Ancient Gene Duplication Provided a Key Molecular Step for Anaerobic Growth of Baker’s Yeast

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Abstract

Mitochondria are essential organelles required for a number of key cellular processes. As most mitochondrial proteins are nuclear encoded, their efficient translocation into the organelle is critical. Transport of proteins across the inner membrane is driven by a multicomponent, matrix-localized “import motor,” which is based on the activity of the molecular chaperone Hsp70 and a J-protein cochaperone. In Saccharomyces cerevisiae, two paralogous J-proteins, Pam18 and Mdj2, can form the import motor. Both contain transmembrane and matrix domains, with Pam18 having an additional intermembrane space (IMS) domain. Evolutionary analyses revealed that the origin of the IMS domain of S. cerevisiae Pam18 coincides with a gene duplication event that generated the PAM18/MDJ2 gene pair. The duplication event and origin of the Pam18 IMS domain occurred at the relatively ancient divergence of the fungal subphylum Saccharomycotina. The timing of the duplication event also corresponds with a number of additional functional changes related to mitochondrial function and respiration. Physiological and genetic studies revealed that the IMS domain of Pam18 is required for efficient growth under anaerobic conditions, even though it is dispensable when oxygen is present. Thus, the gene duplication was beneficial for growth capacity under particular environmental conditions as well as diversification of the import components.

Key words: J-protein, mitochondria, protein translocation, Hsp70, gene duplication.

Introduction

Translocation of proteins from the cytosol into the mitochondrial matrix is a critical process, as most mitochondrial proteins are encoded by nuclear DNA and synthesized on cytosolic ribosomes. Transfer across the impermeable inner mitochondrial membrane is achieved by the action of a highly conserved, multiprotein complex composed of two parts, a translocase through which the proteins pass and an import motor that drives their translocation into the organelle (Chacinska et al. 2009; Mokranjac and Neupert 2009). The driving force of the import motor is provided by an Hsp70 of the mitochondrial matrix (mtHsp70, known as Ssc1 in Saccharomyces cerevisiae). Ssc1 is tethered to the translocon and binds exposed hydrophobic amino acid segments as the unfolded polypeptide enters the matrix while ATP hydrolysis drives forward movement of the polypeptide.

This binding of Ssc1 to the translocating polypeptide requires the intrinsic ATPase activity of Ssc1, which is stimulated by a partner J-protein via its highly conserved J-domain. In S. cerevisiae, two J-protein partners of Ssc1, Pam18 and Mdj2, are found associated with the translocon (Mokranjac et al. 2005). Pam18 is the principal J-protein functioning with Ssc1 in protein import (D’Silva et al. 2003; Mokranjac et al. 2003; Truscott et al. 2003). An essential protein that spans the inner membrane, Pam18, has three domains (fig. 1A): an N-terminal 60-amino acid domain localized to the intermembrane space (IMS), an internal membrane-spanning domain, and a C-terminal J-domain, which extends into the matrix. Although the J-domain and membrane-spanning domain were found to be essential, no phenotypic effect was found upon deletion of the N-terminal IMS domain (Mokranjac et al. 2007; D’Silva et al. 2008). Mdj2, like Pam18, contains a single transmembrane-spanning domain and a C-terminal J-domain localized in the matrix but lacks an N-terminal IMS domain (fig. 1A). Although Pam18 is essential, the absence of Mdj2 has no known phenotype (Westermann and Neupert 1997). However, when overexpressed, Mdj2 allows growth of cells lacking Pam18 (Mokranjac et al. 2005). The presence of two J-proteins associated with the inner mitochondrial matrix translocase in S. cerevisiae is perhaps not surprising, as the progenitor of S. cerevisiae underwent a whole genome duplication (Wolfe and Shields 1997; Dietrich et al. 2004; Dujon et al. 2004; Kellis et al. 2004). Following genome duplication, two gene copies were maintained in at least one species in 20–26% of the cases analyzed (Byrne and Wolfe 2005), likely resulting in specialization, redundancy, or distribution of functions between the duplicate genes.

To better understand the function of J-proteins in mitochondrial import, we undertook evolutionary and molecular
genetic analyses. We found that the PAM18/MDJ2 gene pair may have been one of many key steps leading to functional diversification in respiration and mitochondrial function observed between the *Saccharomyces* and *Pezizomycotina* clades.

### Materials and Methods

#### Identification of PAM18 and MDJ2 Orthologs by Reciprocal BLAST Analysis

Using protein sequences of *S. cerevisiae* PAM18 and MDJ2 as queries, basic local alignment search tool (BLAST) searches (Altschul et al. 1990, 1997) were performed with National Center for Biotechnology Information server (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the Sanger Institute (http://www.sanger.ac.uk/DataSearch/blast.shtml). To assess gene presence and absence, BlastP, TBlastN, and BlastX were utilized. Unannotated, putative open reading frames (ORFs) were identified with ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Results from each BLAST search that were statistically significant were then used as query sequences in the BlastP search against the *S. cerevisiae* protein database to identify reciprocal-best-blast sequences as orthologs.

