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Evidence that phosphate specific transporter is amplified in a fluoroquinolone resistant *Mycobacterium smegmatis*

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We reported in an earlier study that active efflux of drug has a predominant role in conferring resistance in a laboratory-generated ciprofloxacin-resistant mutant of *Mycobacterium smegmatis*. This mutant exhibited mRNA level overexpression, as well as chromosomal amplification, of the gene *pstB*, encoding the putative ATPase subunit of phosphate specific transport (Pst) system. We demonstrate here that this mutant shows enhanced phosphate uptake and that inactivation of *pstB* in the parental strain results in loss of high affinity phosphate uptake and hypersensitivity to fluoroquinolones. These findings suggest a novel role of the Pst system in active efflux, in addition to its involvement in phosphate transport.

Keywords: ABC transporter; drug resistance; fluoroquinolone; mycobacteria; phosphate specific transporter.

Phosphate specific transporter (Pst) is a member of the ATP-binding cassette (ABC) family of permeases. It has been reported that in bacteria, such as *Bacillus subtilis* [1], *Escherichia coli* [2], *Mycobacterium tuberculosis* [3], *Streptococcus pneumoniae* [4], etc., the Pst system is involved in phosphate transport. The import function of this multisubunit transporter can, for technical reasons, be measured only during phosphate limitations [2] although the functional Pst system is present in phosphate excess conditions [5]. In *E. coli*, expression of this transporter is operon controlled. PstB, the nucleotide binding subunit of the Pst system, has been reported to exhibit ATP-ase activity [6].

We have recently observed mRNA level overexpression [7] as well as chromosomal amplification [8] of *pstB* in a laboratory generated ciprofloxacin resistant *Mycobacterium smegmatis* clone, CIP^r (MIC = 64 $\mu g \cdot mL^{-1}$ as opposed to 0.5 $\mu g \cdot mL^{-1}$ in wild-type). Here active efflux of drug appears to play a predominant role in conferring high level of resistance [9]. As the role of ABC transporters in conferring drug resistance in both prokaryotes [10,11] and eukaryotes [12] is well documented, we were interested in elucidating the involvement of the Pst system in the process of efflux mediated fluoroquinolone resistance in CIP^r.

In this communication, we report that CIP^r shows an increase in the initial rate of phosphate uptake as a consequence of transporter gene amplification. Furthermore, in gene knockout experiments with the parental strain, our results argue for a novel function of at least the PstB subunit of the Pst system in the process of drug resistance in addition to its involvement in phosphate scavenging.

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Abbreviations: ABC, ATP binding cassette family; c.f.u., colony forming unit; kan, kanamycin resistance gene; MIC, minimal inhibitory concentration; Pst, phosphate specific transporter.

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MATERIALS AND METHODS

Bacterial strains, vectors and drug susceptibility testing

M. smegmatis, strain mc^2 155 (wild-type) was grown on plates or in liquid media at 37 °C using Middlebrook 7H9/7H10 (Himedia, India or Difco, USA) or Sauton's medium [9] with or without 0.01% Tween 80 (Sigma). The ciprofloxacin resistant mutant clones, CIP^{ir} (MIC = 14 $\mu g \cdot mL^{-1}$) and CIP^r (MIC = 64 $\mu g \cdot mL^{-1}$) were generated by serial adaptation of the wild-type colony from low to high concentrations of the drug as reported earlier [9]. The growth patterns of wild-type and wild-type *pstB* disrupted (WT^d) clones were monitored by absorbance (D_{640}) as well as by scoring colonies on plates after incubating the cultures ($\approx 3 \times 10^6$ c.f.u.$\cdot mL^{-1}$) with different fluoroquinolones (ciprofloxacin, ofloxacin and sparfloxacin) for 48 h at 37 °C in a shaker (200 r.p.m.). Fluoroquinolones used in this study were obtained as gifts from Ranbaxy (Gargaon, India) and Dr Reddy's Laboratory Ltd (Hyderabad, India). All other antibiotics used were procured from Sigma.

