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Functional complementation between transmembrane loops of *Saccharomyces cerevisiae* and *Candida albicans* plasma membrane H^+-ATPases

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Abstract

Saccharomyces cerevisiae PMA1 sequences encoding a putative antifungal target site comprising transmembrane loops 1 + 2 and/or 3 + 4 were replaced with the homologous sequences from *Candida albicans PMA1* by using PCR-mediated domain transfer. The chimeric *pma1* mutants and an isogenic wild type *S. cerevisiae* strain had similar growth rates, growth yields, glucose-dependent proton pumping rates, acid-activated omeprazole sensitivities, salt tolerances and antifungal sensitivities. The yields and kinetic properties of H^+-ATPases in plasma membranes of mutant and wild type strains were comparable. Single heterologous transmembrane loops caused deleterious phenotypes at low pH and elevated temperature. Inclusion of both heterologous transmembrane loops fully suppressed the temperature sensitivity caused by heterologous transmembrane loop 1 + 2, partially suppressed the pH sensitivity and gave *Candida*-like in vitro sensitivity to vanadate, suggesting that the loops operate as a domain. The fully functional chimeric H^+-ATPase containing *C. albicans* transmembrane loops 1 + 2 and 3 + 4 demonstrates this domain's complementarity to the equivalent region of the *S. cerevisiae* enzyme and validates the wild type *S. cerevisiae* H^+-ATPase as an antifungal screening target.

Keywords: ATPase, H^+-; Plasma membrane; Chimeric enzyme; Yeast

1. Introduction

The *S. cerevisiae PMA1* gene encodes a proton pumping ATPase that regulates growth and is essential for cell viability [1–3]. Located in the yeast plasma membrane, the enzyme maintains cellular ionic balance, intracellular pH and generates the membrane potential that powers the uptake of numerous nutrients [1,4]. The similarity between *S. cerevisiae PMA1* and other fungal *PMA1* homologues, about 70% at the deduced amino acid sequence level [5–7], suggests that structure-function relationships within the enzyme have been highly conserved despite the evolutionary divergence of fungal genera.

While the fungal plasma membrane proton pumps contain catalytic, structural and topological features that are common to all members of the extended family of P-type ATPases [8], they also possess distinctive cell surface structural elements at turns between putative transmembrane helices [6]. The cardiac glycosides [9] and omeprazole [10] are examples of P-type ATPase inhibitors that are already in widespread clinical usage for the treatment of heart attack and gastric ulcers, respectively. Both classes of drugs interact with surface-exposed, transmembrane loops in their target enzymes, the Na,K- and gastric H,K-ATPases. Thus, these highly specific and efficacious therapeutics constitute encouraging precedents for the development of novel antifungal compounds directed against the equivalent but structurally distinct surface regions of the fungal proton pumping ATPase [6].

Several lines of evidence, including extensive mutational analysis and molecular modeling studies [6,11–14], suggest that the transmembrane loop TM1 + 2, comprising transmembrane segment 1, a short intervening turn and transmembrane segment 2, is a conformationally sensitive

Abbreviations: PCR, polymerase chain reaction; TM1 + 2, transmembrane loop comprising transmembrane segments 1 and 2 plus the intervening turn of the H^+-ATPase; TM3 + 4, transmembrane loop comprising transmembrane segments 3 and 4 plus the intervening turn of the H^+-ATPase; PMSF, phenylmethylsulfonyl fluoride

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region of the *S. cerevisiae* plasma membrane proton pump which communicates with the cytoplasmic active site of the molecule. Genetic evidence provided by suppressor mutants [13] further indicates that this region is closely associated with the transmembrane loop TM3 + 4 which is bounded by transmembrane segments 3 and 4. In the *S. cerevisiae* ATPase, the structural complex formed by TM1 + 2 and TM3 + 4 may therefore have the attributes of a cell surface antifungal target site. Given the high level of inter-generic fungal H^+-ATPase sequence conservation, might this region constitute a general antifungal target that could be exploited like the ouabain binding site in the Na^+,K^+-ATPase? This possibility was tested by considering the corresponding region of the proton pumping ATPase from the opportunistic pathogenic fungus *C. albicans*.

Because no other fungus has been so thoroughly characterized at the genetic, physiological and biochemical levels, *S. cerevisiae* is the most obvious test organism for antifungal development and drug screening procedures. This approach often requires the expression of an heterologous gene in *S. cerevisiae*, with the implicit assumption that information obtained will be directly applicable to the target as it is expressed in other fungi. Although low level, functional expression of the *Neurospora crassa* ATPase in *S. cerevisiae* has been reported using a plasmid-based expression system [15], when the same plasmid and host strains were used to express the entire coding region of the *C. albicans PMA1* gene, the foreign ATPase was unable to support cell growth rates that could be utilized in subsequent testing (Mason, unpublished observations).

As an alternative strategy, PCR-driven domain transfer was used to engineer chimeric *PMA1* genes that contained DNA sequences encoding putative transmembrane loops (TM1 + 2 and/or TM3 + 4) from *C. albicans PMA1*, precisely transplaced into the *S. cerevisiae PMA1* gene. These chimeric enzymes allowed us to directly assess whether or not homologous regions of separate fungal ATPases were functionally complementary and thereby validate the use of *S. cerevisiae* as an appropriate organism to screen for broad-spectrum antifungal compounds that might target this part of the molecule. In vivo and in vitro characterizations of the chimeric *pma1* mutants show that the inclusion of transmembrane loops TM1 + 2 and/or TM3 + 4 from *C. albicans* produces enzymes which are only subtly perturbed compared with the wild type *S. cerevisiae* enzyme and provide evidence that TM1 + 2 and TM3 + 4 act as a domain.

