DISCUSSION

Genetic basis of membrane structure and function with culture of microbial cells begins with the isolation of mutant cells, exhibiting altered membrane properties, or with the isolation of specific genes that code for membrane components. Spontaneous mutation rates are characteristically very low, typically falling in the range of $10^5$ to $10^9$ per haploid cell per generation. In a diploid cell which generally has two copies (alleles) of each gene, the rate of appearance of recessive mutations is even lower, requiring the occurrence in the same cell of both a mutation and a second low frequency event (i.e. mutation, inactivation, or loss of the other allele). It is thus customary to raise the general rate of mutation by treating the cell population with a mutagenic agent and then bring the two mutated allele together with mild treatment, which allow the selection or detection of the desired mutant class. Accordingly, diploid C. albicans was subjected to two step mutagenesis procedure for the isolation of desired mutants. The rationale being used in two step process was to mutate the gene of interest in the first phase and then induce mitotic crossing over to bring the mutated alleles homozygous (+ + ---> + - ---> - -) (Poulter, 1990) (Fig. 3 and 17).

We have used orthovanadate to isolate mutants resistant to it. Orthovanadate has earlier been used for biochemical characterization of PM H$^+$-ATPase of several yeast and fungi (Goffeau and Slayman, 1981). Since orthovanadate is a transition state analogue of phosphate, it modifies phosphorylation or phosphatase or nucleotide binding domain(s) of PM H$^+$-ATPase protein. The mutants resistant to orthovanadate would be expected to modify the PM H$^+$-ATPase protein and its activity by altering one or all of these domains. Studies related to modified orthovanadate sensitivity should provide valuable information about the reaction mechanism of PM H$^+$-ATPase.

As discussed in earlier section, a two step mutagenized cultures (Fig. 17) of C. albicans were plated on SD plates containing 15 mM orthovanadate and after seven days of incubation at 30°C, resistant colonies were picked up. The resistant clones of different origin from three of the stable prototrophic strains (ATCC 10261, ATCC 307 and WT 286) of C. albicans were subjected
to 15-20 times subculturing on YEPD rich medium. Such repeated transfers of \textit{van} mutants on YEPD plates did not lead to any alterations in resistance to orthovanadate and thus excluding the possibility of any adaptive resistance. The frequency with which a particular type of mutant is isolated, theoretically depends in part on the position of the gene on the chromosome (Poulter, 1990). Frequency of orthovanadate resistant mutants isolated in this study falls in the range of $5 \times 10^{-5}$, although strain specific variation can be observed (Table 3). Similar type of particular resistant mutant class in \textit{C. albicans} have not been isolated earlier. Polyene and azole antifungal resistant mutants, however, were isolated with a reasonable frequency (Hitchcock \textit{et al.}, 1987). Different auxotrophic mutants of \textit{C. albicans} have also been isolated with the frequency of $1/10^3$ to $1/10^4$, although particular type of auxotrophs (\textit{ade}1 or \textit{ade}2) usually falls in the range of $1/10^5$ to $1/10^6$ (Poulter, 1990).

