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Targeting the fungal plasma membrane proton pump*

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The need for new mechanistic classes of broad spectrum antifungal agents has prompted development of the membrane sector and ectodomain of the plasma membrane proton pumping ATPase as an antifungal target. The fungal proton pump is a highly abundant, essential enzyme in *Saccharomyces cerevisiae*. It belongs to the family of P-type ATPases, a class of enzymes that includes the Na^+,K^+-ATPase and the gastric H^+,K^+-ATPase. These enzymes are cell surface therapeutic targets for the cardiac glycosides and several anti-ulcer drugs, respectively. The effects of acid-activated omeprazole show that extensive inhibition of the *S. cerevisiae* ATPase is fungicidal. Fungal proton pumps possess elements within their transmembrane loops that distinguish them from other P-type ATPases. These loops, such as the conformationally sensitive transmembrane loop 1+2, can attenuate the activity of the enzyme. Expression in *S. cerevisiae* of fully functional chimeric ATPases that contain a foreign target comprising transmembrane loops 1+2 and/or 3+4 from the fungal pathogen *Candida albicans* suggests that these loops operate as a domain. The chimera containing *C. albicans* transmembrane loops 1+2 and 3+4 provides a prototype for mutational analysis of the target region and the screening of inhibitors directed against opportunistic fungal pathogens. Panels of mutants with modified ATPase regulation or with altered cell surface cysteine residues are also described. Information about the ATPase membrane sector and ectodomain has been integrated into a model of this region.

In parallel with the spread of HIV and accentuated by several aspects of modern medical practice, the frequency of opportunistic fungal infections has risen dramatically in recent years [1]. Most fungal infections occur in predisposed hosts when innate host defenses have been compromised [2–5]. *Candida albicans*, a normal resident of mucosal surfaces in the gastrointestinal tract and female reproductive system, is responsible for about 60% of all fungal infections and 10–15% of nosocomial bloodstream infections [1]. Its ability to adhere and proliferate on mucosa is usually kept in check by competition with the endogenous microflora, secretions associated with these surfaces and by the immune system [6]. When host defenses

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Abbreviations: BCIP, 5-bromo-4-chloro-3-indolyl phosphate; CSM-URA, complete synthetic medium lacking uracil; DMSO, dimethyl sulfoxide; NBT, nitro blue tetrazolium; NEM, *N*-ethylmaleimide; PMSF, phenylmethyl sulfonylfluoride; SDS, sodium dodecyl sulfate.

are immature, weakened, modulated by hormonal change, or when antibiotics have been applied, mucosal surfaces can be more severely colonized, giving rise to conditions such as vaginitis or oral and oesophageal thrush, as is frequently seen in premature babies, AIDS patients and in cancer patients undergoing chemotherapy. As AIDS patients become immunocompromised there is an increased likelihood of respiratory tract infection with life-threatening opportunistic fungal pathogens such as *Pneumocystis carinii*, while infection by *Cryptococcus neoformans* can lead to meningitis [5]. Hematogenously disseminated fungal infection can also result in patient mortality and most frequently occurs after surgical intervention and in association with the use of indwelling catheters. *C. albicans* is most commonly involved, but other opportunistic fungal pathogens such as *Aspergillus fumigatus* place surgery patients at even greater risk, while *Candida tropicalis* has emerged as the most significant fungal threat to neutropenic patients [7].

Unfortunately the repertoire of therapeutically useful antifungal agents is limited [8], with most targeting either the high content of ergosterol found in the fungal plasma membrane or the sterol biosynthesis pathway. The fungicidal polyene antibiotics remain the "gold standard" amongst the antifungals. Nystatin successfully treats most topical and mucosal infections but its poor solubility restricts its use to superficial tissues. Amphotericin B is still the preferred treatment for disseminated infections, but it is nephrotoxic, with frequent undesirable side-effects during infusion and it is only about 50% effective. Fluconazole, which is an inhibitor of sterol biosynthesis, is about as effective as amphotericin B. It has fewer side-effects, but it is only fungistatic and prolonged treatment can lead to induced resistance or superinfection by naturally resistant yeast such as *Candida glabrata* and *Candida krusei* [9].

Given these problems, new mechanistic classes of broad spectrum antifungal agents are urgently required [8, 10]. What are the desirable attributes of a target for broad spectrum antifungals and why should the plasma membrane proton pumping ATPase be considered a valid target?

A preferred antifungal target should be required for growth. This property that can initially be examined with gene disruption experiments in a genetically amenable organism like *Saccharomyces cerevisiae*. The plasma membrane proton pump specified by the *PMA1* gene is still the only antifungal target which has been demonstrated as essential by gene disruption [11]. Similar experiments have shown other potential antifungal targets to be non-essential or have revealed a multiple gene family which contains several members that need to be disrupted before cell growth is deleteriously affected. Such putative antifungal targets include enzymes involved in cell wall biosynthesis and degradation [12–15], and secreted proteases [16, 17] which are thought to be virulence factors. The involvement of a multiple gene family is not necessarily a problem provided that the targeting feature within the gene products remains constant. If this is not the case, however, drug resistance is more likely to be generated by gene conversion. In addition, the demonstration of essentiality by gene disruption does not necessarily mean that a gene is required for vegetative growth, because its product could have a specialized role in processes such as meiosis or sporulation. Genetic experiments, in which the activity of the plasma membrane ATPase has been directly manipulated by mutation [18] or the transcription of the *PMA1* gene has been modified [19], have shown that the *S. cerevisiae* pump can define the growth rate of vegetatively dividing cells. These observations are consistent with the physiological role of the fungal plasma membrane proton pump, which maintains intracellular pH and ionic composition and generates the electrochemical gradient that powers the uptake of several essential nutrients [10, 20].

Fungi are eukaryotes and share most of the genetic and biochemical properties of their animal hosts. Although genome sequencing projects and an understanding of biosynthetic pathways such as those involved in cell wall biosynthesis may reveal gene products specific to fungi, the pursuit of such targets is a longer term option. We have therefore selected a target from within a family of molecules that is shared by fungi and their human host, but which has features that are specific to fungi.

The fungal plasma membrane proton pump is a member of the extended family of P-type ATPases, a class of molecules that includes the Na^+,K^+-ATPases of mammalian cells, the gastric H^+,K^+-ATPase, the Ca^{2+}-ATPases of red

blood cells and the sarcoplasmic reticulum, the copper transporting ATPases implicated in Wilson's and Menkes' diseases, the proton pumping plasma membrane ATPases of some lower eukaryotic parasities, plant cells and fungal cells, as well as ion specific ATPases of some prokaryotic cells [10, 21].