2. Material and methods

2.1. Yeast strains and cell culture

The *S. cerevisiae* strains used in this study were isogenic derivatives of the wild type strain Y55 (*HO gal3 MAL1 SUC1*) [16]. Yeast transformations were performed using *S. cerevisiae* strain SH122 (*HO / HO MATa / MATα ade6-1 / ade6-1 trp5-1 / trp5-1 leu2-1 / leu2-1 lys1-1 / lys1-1 ura3-1 / ura3-1 pma1Δ:LEU2 / PMA1*). The control strain T48 (*HO ade6-1 trp-5-1 leu2-1 lys1-1 ura3-1 PMA1-URA3*) was selected as a *leu URA* spore from SH122 in which the *LEU2*-disrupted *pma1* gene was replaced with *PMA1-URA3* [11]. 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; Bio101, Vista, CA, USA) 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 this medium adjusted to the indicated pH with concentrated HCl or NaOH. In other experiments CSM-URA medium was supplemented with 10 mM MES and adjusted to the indicated pH with Tris or HCl.

2.2. Engineering of chimeric constructs

Recombinant DNA was cloned and propagated using *Escherichia coli* strain XL1-Blue (Stratagene, La Jolla, CA, USA). Fig. 1 gives an overview of the method used to generate the chimeric *PMA1-URA3* constructs. The high fidelity polymerase Pfu (Stratagene) was used in PCR to amplify *C. albicans PMA1* DNA sequences encoding putative TM1 + 2 or TM 3 + 4 from the plasmid pJAM25 [17]. 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, USA). The single stranded DNA products, which included at least 16 nucleotides at their 5′ and 3′ termini that were homologous to *S. cerevisiae PMA1*, were used individually as mutagenic primers in a Kunkel mutagenesis procedure [18,19] on a single stranded, uracil-containing template derived from plasmid pGW101, previously described by Na et al [13]. This plasmid contains *S. cerevisiae PMA1-URA3* in a pGEM-13Zf(+) vector (Promega, Madison, WI, USA). Mutant plasmids pCTM1 + 2 and pCTM3 + 4, initially detected by screening for the loss of an EcoRI or a XcmI restriction site, respectively, were obtained at modest frequency (~ 1 in 50 transformants). *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 containing transmembrane segments 1–4 from *C. albicans* (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 (Fig. 1) 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 *pma1Δ:LEU2* allele of *PMA1* had been replaced by homologous recombination (described by Harris et al. [11]. Sporulation and tetrad dissection of these transformants yielded four viable spores which segregated 2:2 for *PMA1-ura3: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. The amino acid sequences for transmembrane loops TM1 + 2 and TM3 + 4 of plasma membrane ATPases from *S. cerevisiae*, *C. albicans* and the chimeras are illustrated in Fig. 2.

2.3. Drug challenge growth assays

Yeast were grown to late-log phase (A_{600nm} ~ 3 for *S. cerevisiae* and chimeras and A_{600nm} ~ 7 for *C. albicans*) in CSM-URA and diluted to an A_{600nm} ~ 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. Hygromycin B (400 μg/ml), amphotericin B (4 μg/ml), fluconazole (40 μg/ml) or acetic acid were added to CSM-URA and adjusted to the indicated pH. Drug challenge growth assays were conducted in covered, sterile, flat-bottomed 96 well microtiter plates (Greiner Labortechnic, Frickenhausen, Germany). Each well contained a total of 200 μl of CSM-URA at the indicated pH and included 50 μl of the diluted cell suspension (final A_{600nm} = 0.05). Plates were agitated on a gyratory shaker at 200 rpm for 40–44 h at 30°C and the A_{590nm} of the resultant cell suspensions determined using an EL 340 Bio Kinetics Microplate Reader (Bio-Tek Instruments, Winooski, VT, USA). All readings were in duplicate, with duplicate absorbances falling within 5% of each other.

2.4. Glucose-induced medium acidification

Cells grown into log phase (A_{600nm} ~ 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 48 h incubation at 4°C in distilled water. The starved cells were recovered by centrifugation and concentrated to an A_{600nm} ~ 40. The concentrated cells were diluted to an A_{600nm} = 4.4 in 1.8 ml of reaction medium (in a 3 ml stirred cuvette) containing 111 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