To determine whether an IMS domain was present in each orthologous protein, the amino acid sequence identified as a PAM18 or an MDJ2 orthologous protein was further analyzed to specify the location of a membrane-spanning segment. The putative transmembrane position across a protein sequence was located by comparing the results from three prediction methods of transmembrane spanning segments: HMMTOP2 (Tusnady and Simon 1998, 2001), Split4 (Juretic et al. 2002), and TMpred (Hofman and Stoffel 1993). Then, the presence or absence of an IMS domain in an orthologous protein was comprehensively evaluated in view not only of its transmembrane position but also of sequence comparison with the other orthologous proteins. The full-length sequence data of PAM18 and MDJ2 orthologous proteins were aligned with the ClustalW program (Thompson et al. 1994) and corrected by manual inspection.

#### Sequence Data Set for Phylogenetic Reconstruction

The coding sequence data of PAM18 and MDJ2 for phylogenetic reconstruction were obtained from 21 fungi in the subphylum *Saccharomycotina*. Five sequences of the PAM18/MDJ2 orthologs from non-*Saccharomycotina* fungi were included as outgroups (subphylum *Taphrinomycotina*—*Schizosaccharomyces pombe*; subphylum *Pezizomycotina*—*Aspergillus fumigatus*, *Coccidioides immitis*, *Gibberella zeae*, and *Neurospora crassa*). To construct the phylogenetic trees from sequence data, the coding sequences were first translated to amino acid sequences, and the IMS domains were removed from PAM18 orthologs. Alignments were then generated using Dialign-TX, which has a segment-
Fig. 2. Phylogeny of PAM18 and MDJ2. The number along branches refers to the Bayesian posterior probability (X100) supporting the respective branch. The number of samples in each clade is given in parentheses. The orthologs from the Saccharomycotina species were divided into the CTG clade and the Saccharomycetes complex. The scale bar represents 0.2 substitutions per site. The branches of each ortholog are colored: red, MDJ2; blue, post-duplication PAM18 from the Saccharomycotina fungi; and black, PAM18 from the non-Saccharomycotina fungi. For the complete phylogenetic tree, see supplementary figure S1, Supplementary Material online.

Phylogenetic Tree Construction of PAM18 and MDJ2

Based on the gap-free multiple alignment described above, the optimal gene tree was derived using MrBayes ver. 3.1.2 (Ronquist and Huelsenbeck 2003) under a general time reversible model (GTR) with a proportion of invariant sites, assuming gamma distribution of rate variation across sites (GTR + I + G model). This model was estimated as the best fitting substitution model by MrModeltest version 2.3 (Nylander et al. 2004). Furthermore, the site-specific rate model was used to analyze the protein-coding sequences of the present study, allowing each codon position to evolve under different rates. Other model parameters (statefreq, revmat, shape, and pinvar) were also unlinked across each codon position. The Bayesian analyses were performed with two independent runs (each with four chains), sampling each 100 generations. The analyses were considered finished when the average standard deviation of split frequencies between two runs dropped below 0.01, which required 4,000,000 generations, and the first 25% of sampled trees were discarded as burn-in before estimation of posterior probabilities for branch support. The obtained phylogenetic trees were drawn using Tree Explorer of Molecular Evolutionary Genetics Analysis (MEGA) software 4.0 (Tamura et al. 2007). In the reduced phylogenetic tree of figure 2, the clades of orthologs from the Saccharomycotina species were further divided into two major clades: the Saccharomyces complex and the CTG clade, which is composed of a monophyletic group of taxa that translate the CTG codon into a serine rather that leucine (Kurtzman and Robnett 2003; Fitzpatrick et al. 2006; Moura et al. 2010).