In several cases two subunits contribute to the overall enzyme complex, but catalytic activity appears to reside in the approx. 100 kDa α-subunit of the fungal ATPase [22]. The yeast enzyme may associate with low molecular mass proteolipid proteins that are the products of two separate genes [23]. The function of the proteolipid proteins is poorly understood, but disruption of both genes in *S. cerevisiae* significantly reduces ATPase activity. Cloning has yielded a data-base of at least fifty P-type ATPase α-subunit primary sequences, including sequences for the *PMA1* gene specifying plasma membrane proton pumps in seven fungal species and two sequences for the closely related *PMA2* gene [10, 24].

Biochemical, molecular genetic and immunological experiments have provided a useful understanding of P-type ATPase topology which appears to be strongly conserved [25–30]. The consensus view is that the fungal ATPase spans the plasma membrane ten times with five transmembrane loops [10]. The transmembrane loops divide the ATPase into at least four functionally distinct cytoplasmic domains, contribute intramembrane elements involved in translocating protons across the lipid bilayer and present an ectodomain of short intervening turns to the cell exterior. Comparative alignment of ATPase amino-acid sequences not only shows that the fungal proton pump transmembrane region and ectodomain are structurally well conserved among divergent groups of fungi, but also demonstrates that this region significantly differs from the comparable structures in the P-type ATPases of mammals and even the plant plasma membrane proton pumps, which are the closest functionally-related members of the P-type ATPase family [10]. Targeting an essential fungal cell surface enzyme, which has conserved biochemical features that distinguish it from related enzymes in the human host, should have major advantages in limiting the possibility of side-effects, since the inhibitors can act from outside the cell. Furthermore, resistance pathways which utilize membrane pumps to remove inhibitors from the cell, such as those involved in yeast multidrug resistance [31], should be of little consequence.

Surface elements of P-type ATPases are specific targets of several therapeutic reagents. Extracts from the flowering plant foxglove have been used to treat patients with congestive heart failure since 1775. The active principals of the extracts are the cardiac glycosides, a complex group of steroid-like molecules that lower the activity of the plasma membrane Na^+,K^+ pump in cardiac muscle. Ouabain and the cardiac glycosides exhibit exquisite specificity for the Na^+,K^+-ATPase, inhibiting most strongly the α_1-isoform of the enzyme, and having no effect on other P-type ATPases including the fungal P-type ATPases [32–34]. They interact, at least in part, with exposed cell surface sites that become available during the reaction cycle. Studies with interspecies Na^+,K^+-ATPase chimeras show that only the N-terminal portion of the molecule, and more specifically transmembrane segments 1 and 2, appear essential for the binding and conferral of ouabain sensitivity [35, 36]. Mutational analysis of the Na^+,K^+-ATPase α-subunit suggests that many surface residues can also modify the interaction between the ATPase and ouabain, but most of these effects appear to be indirect [37]. The precise nature of the interaction between cardiac glycosides and the Na^+,K^+-ATPase has yet to be resolved but, consistent with recent cross-linking studies [38], an attractive suggestion is that ouabain interacts with the $\alpha 2\beta 2$ dimer of the ATPase by binding in a cleft between transmembrane loop 1+2 pairs [37]. Another plant-derived inhibitor of a P-type ATPase is the sesquiterpene lactone thiapsigargin. This compound is a potent and specific antagonist of the Ca^{2+}-ATPase. Analysis of chimeric mutants indicates that transmembrane segment 3 is required for its activity [39].

The gastric H^+,K^+-ATPase is the site of action of omeprazole, a pyridinyl-2-methylenesulphinyl-2-benzimidazole derivative, which selectively inhibits the enzyme by exploiting the extremely acidic pH in the surface pits of the gastric parietal cells to activate the drug [40]. The sulphenamide derivative thus produced covalently reacts with the cell surface sulphydryl groups on the ATPase. The fast proximal activation of the drug, the membrane-

-excluding positive charge of the sulphydryl-scavenging sulphenamide and its rapid reductive inactivation by the high concentration of intracellular glutathione and by neutral intracellular pH give omeprazole considerable targeting specificity. A study [41] using pantoprazole, a slightly more acid-stable relative of omeprazole, suggests that its sulphenamide alters enzyme conformation and aggregates the enzyme by reacting with the two cysteines in transmembrane loop 5+6.

Other classes of gastric H^+,K^+-ATPase blockers include the non-covalent K^+-competitive drugs, SCH 28080 and SK&F 96067 [42]. Both drugs appear to act on the luminal surface of the parietal cell and although structurally dissimilar, they seem to target the same site. MeDAZIP, which is a photoaffinity analog of SCH 28080, labels the first transmembrane loop 1+2 of the ATPase sequence [43]. Molecular structure modeling of this region predicts that the inhibitor contacts F124 in transmembrane segment 1 and D136 in the adjacent transmembrane segment 2 of the gastric ATPase.

The highly specific interactions between particular P-type ATPases and inhibitors like ouabain, omeprazole, thiapsigargin and SCH 28080 provide both rationale and precedent for the notion that the membrane sector and ectodomain of the fungal ATPases is a suitable and potentially highly specific target for the development of effective antifungals [10]. Mutational studies have demonstrated that modification of particular amino-acid residues located in transmembrane loop 1+2 of the *S. cerevisiae* plasma membrane proton pump can be lethal, give partially impaired cellular phenotypes or suppress phenotypes caused by mutations near the active site of the enzyme [44–46]. Transmembrane segments 1 and 2 and their short intervening turn appeared tightly folded into a conformationally sensitive transmembrane loop, with structural perturbations in this region impairing ATPase activity and cell growth. A structural model of the region has been developed and comparative analysis of dynamic simulations of wild type and mutant structures support the idea that the region is conformationally sensitive [47].

We have used several approaches to develop the membrane sector and ectodomain of the plasma membrane proton pump as a target for broad spectrum antifungals. Acid-activated omeprazole inhibits the growth of yeast and this observation has been extended to demonstrate that the agent is fungicidal and modifies ATPase structure and function. Expression in *S. cerevisiae* of *pma1* mutants which contain transmembrane loops 1+2 and/or 3+4 specified by the *C. albicans PMA1* gene has given a set of fully functional chimeric ATPases which suggest that interactions occur between transmembrane loops. A third approach using site-directed mutagenesis has led to panels of mutants that will help in antifungal screening and analysis of residue accessibility. Finally, information about the membrane sector and ectodomain suggests a useful hypothetical view of the surface of the enzyme.