As expected, \textit{van} mutants had altered PM H$^+$-ATPase activity i.e., except mutant M3A10261 and M286, all had reduced specific activities (Table 8). Irrespective of the percentage reduction in specific activity, all the mutants exhibited PM H$^+$-ATPase activity which was resistant to orthovanadate (Fig. 25 and Table 8). Based on these results, mutants could be clustered into two groups; one group included mutants which showed reduction in PM H$^+$-ATPase activity as well as resistance to orthovanadate whereas, the other group included mutants which did not show any significant reduction in enzyme activity but exhibited resistance to orthovanadate. \textit{van} mutants of \textit{S. cerevisiae} were isolated spontaneously. The characterization of two of the mutants showed enhanced PM H$^+$-ATPase activity in one case which was vanadate sensitive; while the other had unaltered PM H$^+$-ATPase activity but highly resistant to vanadate (Kanik-Ennulat and Neff, 1990). Mutations leading to vanadate resistance of ATPase activity or location of residues involved in vanadate sensitivity have been predicted in the yeast \textit{S. cerevisiae}. A limited set of amino acid substitutions in three discrete regions of the PM H$^+$-ATPase have been found to produce vanadate resistance in \textit{pma}1 mutants of \textit{S. cerevisiae} (Van Dyck \textit{et al}, 1990): the transduction or phosphatase domain (Thr231, Gly270), the phosphorylation domain (Ser368), and the nucleotide binding domain (Ala608, Asp634, Pro640) (Fig. 47). The \textit{van} mutants of \textit{C. albicans} which had PM H$^+$-ATPase activity resistant to
Fig. 47: Location of residues involved in vanadate sensitivity from the H⁺-ATPase of *S. cerevisiae*. The predicted topography is based on the fragments of transport ATPase genes. The black boxes are regions which show more than 15% mean identity in pairing comparisons of sequence segments of skeletal Ca²⁺-ATPase, sheep Na⁺/K⁺-ATPase, yeast H⁺-ATPase and *E. coli* K⁺-ATPase. The circled residues are those from *S. cerevisiae PMA1*, which after mutation lead to vanadate resistance of ATPase activity (Van Dyck et al., 1990).
orthovanadate (Fig. 25) might also have a defect in one of these three domains.

The pma1 mutants in S. cerevisiae and S. pombe were isolated by the use of Dio-9, an antibiotic of unknown structure and found that mutants resistant to Dio-9 had defective PM H⁺-ATPase activity which was resistant to vanadate in vitro (Ulaszewski et al., 1983; 1986). Additional pma1 mutants of S. cerevisiae were isolated by the use of aminoglycoside inhibitor hygromycin-B with pleiotropic phenotypic effect including reduction in PM H⁺-ATPase activity (McCusker et al., 1987). In each case, the mechanism of resistance appeared to be linked to a reduced PM H⁺-ATPase activity, probably leading to a decrease in drug uptake. Defined mutations within the PMA1 gene have also been produced in S. cerevisiae by recombinant DNA techniques with defect in PM H⁺-ATPase activity, more sensitive to acidification and more resistant to drug than wild type yeast (Vallejo and Serrano 1989).

van mutants of C. albicans were slow grower as compared to their wild type cells in both rich and minimal medium. It was evident by their increase in generation time and decrease in specific growth rate (Figs. 19, 20, Table 4 and 5). van mutants of S. cerevisiae had lower viability, low levels of sporulation and inability to arrest in G₁ phase of the cell cycle (Kanik-Ennulat and Neff, 1990). Growth control defective ATPase deficient mutants of S. cerevisiae were produced by in vitro mutagenesis (Vallejo and Serrano, 1989). It was suggested that the slow growth of ATPase deficient mutants might be caused by a defect in its ability to regulate intracellular pH. That those mutants were defective in pH regulation was confirmed by their increased sensitivity to low pH (Vallejo and Serrano, 1989). PM H⁺-ATPase deficient mutants (pma1) generated by random mutagenesis using hygromycin-B (aminoglycoside inhibitor) were also found to be slow grower (McCusker et al., 1987). From our study, the reduced growth of van mutants of C. albicans can be correlated to PM H⁺-ATPase defect although, the pleiotropic effect of mutants on growth control caused by vanadate resistance can not be ruled out.

ATPase deficient mutants of S. cerevisiae both randomly isolated (McCusker et al., 1987) or generated by in vitro mutagenesis (Vallejo and
Serrano, 1989) exhibited abnormal morphologies. Hygromycin-B resistant pma1 mutants of S. cerevisiae have a very unusual phenotype: large mother cells with many buds surrounding the periphery, which was highly dependent on growth medium (McCusker et al., 1987). Vallejo and Serrano had isolated mutants by modifying the PM H⁺-ATPase promoter sequences and have shown anomalous morphology of the PM H⁺-ATPase deficient mutants, the cells were very elongated with constriction in the middle. Their observation of the morphology in cultures grown at pH 4 had confirmed that the morphological changes were due to PM H⁺-ATPase defect and were not due to their slow growth. Similarly, SEM pictures of van mutants of C. albicans have revealed altered cellular morphologies compared to their wild types (Fig. 21, plate I and II). All the van mutants had elongated shape and all were larger in size than their parent strain. What emerges from this study is that all the mutants which had maximum reduction in PM H⁺-ATPase activity were slow grower and had abnormal morphologies. Although, the exact defect in PM H⁺-ATPase protein is yet to be determined, but in many respect, van mutants of C. albicans appeared to be very much similar to S. cerevisiae pma1 mutations (Vallejo and Serrano, 1989).

