Multidrug resistance (MDR) poses a serious problem in clinical medicine and is usually mediated by an overexpression of the polytopic membrane pumps belonging to the superfamily of ATP-binding cassette (ABC) transporters (Gottesman and Pastan 1993). These traffic ATPases are involved not only in the transport of various compounds but also in many cellular processes and their regulation. (Gottesman and Pastan 1993, Prasad *et al.*, 1996, Borst and Schinkel 1997). However, inter- and intra domain protein-protein interactions in these membrane transporters are crucial for their correct function. Earlier studies have speculated that the transport and other biochemical functions are affected in the absence of physical interactions between the domains of these transporters (Gao *et al.*, 1996, Ostedgaard *et al.*, 1997, Mourez *et al.*, 1998). To gain further insight into these protein interactions, the major multidrug resistance protein of *S. cerevisiae* Pdr5p was analysed in this study using the yeast two-hybrid system. The possible cytosolic loops and cytoplasmic tail of this protein were cloned in the bait vector as LexA fusions and the clones were confirmed by the DNA sequencing for their inframe fusion with the LexA DNA-binding domain. Two-hybrid control experiments demonstrated that all the fusions were suitable for use in the two-hybrid screen except for the CL2 bait, which showed auto-activation. Probably the auto-activation is the result of the fusion product LexA-CL2, since the CL2 as an AD-fusion does not show any activation.



**Protein-protein interactions of the cytosolic loop1 of Pdr5p**

The cytosolic loop1 of Pdr5p has free N-terminal end and contains the first nucleotide binding domain (NBD1). Extensive two-hybrid screening of CL1 resulted in the isolation of nine potential candidate proteins and the strength of interactions revealed that seven candidate proteins *viz. RAX2*, *YDR105c*, *ECM27*, *PRY3*, *YOR071c*, *HKR1* and ***PDR5*** probably had strong interaction with the CL1 of Pdr5p whereas the other two *viz.*, *RED1* and *SPO21* had weak interaction only. Although it was not obvious whether any of these interactions had any physiological significance, the interaction of CL1 with Library-*PDR5* is interesting. Yeast protein database revealed that most of the candidate proteins were having their predicted phenotype. To determine if any signals were responsible for these interactions, the

protein sequences were analysed using PSORTII program. Results of these studies showed presence of atleast one TM domain in the interacting regions of the strongly interacting candidates. This suggests the possibility that the CL1 domain of Pdr5p may interact with TMDs of other proteins. However, the physiological significance of this observation still needs to be verified. It is possible that the transmembrane domains might play a role in the transport process, in a manner similar to the physiological association followed by disengagement seen with the bacterial ATPase subunit, HisP and the membrane spanning domain subunits (Liu *et al.*, 1999). Similar to the bacterial system, to find out the role of NBD interaction with respect to TMDs, the candidate proteins were interacted with CL4 bait, which contains NBD2, a homologous domain of NBD1 located in CL1. Interaction studies revealed that none of the proteins exhibited any interaction with the NBD2. To further analyse the role of C-terminal end of CL1, the candidates were tested for the interaction with the CL1D(*SalI-XhoI*) bait, which contains a deletion of 56 aa from the C-terminal end of CL1. It was observed that all the proteins interacted as strongly as the full-length CL1 protein indicating that the interactions were specific to the N-terminal end of CL1. It can be speculated that some of these interactions may have significance in a manner similar to the N-terminal end of CFTR, a protein structurally identical to Pdr5p, which has been shown to interact with the membrane protein, Syntaxin-1 (Naren *et al.*, 1997). Overall, these results indicated that the free N-terminal region of CL1 is perhaps driving the CL1 interactions with the transmembrane proteins.

**Protein-protein interactions of the cytosolic loop4 of Pdr5p**

The fourth cytosolic loop of Pdr5p has a closed loop structure in the cytosol and contains the second nucleotide binding domain. Extensive two-hybrid screening with the two-hybrid library revealed that none of the proteins interacted with the CL4 domain. This result corroborates the earlier findings on the interaction between the CL4 bait and the CL1 candidate proteins. It may be speculated that the closed CL4 loop does not interact with any other proteins in physiological condition or LexA-

CL4 fusion may be stearically hindered in pulling out any interacting protein from the two-hybrid library.