METHODS

Yeast strains and cell culture. The *S. cerevisiae* strains used in this study were isogenic derivatives of the wild type strain Y55 (*HO gal3 MALI SUCI*) [48]. Yeast transformations were performed using *S. cerevisiae* strain SH122 (*HO/HO MATα/MATα ade6-1/ade6-1 trp5-1/trp5-1 leu2-1/leu2-1 lys1-1/lys1-1 ura3-1/ura3-1 pmaΔ::LEU2/ /PMA1*). The control strains SN236 and T48 (*HO ade6-1 trp5-1 leu2-1 lys1-1 ura3-1 PMA1-URA3*) were selected as *leu URA* spores from SH122 in which the *LEU2*-disrupted *pma1* gene was replaced with *PMA1-URA3* [44]. The *C. albicans* strain used in this study was the wild type strain ATCC10261. *S. cerevisiae* cells were maintained on solid complete synthetic medium lacking uracil (CSM-URA) while the *C. albicans* strain was maintained on solid YPD (1% yeast extract, 2% peptone, 2% dextrose) medium. For growth experiments and biochemical studies cells were grown in YPD at pH 5.7 or in medium adjusted to the indicated pH with concentrated HCl or NaOH.

Engineering of chimeric constructs. Cloning and propagation of recombinant DNA was accomplished using *Escherichia coli* strain XL1-blue (Stratagene). PCR amplification of appropriate sequences from the pJAM25 plasmid template [49] using the high fidelity polymerase Pfu (Stratagene) produced DNA encoding putative TM1+2 or TM3+4 from *C. albicans PMA1*. One-sided PCR, using these double stranded amplimers as template and with the appropriate phosphorylated oligonucleotide

primers, gave single stranded DNA products that were isolated by electrophoresis in low melting temperature agarose (Seaplaque GTG agarose, FMC Bioproducts, Rockland ME 04841). The single stranded DNA products, which included at least 16 nucleotides at both their 5′ and 3′ termini that were homologous to *S. cerevisiae PMA1*, were used individually as mutagenic primers in a Kunkel mutagenesis procedure [50, 51] on a single stranded, uracil-containing template derived from plasmid pGW101 [45]. This plasmid contains *S. cerevisiae PMA1-URA3* in a pGEM-13Zf(+) vector (Promega, Madison, WI, U.S.A.). Bacterial transformants carrying the mutant plasmids pCTM1+2 (containing sequences specifying *C. albicans* transmembrane loop 1+2) and pCTM3+4 (containing sequences specifying *C. albicans* transmembrane loop 3+4) were initially detected by screening for the loss of *Eco*RI or *Xcm*I site, respectively. *C. albicans PMA1* sequences and the junctions with *S. cerevisiae PMA1* DNA in pCTM1+2 and pCTM3+4 were verified by DNA sequence analysis of both strands. The construct pCTM1+2+3+4 was made using a 0.8 kb *Bst*EII/*Bam*HI fragment from pCTM3+4 to replace the equivalent fragment from plasmid pCTM1+2. The recombinant *pma1* gene in pCTM1+2+3+4 therefore included sequences encoding the first two putative *C. albicans* transmembrane loops separated by the *S. cerevisiae PMA1* transduction domain.

The 6 kb *Hin*dIII *pma1-URA3* fragment from each of the three chimeric plasmids was used to transform the diploid yeast strain SH122. *URA* transformants were tested for leucine auxotrophy to confirm that the *pma*Δ*:LEU2* allele of *PMA1* had been replaced by homologous recombination [44]. Sporulation and tetrad dissection of these transformants yielded four viable spores which segregated 2:2 for *ura3 PMA1:pma1-URA3*. For each chimeric mutant (CTM1+2, CTM3+4 and CTM1+2+3+4), the region(s) derived from *C. albicans PMA1* and its overlap with *S. cerevisiae PMA1* were amplified from yeast chromosomal DNA by PCR and the products checked by DNA sequence analysis. The entire *PMA1* coding region of the CTM1+2+3+4 mutant was similarly validated by DNA sequence analysis.

Drug challenge growth assays. Yeast were grown to late-log phase (A_{600} approx. 2 for *S. cerevisiae* and chimeras and A_{600} approx. 7 for *C. albicans*) in CSM-URA and diluted to an A_{600} approx. 0.2 in CSM-URA at the pH used for subsequent growth. Omeprazole, from a fresh stock at 50 mg/ml in 90% DMSO, was diluted to 400 μg/ml in CSM-URA adjusted to pH 3.5 and allowed to activate for 1 h before use. Drug challenge growth assays were conducted in covered sterile float-bottomed 96 well microtitre plates (Greiner Labortechnic, Frickenhausen, F.R.G.) with each well containing in total 200 μl of CSM-URA at the indicated pH, and with test wells including 50 μl of the diluted cell suspension (final A_{600} = 0.05). The cells were agitated on a gyratory shaker at 200 r.p.m. for 20–24 h at 30°C and the A_{620} of the cell suspensions determined using an EAR 340 microtitre reader (SLT Instruments, Austria) All readings were in duplicate, with duplicate absorbances falling within 5% of each other.

Glucose-induced medium acidification. Cells grown into log phase (A_{600} approx. 2–3) in 50 ml of YPD were harvested by centrifugation at 3500 × *g* for 10 min. The culture was washed twice with an equal volume of distilled water and carbon starved by 24 h incubation at 4°C in 50 mM KCl. The starved cells were recovered by centrifugation and concentrated to an A_{600} approx. 40 in 50 mM KCl at pH 4.8. The concentrated cells were diluted to an A_{600} = 4.4 in 1.8 ml of reaction medium (in a 3 ml stirred cuvette) containing 50 mM KCl and adjusted with 10 mM HCl to pH 4.8. After pH stabilization, medium acidification was initiated by adding 0.2 ml of 20% glucose. The pH of the stirred medium was monitored using a rapidly responding semi-micro ROSS combination pH electrode (Orion, Boston, MA 021129, U.S.A.) connected *via* a Radiometer pH Meter 26 (Radiometer, Copenhagen, Denmark) and a MacLab (Analog Digital Instruments, Dunedin, New Zealand) to a Macintosh LCII for data acquisition. Data was recorded using Chart software (Analog Digital Instruments, Dunedin, New Zealand).