Calculation of the Substitution Nucleotide Rates

Non-synonymous substitution rates (the number of substitutions per non-synonymous site, dn) and synonymous substitution rates (the number of substitutions per synonymous site, ds) were determined for each branch of the nucleotide tree (fig. 2) by using maximum likelihood approach with codeml program in PAML package ver. 4.2b (Yang 2007). Codon frequencies were calculated from the average nucleotide frequencies at the three codon positions (codon frequency model F3 × 4). To avoid falling into suboptimal likelihood peaks, the program was run multiple times in each analysis of this study, using different initial parameters (initial dn/ds ratio or ω = 0.01 or 1, and initial transition to transversion rate ratio or κ = 0.4 or 4). Under the branch-specific model estimating different dN and dS values among a priori specified branches (Yang 1998; Yang and Nielsen 1998), three models of sequence evolution were considered: 1) one-ratio model (a single ω ratio, assuming that all branches have the same ratio of ω), 2) two-ratio model (a single ω ratio for PAM18 and a second ω ratio for MDJ2), and 3) three-ratio model (two separate ω ratios for PAM18 of the Saccharomycotina and of the non-Saccharomycotina and a single ω ratio for MDJ2). Statistical significance was determined by likelihood ratio tests (a statistical test of the goodness-of-fit between two models) assuming a chi-square distribution. The two-ratio model provided a significantly better fit to the data than the one-ratio model (P < 0.05) in the likelihood ratio test. On the other hand, the three-ratio model was not a better model than the two-ratio model statistically (P > 0.05). Therefore, the dS and dN parameters of branches in the result of the two-ratio model were adopted.

Codon Usage Bias Estimation

Assuming that optimal codons are significantly common among closely related Saccharomycotina species, the codon adaptation index values of their PAM18 and MDJ2 coding sequences were calculated with CodonW program (Peden 1999) (http://codonw.sourceforge.net/) using the reference set for the optimal codons of S. cerevisiae (Sharp and Cowe 1991).

Yeast Strains, Media, and Growth Conditions

In this study, strains of S. cerevisiae were derived from PJ53 (James et al. 1997), which is isogenic to W303: trp1-1/ trp1-1 ura3-1/ura3-1 leu2-3,112/leu2-3,112 his3-11,15/his3-11,15 ade2-1/ade2-1 can1-100/can1-100 GAL2-Δ/GAL2-Δ met2- Δ1/met2-Δ1 lys2-Δ2/lys2-Δ2. haploid strains were constructed by tetrad dissection of appropriate diploid strains. Yeast strains deleted for PAM18 (pam18::TRP1), TIM44 (tim44::LY52), and SSC1 (ssc1::LEU2) have been described previously (Maarse et al. 1992; Gambill et al. 1993; D’Silva et al. 2003). Strains having deletions of PAM17 and TIM21 were constructed by replacing their coding regions with.
a hygromycin B resistance (hph) gene (Goldstein and McCusker 1999) yielding pam17::HPH and with a KanMX module gene yielding tim21::kanMX, respectively. To obtain single deletion mutant of MDJ2, the DNA between the BamHl and Mfel sites in MDJ2 (−47 to +465) was replaced with the TRP1 gene yielding mdj2::TRP1. For the double deletion mutant of PAM18 and MDJ2, pam18::TRP1 was further genetically modified by replacing MDJ2 with a KanMX module to generate pam18::TRP1 mdj2::kanMX.

Yeast cells were cultivated, where appropriate, in yeast extract/peptone/dextrose media, synthetic complete media (SC), synthetic dropout media, sporulation media, or extract/peptone/dextrose media, synthetic complete media. Yeast genetically modified by replacing the GPD promoter.

Construction of Plasmids
As described previously (D’Silva et al. 2003), PAM18 was obtained by polymerase chain reaction (PCR)-amplifying genomic DNA from position −303 to +702 and cloned into pRS315 (CEN6 LEU2) or into pRS316 (CEN6 URA3) (Sikorski and Hieter 1989). Mutants of PAM18 were constructed by site-directed mutagenesis by using QuickChange protocol (Stratagene). The construction of the Pam18Δ1−60 protein made use of existing an AUG codon at amino acid position 61 within the sequence while the start codons were introduced to allow translation of the Pam18Δ1−25 and Pam18Δ1−43 proteins. The plasmid construction of pRS314 (CEN6 TRP1) carrying the wild-type or P419S-mutated SSC1 was described previously (Miao et al. 1997; Liu et al. 2001). The plasmid construction of pRS314 carrying the wild-type or R180A-mutated TIM44 was also described previously (Liu et al. 2003; Schiller et al. 2008). For overexpression of Pam18Δ1−60 (amino acids 61−168), the corresponding coding sequence was PCR amplified and cloned into the BamHI/XhoI sites of p415 (CEN6 LEU2) placing it under the control of a constitutive promoter derived from the gene encoding translation elongation factor 1α (TEF promoter) (Mumberg et al. 1995). For overexpression of Mdj2, the full-length coding sequence of MDJ2 was PCR amplified from genomic DNA and cloned into the BamHI/XhoI sites of p416 (CEN6 URA3) placing it under the control of a constitutive promoter derived from the gene encoding glyceraldehyde-3-phosphate dehydrogenase (GPD promoter).