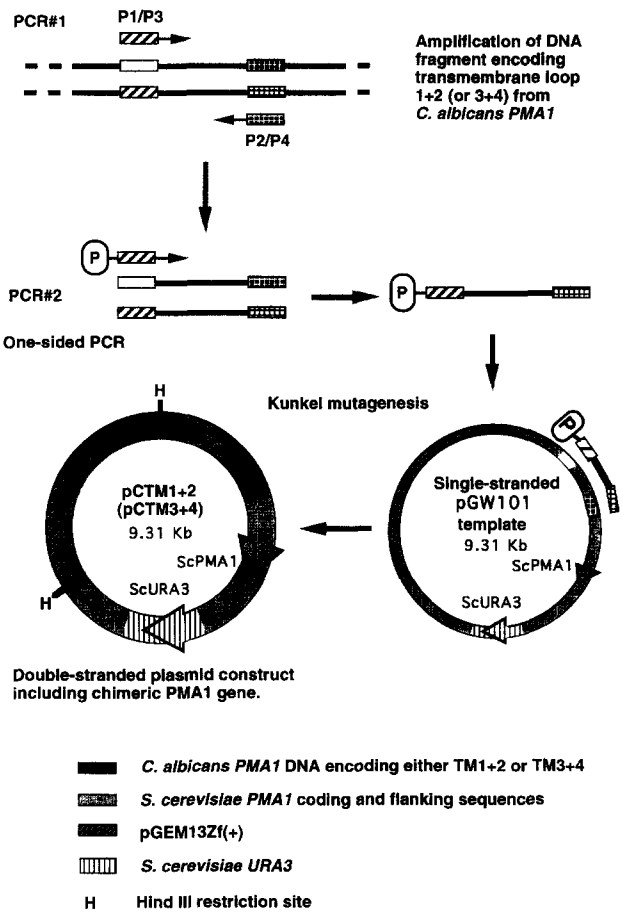

Fig. 1. PCR-mediated transfer of sequences encoding transmembrane loop domains from *C. albicans PMA1* into *S. cerevisiae PMA1*. PCR-mediated domain transfer was carried out as described in Materials and methods. In Reaction 1 (PCR#1) using the oligonucleotide primer pair P1/P2 on the pJAM25 plasmid template gave a 133 base pair amplimer while the P3/P4 oligonucleotide primer pair produced a 163 base pair amplimer. The oligonucleotide primer sequences were based on the *C. albicans PMA1* sequences: P1, 5′-CGTTATGGAAGCCGCTGCT-3′; P2, 5′-ATCGACAATAGAACCAGCTTG-3′; P3, 5′-TTGAACGGTATTG-GTAcTacC-3′; P4, 5′-TGGTAACGACAGCTGGCA-3′ (Nucleotides given in lower case represent differences between *C. albicans PMA1* and *S. cerevisiae PMA1* sequences). The double stranded PCR products were purified using a Magic Prep PCR spin column (Promega, Madison, WI, USA) and quantitated by agarose gel electrophoresis. In Reaction 2, (PCR#2) the phosphorylated oligonucleotide primers P1 and P3 were used with the 133 bp and 163 bp templates, respectively. The resultant single stranded products were recovered by electrophoresis on 1.5% low melting point agarose and ~ 0.25 ng samples annealed with the single stranded uracil-containing pGW101 template which contains the *S. cerevisiae PMA1* negative strand. PCR and one-sided PCR were carried out in an MJ 96V thermal cycler (MJ Research, Watertown, MA, USA). Each 100 μl reaction mix contained Pfu polymerase buffer (Stratagene), 200 μM dideoxynucleoside triphosphates, 5 μM oligonucleotide primers or phosphorylated oligonucleotide primer, 400 ng pJAM25 template or 3.5–7 ng of double stranded PCR product, and 2.5 units Pfu polymerase (Stratagene)) added under hot start conditions. The reaction mix was heated for 30 s at 94°C and 2 min at 92°C followed by 3 cycles of annealing at 40°C, and then by 27 cycles of annealing at 50°C. Extensions were for 1 min at 75°C plus a final extension for 5 min at 75°C.

medium was monitored using a rapidly responding semi-micro ROSS combination pH electrode (Orion, Boston, MA, USA) 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).

2.5. Biochemical characterization of plasma membrane ATPase

Cells grown in YPD at pH 6.5 were harvested at the indicated density, 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. Plasma membranes were purified by sucrose gradient centrifugation and assayed for vanadate-sensitive ATPase activity [17].

Protein was estimated using the Bio-Rad microassay with bovine γ-globulin as standard [20]. 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 μg/ml bromophenol blue, 40 mM dithiothreitol) were separated by SDS-PAGE according to Laemmli [21] using the Bio-Rad minigel system (Bio-Rad, Richmond, CA, USA). Prestained molecular weight markers (M_r range, 14000–200000; GibcoBRL, Gaithersburg, MD, USA) were used as standards. Gels were either stained with Coomassie blue R250 or blotted onto Hybond C nitrocellulose (Amersham, UK). The blots were blocked with 0.2% gelatin and 0.1% Tween 20 in Tris-buffered saline and probed using 1:10000 dilution of rabbit anti-native yeast plasma membrane ATPase antibody [22,23]. Antigen-antibody complexes were detected using a 1:5000 dilution of goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma, St. Louis, MO, USA) developed with BCIP and NBT [22]. ATPase antigen was quantitated by dot blotting onto nitrocellulose; up to 10 ng plasma membrane protein were applied per well. Tween 20/gelatin-blocked dot blots were probed with 1:20000 dilution of rabbit anti-native yeast plasma membrane ATPase antibody and immune complexes detected using a 1:8000 dilution of goat anti-rabbit IgG phosphatase conjugate. The developed blots and gels were placed on an ochre overhead transparency sheet and scanned in grey scale into Adobe Photoshop using a Microtek ScanMaker 600Z flat-bed scanner. Images were analyzed using NIH Image version 1.5 on a Macintosh LCIII computer.