DNA manipulations, PCR, construction of gene disruption cassette and electrotransformation

M. smegmatis genomic DNA was isolated by lysis of spheroplasts as described elsewhere [7]. All manipulations with DNA were performed by standard methods [13]. Restriction/modifying enzymes and other molecular biological reagents were obtained from Boehringer Mannheim, Germany, New England Biolabs, or Promega Corporation, USA.

As reported earlier [7], *mtp1* fragment (579 bp) was PCR-amplified at 72 °C annealing temperature with primers P1 (5′-GGCTGCTCGGGCTGCGGAA-3′) and P2 (5′-GTCCA-GAGCAGAGGTGGCCTC-3′) using wild-type genomic DNA as template. *mtp1* was subcloned in pUC18. The gene disruption cassette pmtp1-kan was constructed by subcloning the *Eco*RI–*Xba*I fragment from pUC18 containing *mtp1* into pGEM-7Z(f +), followed by insertion of ≈ 1.3 kb kanamycin resistance gene (kan) at the unique *Pst*I site of *mtp1*. Two

PCR primers, P3 (5′-GACGTGTCACTCGCGGT-3′) and P4 (5′-GATCTTCTCGGTGTCGTCG-3′) were designed based on the flanking sequence of *mtp1* in the genome; the third primer, N (5′-CACCGAGGCAGTTCCATAGG-3′), was from the internal region of the kanamycin cassette. These primers (P3, P4 and N) were used for analyses of recombination events by PCR (annealing at 60 °C). Transformation of the pmtp1-kan cassette in *M. smegmatis* was carried out through electroporation [8] using a Biorad Gene Pulser.

Southern hybridizations

Standard protocols [13] were followed in carrying out Southern hybridization of the genomic DNA and PCR products. Probes were radiolabelled by random priming using [α^{32}P]d-CTP (BARC, Mumbai, India). To ensure that desired amounts of DNA were loaded for the Southern analysis in gene amplification studies, following restriction digestion, samples were ethanol precipitated, reconstituted in Tris/EDTA buffer (10 mM Tris, pH 8.0 and 1 mM EDTA) and quantitated. To determine fold amplification, autoradiographs were scanned through a densitometric scanner (LKB).

Phosphate uptake studies

M. smegmatis cultures (wild-type or WT^d or CIP^r) were grown in Sauton's medium (3.7 mM phosphate) to D_{640} of ≈ 1.0 (cell density = ≈ 10^9 mL^{-1}, wet weight = ≈ 10 mg·mL^{-1}). Cells were harvested, washed and suspended in phosphate free (ensured by assaying inorganic phosphate) Sauton's medium (D_{640} ≈ 0.4). They were maintained at 37 °C in phosphate starved condition for different time periods (0–2 h). Phosphate uptake was measured following incubation (0–15 min) with ^{32}P-labeled orthophosphate (final concentration 0.5 μM, specific activity 200 mCi·mM^{-1}; BARC, India) as described elsewhere [3].

RESULTS AND DISCUSSION

We generated a ciprofloxacin resistant mutant of *M. smegmatis* (CIP^r) by gradually adapting a wild-type colony from low to high concentrations of the drug. In an earlier study, we reported that drug efflux has a predominant role in conferring resistance in CIP^r [9]. Furthermore, CIP^r exhibited mRNA level overexpression of a ≈600-bp DNA fragment (*mtp1*) showing homology to the nucleotide binding component of an ABC transporter [7]. This finding was consistent with the behaviour of the transporters known to be involved in drug efflux [14]. The efficiency of these transporters seems to be modulated by need, induction [14,15] and/or amplification [14–16] of the relevant transporter genes have been reported.

