The low-affinity phosphate transporter PitA is dispensable for in vitro growth of Mycobacterium smegmatis

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Background: Mycobacteria have been shown to contain an apparent redundancy of high-affinity phosphate uptake systems, with two to four copies of such systems encoded in all mycobacterial genomes sequenced to date. In addition, all mycobacteria also contain at least one gene encoding the low-affinity phosphate transporter, Pit. No information is available on a Pit system from a Gram-positive microorganism, and the importance of this system in a background of multiple other phosphate transporters is unclear.

Results: The aim of this study was to determine the physiological role of the PitA phosphate transporter in Mycobacterium smegmatis. Expression of pitA was found to be constitutive under a variety of growth conditions. An unmarked deletion mutant in pitA of M. smegmatis was created. The deletion did not affect in vitro growth or phosphate uptake of M. smegmatis. Expression of the high-affinity transporters, PstSCAB and PhnDCE, was increased in the pitA deletion strain.

Conclusion: PitA is the only low-affinity phosphate transport system annotated in the genome of M. smegmatis. The lack of phenotype of the pitA deletion strain shows that this system is dispensable for in vitro growth of this organism. However, increased expression of the remaining phosphate transporters in the mutant indicates a compensatory mechanism and implies that PitA is indeed used for the uptake of phosphate in M. smegmatis.

Background

Uptake of phosphate by bacteria most commonly occurs via two systems, the low-affinity, constitutively expressed Pit system, and the high-affinity, phosphate-starvation induced Pst system [1,2]. Pit systems consist of a single membrane protein, encoded by pitA or pitB, and are energized by the proton motive force [2,3]. Pst systems are multi-subunit ABC transporters, usually encoded by a four-gene operon, pstSCAB [1,2]. Several bacterial species also contain additional transporters for the uptake of alternative phosphorus-compounds. Examples include the Ptx and Htx systems of Pseudomonas stutzeri, which transport phosphonates, phosphite and hypophosphite [4,5], and the Phn-system for the uptake of phosphonates in E. coli and several other Gram-negative bacteria [6-8].
Mycobacteria appear unique in that they contain several copies of high-affinity systems specific for phosphate: In the pathogenic species, such as *M. tuberculosis*, *M. bovis* and *M. leprae*, this is due to duplication of the *pst* genes [9]. For example, *M. tuberculosis* contains three different copies of *pstS*, two copies each of *pstC* and *pstA*, and one copy of *pstB* [10], plus a homologous gene, *phaI*, which has been shown to fulfill the same function as *pstB* in *M. bovis* [11]. Expression of all three copies of *pstS* under phosphate-limited conditions has been shown for *M. bovis* BCG [9], although a recent microarray analysis of phosphate-limited *M. tuberculosis* only found one of the *pst*-operons to be upregulated [12].

The environmental species *M. smegmatis* possesses only a single copy of the *pst*-operon, but it also contains a second operon, *phnDCE*, which encodes another phosphate-specific high-affinity transporter [13]. Furthermore, a third, as yet unidentified, high-affinity phosphate transport system may be present in *M. smegmatis*, because a *phnD/pstS* double deletion mutant still retained phosphate uptake activity with a K_m-value of around 90 μM, which is similar to the values of the Pst and Phn systems [13].

Despite this abundance of high-affinity transporters, all mycobacterial genomes available to date also contain a *pitA* gene, encoding the low-affinity system, with the genome of *M. tuberculosis* containing a second Pit system, encoded by *pitB* [14]. The present study was directed at investigating the role of the low-affinity phosphate transporter in a bacterium containing at least two high-affinity systems, using the model of *M. smegmatis*.

**Results and Discussion**

**PitA is constitutively expressed**

Previous studies of Pit systems have focused on Gram-negative bacteria, where *pitA* expression is independent of phosphate concentrations [1,15], while *pitB* of *E. coli* and the *pit*-like gene of *Sinorhizobium melliloti* are repressed at low phosphate concentrations [16,17]. To study the expression of *M. smegmatis* *pitA*, a low-copy number transcriptional *pitA-lacZ* fusion (pAH1) was introduced into wild-type *M. smegmatis*. The resulting strain had β-galactosidase activities of about 135 Miller Units (MU), which is similar to the values of the Pst and Phn systems [13].