3. Results

3.1. Phenotypic characteristics of chimeras

The growth characteristics of *S. cerevisiae* strain T48, wild type *C. albicans* strain ATCC10261 and strains with chimeric ATPases (Fig. 2) are shown in Table 1. The chimeric strains had growth rates and growth yields that were comparable to the isogenic control strain T48 in YPD medium at pH 5.7. The chimeras had a mean generation time of approximately 1.7 h at 28°C, reaching stationary phase at an A_{600nm} ~ 5.5. The *C. albicans* strain ATCC10261 showed a mean generation time of 1.5 h but reached stationary phase at an A_{600nm} ~ 25 as expected for this obligate aerobe. In the same medium at pH 3, ATCC10261 and T48 had almost identical generation times of 1.6 h and 1.7 h respectively while the chimeras CTM1 + 2, CTM3 + 4 and CTM1 + 2 + 3 + 4 had generation

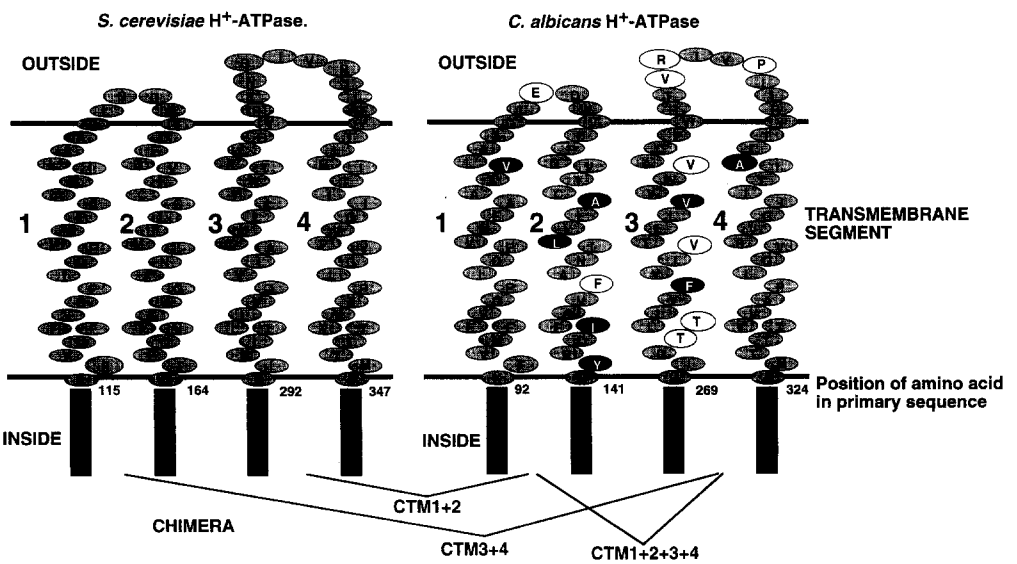

Fig. 2. Transmembrane loops TM1 + 2 and TM3 + 4 in *S. cerevisiae*, *C. albicans* and chimeric mutants. A diagrammatic representation of the amino acid sequences and proposed topology of transmembrane loops TM1 + 2 and TM3 + 4. Non-conserved amino acid residues found in the transmembrane loops from *C. albicans* but not *S. cerevisiae* are shown on a dark (conservative changes) or light (non-conservative changes) background.

Table 1
Growth of *S. cerevisiae, C. albicans* and chimeric strains in liquid culture

Strain	Mean generation time at pH 5.7 (h)	Growth yield at pH 5.7 (A_{600nm})	Mean generation time at pH 3.0 (h)	pH-dependent growth inhibition (pH of I_{50})	I_{50} of growth inhibition (mM)		
					Acetate	$(NH_4)_2SO_4$	NaCl
T48	1.7	5.9	1.7	2.3	65	200	100
CTM1+2	1.7	5.5	2.8	2.6	45	80	90
CTM3+4	1.7	5.2	2.5	2.6	60	165	120
CTM1+2+3+4	1.7	5.5	2.2	2.7	40	200	90
ATCC10261	1.5	26	1.6	2.1	30	> 750	> 750

Generation times and growth yields were determined at 28°C in YPD medium buffered to the indicated pH with HCl. The response of cells to acetate was determined in CSM-URA buffered with MES-HCl to pH 3.5. The effect of $(NH_4)_2SO_4$ and NaCl were determined in CSM-URA buffered with MES-Tris to pH 5.

times of 2.8 h, 2.5 h and 2.2 h, respectively. The *C. albicans* wild type and *S. cerevisiae* control strains were relatively insensitive to acidic growth conditions, primarily due to the proton pump's ability to regulate intracellular pH as large numbers of protons enter the cytoplasm. In liquid culture the growth yield of *C. albicans* ATCC10261 became pH sensitive below pH 2.2, while *S. cerevisiae* T48 showed pH sensitivity below pH 2.4 (Table 1). The chimeric strains' growth yields were only slightly more sensitive to acidic pH than *S. cerevisiae* T48 and became acid sensitive at about pH 2.6.

The sensitivity of *S. cerevisiae* to the protein synthesis inhibitor hygromycin B requires that the cellular membrane potential be sufficient to drive drug uptake [24]. Hygromycin B resistance is strongly linked to *PMA1* although mutations in other genes can produce the same phenotype [16]. Mutations in the *S. cerevisiae PMA1* gene are known to induce prominent hygromycin B resistance phenotypes [11,13,14,16]. These are thought to reflect either a cell with an ATPase of low activity or a restricted ability to generate a membrane potential. During growth in CSM-URA liquid medium *C. albicans* was resistant to hygromycin B concentrations 2–5 fold greater than those tolerated by *S. cerevisiae* T48 (data not shown). Both species also showed enhanced hygromycin B sensitivity as the pH approached 7. Between pH 5 and pH 7 the chimeric ATPase strains were all significantly more sensitive to hygromycin B than *C. albicans* and showed a pattern of sensitivity much like that of T48.

Weak acids, such as acetate, can be used to acidify the yeast cytoplasm, providing a relative measure of the kinetic capacity of the proton pump to transport protons and regulate intracellular pH. At pH 3.5 in CSM-URA medium 65 mM acetate was required for 50% growth inhibition of the control strain T48 (Table 1) and the S139E mutant of *S. cerevisiae* [14]. The CTM3 + 4 mutant similarly required 60 mM acetate for 50% inhibition of growth, while strains CTM1 + 2, CTM1 + 2 + 3 + 4 and *C. albicans* strain ATCC10261 were 50% inhibited by 45, 40 and 30 mM acetate, respectively.