To examine transporter gene amplification, genomic restriction patterns of wild-type and drug resistant clones were compared. Besides wild-type and CIP^r, genomic DNA was also extracted from a colony (CIP^{ir}) that showed drug resistance to an intermediate concentration of ciprofloxacin (MIC = 14 μg·mL^{-1}) and was obtained in course of generating CIP^r. Southern hybridization was carried out following restriction digestion of genomic DNAs with different enzymes (*Bam*HI, *Bgl* I, *Pst* I and *Sal* I) using *mtp1* as a probe. Comparison of the results indicated progressive amplification of *mtp1*-recognized bands as determined by densitometric scan, with CIP^{ir} showing intermediate level (≈ twofold compared to wild-type) and CIP^r exhibiting maximum (≈ fourfold compared to wild-type) band intensity (Fig. 1). Such an event does

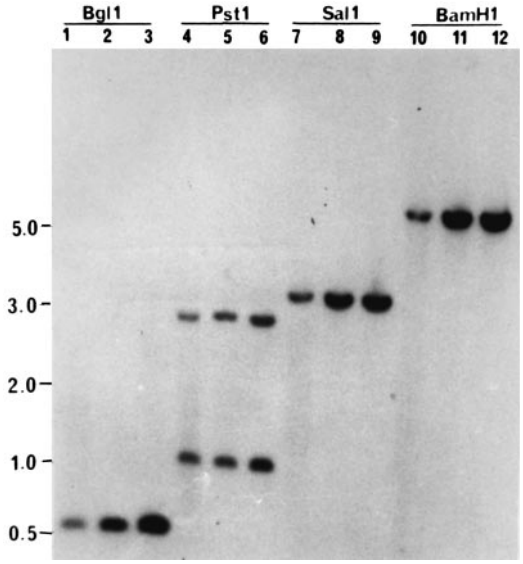


Fig. 1. Amplification of pstB in CIP^r clone. Southern hybridization profile of genomic DNA (5 μg per slot) from wild-type (lanes 1, 4, 7 and 10), CIP^{ir} (lanes 2, 5, 8 and 11) and CIP^r (lanes 3, 6, 9 and 12) following digestion with different restriction enzymes as indicated. *mtp1 has* been used as a probe. Numbers on left indicate positions of molecular mass markers.

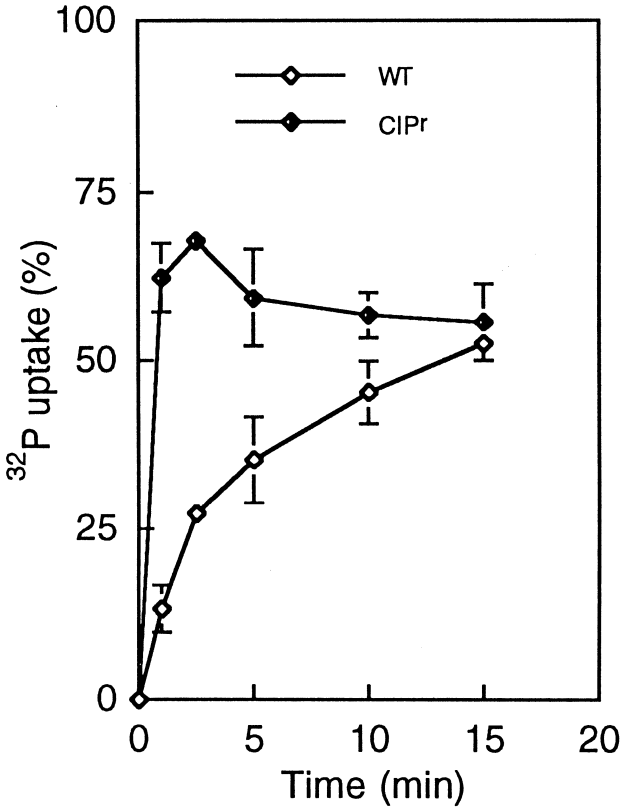
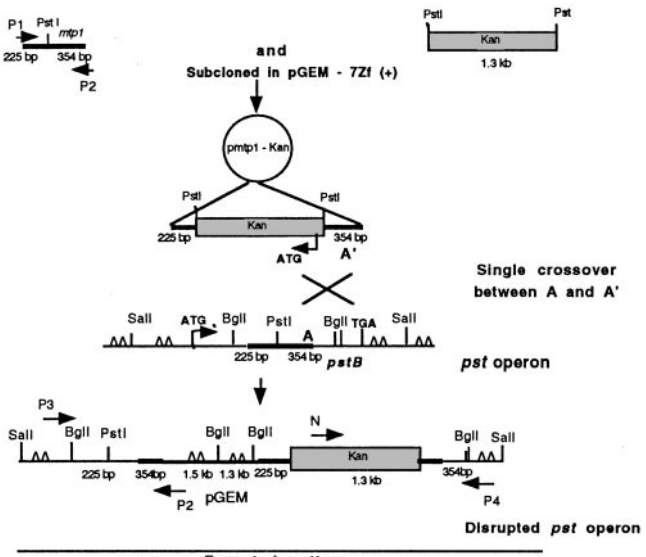