Analysis of Precursor Accumulation In Vivo
Yeast cells carrying either wild-type or mutant form of PAM18 were grown on SC solid media supplemented with Ergosterol and Tween 80 (SC + Erg/Tw) under anaerobic conditions at 30 °C for 2.25 days. After growth on solid media, cells were scraped from the plates, the cultured cells were subjected to alkali treatment, followed by 3 min boiling in SDS sample buffer. Total cell lysates were analyzed by Laemmli–SDS-PAGE (Laemmli 1970) and by immunoblot analysis using Hsp60-specific antibody.

Antibodies
Preparation of antibodies against Pam18, Tim23, and Hsp60 were described previously (D’Silva et al. 2003, 2008). Because the Pam18 antibody was prepared against the C-terminus of the protein (amino acids 80−168), it reacts equally well with full-length Pam18, Pam18Δ1−60 (amino acids 61−168), and the other truncated Pam18 variants (Pam18Δ1−25 amino acids 26−168; Pam18Δ1−43, amino acids 44−168). For production of antibody against Mdj2, 6XHis-tagged coding sequence of Mdj2 protein (amino acids 20−146) was cloned into pET-3a vector (Novagen). The protein expression from the plasmid construct was induced with isopropyl-β-d-thiogalactopyranoside in Escherichia coli C41 (DE3) strain (Miroux and Walker 1996). After purification, the His-tagged protein was injected into rabbits for Mdj2-specific polyclonal antibody production.

Miscellaneous
Yeast transformation with exogenetic DNA was conducted by the lithium acetate/single-stranded carrier DNA/polyethylene glycol method (Gietz and Woods 2002). Transformed cells were selected on the appropriate selective media.
Results

**The PAM18/MDJ2 Pair Arose by Duplication in a Common Ancestor of the Saccharomycotina**

As a first step in elucidating the origin of the PAM18 and MDJ2 paralogs, located on chromosomes XII and XIV, respectively, we determined their phylogenetic distribution in fungal genomes using BLAST analyses (supplementary table S1, Supplementary Material online). Within Saccharomycotina, all species exhibited the presence of both PAM18 and MDJ2. However, outside the subphylum, each species exhibited only a single ORF when queried using either PAM18 or MDJ2 sequence. Reciprocal BLAST matches of each single copy gene against the S. cerevisiae proteome were consistently more similar to PAM18 than to MDJ2. For each species in Saccharomycotina, two ORFs were identified that resulted in significant BLAST e-values when probed with either PAM18 or MDJ2. In each case, reciprocal best BLAST analysis consistently distinguished between PAM18 and MDJ2 putative orthologs.

Based on the above BLAST results, we concluded the duplication event that gave rise to the PAM18 and MDJ2 pair occurred prior to the whole genome duplication in a common ancestor of the Saccharomycotina clade (fig. 18). However, it was also possible that the presence of only a single mitochondrial J-protein in non-Saccharomycotina fungal species was due to multiple losses of one member of the gene pair that originated from an earlier duplication event. Therefore, we determined whether a single copy J-protein was present in nonfungal eukaryotes via BLAST analysis in well-annotated genomes among plantae, metazoa, and protozoa. The BLAST search using the sequence of *S. cerevisiae* MDJ2 and PAM18 as a query provided one or more significant hits in each surveyed species. However, the reciprocal BLAST search against *S. cerevisiae* database revealed that they were all more closely related to PAM18 than to MDJ2 (supplementary table S1, Supplementary Material online), indicating homology to PAM18 outside the fungi.

Next, we analyzed the phylogenetic relationship of PAM18 and MDJ2 orthologs from Saccharomycotina fungi as well as selected single copy orthologs from non-Saccharomycotina fungi. Results of Bayesian reconstruction of the nucleotide-based phylogeny indicated that orthologs formed strongly supported monophyletic clades among taxa within the Saccharomycotina (fig. 2 and supplementary fig. S1, Supplementary Material online), consistent with a single gene duplication event. Congruent monophyletic patterns were also observed with maximum likelihood methods (data not shown). Taken together, our analyses indicate that duplication of the ancestral single copy PAM18/MDJ2 gene gave rise to the two J-proteins, PAM18 and the fungal-specific Mdj2, prior to the diversification of the Saccharomycotina clade.