The sensitivities of the chimeras to a range of growth inhibitors was used to assess whether the expression of the chimeric ATPases more widely affected cellular functions. In CSM-URA at pH 5, $(NH_4)_2SO_4$ and NaCl strongly inhibited the growth of *S. cerevisiae* but showed weak or no inhibition of *C. albicans* (Table 1). Each chimera was inhibited at similar or slightly lower ion concentrations than those which inhibited *S. cerevisiae*. Although other mechanisms might cause ammonium toxicity, uncharged ammonia is thought to cross the plasma membrane and raise intracellular pH by combining with intracellular protons. The in vivo response to a weak base like ammonia may therefore suggest that *S. cerevisiae* and the chimeras are less tolerant than *C. albicans* of intracellular alkalinization. Since *C. albicans* is a commensal of mammals its relative tolerance of sodium ions (at least 5 times greater than *S. cerevisiae*) is not surprising, although the mechanism for this is unknown. The tolerance of *S. cerevisiae* to Na^+ is primarily determined by a family of P-type ATPases (specified by the genes *ENA1-ENA4*), and possibly to a lesser extent by an Na^+/H^+ exchange mechanism [25]. The ammonium and sodium ion sensitivities of the chimeras suggest that the chimeric ATPases do not significantly alter the cellular environment of their *S. cerevisiae* host. *S. cerevisiae* and the chimeras (I_{50} 20–50 μM) showed similar growth sensitivity to fluconazole, a drug which affects ergosterol biosynthesis, but were an order of magnitude more resistant than *C. albicans* (I_{50} = 1.3 μM). Amphotericin B inhibited the growth of *C. albicans*, *S. cerevisiae* and the chimeras at about 2 μM. This drug inhibits cell growth by interacting with ergosterol (the principal sterol in most fungal plasma membranes), forming pores that allow leakage of intracellular ions. Our results imply that sterol biosynthesis and the ergosterol content of the plasma membrane are not significantly modified in *S. cerevisiae* strains with a chimeric H^+-ATPase.

The growth inhibitory and fungicidal activity of acid-activated omeprazole on *S. cerevisiae* was recently demonstrated [26]. Biochemical evidence strongly correlated inhibition of cell growth with inhibition of proton pumping and plasma membrane ATPase activity. The predicted amino acid sequence of the first two transmembrane loops of the *C. albicans* ATPase includes cysteines at positions equivalent to C148 and C312 of the *S. cerevisiae* ATPase. Consequently, all of the chimeric ATPases should have retained the full complement of cysteine residues. Unless the inclusion of foreign ATPase domains had caused anomalous folding of the mature enzyme, strains expressing chimeric ATPases were expected to remain fully sensitive to acid-activated omeprazole. The omeprazole sensitivities of *S. cerevisiae*, *C. albicans* and the chimeras at pH 3.5 ranged from 72–106 μM (data not shown) and were therefore probably not significantly different.

Glucose-induced proton pumping (medium acidification) by starved yeast cells at pH < 5 provides a qualitative measure of the activity of the plasma membrane proton pump [24]. Glucose-dependent proton pumping by the *C. albicans* and the *S. cerevisiae* strains was initiated by adding 2% glucose to glucose starved cells in unbuffered 100 mM KCl medium at pH 4.8 (to minimize the effects of membrane potential and organic acid transport). *S. cerevisiae* T48 and the chimeric strains had comparable rates of proton pumping (data not shown). These rates were about twice that of *C. albicans* under the same conditions, and yet *C. albicans* was still able to acidify the medium to below the final pH reached by the *S. cerevisiae* strains. The latter result may indicate that the *C. albicans* plasma membrane proton pump is less susceptible than the *S. cerevisiae* enzyme to negative regulation by the transmembrane electrochemical gradient [26].

In the chimeric strains TM1 + 2 and/or 3 + 4 from the *C. albicans* enzyme should interact with other transmem-

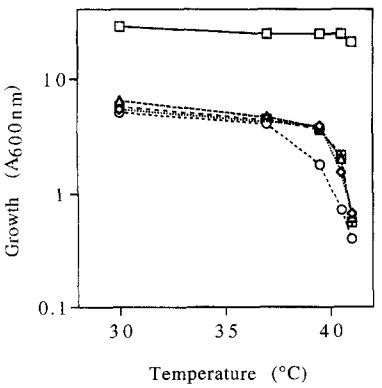

Fig. 3. Temperature-dependence of growth of *S. cerevisiae*, *C. albicans* and chimeric strains. The thermotolerance of the cells was measured turbidometrically after growth in 50 ml YPD cultures at the indicated temperature. ATCC10261 (□), T48 (◇), CTM1 + 2 (○), CTM3 + 4 (△), CTM1 + 2 + 3 + 4 (crossed square). The values obtained at each temperature are averages from at least three separate experiments.

