SUMMARY

Active efflux of drugs as a major contributing factor in bacterial drug resistance has been realised in recent years (Banerjee *et al.*, 1996; Takiff *et al.*, 1996; Neyfakh, 1997). The efflux of drugs conferring antimicrobial resistance in bacteria have been reported to be mediated by several transport proteins, grouped into major facilitator (MFS), resistance-nodulation-cell division (RND), small multidrug resistance (SMR) and ATP-binding cassette (ABC) super families (Levy, 1992; Nikaido, 1994; Saier *et al.*, 1997) and the encoded genes are mostly chromosomal. Most of the bacterial systems characterized so far use transporters energized by proton motive force (Nikaido, 1994, 1996). Recently, a transporter of this family has been shown to be involved in conferring low level of fluoroquinolone resistance in *Mycobacterium smegmatis*.

ABC transporters, which have been reported to utilize ATP as the driving force in drug trafficking in eukaryotes (Gottesman and Pastan, 1993), have largely been shown to be responsible for metabolite transport in prokaryotes (Higgins, 1992). Recently, LmrA belonging to the ABC family of transporters, has been shown to be directly effluxing out drugs and thus causing multidrug resistance in *Lactococcus lactis* (van Veen *et al.*, 1998). However, the exact mechanism action of ABC transporters remains unclear. Although direct drug-efflux pump mode of action of eukaryotic transporters have been suggested (Gottesman and Pastan, 1993), there are reports which propose their indirect involvement in the process by affecting transmembrane transport of drugs (Roepe *et al.*, 1993). Even interactions among ABC transporters in regulating efflux mediated drug resistance has been noticed (Nourani, 1997). The efficiency of these transporters in eukaryotes has been shown to be modulated according to the need and as a consequence, induction (Cole *et al.*, 1992), amplification (Henderson *et al.*, 1992) or induction followed by amplification (Shen *et al.*, 1986) of the relevant transporter genes have been reported.

Emergence of drug resistant mycobacterial strains is one of the prime cause of rapid resurgence of tuberculosis worldwide. Recent evidence implicate mutation in different target genes (*rpoB* for rifampin; *rpsL* and *rrs* for streptomycin; *inhA* and *katG* for isoniazid; *pncA* for pyrazinamide; *gyrA* for fluoroquinolones) to be responsible for resistance to individual antituberculosis drugs in mycobacteria (Musser, 1995; Blanchard, 1996; Scorpio and Zhang, 1996) and collective mutations in different loci is primarily thought to be the cause for multidrug resistant phenotype (Morris *et al.*, 1995). However, the other

mechanisms, like differential uptake of the drug and their active efflux has not yet received proper attention in mycobacteria. Therefore in this study, we have attempted to evaluate the role of an ABC transporter in the process of efflux mediated drug resistance in mycobacteria.

We have utilized a saprophyte, *M. smegmatis* mc^2155 in our study which has often been used as a model for genetic studies for *M. tuberculosis* (Takiff *et al.,* 1996). We obtained CIP^r (MIC = 64 μg/ml) by serially passaging a wildtype colony from lower to higher concentrations of ciprofloxacin. The existing reports indicated that mutations in DNA-gyrase A are the prime cause for fluoroquinolone resistance in mycobacteria (Musser, 1995). Strikingly, we noticed that despite mutations in DNA-gyrase A locus (Fig.3.7), CIP^r exhibited reversal of drug resistance when incubated with verapamil, a calcium channel blocker (Fig.3.2). This observation was similar to the findings in studies of multidrug resistance in eukaryotes (Gottesman and Pastan, 1993) where ABC transporters are involved. Verapamil has been reported to inhibit active efflux of drugs apparently by sterically blocking the drug transporters (Endicott and Ling, 1989) and thereby increasing intracellular drug pool. Therefore, we presumed that active efflux of drugs through transporters could be one of the factors conferring such resistance in CIP^r. These consequences also led us to suspect the involvement of an ABC transporter in the process, although it is quite atypical for fluoroquinolone resistance in prokaryotes (Levy, 1992).