**MDJ2 Is Rapidly Evolving**

Next, we compared rates of evolution of PAM18 and MDJ2. The nucleotide-based tree and the amino acid–based tree (supplementary fig. S2, Supplementary Material online)

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**Fig. 3.** Analysis of codon usage in PAM18 and MDJ2. (A) Codon-based phylogenies of PAM18 and MDJ2 orthologs. Shown is the nonsynonymous substitution rate tree (dN tree, top) or synonymous substitution rate tree (dS tree, bottom) whose branch lengths are proportional to nonsynonymous or synonymous substitution rates, respectively. Scale bars indicate substitution rates per site. Red, MDJ2; blue, post-duplication PAM18 from the Saccharomycotina fungi; and black, pre-duplication PAM18 from the non-Saccharomycotina fungi. (B) Codon adaptation index (CAI) values of PAM18 and MDJ2 of Saccharomyces species. The CAI values of the PAM18 and MDJ2 coding sequences from closely related Saccharomyces species (*S. cerevisiae*, *S. paradoxus*, *S. mikatae*, *S. kudriavzevii*, and *S. bayanus*) were calculated with CodonW program using the reference set of highly expressed genes in *S. cerevisiae* cells. Data are expressed as the mean and standard deviation of the CAI values from the five species (*P < 0.01, two-tailed t-test*). The CAI calculation using the codon usage tables of each species genome (Nakamura et al. 2000) as the references also produced the same result in that the CAI values of MDJ2 are statistically lower than those of PAM18 among the Saccharomyces species (data not shown).
exhibited longer branches for the MDJ2 clade compared with PAM18. The analysis of codon evolution further indicated that both synonymous and nonsynonymous rates of evolution are higher for the MDJ2 gene than the PAM18 gene (fig. 3A). In addition, our analyses of average levels of codon bias (fig. 3B) and expression studies (Holstege et al. 1998) indicate that MDJ2 is expressed at lower levels than PAM18. The observed lower levels of expression, reduced codon bias, and rapid rate of evolution for MDJ2 relative to PAM18 are consistent with previous observations that genes with lower levels of expression tend to evolve more rapidly (Drummond et al. 2005; Drummond and Wilke 2009). This accelerated rate of evolution for MDJ2 also explains why nonfungal mitochondrial J-proteins have higher BLAST similarity scores for PAM18.

**Acquisition of Fungal Pam18 IMS Domain Coincides with the Gene Duplication**

Upon further examination of the PAM18/MDJ2 pair, we noted that all post-duplication Pam18 orthologous proteins have an extension N-terminal to the membrane-spanning region (figs. 1B and 4, supplementary table S1, Supplementary Material online). A qualitative comparison among these N-terminal extensions revealed a high degree of conservation, indicating ancient acquisition and subsequent negative selection for the maintenance of this domain. In addition, BLAST analyses using only the IMS domain of Pam18 as a query failed to return any significant matches among fungi, indicating that this novel domain is unlikely to have originated from domain shuffling of previously existing polypeptides.

To investigate whether any nonfungal Pam18 orthologous proteins had an N-terminal extension sharing homology to fungal Pam18 IMS domains, we conducted a BLAST analysis of well-annotated genomes among plantae, metazoa, and protozoa (supplementary table S1, Supplementary Material online). Most nonfungal Pam18 orthologs lacked an N-terminal extension. An exception was a metazoan group consisting mostly of vertebrates. Each genome of the surveyed vertebrates encoded a pair of Pam18 homologs; in each case, one had a short N-terminal extension, approximately 35 amino acids in length. However, the sequence alignment of the fungal IMS domains (50–70 amino acids in length) and the nonfungal IMS domains showed no detectable homology (data not shown), suggesting that fungal and vertebrate IMS domains of Pam18 evolved independently from each other.

In addition to the novel domain found among all post-duplication Pam18 orthologous proteins, we also noted an additional amino acid segment present in all post-duplication Mdj2 orthologous proteins, situated between the membrane-spanning domain and C-terminal J-domain (fig. 4B). Although the gain of this domain within Mdj2 is of interest, MDJ2 loss-of-function alleles have no known phenotype (Westermann and Neupert 1997); hence, we decided to focus on the novel IMS domain of Pam18.

**The IMS Domain of Pam18 Is Required for Normal Growth and Protein Import in the Absence of Oxygen**

The IMS domain of *S. cerevisiae* Pam18 has been reported to be unimportant for efficient protein import and thus...
Hsp60 are indicated. Protein extract from a mutant known to be defective in Hsp60 import (a loading control. (A) Growth of cells expressing truncations of IMS domain of Pam18 under anaerobic conditions. Ten-fold serial dilutions of the cell suspensions were spotted on synthetic complete media supplemented with Ergosterol and Tween 80 (SC + Erg/Tw). After plating, cells were grown aerobically (+O₂) or anaerobically (−O₂) at the indicated temperatures. Culture periods: 5 days at 18 °C, 2 days (+O₂) or 2.25 days (−O₂) at 30 °C, 2.5 days (+O₂), or 3 days (−O₂) at 37 °C. (B) Expression levels of wild-type and truncated Pam18 proteins in anaerobically grown cells. After growth on SC + Erg/Tw media at 30 °C for 2.25 days under anaerobic conditions, extracts were prepared from cells that were scraped off agar-plates. The extracts were subjected to electrophoresis and analyzed by immunoblotting with an antibody against Pam18, which recognizes all Pam18 proteins with equal efficiency (see Materials and Methods). Tim23 protein was also detected as a loading control. (C) Accumulation of a mitochondrial precursor protein in vivo. Total protein extracts prepared from cells grown anaerobically on SC + Erg/Tw media at 30 °C for 2.25 days were subjected to immunoblot analysis using an antibody specific for Hsp60. Protein extract from a mutant known to be defective in Hsp60 import (pam18_150W) was used as a positive control. pam18_150W extract was made from cells grown aerobically in rich liquid media at 23 °C and heat-shocked at 37 °C for 7 h. Precursor (P) and mature (M) forms of Hsp60 are indicated.