In addition to vanadate resistance, several pleiotropic characteristics of van mutants were also observed which were similar to pma1 mutations of S. cerevisiae. The van mutants of C. albicans exhibited a number of other phenotypes (Figs. 22, 23 and Table 6), some of which might have been predicted due to mutations in PM H⁺-ATPase enzyme e. g., low pH sensitive growth, osmotic pressure sensitive growth, morphological abnormalities and altered germ tube induction capability (dimorphic transition) etc. Similar pleiotropic PM H⁺-ATPase mutations of S. cerevisiae have also been reported (McCusker et al., 1987), and found that since these phenotypes were complemented by a cloned copy of the PMA1 gene, they were related to its mutation. The involvement of a second site mutations that enhanced and suppressed various phenotypes of pma1 mutants was also envisaged (McCusker et al., 1987).

PM H⁺-ATPase of yeast may be modified by a number of other gene products; for example pleiotropic drug resistance locus PDR (Rank et al., 1975a,b; Sounders and Rank, 1982). pdr1 mutants were reported to have
decreased PM H^+-ATPase activity (Rank et al., 1977, McCusker et al., 1987). With the isolation of PDR and PMA mutants it is now confirmed that these two loci are genetically distinct, although, they mapped in the same chromosome (Balzi and Goffeau, 1991). The van mutants of C. albicans were also tested for their cross resistance with other drugs. Surprisingly, most of the van mutants were found to be sensitive to antibiotic hygromycin-B and to inhibitors, X-bag, protamine sulfate etc. Interestingly, no difference was observed between wild type and mutant's growth on a variety of drugs (cycloheximide, erythromycin, tetracycline, chloramphenicol, ampicillin etc.) to which pdr1 mutants of S. cerevisiae showed cross resistance. Hygromycin-B sensitivity of van mutants of C. albicans combined with their defect in PM H^+-ATPase activity is an unusual phenomena. This antibiotic was earlier used to isolate PM H^+-ATPase defective mutants of S. cerevisiae (McCusker et al., 1987). Very recently, it has been shown that in vitro mutagenesis of C. albicans PMA1 gene leads to more resistance to hygromycin-B (Monk et al., 1991; cited as unpublished observations). On the other hand, spontaneously isolated recessive van mutants of S. cerevisiae were found to be associated with enhanced hygromycin-B sensitivity (Ballou et al., 1991). van mutants of C. albicans except M3A10261 and M286 (Fig. 24) were sensitive to protamine sulphate. Similar sensitivity to protamine sulphate has been shown for Dio-9 resistant pma1 mutants of S. pombe which was allelic to modified PM H^+-ATPase activity and was used as a marker for genetic mapping (Ulaszewski et al, 1986). Since van mutants of C. albicans do not elicit cross resistance to several drugs but rather becomes more sensitive to many drugs e.g., hygromycin-B, X-bag and protamine sulphate etc., the van locus of C. albicans appears to be unrelated to pdr.

PM H^+-ATPase of yeast corresponds to the proton pump (Serrano, 1980), which is inhibited by inhibitors like DCCD or DES etc. In this study, it was ascertained if reduction in PM H^+-ATPase activity of van mutants was also reflected in proton translocating ability of C. albicans cells. From our result, it is evident that there was no apparent correlation between proton efflux rate of whole cells and levels of PM H^+-ATPase activity (Table 10, Figs. 28a and 28b). Our measurements of H^+ efflux do not distinguish between PM H^+-ATPase activity and fluxes of organic acids but has been used in past as an
index of PM H⁺-ATPase function (Serrano, 1980). It may be mentioned that C. albicans cell wall acts as a cation exchanger and hampers H⁺-symport measurements (Prasad, 1991), therefore, a lack of correlation between H⁺ efflux and PM H⁺-ATPase activity may not be a surprising. No direct correlation between the rate of H⁺-efflux and ATP hydrolysis was found in pma1 mutants of S. cerevisiae isolated by site directed mutagenesis (Portillo and Serrano, 1989). Two of the hygromycin-B resistant pma1 mutants of S. cerevisiae also did not show any significant difference in proton transport, as deduced from whole cell medium acidification although they had reduced levels of PM H⁺-ATPase activity (Perlin et al., 1989).