Biochemical characterization of plasma membrane ATPase. Cells in mid-log phase were harvested by centrifugation, resuspended in 10 ml ice cold homogenization medium (50 mM Tris, pH 7.0, 0.5 mM EDTA, 1 mM PMSF and 2% glucose) and disrupted by vortexing with glass beads. The homogenate was adjusted to pH 7.0 with 1 M Tris and plasma membranes

were purified by sucrose gradient centrifugation as described previously [49]. Microscale plasma membrane ATPase assays were conducted as previously described [49]. Protein was estimated using the Bradford microassay with bovine γ-globulin as standard [52]. Purified plasma membranes dissolved at room temperature in SDS-lysis buffer (2% SDS, 50 mM Tris/HCl, pH 6.7, 10% glycerol, 2.5 mM EDTA, 0.01% PMSF, 1 mg/ml bromophenol blue, 40 mM dithiothreitol) were separated by SDS-PAGE [53] using the Bio-Rad minigel system (Bio-Rad, Richmond, CA, U.S.A.). Prestained molecular weight markers (M_r range, 14000–20000; Gibco-BRL) were used as standards. Gels were either stained with Coomassie blue R250 or electrotransferred to Amersham Hybond C nitrocellulose (Amersham, U.K.) Blots were blocked with 0.2% gelatin and 0.1% Tween-20 in Tris-buffered saline and probed with a 1/400 dilution of affinity purified dilution of rabbit anti-native yeast plasma membrane ATPase or anti-carboxyl terminal antibody [28, 29]. Antigen-antibody complexes were detected using a 1/5000 dilution of goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma) developed with BCIP and NBT [28]. The developed blots and gels were placed on an ochre overhead transparency sheet and scanned in grey scale into Adobe Photoshop using a Microtek ScanMarker 600Z flat-bed scanner. Images were analyzed using NIH Image version 1.5 on a Macintosh IIVX computer

RESULTS

Omeprazole and the plasma membrane proton pump

Table 1 shows that acid-activated omeprazole above 0.1 mM in YPD medium at pH 3.5 inhibited the growth of *S. cerevisiae* and that growth was completely inhibited by 0.4 mM omeprazole. More extensive analysis showed that the growth inhibitory effect depends on the sulphenamide of omeprazole. Thus growth inhibition required pH-dependent activation of omeprazole to the sulphenamide and the inhibitor was unstable at pH > 4. The sulphenamide is a sulphydryl scavenging reagent and the action of acid-activated omeprazole against

Table 1
Effect of omeprazole on the growth and cell viability of S. cerevisiae in YPD medium at pH 3.5

	Omeprazole (mM)				
	0	0.1	0.2	0.4	0.8
Growth (% control)	100	97	27	3	2
Plating efficiency (% control)	100	98	92	26	1

S. cerevisiae cells were grown for 24 h in wells of a microtitre plate containing 200 µl of YPD at pH 3.5 with the indicated concentration of omeprazole. The relative growth was estimated from the A_{620} (taking into account a small contribution from the omeprazole). Cell viability was estimated by plating replicate dilutions the cultures onto a solid YPD medium and calculated according to the formula 100 × (percent plating efficiency treated cells)/(percent plating efficiency of control cells not treated with omeprazole).

fungal cells was quantitatively blocked by preincubation with the sulphydryl donor 2-mercaptoethanol. Testing for the viability of cells recovered from growth inhibition assays showed that the omeprazole was fungicidal. Slightly higher concentrations of omeprazole were required to kill cells than were needed to inhibit growth, with at least 70% of the growth capacity blocked before significant cell death occurred (Table 1), consistent with a study which found that viable *S. cerevisiae pma1* mutants retain at least 25% of the wild type ATPase activity [18].

The principal *in vivo* activity of the plasma membrane proton pump was qualitatively measured in starved cells as glucose-dependent proton pumping at pH < 5. Treatment of cells with acid-activated omeprazole cause a dose-dependent inhibition of proton pumping, with the kinetics of the inhibition indicating a requirement for the ATPase to proceed through the reaction cycle. Thus inhibition was independent of preincubation time and only occurred once pumping was initiated. This experiment was scaled up and plasma membranes were purified from control cells and cells treated with various concentrations of acid-activated omeprazole during glucose-dependent proton pumping. A good correlation was observed between the extent to which steady state proton pumping was inhibited and the inhibition of enzyme activity. The kinetic characteristics of the partially inhibited ATPase

Table 2
Properties of C. albicans, S. cerevisiae and pma1 chimeras expressed in S. cerevisiae

Strain	ATCC10261	T48	CTM1+2	CTM3+4	CTM1+2+3+4
a) *In vivo* properties					
Growth rate (h/generation)	1.5	1.7	1.7	1.7	1.7
Growth yield (A_{600})	26	5.9	5.5	5.2	5.5
Omeprazole (I_{50} pH 3.5, μM)	106	86	72	100	100
b) *In vitro* properties					
100 kDa band (% plasma membrane protein)	29	28	27	28	25
V_{max} (μmole/min per mg)	1.5	3.1	2.3	1.6	1.5
k_{cat} (s^{-1})	8.6	19	14	9	10
K_m Mg-ATP (mM)	3.9	2.2	2.2	1.9	1.7
K_i Vanadate (μM)	1.5	6	4	9	2
K_i Diethylstilbestrol (μM)	30	25	15	50	30

recovered in this experiment showed that only the V_{max} but not the K_m for Mg^{2+}-ATP was affected by *in vivo* treatment with acid-activated omeprazole. Two sites in the ATPase carboxyl terminal domain are thought to be phosphorylated in the presence of glucose, giving an activated enzyme with a lowered K_m for Mg^{2+}-ATP and a high V_{max}. Thus, omeprazole inhibition did not alter the glucose-induced increase in the affinity of the enzyme for its substrate. However, this result did not exclude either omeprazole-mediated activation of the protein phosphatase or deactivation of the calmodulin-dependent protein kinase which modify the site determining the V_{max}. The properties of an *S. cerevisiae pma1Δ901-918* mutant, which eliminates the consensus R909-T912 calmodulin-dependent protein kinase phosphorylation site, were therefore investigated. The mutant supported normal growth in YPD medium and contained an ATPase that had a high V_{max} and low K_m for Mg^{2+}-ATP (Table 3). Mutant and wild type cells were comparably sensitive to acid-activated omeprazole at pH 3.5, indicating that the kinase and phosphatase pair affecting the V_{max} *via* the R909-T912 site had no impact on omeprazole inhibition of the pump. These results show that *in vivo* omeprazole inhibition of the ATPase is independent of ATPase activation by glucose metabolism, and imply that either the ATPase itself or some other essential associated element is the target of omeprazole action.