Fig. 5. Importance of the IMS domain of Pam18 for growth and protein translocation under anaerobic conditions. Analysis of pam18Δ cells carrying a centromeric plasmid (pRS315) with the indicated PAM18 alleles: full-length PAM18 (WT); pam18Δ1-60 (Δ1–60); pam18Δ1-25 (Δ1–25); and pam18Δ1-43 (Δ1–43). (A) Growth of cells expressing truncations of IMS domain of Pam18 under anaerobic conditions. Many mitochondrial proteins encoded by nuclear DNA, such as Hsp60, are synthesized as preproteins, having a mitochondrial targeting sequence that is cleaved by a processing protease upon entrance into the matrix. Thus, the accumulation of the precursor form of such proteins can be taken as a loading control. (C) Accumulation of a mitochondrial precursor protein in vivo. Total protein extracts prepared from cells grown anaerobically on SC + Erg/Tw media at 30 °C for 2.25 days were subjected to immunoblot analysis using an antibody specific for Hsp60. Protein extract from a mutant known to be defective in Hsp60 import (pam18_150W) was used as a positive control. pam18_150W extract was made from cells grown aerobically in rich liquid media at 23 °C and heat-shocked at 37 °C for 7 h. Precursor (P) and mature (M) forms of Hsp60 are indicated.

Fig. 5.

vibrant cell growth (Mokranjac et al. 2007). However, the sequence conservation among post-duplication Pam18 IMS domains suggests that acquisition of the IMS domain could have important functional consequences. Therefore, we reassessed the ability of a Pam18 variant lacking the N-terminal 60 amino acids (Pam18Δ1–60) to grow under a variety of conditions. We tested growth on media containing different carbon sources at various temperatures. In addition, because previous comparative genomic studies have noted that differences in gene presence/absence between the Saccharomycotina and Pezizomycotina clades are related to mitochondrial and respiratory function, we also examined growth in anaerobic conditions. Even though no oxidative phosphorylation occurs in the absence of oxygen, other mitochondrial functions, such as Fe-S cluster biogenesis, are critical under these conditions (Groot et al. 1971; Trocha and Sprinson 1976; Lill and Muhlenhoff 2008). Consistent with previous reports (Mokranjac et al. 2007), we found no deleterious effect of the absence of the IMS domain of Pam18 under aerobic conditions (data not shown).

In sharp contrast, under anaerobic conditions pam18Δ1–60 cells grew more slowly than cells expressing full-length Pam18 at all temperatures tested (fig. 5A and data not shown). This difference was especially apparent at low (18 °C) and at high (37 °C) temperatures.

To determine the region of the IMS domain critical for robust growth under anaerobic conditions, we created two smaller nested N-terminal truncations, lacking the first 25 (Pam18Δ1–25) or 43 amino acids (Pam18Δ1–43). We compared the growth of cells expressing these two variant alleles with cells expressing full-length Pam18 or the variant allele lacking the N-terminal 60 amino acids, Pam18Δ1–60. Both pam18Δ1–25 and pam18Δ1–43 cells grew similarly to cells expressing full-length Pam18 under anaerobic, as well as aerobic conditions (fig. 5A).

These results are consistent with amino acids 44–60, a conserved segment of the IMS domain of Pam18, being required for robust cell growth under anaerobic conditions. However, to ensure that the effects on growth were not due to differences in expression levels of the variants, we prepared cell extracts from anaerobically grown cells and examined the levels of the Pam18 proteins using antibodies specific for the matrix domain of Pam18 (fig. 5B). We found that full-length Pam18, Pam18Δ1–60 and Pam18Δ1–43 were all expressed at similar levels. Because only the cells expressing Pam18Δ1–60 exhibited an anaerobic growth defect, we conclude that the IMS domain plays an important function under these conditions, particularly at suboptimal growth temperatures.