Intracellular pH (pHi) maintained by the ejection of protons from the cytosol, could be a mechanism of growth control in yeast (Serrano, 1989). Yeast growth rate was found to be optimal at neutral pHi and decreased as the cell became more acidic (Gillies et al., 1981). A defect in pH regulation was also evident by the increased sensitivity of pma1 mutants to low pH (Vallejo and Serrano, 1989). Mutants of S. pombe with reduced PM H⁺-ATPase activity also showed decreased inability to efflux protons, slow growth and lowered pHi (Ulaszewski et al., 1987b). We observed that in unbuffered rich medium, the pHi of the van mutants of C. albicans was lower than their corresponding wild types (Fig. 29). The inability to sustain similar level of pHi by these van mutants could well be related to their defect in PM H⁺-ATPase activity.

Amino acid transport was undertaken in van mutants of C. albicans to investigate the role of PM H⁺-ATPase in secondary transport systems. van mutants of C. albicans had altered uptake rates of L-glu, L-lys, L-Pro and gly (Figs. 38, 39, and 40). The uptake of all the stated amino acids was significantly reduced in all van mutants except mutant M2307. The reduced uptake rate of amino acids in van mutants was suggestive of a role and importance of PM H⁺-ATPase in overall secondary transport systems of C. albicans. Some of the van mutants e.g. M3A10261 and M3B10261 could not form germ tube (could form pseudohyphae) had reduced rate rate of L-pro uptake (Fig. 40). An interesting observation with mutant M2307 needs special mention. This mutant showed maximum reduction in PM H⁺-ATPase activity, the reduced activity was resistant to vanadate, could not form germ
tube and had increased rate of uptake of all the amino acids tested (L-glu 60%, L-lys 12%, L-pro 42%, gly 41%). This enhanced rate of uptake could be due to a pleiotropic effect of *van* mutants. On the other hand, it could also be due to a simultaneous unlinked mutation of general amino acid permease or membrane components which could modify the permease, because the selection medium which was used to isolate the mutants was limiting (SD minimal medium) in which mutants with increased transport capacity could arise (Adelberg and Slayman, 1988).

Vanadate is known to enter the cell via the phosphate uptake system(s) (Cantley *et al.*, 1978; Bowman, 1983). The result of present work demonstrated that *C. albicans* only has one type of phosphate uptake system which is pH dependent (Figs. 31 and 33) and derepressible (Figs. 36a, 36b and Table 13) *van* mutants of *C. albicans* showed between 20 and 70% reduction in the rate of phosphate uptake (Table 11). Orthovanadate has been found to competitively inhibit phosphate uptake in *C. albicans*, thus confirming that both share a common permease. This is also in accordance with an earlier observation where *van* mutants of *N. crassa* were shown to be defective in vanadate uptake which shared a high affinity phosphate uptake system (Bowman *et al.*, 1983). Although, there has been very little experimental support for the assumption that vanadate is directly taken up into the cells, our results have demonstrated that *van* mutants might have developed resistance to orthovanadate by modifying the rate of its entry. This conclusion is substantiated by our observations that *van* mutants exhibited slow growth (discussed in earlier section) in high phosphate medium, and elicited a considerable reduction in the rate of phosphate uptake and that the uptake was inhibited competitively by orthovanadate.