How might omeprazole inhibit the plasma membrane proton pump? Acid-activated omeprazole completely inhibited the activity of the ATPase in isolated plasma membranes at 10 μM and this effect was fully protected with Mg^{2+}-ATP. The site affected therefore seemed to be the previously characterized *N*-ethylmaleimide (NEM) binding site (C533 in *S. cerevisiae*) within the cytoplasmic ATP-binding domain [54]. On the other hand, inhibition of cell growth and proton pumping required much higher concentration of omeprazole. Since acid-activated omeprazole is positively charged and is therefore considered unlikely to cross the plasma membrane, sulphydryl groups located at the cell surface (C312) and in the membrane sector (C148 and C867) of the plasma membrane proton pump were assessed as potential targets for omeprazole by using a panel of site-directed mutants in which these three sulphydryl groups were individually, pairwise or completely replaced with either serine (C148S, C312S) or alanine (C867A). All of the mutants grew normally, indicating that none of the modified sulphydryl groups were essential, either individually or in combination. Experiments in CSM-URA at pH 3.5 found that all the mutants were at least as sensitive to acid-activated omeprazole as the control wild type strain. These observations showed that

covalent interaction between acid-activated omeprazole and C148 or C312 or C867 was not required for inhibition of the ATPase. Other *pma1* mutants modified in transmembrane loops 1+2 give omeprazole resistance (G156C G158D) or increased omeprazole sensitivity (M128C), indicating a role for this region in omeprazole action. An omeprazole resistant mutant, previously characterized as C148S, contains a second mutation V209I and is being reinvestigated.

Plasma membranes isolated after *in vivo* inhibition of proton pumping with omeprazole contain small amounts of fluorescent omeprazole bound to the approx. 100 kDa ATPase band. Omeprazole binding also caused an aggregation of the ATPase that was partially reversed by treatment with dithiothreitol (Fig. 1). This study suggested that omeprazole can bind directly to the ATPase *in vivo* and that the molecular properties of the enzyme were altered when the enzyme was inhibited. Omeprazole-dependent aggregation of the *S. cerevisiae* ATPase is consistent with reports of omeprazole

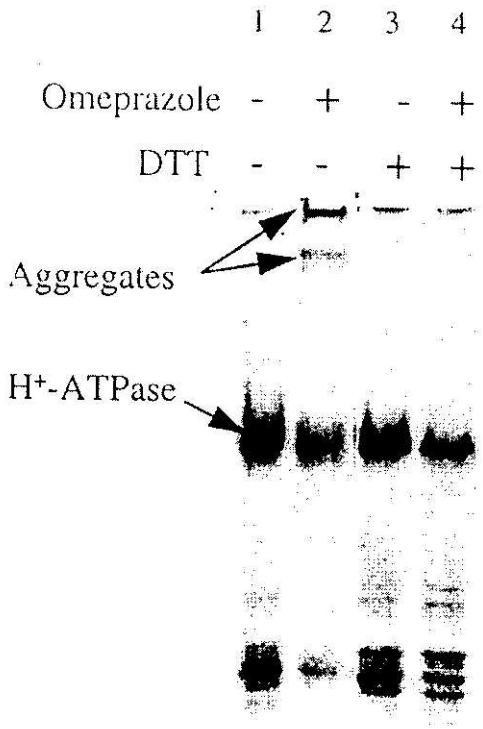

Fig. 1. Effects of in vivo labeling of plasma membranes with acid-activated omeprazole.
Plasma membrane samples (1 μg) from the wild type strain SN236 that was either untreated (lanes 1 and 3) or treated *in vivo* with 360 μM acid-activated omeprazole at pH 3.5, were separated by SDS-PAGE and Coomassie stained. Dithiothreitol (40 mM) was included in the SDS sample preparation buffer where indicated.

and pantoprazole-mediated aggregation of the gastric H^+,K^+-ATPase [40, 41]. The molecular basis of the interaction between omeprazole and the yeast ATPase remains unresolved. Sufficient amounts of omeprazole could cross the plasma membrane and inactivate the ATPase by interacting with one of the six cytoplasmic sulphydryl groups in the ATPase, before itself being inactivated by intracellular glutathione and the high intracellular pH. Alternatively, omeprazole might interact with the sulphydryl-bearing proteolipid molecules that associate with the proton pump [23]. Covalent modification of the proteolipid proteins might facilitate aggregation and inactivation of the ATPase. These possibilities are being investigated.

Presentation of foreign transmembrane loops 1+2 and 3+4 in *S. cerevisiae*

In 1991, the cloning and characterization of the *C. albicans PMA1* gene was reported [49]. Since then only two other potential *PMA1* genes from pathogenic fungi (*Histoplasma capsulatum* and *P. carinii*) have been described [55, 56]. Unfortunately many pathogenic fungi are either difficult to culture or would present a biohazard in laboratories wishing to screen for antifungal agents. The *S. cerevisiae* system offers a suitable test bed for screening broad spectrum antifungal agents directed against the ATPase. One approach would be to express foreign *PMA1* genes in *S. cerevisiae*. This approach has proved problematic, with a plasmid-based system giving low level expression of *Neurospora crassa PMA1* [57]. Using a similar system, *C. albicans PMA1* expression gave growth rates too low to support screening procedures. As an alternative strategy chimeric ATPases were construc- ted by transplacing the sequences specifying transmembrane loops from *C. albicans PMA1* into *S. cerevisiae PMA1*. Single stranded copies of the sequences encoding transmembrane loops 1+2 and/or 3+4 from *C. albicans PMA1* were produced by PCR and a modification of the Kunkel mutagenesis procedure allowed the transplacement of these sequences into the *S. cerevisiae PMA1* gene. Homologous recombination followed by meiosis of selected transformants gave *S. cerevisiae* cells which expressed chimeric plasma membrane ATPase, without the complication of the background of endogenous *S. cerevisiae*

wild type ATPase. The chimeric mutants showed essentially normal growth rates, growth yields and comparable responses to acid-activated omeprazole (Table 2). The plasma membrane ATPase was recovered in the membranes at wild type or near wild type levels (24–29% of plasma membrane protein) and the enzymes had essentially normal kinetic characteristics. However, Fig. 2 showed that the growth of chimeras which separately include *C. albicans* transmembrane loops 1+2 or 3+4 was markedly less thermotolerant than the control *S. cerevisiae* strain. The chimera containing *C. albicans* transmembrane loops 1+2 or 3+4 was at least as thermotolerant as *S. cerevisiae* but was not as thermotolerant as *C. albicans*. These data suggest that transmembrane loops 1+2 and 3+4 operate together as a functional domain, while the presence of individual *C. albicans* transmembrane loops modestly destabilize the structure of the *S. cerevisiae* ATPase.