Fig. 2. Phosphate uptake in wild-type and CIP^r clones. Cultures were grown in Sauton's media. Cells were pelleted, washed and incubated for 2 h at 37 °C in phosphate-free media. [^{32}P]orthophosphate uptake was monitored at different time periods (0–15 min) of incubation. Results obtained were calculated as the percentage uptake of total [^{32}P]orthophosphate used for each reaction. Values plotted as mean ± SD from three or four independent experiments and wild-type is denoted by WT.



Expected patterns

Southern Enzyme	Probe mtp1	Probe kan	Probe pGEM	PCR Primers	Size	Probe
BglII	2 kb	2 kb	2 kb 1.3 kb	N - P4	1.2 kb	mtp1, kan
SalI	7.6 kb	7.6 kb	7.6 kb	P3 - P2	620 bp	mtp1

Fig. 3. Scheme for disruption of pst operon in wild-type. Part of the *pst* operon showing the *pstB* ORF (5′ and 3′ ends denoted by ATG and TGA, respectively), the targeting construct pmtp1-Kan (indicating restriction sites as well as position of different PCR primers), inactivated operon with expected restriction fragments and PCR amplified products. Open triangles indicate alteration in scale.

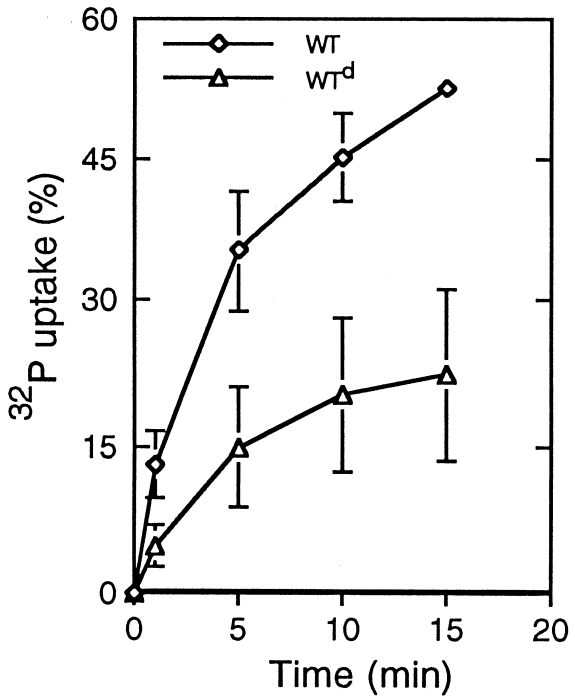


Fig. 5. [^{32}P]Orthophosphate uptake profile in wild-type and WT^{d} clones. Phosphate uptake capability of wild-type (WT) and WT^{d} cultures following incubation in phosphate-free media for 2 h at 37 °C. Results expressed as the percentage uptake of total [^{32}P]orthophosphate used for each reaction. Values are in mean ± SD and the reproducibility was checked in four independent experiments.

not seem to be an experimental artifact, as the ethidium bromide stained gel which served as a loading control did not show any discrepancy in sample loading (data not shown). Furthermore, inactivation of the transporter gene in CIP^{r} resulted in colonies, genotype analyses of which revealed partial disruption of the locus [8]. The simplest explanation of such a finding would be the presence of more than one copy of the gene in the CIP^{r} clone. The fold amplification of *mtp1*, however, was not remarkably high in these drug resistant clones. In fact, the available literature does not indicate the existence of any minimum threshold of gene copy numbers for a transporter in conferring drug resistant phenotype and, it seems, this number varies from cell to cell [17].