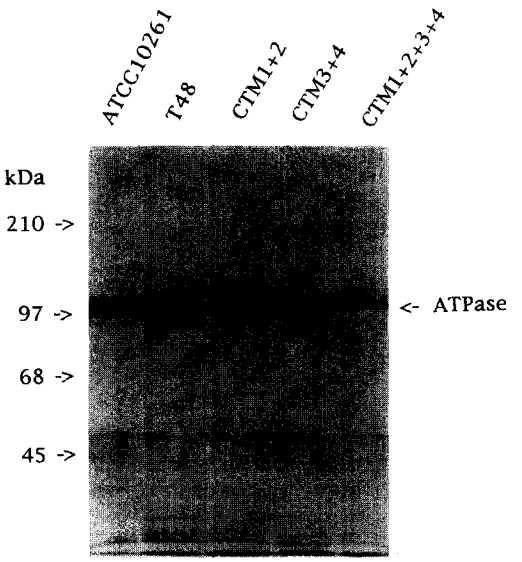

Fig. 4. Polypeptide composition of purified plasma membranes from *S. cerevisiae*, *C. albicans* and chimeric strains. Gradient purified plasma membranes (10 μg protein) from the indicated strains were separated by SDS PAGE in 8% acrylamide gels and stained with Coomassie blue R250 as described in Section 2.

brane loops of the *S. cerevisiae* ATPase and possibly with the lipid bilayer. Incompatible interactions might be expected to produce a less stable or possibly less thermotolerant enzyme which, in turn, should affect the ability of cells to grow at elevated temperatures. Fig. 3 reports the growth thermotolerance of *C. albicans*, *S. cerevisiae* and the chimeric strains in liquid YPD medium at pH 5.7. *C. albicans* continued to grow at 41°C and was more thermotolerant than *S. cerevisiae*, which failed to grow at 41°C. Chimera CTM1 + 2, which contains a single *C. albicans* transmembrane loop, gave growth yields less than half of those of *S. cerevisiae* strain T48 at temperatures of 39.5°C and above. Chimeric strains CTM3 + 4 and CTM1 + 2 + 3 + 4 both grew well at 40.5°C and were at least as thermotolerant as T48 but were less thermotolerant than *C. albicans* ATCC10261. Similar results were obtained on solid YPD medium (data not shown).

3.2. In vitro characterization of chimeric ATPases

SDS-PAGE analysis of gradient-purified plasma membranes from *C. albicans* (ATCC10261), *S. cerevisiae* (T48) and the three chimeric *pma1* mutants (Fig. 4) showed the expected high content of the ~ 100 kDa ATPase molecule in each membrane preparation, which constituted between 24 and 29% of total plasma membrane protein (Table 2). The *C. albicans* ATPase band is slightly smaller and therefore migrated slightly faster than the corresponding band in the T48 and chimera preparations [17]. Membranes from CTM1 + 2 and CTM3 + 4 contained levels of 100 kDa ATPase band that were comparable to T48 while CTM1 + 2 + 3 + 4 contained about 90% as much ATPase. Western blot and dot blot analysis using anti-*S. cerevisiae* H^+-ATPase antibody (data not shown) confirmed these observations, although slightly less antigen was detected in the *C. albicans* membranes, as previously reported [17].

The highest rate of ATP hydrolysis measured in vitro at 30°C (V_{max} = 3.0 μmol $min^{-1}mg^{-1}$ plasma membrane protein) was recorded for 'wild type' plasma membranes recovered from *S. cerevisiae* T48 (Table 2). Plasma membranes prepared from the chimeric ATPase strains and *C. albicans* ATCC10261 had 23%–57% less vanadate-sensitive ATPase activity when measured under the same conditions. However, when *C. albicans* membranes were assayed at 37°C, the V_{max} value increased to 3.2 μmol

Table 2
H^+-ATPase enzyme in wild type *PMA1 S. cerevisiae* and *C. albicans* and in chimeric *pma1* strains

Strain	100 kDa band (% tpmp *)	V_{max} (μmol $min^{-1}mg^{-1}$)	K_{cat} (s^{-1})	K_m Mg-ATP (mM)	K_i vanadate (μM)	K_i DES (μM)
T48	27.5	3.0 ± 0.2	18.2	1.9 ± 0.2	5.7 ± 0.1	24 ± 2
1 + 2	26.9	2.3 ± 0.2	12.4	2.0 ± 0.3	4.7 ± 0.1	16 ± 1
3 + 4	28.3	1.5 ± 0.1	8.8	1.8 ± 0.2	8.7 ± 0.9	51 ± 5
1 + 2 + 3 + 4	24.5	1.4 ± 0.1	9.5	1.5 ± 0.2	2.8 ± 0.1	21 ± 1
ATCC10261	29.1	1.3 ± 0.1	7.5	4.2 ± 0.8	2.6 ± 0.2	26 ± 2

Assays were conducted at 30°C on replicate preparations of plasma membranes as described in Section 2. Cells were harvested in late log phase (A_{600nm} = 3 for *S. cerevisiae* and the chimeras and 8.7 for *C. albicans*). V_{max}, K_m and K_i values were determined by linear regression analysis of Lineweaver-Burke plots of vanadate sensitive ATPase activity at pH 6.5, with each assay point measured in duplicate.

* % of total Coomassie stained plasma membrane protein.

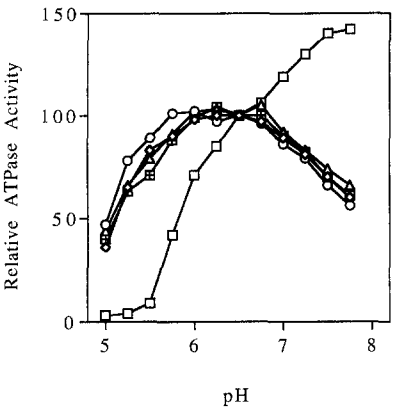

Fig. 5. pH-dependence of vanadate-sensitive plasma membrane ATPase activity of *S. cerevisiae*, *C. albicans* and chimeric strains. ATPase activity measurements were conducted on 1 μg aliquots of purified plasma membrane protein incubated for 30 min in 15 mM Mg-ATP in 50 mM MES buffer adjusted to the indicated pH with Tris. The reaction medium contained 5 mM sodium azide, 0.2 mM sodium molybdate and 50 mM potassium nitrate in order to eliminate other ATPase activities. The remaining ATPase activity was completely sensitive to 100 μM sodium vanadate. ATCC10261 (□), T48 (◇), CTM1+2 (○), CTM3+4 (△), CTM1+2+3+4 (crossed square).