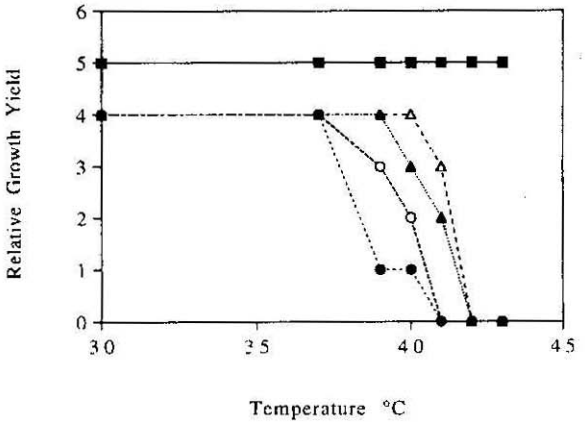

Fig. 2. Thermotolerance of C. albicans, S. cerevisiae and chimeric mutants.
Triplicate 2 µl samples of cells (A_{600} = 0.20) spotted on solid YPD medium were visually scored for growth after 24 h at the indicated temperature, in two separate experiments at each temperature. ATCC10261 (■), T48 (▲), CTM1+2 (●), CTM3+4 (○) and CTM1+2+3+4 (Δ).

Comparison of seven fungal *PMA1* sequences specifying transmembrane loops 1+2 and 3+4 showed that most residues are absolutely conserved (Fig. 3). Conservative and non-conservative substitutions were found in both loops, with several substitutions predictably occurring in the connecting turns. If the transmembrane sequences are indeed helices, as is predicted for transmembrane segments 1–3, than most of the resident conservative and non-conservative substitutions occurred in single strips along each transmembrane segment. The remaining changes found at the extracytoplasmic end of transmembrane segment 3 seemed to involve intrasegment complementarity

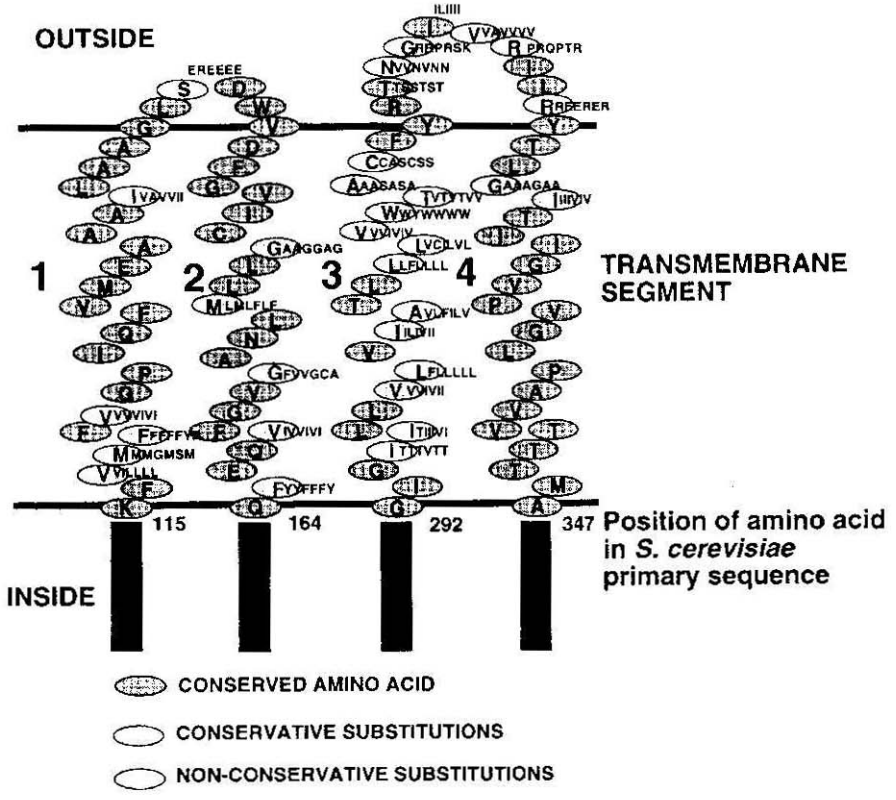

Fig. 3. Comparison of transmembrane loops 1+2 and 3+4 of seven plasma membrane proton pumps.
The protein sequences were deduced from the sequences of *PMA1* genes from *S. cerevisiae*, *C. albicans*, *Schizosaccharomyces cerevisiae*, *Neurospora crassa*, *Zygosaccharomyces rouxii*, *Histoplasma capsulatum* and *Kluyveromyces lactis*, and where substitutions occur, they are given in that order.

amongst resident polar amino acids (C, Y, W and T) [47]. It is generally thought that highly conserved complementary helix side chains are likely to be in close contact with other protein structural elements rather than with the lipid bilayer, while non-conserved regions are likely to be in contact with the lipid bilayer. However, some of the variation that occurs in transmembrane segments 2 and 3 seems to involve compensatory changes (M152(LMLFLF)--A303-(VLFILV),F163(YYFFFY)--I296(TIIIVI),G-156-(FVVGCA)--L300(FLLLLL)) in either the bulk, hydrogen bond-forming potential or the aromatic nature of side chains. The compensatory residues appear disposed so that the non-conserved strip on transmembrane helix 2 interacts with one edge of the non-conserved strip on transmembrane helix 3. This juxtaposition of the transmembrane helices is consistent with the relative thermotolerance of the chimeric mutants and the finding that the S139E mutation in the turn between transmembrane segments 1+2 does not diminish the thermotolerance of *S. cerevisiae*. Experiments are in progress to measure the relative thermotolerance of the ATPase recovered in plasma membranes from the wild type strains and chimeric mutants.