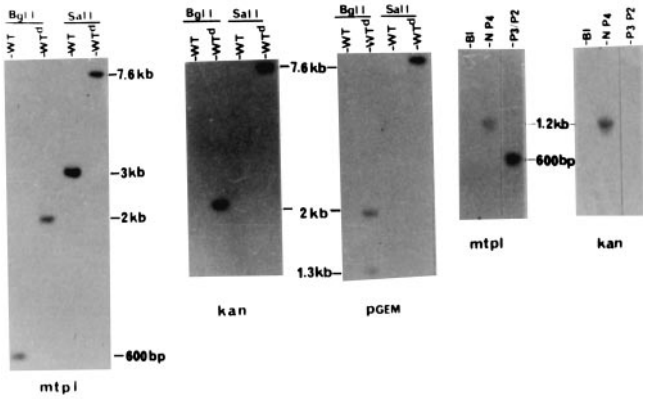


Fig. 4. Southern and PCR analyses with wild-type and WT^{d} genomic DNA. WT^{d} genomic restriction patterns with *Bgl*I and *Sal*I compared to wild-type using different probes (left and middle panels). Southern hybridization of PCR amplification products with WT^{d} genomic DNA using primers N/P4 and P3/P2 (right panels). Probes (*mtp1* or kan or vector) used are indicated at the bottom of each blot. pGEM, pGEM-7Z(f+); Bl, PCR blank.

We further screened the *M. smegmatis* mc^{2}155 genomic library (in cosmid pHC79) using *mtp1* as probe and isolated a ≈ 3 kb fragment spanning *Sal*I sites. Comparison of the 777 bp ORF of this fragment with other GENBANK sequences revealed *mtp1 to* be PstB (≈ 47% overall homology compared to reported sequences of the same subunit from *E. coli*, *M. tuberculosis* and *M. leprae*), the ATP binding subunit of Pst [7].

It has already been suggested that this multi-subunit Pst system in mycobacteria is involved in phosphate transport, as it is in other prokaryotes [3,18]. As the *mtp1* ORF was overexpressed [7] as well as amplified in CIP^{r} (Fig. 1), we evaluated the phosphate scavenging status of this importer in both wild-type and CIP^{r} by monitoring [^{32}P]orthophosphate uptake. It was necessary to expose cultures to phosphate free media for two hours prior to measurement of phosphate uptake, because preliminary data showed that starvation for shorter times resulted in lower uptake rates, apparently due to high endogenous phosphate levels (data not shown). Phosphate uptake profiles in these cultures were subsequently monitored following incubation with [^{32}P]orthophosphate for different time periods (0–15 min). The phosphate uptake kinetics, plotted as a function of time, indicated a sharp increase in the initial rate of uptake in CIP^{r} compared to the wild-type and the slopes at initial time period were distinct from each other (Fig. 2). This observation confirms that higher phosphate uptake in CIP^{r} was due to enhanced expression of the

