting in a final concentration of 165 pM to 1.65 nM of radioactive P_i in the assay, depending on the age of the radioactive stock. The cell suspension aliquots (200 μ l) were pre-incubated at 37 °C in 5 ml polystyrene test-tubes. Transport was started by addition of 7 μ l of the appropriate mix of ³³P and unlabelled P_i, 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 μ m HA MF membrane filters (Millipore), using a vacuum manifold (Millipore) with an applied vacuum of approximately 80 p.s.i. 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 ¹⁴C-window and counting each vial for 1 min. The amount of P_i taken up by the cells was calculated from the counts on the filters of each time-point relative to a *t*₀ control (i.e. simultaneous addition of ³³P and LiCl), the total counts initially added to the assay, and the excess of unlabelled P_i over ³³P. Rates of P_i uptake were expressed as nmol P_i min⁻¹ (mg protein)⁻¹. Controls for non-specific binding of ³³P were carried out by pre-incubating cells with nigericin and valinomycin (15 μ M each) for 30 min as previously described (Rao *et al.*, 2001).

The protein 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 μ g ml⁻¹, and this value was the same for the wild-type and all

mutants. $V_{\max}$ 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 μ M and 100 μ M P_i . Under these conditions, both the specific growth rate (μ) and the cell yield (expressed as final OD₆₀₀) of the cultures increased with increasing P_i concentration (Fig. 1a). OD₆₀₀ 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 μ M 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), μ is plotted as a function of P_i concentration, according to the Monod equation. The same data plotted as μ 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 μ M. At concentrations above 30 μ M, the apparent saturation constant, which is the P_i concentration that supports half-maximal growth rate (Pirt, 1975), was 17 μ M, 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 μ M, 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 phosphite 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, β -galactosidase activities and OD₆₀₀ 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 OD₆₀₀ between 1.5 and 2.5. Stationary-phase cultures had an OD₆₀₀ 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, β -galactosidase activities increased significantly once the P_i concentration in the medium dropped below values of approximately 40 μ M. 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 t_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

Fig. 1. Phosphate-limited growth of *M. smegmatis*. (a) Growth, expressed as OD₆₀₀, in ST medium with 100 μ M (◆), 60 μ M (▲), 40 μ M (●), 10 μ M (▼), 2.5 μ M (■) P_i . (b) Growth rates (μ) plotted as a function of P_i concentration. (c) Data from (b) plotted as μ versus μ divided by P_i concentration in μ M. Results were reproducible in two independent experiments. Panels (b) and (c) show the data from both experiments.

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 OD₆₀₀ (■), 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.

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 and transformed into *M. smegmatis* mc²155. 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 *Sma*I 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* mc²155, 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 *Eco*RI 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 *Eco*RI-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 *Eco*RI 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. 3. Construction of *pstS* and *phnD* deletion mutants of *M. smegmatis* *mc*²¹⁵⁵. (a) Schematic diagram of the two-step approach for allelic replacement of *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 *phnD* with the kanamycin cassette (KO) are shown. Restriction sites of *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 *phnD* deletion construct. *SmaI* digests of genomic DNA from candidate colonies of the wild-type *mc*²¹⁵⁵ carrying the integrated deletion construct for *phnD* in the right or left flank (lanes 1 and 2 respectively), from the wild-type (lane 3) and from the *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 *pstS* with the gentamicin marker. *EcoRI*-digests of genomic DNA from the wild-type (lane 1) and *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 *pstS* in strain SG34. *EcoRI*-digested genomic DNA from candidate colonies of SG34 carrying the integrated deletion construct for *phnD* in the left or right flank (lanes 1 and 2 respectively), from strain SG95 (lane 3), and from the *phnD pstS* double deletion mutant SG118 (lane 4) were probed with radiolabelled left flank PCR product of the *pstS* deletion construct. Molecular masses of detected bands are indicated in kb.

of SG34 with pSG38, which supplied a single copy of *phnD* to the cell, resulted in strain SG106. Transformation of strain SG95 with pSG43, supplying a single copy of *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

Fig. 4. Growth phenotype of the Pst and Phn system mutant strains. (a) Growth of the wild-type strain mc²155 (■) and mutant strains SG34 (▲) and SG95 (▼) in ST medium containing 10 mM P_i. (b) Growth of the wild-type (■) and complemented strains SG106 (△) and SG120 (▽) in ST medium containing 0.1 mM P_i. Experiments were carried out in triplicate and representative results are shown.