Expression of a transcriptional *pitA-lacZ* fusion construct in *M. smegmatis*. Wild-type *M. smegmatis* harbouring the *pitA-lacZ* construct pAH1 was grown in ST medium containing 100 mM phosphate (Control), followed by 2 h starvation in phosphate-free (–P) or Mg2+-free (–Mg2+) ST medium, or 2 h exposure to 5 mM EDTA (+ EDTA), pH 4 or pH 9. β-Galactosidase (β-Gal) activities were assayed and are expressed in Miller Units (MU). Results are the mean ± standard deviation of three independent experiments.

A *pitA* deletion mutant has no growth defect in vitro

To determine if *pitA* played a role in growth and phosphate uptake of *M. smegmatis*, we next constructed an unmarked *pitA* deletion strain by an adaptation of the two-step protocol used previously to create a double-kanamycin marked mutant of *M. smegmatis* [20] (Figure 2). In the first step of mutagenesis, the construct was integrated into the chromosome by growth at 40°C. Southern hybridization analysis showed that correct integration had occurred via a cross-over event in the left flank (Figure 2B). Excision of the plasmid backbone through a second cross-over event was then selected for by growth on 10% sucrose. This second cross-over could lead either to reversion to wild-type or to deletion of the target gene. Nine colonies were screened by Southern hybridization, of which four had reverted back to the wild-type pattern, while five displayed the correct band pattern of a *pitA* deletion mutant (Figure 2C). One of the latter was chosen for further characterization.

![Figure 1: Expression of a transcriptional *pitA-lacZ* fusion construct in *M. smegmatis*.](http://www.biomedcentral.com/1471-2180/9/254)
**Figure 2**

**Construction of an unmarked pitA deletion mutant of M. smegmatis mc²155.** A: Schematic diagram of the two-step approach for deletion of pitA. The knock-out construct consisted of two fragments flanking pitA on the left (LF) and right (RF) in pX33. Integration of the vector (thick grey line) into the chromosome (thin black line) via the left flank (Int LF) or right flank (Int RF) and subsequent deletion of pitA (KO) are shown. Restriction sites of BamHI (B) and fragment sizes as detected in Southern hybridization are indicated. Drawing not to scale. WT, wild-type. B: Southern hybridization analysis of the integration event. BamHI-digests of genomic DNA of wild-type mc²155 (lane 1) and a candidate colony (lane 2) were probed with radiolabeled right flank PCR product of the deletion construct. C: Southern hybridization analysis of pitA deletion. Analysis of wild-type mc²155 (lane 1) and the pitA deletion strain (lane 2) was performed as in panel B. Molecular masses are indicated in kb.
Growth experiments showed no difference between wild-type and pitA mutant in LBT medium or ST medium, either under phosphate-replete conditions (100 μM to 100 mM phosphate) or phosphate-limited conditions (10 μM or 50 μM phosphate) (not shown). This characteristic of the pitA mutant is markedly different from the previously created M. smegmatis mutants in the high-affinity phosphate transporters, which were unable to grow in minimal medium at 10 mM phosphate or below [13]. As mentioned above, Pit systems of Gram-negative bacteria transport a metal-phosphate complex. While no information regarding their substrate is available for Pit systems of Gram-positives, a mutant of Bacillus subtilis carrying an uncharacterized mutation in phosphate uptake was also defective in uptake of metal ions [21], suggesting an interrelation between uptake of phosphate and metals. The biological role of Pit in a bacterium with a plethora of high-affinity phosphate transporters may therefore be in uptake of divalent metal ions. To test this, we performed growth experiments in Mg2+–limited ST medium (2 μM to 2 mM MgCl2), but could not discern a difference between the pitA and wild-type strain (not shown). Because the distribution of MeHPO4 versus free phosphate depends on the medium pH, with MeHPO4 being the predominant species at high pH values [19], it was conceivable that the physiological role of Pit is to act under conditions where most phosphate is present as MeHPO4. To simulate such a condition in vitro, we modified the ST medium to contain a high concentration of MgCl2 (8 mM) and low concentration of phosphate (100 μM) and adjusted the pH to 8.5 (buffered with 100 mM Tricine). No difference was found between the wild-type and pitA mutant strains (not shown). An E. coli pitA mutant displayed increased resistance to toxic divalent cations (Zn2+ and Cd2+), due to reduced uptake of these ions [22]. The M. smegmatis pitA mutant and wild-type strain were therefore grown on solid media (ST agar, 50 mM MES [pH 7], 1 mM phosphate) containing 1-15 mM ZnSO4 or CuSO4. Both strains were able to grow in the presence of 1 mM of either salt, but could not grow at concentrations of 5 mM or higher. Taken together, the data presented here suggest that either PitA of M. smegmatis does not transport MeHPO4, or that one or both of the high-affinity systems also recognize such a complex as substrate. It should be noted that no substrate specificities have been determined to date for a Pst system from a Gram-positive bacterium, or for a Phn system.