**Protein-protein interactions of the cytoplasmic tail of Pdr5p**

The C-terminal end of Pdr5p has a short tail of 16 aa and is localised in the cytosol. Extensive two-hybrid library screening resulted in the isolation of six potential candidate proteins. Of these six proteins, five proteins, *viz.*, *YJL109c*, *YPL136w*, *YFL044c*, *YAP3* and *SCP160*, showed strong interactions whereas one protein, *YBR160w* showed weak interaction with the C-tail bait. Since this short tail pulled out six protein candidates whose physiological relevance was not apparent, further tests were carried out to rule out the possibility of false positives. In the auto-activation analysis, none of the proteins displayed auto-activation. Furthermore, these proteins did not show any interaction with CL1 or CL4, indicating that the interactions were both specific, and real, at least as adjudged by the yeast two-hybrid analysis.

**Intra-domain interactions of Pdr5p**

Earlier studies have suggested that the two NBDs in multidrug transporters cooperatively function perhaps through their close interactions in the native membrane (Carson *et al.*, 1995, Wilkinson *et al.*, 1996, Gadsby and Nairn 1999, Zeltwanger *et al.*, 1999). This has been further supported by the recent study (Qu and Sharom 2001), which showed closed interactions between the NBD1 and NBD2 domains of Pgp, by FRET analysis. However, two-hybrid interaction between the CL1 and CL4 baits indicated that NBD1 of CL1 does not interact directly with NBD2 of CL4. This was further confirmed from the studies of two-hybrid interaction between the CL4 bait and the library-*PDR5* (a CL1 candidate protein containing NBD1) where the two-hybrid analysis failed to detect any interaction between the CL1 and CL4 of Pdr5p. The possible reasons for this difference between these two studies are however, not clear at this moment.

To study the interaction between the CL1 - CL2 and CL4 - CL2, the CL2 domain was cloned in prey vector and tested for the auto-activation since the CL2

domain showed self-activation on its own in the bait vector. Results showed that CL2 does not show any auto-activation in the prey vector. Two-hybrid interaction studies between these loops revealed that there is no possible intra-domain interaction between these cytosolic loops.

**Two-hybrid interaction between NBD1-NBD1 domains - a possible homodimer**

The isolation of a Pdr5p encoding clone (clone S20) among the proteins interacting with the CL1 was very intriguing. It indicated the possibility that it may have a role in either dimerization or interaction with the transmembrane domains adjacent to the NBD1. Initial restriction analysis and DNA sequencing studies revealed that S20 clone does not contain any additional sequence besides the CL1 domain. This result suggested that perhaps a recombination event had occurred between the CL1 bait and the Library-*PDR5* prey resulting in the isolation of S20 clone. To further analyse this domain-domain interaction, several deletion mutants of CL1 bait were constructed and the two-hybrid interaction between the mutant baits and the S20 were studied. Except for CL1D(*Sal*I-*Xho*I) bait which contains a deletion of 56 aa from the C-terminal end, none of the other deletions interacted with S20 prey. In addition, the bait pEG202-CL1(*Eco*RI-*Bgl*II), which contains only NBD1 region and the bait pEG202-CL1(*Bgl*II-*Xho*I), which does not contain NBD1 showed negative two-hybrid interaction with S20 prey. These studies indicated that full length of NBD1 alongwith a small downstream C-terminal region (total ~ 460 aa) was important for the homodimerization. Surprisingly, the two-hybrid interaction between the CL1-CL1 domains in the conventional bait and prey plasmids showed negative (data not shown). It is therefore possible that CL1-CL1 interaction is context dependent, which can only be authenticated by biochemical data.

In conclusion, this study is perhaps the first of its kind wherein the different cytoplasmic loops of a multiple membrane spanning protein has been systematically analysed for interacting partners within the cell, as well as for interaction within the different domains of the same protein. Furthermore, it provides preliminary evidence that Pdr5p, through the CL1, might have been interacting with another Pdr5p molecule. In addition, it suggests that both the CL1 and the C-terminal tail of Pdr5p

could be involved in mediating the interaction of this protein with other proteins. These results may therefore provide a useful road map to other investigators for further analysis. Although the yeast two-hybrid system is an excellent system for rapidly obtaining the potential interacting proteins, it needs to be rigorously followed up with biochemical studies.

Attempts to carry out biochemical studies using the purified CL1 protein have so far been unsuccessful due to the tendency of CL1 to form inclusion bodies. Despite varying several parameters such as growth temperatures, different solubilization conditions and unfolding-refolding strategies, that have been successfully applied for the NBD regions of higher eukaryotes, we have not been successful in obtaining a functional, solubilized (or resolubilized) CL1 protein suitable for use in *in vitro* studies (data not shown). Newer approach may therefore be needed to authenticate the results obtained by the yeast two-hybrid based analysis.