$min^{-1}mg^{-1}$ protein. The affinity of the *S. cerevisiae* T48 and chimeric ATPase preparations for Mg-ATP varied only modestly (K_m = 1.5–2.0 mM). The substrate affinity of the *C. albicans* enzyme was also temperature dependent, having a lower affinity at 30°C (K_m = 4.2 mM) than at the physiologically relevant temperature of 37°C (K_m = 2.4 mM). From the estimated amounts of 100 kDa ATPase band in each preparation, the turnover rate for each ATPase (k_{cat} = 7.5–18.2 s^{-1}) appeared to be similar; once again, the *C. albicans* and *S. cerevisiae* wild type enzymes constituted the lower and upper limits, respectively, of the range of values (Table 2). All mutant and wild type enzymes could be completely inhibited with either vanadate or diethylstilbestrol; the concentrations required for enzyme inhibition differed only modestly between strains (Table 2). Of the chimeras, CTM1 + 2 alone showed increased sensitivity to both inhibitors relative to T48 while CTM3 + 4 gave increased resistance. CTM1 + 2 + 3 + 4 showed inhibitor sensitivities, particularly to vanadate, that most closely matched those of the *C. albicans* enzyme. The pH profiles for ATP hydrolysis by the *S. cerevisiae* control and chimeric enzymes (Fig. 5) were superimposable. The *C. albicans* enzyme, as previously reported [17], showed no apparent optimum in the pH range 5.5 to 7.5.

4. Discussion

Chimeric *pma1* genes containing DNA sequences that encode putative transmembrane loops 1 + 2 and/or 3 + 4 of the *C. albicans* ATPase were introduced into the *S. cerevisiae* genome by targeted integration via homologous recombination and expressed in homozygous mutant strains [11], obviating complications that might be caused by the expression of a background copy of *PMA1* [1]. The first two membrane loop domains from the *C. albicans* plasma membrane proton pumping ATPase functionally substituted for the equivalent native regions of the *S. cerevisiae* ATPase. A combination of PCR and mutagenesis procedures allowed for seamless transfer of the heterologous genetic material in creating the chimeric constructions (Fig. 1), with no need for the introduction of silent restriction sites. The flexibility of the method should allow for construction of chimeras with foreign domains of various sizes and from different parts of the ATPase molecule that might subsequently be identified as potentially vulnerable drug targets.

At 30°C, the growth rates and growth yields of *S. cerevisiae* cells expressing the chimeric ATPases were essentially the same as those of the *S. cerevisiae* T48 isogenic control strain. *C. albicans* had a similar growth rate but its growth yield was about 4–5 times higher than any of the *S. cerevisiae* strains, probably because this organism is an obligate aerobe. Measurement of glucose-activated proton pumping indicated that all three yeast mutants with chimeric *pma1* genes expressed fully functional plasma membrane ATPase molecules which behaved like the *S. cerevisiae* enzyme. Although organic acid secretion and K^+ uptake could contribute to the slower initial pumping rate of *C. albicans*, this property may reflect the use of oxidative metabolism to convert glucose into energy currency and biomass more efficiently than *S. cerevisiae*. For example, yeast cells that are oxidizing glucose take up this substrate several times more slowly than cells which are only fermenting [27]. A lower rate of proton pumping by *C. albicans* might partially explain the modest hygromycin resistance of this organism. *S. cerevisiae pma1* mutants with low ATPase activity are also hygromycin resistant [24].

When the deduced amino acid sequences of the first four transmembrane helices of the *C. albicans* ATPase are compared with the corresponding sequences of the *S. cerevisiae* ATPase (Fig. 2) it is apparent that most amino acid substitutions (8/13) are conservative. Non-conservative substitutions are prevalent at the extracellular turns between transmembrane segments 1 and 2 (1/1) and 3 and 4 (3/3) and in transmembrane helix 3 (4/6). It has been observed previously that in fungal H^+-ATPases, conserved residues tend to cluster in strips along closely juxtaposed portions of helices, while non-conserved helical residues tend to be found on surfaces that are in less tight contact, unless compensatory changes are involved [12]. If transmembrane segments 2 and 3 are indeed helices, the altered residues in these helices also occur in strips (Fig. 2). Given these considerations, our finding that the in vivo behaviour of the chimeric plasma membrane ATPases was essentially like that of the wild type *S. cerevisiae* enzyme seems reasonable.

The growth thermotolerances shown by strains with chimeric ATPases and *S. cerevisiae* T48 suggest that transmembrane loops 1 + 2 and 3 + 4 operate as a domain. Thus, in liquid culture *C. albicans* transmembrane loop CTM1 + 2, appeared to be slightly destabilizing in the context of the *S. cerevisiae* enzyme and gave more thermosensitive cells, whereas the inclusion of either CTM3 + 4 or both heterologous loops gave mutants that were at least as thermotolerant as the control *S. cerevisiae* strain. In addition, an S139E mutant of *S. cerevisiae* had a thermotolerance profile similar to that of T48 (data not shown), indicating that the short turn between transmembrane segments 1 and 2 is unlikely to contribute an important thermostabilizing ionic contact in CTM1 + 2 + 3 + 4. These results argue that some altered residues in the intramembrane strips provide compensatory interactions between TM1 + 2 and TM3 + 4 and stabilize this domain. The ATPase from CTM1 + 2 did not appear to be more thermosensitive in vitro when compared with the ATPases from the other chimeras, *S. cerevisiae* or *C. albicans* (data not shown), suggesting that the residue changes in TM1 + 2 affect ATPase biogenesis rather than ATPase function. This aspect is currently under investigation.