Mutations affecting ATPase regulation

The plasma membrane proton pump of fungi is a complex molecule. It contains sequences near the amino terminus that help target the ATPase to the plasma membrane. As a major consumer of ATP, the enzyme is tightly regulated by its carboxyl terminal domain so the metabolic flux determines the kinetic characteristics and conformation of the enzyme. The functions of the amino and carboxyl terminal regions of the ATPase were previously investigated using a plasmid-based differential expression system [58, 59]. These experiments showed that a region of 27 poorly conserved residues between the amino terminus and the membrane targeting element in the *S. cerevisiae* ATPase was not required for ATPase function. Similarly, the carboxyl terminal domain of the ATPase was not needed for normal growth. We extended these observations by constructing a panel of mutants (*pma1Δ2-28*, *pma1Δ901-918*, and *pma1Δ2-28Δ901-918*) in which the mutant plasma membrane pump was expressed in yeast in the absence of any wild type activity. These mutants grew normally in YPD medium showing the non-essentiality of the deleted amino terminal and carboxyl terminal domains (Table 3). The *pma1D2-28* mutant showed glucose-dependent proton pumping at the wild type rate, while the *pma1Δ901-918* and *pma1Δ2-28Δ901-918* mutants pumped proton at about one half and one quarter of the wild type rate, respectively. This indicated that either the activity or expression of the ATPase might be altered in the affected mutants. Cells grown in the YPD medium at pH 5.7, were starved in 50 mM KCl, pH 4.8, overnight, proton pumping

Table 3

Properties of amino-terminal and carboxyl-terminal deletion mutants of S. cerevisiae

Strain	T48	*pma1Δ2-28*	*pma1Δ901-918*	*pma1Δ2-28Δ901-918*
a) *In vivo* properties				
Growth rate (h/generation)	1.6	1.6	1.7	1.8
Growth yield (A_{600})	4.6	3.6	3.6	3.6
Omeprazole sensitivity (I_{50} pH 3.5, μM)	60	45	45	45
Acetate sensitivity (I_{50} pH 5.0, mM)	> 200	140	90	50
b) *In vitro* properties				
Approx. 100 kDa band (% plasma membrane protein)	19	15	12	6
ATPase antigen (% relative to T48)	100	75	69	20
ATPase activity (μmoles/min per mg)	1.3	1.3	1.2	0.45
k_{cat} (s^{-1})	11	15	15	20
K_m Mg-ATP (mM)	1.26	1.50	0.22	0.17

initiated with glucose so that the ATPases were fully activated and plasma membranes isolated. Coomassie stained SDS-PAGE showed, as expected, that the ATPase of the *pma1Δ2-28* mutant migrated significantly faster than the wild type control. The *pma1Δ901-918* mutant ATPase migrated slightly faster than the wild type ATPase while the *pma1Δ2-28Δ901-918* mutant ATPase migrated slightly faster than the ATPase of the *pma1Δ2-28* mutant. An affinity-purified antibody which recognizes the 11 carboxyl terminal amino acids of the *S. cerevisiae* ATPase [28] bound to the ATPase of the wild type and the *pma1Δ2-28* mutant, but did not recognize the ATPase of either the *pma1Δ901-918* or *pma1Δ2-28Δ901-918* mutants. Quantitative analysis of the Coomassie stained SDS-PAGE profile showed that the approx. 100 kDa ATPase band in membranes from wild type cells accounted for 19% of plasma membrane protein. The membranes from the *pma1Δ2-28*, *pma1Δ901-918* and *pma1Δ2-28Δ901-918* mutants contain the comparable protein band at about 3/4, 2/3 and 1/3 of wild type levels, respectively. The ATPase of the *pma1Δ2-28Δ901-918* mutant, which ran at about 96 kDa, appeared to co-migrate with another plasma membrane protein seen in the other membrane preparations. An affinity purified anti-native ATPase antibody was therefore used to quantitate the ATPase. This analysis (Table 3) showed that the *pma1Δ2-28*, *pma1Δ901-918* and *pma1Δ2-28Δ901-918* mutants contained about 3/4, 2/3 and 1/5 the ATPase found in the wild type membranes, respectively. The ATPase from each membrane preparation was completely sensitive to the ATPase inhibitor vanadate, and showed enzyme turnover rates in the range of 11–20 molecules of ATP hydrolyzed per second. The plasma membrane proton pump in the *pma1Δ2-28Δ901-918* mutant grown in YPD at pH 5.7 appeared fully functional apart from its deleted regulatory features, even though the amount of potential enzyme activity was only about 1/3 of that found in membranes from the wild type control. These data imply that the plasma membrane ATPase of wild type cells was only partially active under normal growth conditions. ATPase activity is self-regulated by the generation of membrane potential and by negative regulatory elements in the carboxyl terminal domain [58–61]. Another possible regulatory mechanism is the maintenance of a dynamic equilibrium between active ATPase monomer and multimeric storage forms of the enzyme. The latency of proton pumping capacity is indicated by the much poorer tolerance of the deletion mutants, particularly the *pma1Δ2-28Δ901-918* mutant, to the presence of acetate ions in the growth medium at pH 5 (Table 3). This technique provides a measure of the proton pumping capacity of the ATPase by delivering a flux of protons into the cytoplasm.

Panels of *S. cerevisiae pma1* mutants

A particularly useful feature of *S. cerevisiae*, compared with less tractable fungi like *C. albicans*, is that its genetic system is well defined and it is relatively easy to construct mutants in suitable controlled genetic backgrounds. For example, the ability to selectively remove cell surface sulphydryl groups from the ATPase, either singly or in combination, without significantly affecting the growth of cells means that it will not only be possible to detect cell surface sites specifically affected by sulphydryl reagents but also introduce a new sulphydryl groups that can be used to measure the accessibility of individual amino acids. In addition, once a particularly sensitive feature is identified, the modification of neighboring residues can usefully define the physicochemical nature of its environment and thereby simplify the task of the medicinal chemist.