We utilised polymerase chain reaction (PCR) to identify an ABC transporter that could be involved in conferring drug resistance in CIP^r. We exploited sequence conservation (Chen *et al.,* 1986) of the nucleotide binding regions of ABC transporters to design primers using preferred codon usage for mycobacteria (Fig.4.1). PCR at 72°C annealing resulted in amplification of a ~600 bp band (*mtp1*) in both wildtype and CIP^r clones (Fig. 4.2). However, reverse transcription followed by PCR (RT-PCR) with same primers exhibited increased expression of *mtp1* in CIP^r compared to the wildtype (Fig. 4.3). Using this as a probe, we screened *M. smegmatis* mc^2 155 genomic library in cosmid pHC79 and identified a gene having a 777 bp open reading frame. Sequence analysis of this fragment through BLAST X search revealed an overall ~47% homology

apparent from our study that the Pst system is involved in a high level of resistance. However it is yet unknown how these transporters share functional liabilities among themselves. It seems such interaction is not unusual since apparent cohesion through a regulatory process between two different transporters in exhibiting pleotropic drug resistance has been reported (Nourani, 1997). At this juncture it would be appropriate to mention that among prokaryotes, presence of multicopies of all the components of Pst except PstB subunit has recently been reported only in mycobacteria (Cole *et al.*, 1998). Our results add to it that the *pstB* copy number increases when necessary.

We further explored the possibility of *pstB* overexpression *per se* in imparting drug resistance. Such an assumption is not remote since ATP-binding subunit of ABC transporters have been reported to be associated with diverse biological processes (Hiles *et al.*, 1987). For this purpose we cloned *pstB* in pET-23a(+) multicopy expression vector and transformed in *E.coli* host BL21(DE3). This colony was designated as BPSTB9. Strikingly overexpression *M. smegmatis* PstB in heterologous host resulted in ~ 4 fold enhancement in the level of ciprofloxacin resistance (Fig. 5.13). This colony also exhibited cross resistance to different xenobiotics (Fig 5.14 and Table 5.1) as has been observed in CIP^r (Table 3.1). Furthermore, alteration in the drug sensitivity was accompanied by ~3 fold increase in the efficiency of ciprofloxacin extrusion over the control (Fig. 5.15). Thus functional expression of mycobacterial *pstB* in *E. coli* host unambiguously established its role in efflux mediated drug resistance.

Thus we have provided strong molecular genetic evidence showing involvement of the Pst system in mycobacterial drug resistance. The precise biochemical events and mechanisms in conferring drug resistance, nonetheless remain to be elucidated. Since PstB is an ABC protein, its function is to energise the transport process (Higgins, 1992). Amplification of the Pst system occurs in CIP^r and the PstB component is possibly recruited by a yet unidentified transporter. Alternatively the Pst system may regulate the activity of another drug effluxing pump. These postulations are schematically presented in Fig. 6.1. In this consequence it will be interesting to mention that in *M. tuberculosis* genome several putative ABC transporter sequences have been identified (Cole *et al.*, 1998) and it is not unlikely that ATP-binding subunit might have evolved independently of other

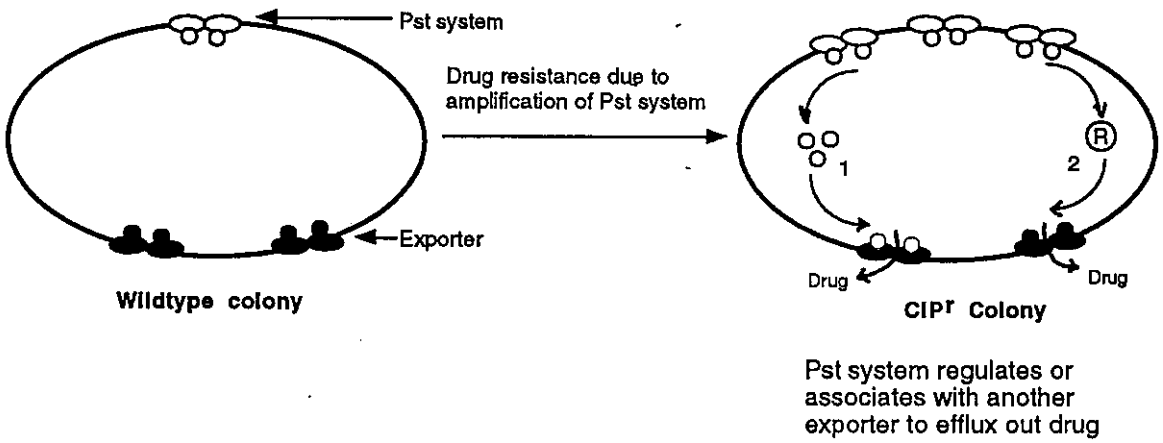

Fig. 6.1 Schematic representation of the possibilities of involvement of the Pst system in efflux mediated drug resistance in mycobacteria. In the CIP^r colony, either the PstB component is recruited another exporter (1), or the Pst system regulates the activity of another transporter to efflux out drug (2) through a hypothetical regulator 'R'.

transporter components and subsequently recruited as per the need. It would certainly be interesting to elucidate this aspect.

Finally, we used a model mycobacterium to unravel a novel role of an already known transporter. Thus our study emphasizes the necessity for critical evaluation of different transporter functions for their use as effective drug targets in the era of bacterial multidrug resistance.