The pitA mutant displays no defect in phosphate uptake

We next determined the rates and kinetics of uptake of [33P]ortho-phosphate, to assess whether the pitA deletion strain had a defect in phosphate uptake. To prevent induction of the Pst or Phn systems, cells were grown in LBT medium as described in the methods section. As shown in figure 3, maximum uptake rates were 12.9 ± 1.6 nmol min−1 mg protein−1 for the wild-type, and 9.9 ± 1.0 nmol min−1 mg protein−1 for the pitA strain. Kd values were similar between the strains, with 50.1 ± 26 μM phosphate for the wild-type and 27.9 ± 16.4 μM phosphate for the pitA strain. Slight differences in transport rates at the higher phosphate concentrations were not significant (p > 0.2 in unpaired, two-tailed t-test).

These kinetic parameters suggest that the rates of transport determined are due to activity of the high-affinity systems, because Kd values of phosphate uptake under phosphate-starved (i.e. Pst and Phn systems induced) conditions were found to be between 40 and 90 μM phosphate [13]. The rates of transport in the present study are about ten-fold lower than those in phosphate-starved cells, consistent with the previously described 20-fold lower expression from the pst and phn promoters under these conditions [13]. PitA of M. smegmatis therefore appears to be either not active, or to have a very low activity, which cannot be detected over the background of the high-affinity systems using the assay employed here. Considering the abundance of phosphate transport systems in M. smegmatis, we hypothesized that loss of PitA is easily compensated for by increased use of the Pst and Phn systems.

Deletion of pitA causes increased expression of the Pst and Phn systems

To address the question whether the pitA deletion mutant employs increased expression of either the Pst or Phn system to compensate for the deletion, we introduced the previously created transcriptional pstS-lacZ (pSG42) and
phnD-lacZ (pSG10) fusion constructs [13] into the pitA deletion background. As shown in figure 4, under phosphate-replete conditions the activity of both promoters was increased by about two-fold in the pitA strain. Complementation of the deletion with a single copy of pitA under control of its native promoter restored expression of pstS-lacZ and phnD-lacZ to wild-type levels. No differences between strains were observed in phosphate-starved cells (data not shown). These data imply that PitA is indeed used for phosphate uptake under high phosphate conditions by M. smegmatis, but that loss of this system is easily compensated for by the remaining phosphate transporters.

Conclusion
In summary, we here show that the PitA system of M. smegmatis is constitutively expressed under a variety of growth conditions, and that deletion of the pitA gene does not appear to affect growth or phosphate uptake in vitro. This is presumably due to compensation of the deletion by increased expression of the high-affinity phosphate transport systems, PstSCAB and PhnDCE.