DISCUSSION

Identification of the plasma membrane proton pump as an antifungal target [10] is a significant step towards obtaining new mechanistic classes of antifungals. The use of P-type ATPases as differential therapeutic targets is well established and there are clear advantages in focusing on a well characterized and abundant plasma membrane protein that is needed for cell growth and that presents a conformationally sensitive membrane sector and ectodomain. We have shown that genetic and biochemical modification of the ATPase can block cell growth or even be fungicidal, but the site(s) affected by acid-activated omeprazole, which does not appear to interact covalently with any of the predicted cell surface sulphydryls, remains to be defined. Further progress in developing the ATPase as an antifungal target is

hindered by the uncertainties inherent in our view of the structure of the membrane sector and ectodomain. Our "best guess" of the structure of this region is presented in Fig. 4. This hypothetical view was constructed by inserting our model of transmembrane loop 1+2 and our concept of its interaction with transmembrane loop 3+4 into a model of the sarcoplasmic reticulum Ca^{2+}-ATPase. The Ca^{2+}-ATPase model was derived from a two dimensional crystallographic analysis which provided information at about 14 Å resolution and which was further refined [62, 63]. Figure 4 shows the fungal ATPase membrane sector and ectodomain seen from outside the cell. It locates the ten transmembrane segments and their extracytoplasmic connecting turns and interfaces the non-conserved strips of transmembrane segments 2 and 3 with each other and with the lipid bilayer. Transmembrane segment 4 is shown as a central region of β-sheet bounded by two helical elements, although it could also be a kinked membrane-spanning helix. The spacing and proximity of the conserved polar glycine and proline residues as well as their separation by larger hydrophobic residues could support the presence of the β-sheet in transmembrane segment 4. Together with transmembrane segment 4, transmembrane helices 5, 6 and 8, which each contains a conserved acidic residue, could form a proton pore. Of these acidic residues only D730 in the *S. cerevisiae* ATPase is known to be essential [18]. Assuming that the conserved acidic side chains project into the proton pore, other residues of interest in transmembrane helices 5, 6 and 8 can be spatially located. The β-sheet in transmembrane segment 4, which includes the essential P345 residue [64], would contribute a slightly polar cytoplasmically-oriented vestibule on one face, with the other face interacting favorably with hydrophobic elements of the sloping transmembrane segment 7. Integrated into this hypothetical structure are the possible locations of several residues at or near the cell surface that may be essential or could affect the properties of the fungal ATPase. The model can be tested, and if found to be valid, should be a useful tool in rationally designing site-directed mutagenesis experiments to identify key residues that affect the properties of the proton pump.

Two other features of our experimental strategy are worthy of mention. The *pmaΔ-28Δ901-918* mutant produces fully active proton pumping ATPase under normal growth conditions, unlike the wild type enzyme which is only partially active. We recently found conditions which allow the insertion of near normal amounts of this mutant ATPase into the plasma membrane. The ability to prepare a single conformer of the mutant fungal ATPase in a form suitable for crystallization is now a major experimental priority. This work will hopefully lead to an informative high resolution structure of the ATPase. The development of the proton pump as an antifungal target has primarily

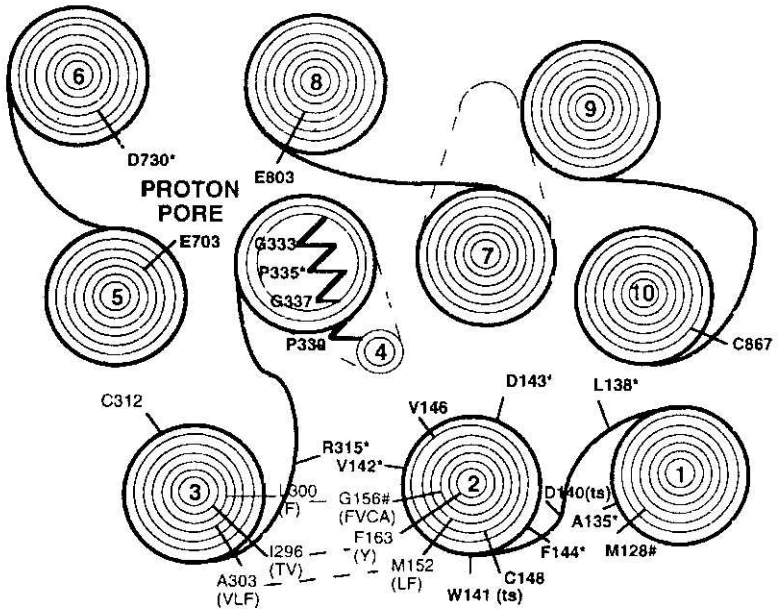

Fig. 4. A hypothetical model of the membrane sector and ectodomain of the S. cerevisiae plasma membrane proton pump viewed from outside the cell.

Transmembrane helices are illustrated by a series of concentric circles, with smaller circles representing helical turns further away from the viewer. Transmembrane segment 4 contains a central portion of β-sheet structure which tilts the orientation of the segment while the tilted orientation of transmembrane helix 7 is indicated by dashed lines. Amino-acid residues indicated in **bold** are conserved, alteration of residues marked with an * can be lethal, modification of those marked with an # affect the sensitivity of cells to acid-activated omeprazole, while changes to residues marked with ts can give a temperature sensitive phenotype.

focused on the *S. cerevisiae* system because of the utility of this organism for mutagenesis and screening. For example, the expression in *S. cerevisiae* of a fully functional chimeric *S. cerevisiae* ATPase containing transmembrane loops 1+2 and 3+4 specified by *C. albicans PMA1* has provided an initial test of the universality of this part of the cell surface target site which can now be extended to other fungal *PMA1* genes. Once the essential conserved elements in the fungal target site are identified, future screening protocols may use suitable *S. cerevisiae* strains and chimeric mutants rather than fungal pathogens such as *P. carinii*. In addition, fully functional mutants which express abnormal levels of ATPase protein have been difficult to obtain. Multicopy plasmids expressing the *S. cerevisiae PMA1* gene are selected against [65], while mutants with less than about 25% of the wild type levels of the ATPase fail to support growth [18]. The *pma1Δ-28Δ901-918* mutant expresses only 20% of wild type levels of ATPase under normal growth conditions and therefore provides a way to differentially screen for high affinity inhibitors of the fungal ATPase by using simple drug challenge assays. When combined with other assays of ATPase function, such mutants will allow rapid screening of libraries of natural and synthetic compounds for antifungals directed against the plasma membrane proton pump. Finally, as exemplified by the set of mutants in which endogenous surface cysteines have been systematically removed, the tactical replacement and insertion of chemically reactive residues can be expected to simplify the task of the medicinal chemist in developing lead compounds. Clearly, the potential of *S. cerevisiae* in the detection and design of broad spectrum of antifungal agents directed against the fungal proton pump has barely been exploited.

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