SUMMARY

Emergence of drug resistance in tuberculosis has become a global menace. To overcome the situation there is a need to understand the molecular mechanism of drug resistance so as to accelerate the pace of drug development. However, active efflux, a well known mechanism for the developing drug resistance has not yet received proper attention in mycobacteria. We therefore, have attempted to elucidate the mechanism of efflux mediated drug resistance in mycobacteria particularly the role of transport protein (s) involved in the process.

We initiated our study by generating a clone of *Mycobacterium smegmatis* mc^2155 that exhibited high level of ofloxacin resistance. This clone (OFL^r) also exhibited cross resistance to the structurally related or unrelated compounds. The rate of drug efflux was accelerated in OFL^r compared to the wildtype strain. Calcium channel blockers, like verapamil, nifedipine, diltiazem enhanced the drug accumulation in OFL^r by diminishing the efflux and thus reversed the resistant phenotype. Sequence analysis revealed a missense mutation in the quinolone resistance determining region of the DNA-gyrase A subunit of OFL^r. Additionally, this phenomenon was specific since a ciprofloxacin resistant clone generated separately also showed similar phenotype. Taken together, these results suggested that drug efflux plays a major role in conferring such a high level of fluoroquinolone resistance in these clones in addition to the mutation in the DNA-gyrase locus.

Although it is quite atypical for fluoroquinolone resistance in prokaryotes, our observations like reversal of resistance in OFL^r and CIP^r in presence of verapamil was similar to the findings in studies of multidrug resistance in eukaryotes where ABC transporters are involved. This led us to look for the involvement of an ABC transporter in this process. We therefore carried out PCR with wildtype genomic DNA using primers derived from consensus sequences of ABC family of transporters and observed amplification of several bands. Among them PCR amplification of a ~600 bp band (*mtp1*) in both the wildtype and fluoroquinolone resistant clones was consistent even at stringent annealing conditions (72°C). The *mtp1* fragment encoded amino acid sequences that exhibited significant homology with different ABC transporters. Screening of a

genomic library using *mtp1* as a probe and subsequent sequence analysis revealed 777 bp open reading frame has homology with B-subunit of phosphate specific transporter (PstB), an ABC protein which is a part of the binding protein-dependent phosphate uptake system (Pst).

Since both the drug resistant clones exhibited similar physiological as well as biochemical characteristics we presumed existence of a common genetic mechanism for the development of drug resistance. Thus we proceeded with the clone CIP^r for further characterization of the resistance mechanism. Southern analysis revealed DNA level amplification at *pstB* locus in this clone. Such alteration was also reflected in phosphate scavenging function of CIP^r. Increased phosphate uptake in this clone also supported the gene amplification event. Since the mycobacterial cultures were grown at media phosphate concentration of 3.7 mM when phosphate import function of this transporter was inoperative, amplification of *pstB* in such a condition did not apparently highlight its role in phosphate scavenging. We therefore evaluated the possibility of the involvement of Pst system in drug resistance by inactivating the locus. Since *pstB* is amplified in CIP^r, we attempted disruption of the operon in wildtype where single-copy of the gene is present.

A gene disruption cassette, pmtp1-kan, was constructed and introduced in wildtype cells by electroporation. The kanamycin resistant clones were selected and colony purified. One such clone (WT^d) was used for further studies. Disruption of *pstB* locus in WT^d clone was confirmed by Southern analysis, PCR and western blot . Phosphate uptake pattern in WT^d was compared with wildtype cells following incubation in phosphate-free media. Unlike wildtype, inability of WT^d to scavenge phosphate in a condition when this function has been induced confirmed the inactivation of the operon and indeed established *mtp1* as a component of the Pst system. In drug sensitivity testing, WT^d exhibited ~2 fold decrease in MIC for ofloxacin, ciprofloxacin and sparfloxacin as compared to the wildtype. Furthermore, WT^d showed hypersensitivity to structurally unrelated xenobiotics that showed cross-resistance to CIP^r and OFL^r. These results therefore strongly argue the contribution of Pst system to the intrinsic level of fluoroquinolone resistance in *M. smegmatis*.