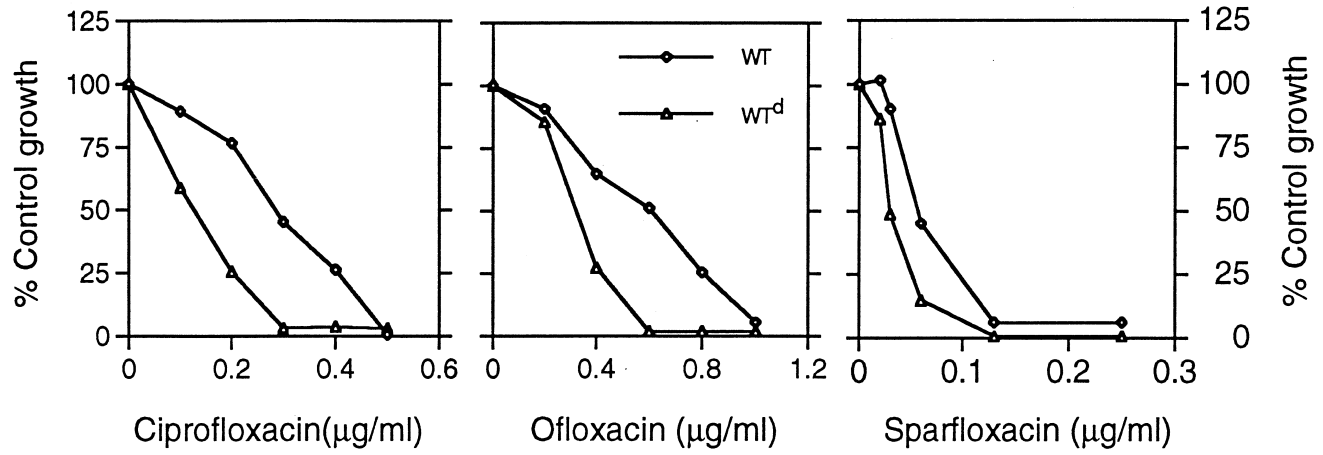


Fig. 6. Effect of fluoroquinolones on the growth profile of wild-type and WT^d clones. Both wild-type (WT) and WT^d clones were grown in liquid cultures in the presence of indicated concentrations of either ciprofloxacin or ofloxacin or sparfloxacin. The cell density was monitored in terms of optical density at 640 nm and the cultures (WT or WT^d) grown in the absence of drug were taken as controls in calculating the percentage of control growth. The results were confirmed by scoring colonies in plates as well. The reproducibility was also checked in three independent experiments.

transporter as a consequence of increase in its copy number.

Apparently duplication of a gene renders flexibility to a system in increasing the levels of its expression for a specific need [19]. Therefore, in CIP^r, if transporter gene amplification is instrumental in efflux mediated fluoroquinolone resistance, it must be an intrinsic function of the Pst system. To elucidate this aspect, we disrupted the *pstB* locus by constructing a gene disruption cassette, pmtp1-kan (Fig. 3) and transformed in the parental strain where a single copy of the gene is present. Six colonies which grew in the presence of kanamycin (10 $\mu g \cdot mL^{-1}$) were subjected to sib-selection on kanamycin plates. One such colony (WT^d) was selected for further studies. Southern hybridization results revealed that both *Bgl*I and *Sal*I restriction enzyme digestion yielded expected shifts in the same band (Fig. 3) recognized by *mtp1*, pGEM-7Z(f+) and kan (Fig. 4, left and middle panels). PCR using WT^d genomic DNA as template with primers N/P4 and P3/P2 showed amplification of bands with expected sizes (Fig. 4, right panel). Thus these two lines of evidence support disruption of the operon in WT^d through single cross-over event between 354 bp homologous sequences of pmtp1-kan and genomic *pstB* (Fig. 3).

The functional consequences of this disruption were determined in WT^d by monitoring phosphate uptake and fluoroquinolone sensitivity patterns. WT^d compared to the parental strain showed a remarkable reduction in its phosphate scavenging ability (Fig. 5). This observation thus confirmed the contribution of the *pst* operon in the process of phosphate import and further established *mtp1* to be a component of the Pst system. In drug sensitivity testing, on the other hand, WT^d exhibited $\approx$ twofold increase in sensitivity compared to the wild-type for all the fluoroquinolones tested (Fig. 6). However, such an effect does not seem to be either coincidental, or an unexplained effect of the integrated plasmid as partial disruption of the operon in the CIP^r clone also resulted in decrease in MIC for ciprofloxacin as well as in observed drug efflux patterns [8]. These results therefore argue that both the activities are mediated by at least the PstB subunit of Pst system in *M. smegmatis*.

Finally, we provide here strong evidence that the Pst system, while being an ABC importer, has a role in promoting drug efflux in the CIP^r clone. The drug efflux ability of this transporter is perhaps accentuated through the process of gene duplication, as has been observed in the CIP^r clone [8,9]. In this regard it would be appropriate to mention that the ABC importer for L-amino acids in *Rhizobium leguminosarum* has also been shown to be involved in solute efflux [20]. Further studies, following expression of the operon, may unravel how both import and export functions are performed by the mycobacterial Pst system.

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