As a component of the import motor, it has been established that Pam18 plays a critical role in the translocation of proteins into the mitochondrial matrix. We next wanted to determine if the growth defect of pam18Δ1–60 we observed in the absence of oxygen is consistent with a defect in protein translocation under this condition. Many mitochondrial proteins encoded by nuclear DNA, such as Hsp60, are synthesized as preproteins, having a mitochondrial targeting sequence that is cleaved by a processing protease upon entrance into the matrix. Thus, the accumulation of the precursor form of such proteins can be taken as
Hayashi et al. absence (on SC fold serial dilutions of the indicated deletion mutants were spotted encoding various components of the translocation apparatus. Ten-defects in mitochondrial import caused by mutations in genes of the essential genes, 2012 (is present at extremely low levels and nearly undetectable a marker for defective protein import, as normally trans-blot analysis of cell extracts. As a control, we used pRS315. In the case of the nonessential genes encoding the indicated amino acid alterations on the plasmid deletion strains carried either the wild-type (WT) or mutant gene WT and complete deletion mutants were compared. p419s/pam18/C0/Erg/TW plates and grown in the presence (O2) or absence (−O2) of oxygen at the indicated temperatures. In the case of the essential genes, SSC1 (A), TIM44 (B), or PAM18 (D), the deletion strains carried either the wild-type (WT) or mutant gene encoding the indicated amino acid alterations on the plasmid pRS315. In the case of the nonessential genes PAM17 and TIM21 (C), WT and complete deletion mutants were compared.

a marker for defective protein import, as normally translocation is so rapid that the precursor form of such proteins is present at extremely low levels and nearly undetectable (Reid and Schatz 1982). Therefore, to assess the efficiency of protein import into the mitochondrial matrix, we monitored the accumulation of Hsp60 precursor by immunoblot analysis of cell extracts. As a control, we used a pam18 mutant that does not grow aerobically at 37 °C. After shift to the nonpermissive temperature, sub-

stantial accumulation of Hsp60 precursor was detected (fig. 5C). Precursor accumulation was also observed in extracts prepared from pam18Δ1–60 cells grown anaerobically at 30 °C, a temperature at which these cells grow more slowly than wild-type cells. However, negligible precursor was detected in pam18Δ1–25 and pam18Δ1–43 cells, as well as wild-type cells under these conditions. This precursor accumulation in pam18Δ1–60, but not the shorter truncation mutants, is consistent with an anaerobic growth defect of pam18Δ1–60 cells, being caused by inefficient import of proteins into mitochondria.

Other Mutants Defective in Protein Import Grow Equally Well in the Presence or Absence of Oxygen
The compromised growth of pam18Δ1–60 under anaerobic, but not aerobic, conditions raises the question as to whether particularly efficient function of the import apparatus of the inner membrane is required in the absence of oxygen. To address this question, we compared aerobic and anaerobic growth of several well-studied strains having mutants in or deletion of genes encoding several components of the import apparatus. All these mutant strains have been documented to have protein translocation defects. First, the effects of conditional mutations in two genes encoding essential components, Ssc1 and Tim44, the protein that tethers Ssc1 to the translocon, were tested. scc1 p419s (scc1-2) cells are defective in protein import into the mitochondrial matrix due to a defective interaction with Tim44 (D’Silva et al. 2004). Consistent with previous results (Gambill et al. 1993; Voisine et al. 1999), scc1 p419s grew poorly at all temperatures and did not form colonies at 34 °C and above in the presence of oxygen. Growth of scc1 p419s under anaerobic conditions was very similar (fig. 6A). The second mutant tested, tim44R180A, is defective in regulation of the Ssc1–Tim44 interaction as well as in stable association with the translocon (Schiller et al. 2008). tim44R180A is temperature sensitive under aerobic conditions, unable to form colonies at 34 °C and above. The growth defect of tim44R180A was slightly more severe under anaerobic than aerobic conditions at all temperatures tested (fig. 6B). However, the difference between aerobic and anaerobic growth was not nearly as drastic as that found with pam18Δ1–60 (fig. 5A). The effects of the absence of two nonessential components of the translocation machinery, Pam17 and Tim21 (van der Laan et al. 2005; Hutu et al. 2008; Popov-Celeketic et al. 2008), which affect the general organization of the components of the transloca-
tion machinery, were also tested. pam17Δ cells and tim21Δ cells grew as well as wild-type cells under both anaerobic and aerobic conditions (fig. 6C). We conclude that defects in the translocation apparatus of the inner mitochondrial membrane do not generally cause enhanced growth defects under anaerobic conditions.