Ramasarma and coworkers have found that non enzymatic oxidation of NADH increased with vanadate (Ramasarma *et al.*, 1981). The oxidation of NADH in presence of vanadate was found to be stimulated by the addition of mouse liver plasma membranes, inhibited by SOD, and generated H$_2$O$_2$ (Ramasarma *et al.*, 1981). A similar NADH oxidation activity has also been reported in rat liver microsomes (Coulombe *et al.*, 1987), erythrocyte membrane (Vijaya *et al.*, 1984), sugar beet microsomal membranes (Briskin *et al.*, 1985) and very recently in plasma membrane of *S. cerevisiae* (Minasi
and Willsky, 1991). We could group our mutants in two classes with respect to vanadate dependent membrane-stimulated NADH oxidation. Mutants M3A10261 and M3B10261 showed more than 50% reduction and rest of the mutants had increased NADH oxidation between 40 and 70% as compared to their parental wild type strains (Fig. 43 and Table 16). The increase in oxidation ability would imply their enhanced capability to metabolize orthovanadate to less toxic form by these mutants. Since some of the mutants also had reduced ability to vanadate dependent membrane stimulated NADH oxidation, the mechanism of resistance to orthovanadate by these mutants may differ from each other.

Several studies have confirmed that the mechanism of resistance to toxic substances is either by the formation of its non toxic form or by rapid efflux of the substrates (similar to ATPase system driven by ATP) or by the reduced influx of the substances. Metabolism of vanadate was found to be the cause of resistance in \textit{van} mutants of \textit{S. cerevisiae} (Willsky \textit{et al.}, 1985). Two bacterial efflux ATPases have been identified; arsenate resistance ATPases and cadmium ATPases. Deduced amino acid sequence of these two ATPases revealed extensive homology between all of the cation-translocating 'P' type ATPases of bacterial and eukaryotic origin (Silver \textit{et al.}, 1989). In \textit{N. crassa}, \textit{van} mutants had defect in the uptake rate of vanadate (Bowman \textit{et al.}, 1983). Similarly, hygromycin-B resistant mutants of \textit{S. cerevisiae} also shown to have defect in the uptake rate of the antibiotic (Perlin and Haber, 1989). \textit{van} mutants of \textit{C. albicans} elicited a considerable reduction in the rate of phosphate uptake (discussed in earlier paragraph), and exhibited slow growth in high phosphate medium. These results point out that \textit{van} mutants could have developed resistance to vanadate by modifying the rate of its entry. This result is also supported by our preliminary finding with Atomic Emission Spectroscopy (AES) method where differential accumulation of vanadate occurs in \textit{van} mutants compared to their wild type cells. On the other hand, the cause of vanadate resistance in atleast some of the \textit{van} mutants which might result from increased activity of membrane stimulated vanadate dependent NADH oxidation can not be excluded. Furthermore, \textit{in vivo} metabolism vanadate observed by using Nuclear Magnetic Resonance (NMR 90 MHz in JOEL FX 90) revealed that a converted form of vanadate did exist in \textit{van} mutants. Thus, \textit{van} mutants might have stimulated the enzymatic
Table 17 PM H⁺-ATPase activity and level of PM H⁺-ATPase protein in pma1 mutant and its wild type strain of S. cerevisiae.

PM H⁺-ATPase was assayed as described for C. albicans strains (Materials and Methods). Protein content was measured using antisera raised against PM H⁺-ATPase protein of C. albicans by ELISA as described in Fig. 30.

<table>
<thead>
<tr>
<th>Strain</th>
<th>ATPase activity (umole pi. min⁻¹. mg⁻¹.protein)</th>
<th>ATPase content (ELISA OD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>W.T. Σ1278b</td>
<td>0.501</td>
<td>0.633</td>
</tr>
<tr>
<td>MG 2129</td>
<td>0.097</td>
<td>0.610</td>
</tr>
</tbody>
</table>
machinery to convert toxic vanadate into less toxic or non toxic form. However, both the data form AES and NMR are of preliminary nature and no definite conclusion could be drawn at this stage. As discussed, the van mutants of *C. albicans* exhibited some characteristics such as, reduced PM H⁺-ATPase activity, activity resistant to vanadate *in vitro*, low pH and osmotic pressure sensitivity, slow growth, and abnormal cellular morphology, low pHi, and alterations in secondary transport system (amino acid) etc. which are very much similar to *pma1* mutants of *S. cerevisiae*. On the other hand, the reduction in PM H⁺-ATPase activity of van mutants was not associated with a decreased content of the protein, which was confirmed by the use of polyclonal antisera raised against purified proteins (Fig. 30). Interestingly, protein content and PM H⁺-ATPase activity did also not correlate in *pma1* mutants of *S. cerevisiae* (MG2129) which had only 20% of the total activity, yet no detectable difference in protein levels (Table 17).