0.1 mM P_i (Fig. 4b), proving the role of *phnD* and *pstS*, respectively, in the observed growth characteristics of the mutants.

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

For assays of P_i uptake, cells were grown in ST medium, harvested, washed and resuspended in P_i-free medium. This was followed by incubation at 37 °C for 2 h to deplete endogenous P_i levels as described previously (Bhatt *et al.*, 2000) and to induce the Phn and Pst transport systems (Fig. 2). P_i transport was measured by following the accumulation of [³³P]orthophosphate (³³P) inside whole cells of *M. smegmatis* over time. As shown for strain mc²155 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 P_i) and subjected to P_i starvation in the presence of 200 µg chloramphenicol ml⁻¹ to prevent synthesis of inducible transport systems, showed no measurable uptake of P_i (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 P_i transport kinetics in the wild-type and *pstS* and *phnD* mutant strains

In previous studies of P_i transport in mycobacteria, uptake of radioactive P_i 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 P_i. To further characterize P_i transport in *M. smegmatis*, we therefore chose to determine the kinetic parameters of P_i uptake at different external P_i

Fig. 5. Uptake of [³³P]orthophosphate into whole cells of *M. smegmatis*. Accumulation of radioactive orthophosphate (³³P, >92.5 TBq mmol⁻¹, Amersham) in cells of *M. smegmatis* mc²155, expressed as c.p.m. (determined by liquid scintillation counting) over time. Data are normalized to a cell suspension in the assay of OD₆₀₀ 1.0. (a) P_i uptake in cells that were incubated in P_i-free medium for 2 h in the presence (□) and absence (■) of 200 µg chloramphenicol ml⁻¹. P_i concentration in the assay was 200 µM. (b) P_i uptake in the presence (▲) and absence (■) of nigericin and valinomycin (15 µM each). P_i concentration in the assay was 100 µM.

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 $\text{nmol } P_i \text{ min}^{-1} (\text{mg protein})^{-1}$, 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 $\text{nmol } P_i \text{ min}^{-1} (\text{mg protein})^{-1}$. 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 $\text{nmol } P_i \text{ min}^{-1} (\text{mg protein})^{-1}$ 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 $\text{nmol } P_i \text{ min}^{-1} (\text{mg protein})^{-1}$]. 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. 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 (^{33}P , $>92.5 \text{ TBq mmol}^{-1}$, Amersham), expressed as $\text{nmol } P_i \text{ min}^{-1} (\text{mg mycobacterial protein extract})^{-1}$, 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 $\text{nmol } P_i \text{ min}^{-1} (\text{mg protein})^{-1}$; 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; Voegelé *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 phosphite) to competitively inhibit P_i uptake at a concentration (1 mM) 10-fold above that of radiolabelled P_i (100 μ M) as described previously (Voegelé *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 (^{31}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 phosphite 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

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

Rates of P_i transport at 100 μ M ^{33}P , expressed as nmol P_i min⁻¹ (mg protein)⁻¹; competitor substrates (final concentration 1 mM) were added 1 min prior to the addition of ^{33}P (final concentration 100 μ M). PnAc, phosphonoacetate; MePn, methylphosphonate; Pt, phosphite; As, arsenate; P_i , phosphate (^{31}P).

Cells	Control*	PnAc	MePn	Pt	As	P_i
mc ² 155	254	252	346	269	27	33
SG34	173	188	158	208	25	16
SG95	175	177	174	213	18	15
SG118	170	178	171	186	21	17

*Rate of transport at 100 μ M ^{33}P without addition of competitor substrates.

transcriptional 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 phosphite 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 μ M. This P_i concentration is consistent with the threshold value (30 μ M 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 μ M and 80 μ M 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 μ M P_i in *E. coli* (Wanner, 1996), and below 10 μ M 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,

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 granaticolor* 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 (Voegelé *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 (Voegelé *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* (Lefèvre *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 phosphite (Imazu *et al.*, 1998; Metcalf & Wanner, 1991; Metcalf & Wolfe, 1998; Voegelé *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 alkylphosphonates. 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 hydrolase (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 ^{33}P . 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; Lefèvre *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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