Analysis of plasma membranes purified from yeast expressing the chimeric ATPases revealed polypeptide compositions much like that of plasma membranes from *S. cerevisiae* T48, including a high content of the ~ 100 kDa proton pump antigen and a plasma membrane ATPase activity that was fully inhibited by vanadate and diethylstilbestrol. The vanadate and diethylstilbestrol inhibition profiles of the chimeric enzymes, although close to the range expected for a wild type enzyme [14], gave sensitivities that are consistent with the TM1 + 2 plus TM3 + 4 operating as a domain. The kinetic properties (K_{cat} and K_m for Mg-ATP) of the ATPases from *C. albicans*, *S. cerevisiae* and the chimeras showed no marked divergence when measured at pH 6.5. On the other hand, while the *S. cerevisiae* wild type and chimeric enzymes showed a broad pH profile with an apparent optimum at pH 6.25, the *C. albicans* enzyme showed steadily increasing activity in the pH range 5.5–7.5. Given the kinetic behaviour of the chimeric enzymes it is unlikely that transmembrane loops 1 + 2 and 3 + 4 contribute to the differential in vitro effects of pH on the ATPase activity of these two fungal enzymes. Furthermore, *C. albicans* was at least 4 times more resistant to the weakly basic growth inhibitor $(NH_4)_2SO_4$ than *S. cerevisiae* and the chimeras. The strong in vivo resistance of *C. albicans* to NH_4^+ could reflect a tolerance of elevated internal pH, a property that may be important because *C. albicans* is known to generate a slightly alkaline internal pH during germ tube formation [28]. The significantly alkaline pH optimum of the *C. albicans* ATPase would seem well suited to these conditions.

The replacement of *S. cerevisiae* transmembrane loops 1 + 2 and/or 3 + 4 with their *C. albicans* homologs gave essentially normal *S. cerevisiae*-like ATPases that pumped protons, were sensitive to the ATPase inhibitor acid-activated omeprazole and generated normal membrane potentials. Although 2–5 fold more resistant to hygromycin B than *S. cerevisiae* and the chimeras, *C. albicans* showed a pH-dependent sensitivity to the inhibitor which paralleled that of *S. cerevisiae* in the pH range 4–7. The comparable hygromycin B sensitivities of *S. cerevisiae* and the chimeras indicate that the weak hygromycin resistance shown by *C. albicans* in CSM-URA medium is not affected by TM1 + 2 or TM3 + 4.

Features expected to be independent of ATPase function appeared normal in the chimeras. Thus the chimeras showed an *S. cerevisiae*-like pattern of sensitivity to NaCl, while *C. albicans* appeared to be extremely resistant. Furthermore, amphotericin B was equally effective against *C. albicans*, *S. cerevisiae* and the chimeras while *C. albicans* was an order of magnitude more sensitive to fluconazole than *S. cerevisiae* and the chimeras. Only when the chimeras were subjected to extreme physiological conditions such as high proton flux and high temperature was it possible to discern phenotypic effects that might reflect incompatibilities between individual transmembrane loops. Importantly, the deleterious effects caused by low pH (down to pH 3) and high temperature could be strongly suppressed by including both *C. albicans* transmembrane loops in the *S. cerevisiae* enzyme.

In CTM1 + 2 + 3 + 4 the foreign domain interacted with other membrane bound and cytoplasmic elements of the *S. cerevisiae* ATPase so that growth characteristics were essentially unaltered. The presence of the two *C. albicans*-derived transmembrane loops did produce detectable phenotypes: increased sensitivity to cytoplasmic acidification by acetate and a diminished growth yield at pH < 2.8. These subtle effects notwithstanding, the chimeric *pma1* construct incorporating a putative antifungal target region from the *C. albicans PMA1* gene could be expressed independently in *S. cerevisiae* as fully functional plasma membrane H^+-ATPase. The nondisruptive fusion of the *C. albicans* H^+-ATPase transmembrane loops within the *S. cerevisiae* H^+-ATPase background confirms the functional complementarity of these structures and also suggests that compounds which might interact with this region of the *S. cerevisiae* H^+-ATPase should interact similarly with the *C. albicans* enzyme. The use of the wild type *S. cerevisiae* ATPase as a relevant target to screen for broad-spectrum antifungal drugs that inhibit the ATPase through interaction with this part of the enzyme is therefore validated. Given additional data from sequence alignment of fungal *PMA1* genes [6,7], which place non-conserved transmembrane helix residues within the compensatory strips discussed above, our results strongly suggest that the incorporation of the domain comprising TM1 + 2 and TM3 + 4 from other pathogenic fungi is also likely to be tolerated by *S. cerevisiae*. Such an approach would further test the concept that the TM1 + 2 plus TM3 + 4

domain is a suitable target for broad spectrum antifungal action and could provide additional templates for antifungal screening. Standard mutagenesis procedures can now also be used to modify the heterologous target site in chimeras like CTM1 + 2 + 3 + 4 in order to test structure/function relationships that cannot readily be studied in *C. albicans* and to provide sets of mutated ATPase templates for screening in a genetically defined *S. cerevisiae* strain background.

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