The lack of phenotype of the pitA mutant under the growth conditions tested here, together with the wild-type levels of phosphate uptake in the mutant strain, raises the question as to why mycobacteria still contain this transporter. This point is further emphasized by the presence of a functional pitA gene in M. leprae, whose genome has undergone reductions and decay to the point where the bacterium is unable to replicate outside of its host [23]. The answer may be found in the energetics of transport: Pit systems transport metal-phosphate in symport with protons at a stoichiometry of 1:1 [3], while the Pst and Phn systems are ABC-transporters and thus likely require hydrolysis of two ATP per substrate transported [24]. Uptake of phosphate via the Pit system is therefore energetically less expensive to the cell, and this could be important under conditions where energy is limiting. However, there was no significant difference in the molar growth yield (mg [dry weight] cells/mmol of substrate consumed) between the pitA deletion mutant and the wild-type when grown under carbon limitation in continuous culture at a dilution rate of 0.01 h⁻¹ (doubling-time of 70 h) (our own unpublished results). We therefore hypothesize that a phenotype for a pitA mutant of mycobacteria may well only manifest itself in vivo under conditions where the cell is exposed to multiple limitations (e.g. carbon, energy, oxygen), such as are commonly found in the intraphagosomal environment of the pathogens or the soil habitat of environmental species.

Methods
Bacterial strains and growth conditions
All strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown in Luria-Bertani (LB) medium at 37°C with agitation (200 rpm). *Mycobacterium smegmatis* strain mc²¹⁵⁵ [25] and derived strains were routinely grown at 37°C, 200 rpm in LB containing 0.05% (w/v) Tween80 (LBT) or in modified Sauton’s (ST) medium [13]. Variations of phosphate and MgCl₂ concentrations and other modifications of the ST medium are given in the text. Cells to be used as inoculum in phosphate-limited ST medium were washed once in phosphate-free medium prior to use. Starvation experiments in phosphate-free ST medium were carried out as described previously [13]. M. smegmatis transformants were grown at 28°C for propagation of temperature-sensitive vectors and at 40°C for allelic exchange mutagenesis. Selective media contained kanamycin (50 μg ml⁻¹ for *E. coli*; 20 μg ml⁻¹ for *M. smegmatis*), gentamycin (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.

DNA manipulation and cloning of constructs
All molecular biology techniques were carried out according to standard procedures [26]. Restriction or DNA mod-

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**Figure 4**

Expression from the pst and phn promoters in the pitA deletion background. Transcriptional phnD-lacZ and pstS-lacZ fusion constructs were introduced into wild-type *M. smegmatis* (open bars), the pitA deletion strain (black bars) and the pitA complemented strain (hatched bars). β-Galactosidase (β-Gal) activities, expressed as Miller Units (MU), were determined from cultures grown in ST medium with 100 mM phosphate and are shown as the mean ± standard deviation from three independent experiments. Significant differences between samples in one-way ANOVA followed by Bonferroni post-test analyses are indicated by two (p < 0.01) or three (p < 0.001) asterisks.
ifying enzymes and other molecular biology reagents were obtained from Roche Diagnostics or New England Biolabs. Genomic DNA of M. smegmatis was isolated as described previously [13]. All primer sequences are listed in Table 1.

To create a transcriptional fusion of the pitA promoter to lacZ, a fragment containing 750 bp of upstream sequence to pitA (MSMEG_1064) was amplified with primers PitA6 and PitA5 and cloned into the BamHI and SphI sites of the low copy-number vector (3-10 copies per cell) pJEM15 [27], resulting in plasmid pAH1. Assays for β-galactosidase activity were carried out as described previously [13].

Cells of M. smegmatis harbouring the empty vector pJEM15 displayed β-galactosidase activities of less than 2 MU. Statistical analysis of reporter-strain experiments after starvation or stress-exposure was performed using one-way ANOVA followed by a Dunnett’s post-test comparison of each sample to the control condition. Data from experiments of the phnD-lacZ and psts-lacZ constructs in various genetic backgrounds were analyzed by one-way ANOVA followed by Bonferroni post-test comparison of all pairs of data-sets. All statistical analyses were performed using GraphPad Prism 4 software.