Based on these analogies, it was expected that the mutation might lie in the *PMA1* gene of *C. albicans*. We did not observe any difference in restriction pattern and karyotypes between mutant and their parents. The hybridization with *PMA1* gene of *C. albicans* also did not reveal any difference. If there is a point mutation conferring resistance to vanadate, it may be possible to detect such difference by employing the recently developed technique of temperature gradient gel electrophoresis. Due to lack of selectable markers in the strains employed for mutant isolation, the complementation of these mutants with the cloned *PMA1* gene could not be done.

As mentioned, it has been shown that resistance to toxic concentrations of vanadate can arise in *S. cerevisiae* by both recessive and dominant spontaneous mutations in a large number of loci. Mutations in two recessive loci *van 1-18* and *van 2-93*, resulted in alterations in phosphorylation of a number of proteins (Kanik-Ennulat and Neff, 1990). The *van 1-18* mutant showed an increase in PM H⁺-ATPase activity while mutant *van 2-93* had normal level of enzyme activity. The PM H⁺-ATPase activity of both the mutants, however, was resistant to vanadate. A vanadate resistant gene has been identified and sequenced in *S. cerevisiae* (Kanik-Ennulat and Neff, 1990). The *VAN1* gene product is involved in the regulation of
Fig. 48: Membrane protein glycosylation of van mutants of C. albicans. Glycosylation was studied using PAS staining procedure with SDS-PAGE as described (Gupta et al., 1991) (A) Coomassie Blue Staining of SDS-PAGE: (B) PAS staining of SDS-PAGE: (1) ATCC 10261 (2) M2 (3) M3A (4) M3B (5) M3C (8) ATCC 307 (7) M1 307 (8) M2 307 (C) (9) WT 286 (10) M 286.
phosphorylation of a number of proteins, some of which appear to be important for cell growth. Whether such pleiotropic effect also leads to vanadate resistance in *C. albicans* remains to be established. At this point, it is not established if the *VAN1* gene of *S. cerevisiae* is homologous to *C. albicans*. Based on the biochemical, physiological and molecular characterization of *C. albicans* *van* mutants, what emerges from this work is that these mutants defects are probably related to *pma1* mutation.

Recently, Ballou and coworkers have found that *van* mutants of *S. cerevisiae* were defective in protein glycosylation which can mimic the previously isolated *mnn* mutants and have suggested that the primary sites of vanadate inhibition impinge directly on the processing of the glycoproteins in the golgi (Ballou *et al.*, 1991). On the other hand, *S. cerevisiae mnn9* mutants which were defective in protein glycosylation were found to be resistant to orthovanadate and sensitive to hygromycin-B (Hitzeman *et al.*, cited as unpublished data in Ballou *et al.*, 1991). Our preliminary study of membrane protein glycosylation with *van* mutants of *C. albicans* by the PAS staining method showed defective glycosylation (Fig. 48). Additional information of the *VAN* gene of *C. albicans* will be very interesting because many vital cellular functions i.e., protein phosphorylation and protein glycosylation, might shed some light on the enigmatic problems (e.g., dimorphism, pathogenicity, host defense mechanism etc.) of this pathogenic yeast.

In conclusion, *van* mutants of *C. albicans* appear to have acquired resistance to orthovanadate (15 mM) by preventing its entry (Fig. 31 and Table 11); converting it to less toxic form and by modifying PM H\(^+\)-ATPase activity (Table 8). Many phenotypic characteristics of *van* mutants of *C. albicans* are very much similar to *pma1* mutants of other yeasts however, these aspects remain to be confirmed genetically.