To create a construct for markerless deletion of pitA, an 833 bp fragment flanking pitA on the left, including 62 bp coding sequence, was amplified with primers PitA1 and PitA2, and a 1022 bp fragment flanking pitA on the right, including 4 bp coding sequence, was amplified with primers PitA3 and PitA4. The two products were fused by PCR-overlap extension [28], cloned into the SpeI site of the pPR23-derived [29] vector pX33 [13], creating pPitAKO, and transformed into M. smegmatis mc2155. Deletion of pitA was carried out using the two-step method for integration and excision of the plasmid as described previously [20]. Correct integration and excision were confirmed by Southern hybridization analysis as described previously [13]. The deletion resulted in loss of 95% of the pitA coding sequence, creating strain NP6.

Table 1: Bacterial strains, plasmids and primers used in this study

<table>
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<tr>
<th>Strain or Plasmid</th>
<th>Description</th>
<th>Source or Reference</th>
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<tr>
<td>E. coli DH10B</td>
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<td>M. smegmatis mc155</td>
<td>Electrocompetent wild-type strain of M. smegmatis</td>
<td>[25]</td>
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<td>NP6</td>
<td>mc155 ΔpitA</td>
<td>This study</td>
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<tr>
<td>NP13</td>
<td>mc155 ΔpitA carrying pCPitA; Hygr'</td>
<td>This study</td>
</tr>
<tr>
<td>Plasmids</td>
<td>pJEM15 E. coli-mycobacteria shuttle vector for the creation of transcriptional promoter fusions to lacZ; Km'</td>
<td>[27]</td>
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<td>pX33 pPR23 [29] carrying a constitutive xylE marker; Gm'</td>
<td>[13]</td>
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<tr>
<td></td>
<td>pUHA267 E. coli vector with mycobacteriophage L5 integrase and attP for integration into L5 attB of mycobacteria; Hygr'</td>
<td>AgResearch, Wallaceville, NZ</td>
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<td>pCPitA pUHA267 harbouring pitA with its native promoter; Hygr'</td>
<td>This study</td>
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<td>pSGL10 pEM15 harbouring a 500 bp phnD-lacZ fusion; Km'</td>
<td>[13]</td>
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<td>pSG42 pEM15 harbouring a 650 bp psts-lacZ fusion; Km'</td>
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<td>PitA3 CGACTTCACTGATGCTAAAGAAAGAGTTGA</td>
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<td>cPitAr AAATTTAAGCTTCAAGCTGTCATGGATTCTTC</td>
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1Km', kanamycin resistance; Gm', gentamycin resistance; Hygr', hygromycin resistance; Sac', sucrose sensitivity; ts, temperature sensitivity. Primer sequences are given in the 5'-3' direction; restriction sites included in the primer sequences are underlined.
**Phosphate transport assays**

Strains of *M. smegmatis* were grown to an OD_{600} of 1 in LBT medium, collected by centrifugation and resuspended to an OD_{600} between 1.5 and 2 in pre-warmed assay buffer (50 mM MOPS [pH 7.5], 5 mM MgCl₂, 0.05% (w/v) Tween80, 0.4% glycerol, 37°C). Initial rates of uptake of [³³P]ortho-phosphate (> 92.5 TBq mmol⁻¹; Amersham) were determined over a range of phosphate concentrations between 25 μM and 500 μM as described previously [13].

**Authors’ contributions**

SG contributed to design of the study, participated in growth experiments, phosphate transport and reporter gene assays and drafted the manuscript. NE carried out the molecular work and participated in all other experimental aspects. GMC contributed to design of the study, participated in phosphate transport assays and helped to draft the manuscript. All authors read and approved the final manuscript.

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**References**