Because the mutants described above did not have enhanced growth defects under anaerobic conditions, we next wanted to test whether the affect of the absence of the IMS domain of Pam18 reflected a general increase in demand for Pam18 function or a specific requirement

![Fig. 6. Similarity of aerobic and anaerobic growth of strains having defects in mitochondrial import caused by mutations in genes encoding various components of the translocation apparatus. Ten-fold serial dilutions of the indicated deletion mutants were spotted on SC + Erg/TW plates and grown in the presence (+O2) or absence (−O2) of oxygen at the indicated temperatures. In the case of the essential genes, SSC1 (A), TIM44 (B), or PAM18 (D), the deletion strains carried either the wild-type (WT) or mutant gene encoding the indicated amino acid alterations on the plasmid pRS315. In the case of the nonessential genes PAM17 and TIM21 (C), WT and complete deletion mutants were compared.](image-url)
Duplication of Mitochondrial J-Protein Gene

were expressed from the pRS315 plasmid. (Fig. 7) pam18ΔΔ cells expressing either full-length PAM18 (WT) or pam18Δ1–60 under the control of its endogenous promoter (Δ1–60) or the TEF promoter (Δ1–60†) for overexpression. After spotting on SC + Erg/Tw media cells were grown at 18 °C for 5 days in the presence (+O2) or absence (−O2) of oxygen. (Right) Extracts of the indicated strains were prepared and examined by immunoblot analysis. The expression levels of the PAM18 proteins were assessed as described in figure 5B.

Fig. 7. Mdj2 does not compensate for lack of the IMS domain of Pam18 in the absence of oxygen. (A) Cells lacking Mdj2 have no anaerobic growth defect. Ten-fold serial dilutions of wild-type (WT) and mdj2ΔΔ cells were spotted on SC + Erg/Tw plates and grown in the presence (+O2) or absence (−O2) of oxygen at the indicated temperatures. (B) No synthetic genetic interaction between mdj2ΔΔ and pam18Δ1–60 mutations. The indicated combinations of mutants were grown in the presence (+O2) or absence (−O2) of oxygen as described in A. In the case of PAM18, the chromosomal copy was deleted, and the control WT Pam18 (WT) and Pam18Δ1–60 (Δ1–60) were expressed from the pRS315 plasmid. (C) Overexpression of Mdj2 does not rescue the anaerobic growth defect of pam18Δ1–60. Cells expressing either full-length Pam18 (WT) or Pam18Δ1–60 (Δ1–60) from pRS315 as well as harboring either p416 having MDJ2 under the control of the GPD promoter (MDJ2†) or p416 control vector (empty) were grown in the presence (+O2) or absence (−O2) of oxygen. SC-Ura-Leu dropout media with Ergosterol and Tween 80 supplement (SC-Ura-Leu + Erg/Tw) were used to ensure plasmid retention. Cells were grown at 34 °C aerobically for 2 days or anaerobically for 2.5 days. (Right) Whole cell lysates prepared from cells cultured on SC-Ura-Leu + Erg/Tw at 30 °C under anaerobic condition were used for the immunoblot analysis with antibodies against Mdj2 and Tim23, as the loading control.

for the IMS domain itself. We therefore tested another PAM18 mutation, pam18L150W, which causes a single amino acid alteration in the matrix domain (D’Silva et al. 2008). pam18L150W has a temperature-sensitive growth defect when grown aerobically (fig. 6D). However, it grew as well in the absence of oxygen as in the presence of oxygen. Thus, under the conditions tested, none of the mutants, with the exception of pam18Δ1–60, was more compromised in the absence of oxygen than in its presence. Thus, our results are consistent with the hypothesis that the IMS domain of Pam18 is particularly required under anaerobic conditions, which indicates that the gene duplication event and concomitant gain of the IMS domain generated some novel advantage under such conditions.

Mdj2 Is Not Required Under Anaerobic Conditions

In addition, we examined the anaerobic growth of cells lacking the nonessential Mdj2, the other descendant of the ancestral duplication, to determine if it plays an important role under anaerobic conditions as well. Consistent with previous reports (Westermann and Neupert 1997; Mokranjac et al. 2005), mdj2ΔΔ cells grew as well as wild-type cells under aerobic conditions. Nor was a growth defect observed in the absence of oxygen (fig. 7A), indicating that Mdj2 is not specifically required under these conditions. To test for any synthetic genetic interaction between the IMS domain of Pam18 and Mdj2, we constructed a double mutant lacking both Mdj2 and the Pam18 IMS domain (pam18Δ1–60 mdj2ΔΔ). No significant growth difference between pam18Δ1–60 mdj2ΔΔ and pam18Δ1–60 cells were observed between aerobic and anaerobic conditions (fig. 7B).

To test whether Mdj2 could compensate for the lack of the Pam18 IMS domain, we then asked if overexpression of Mdj2 could restore the anaerobic specific defect of the pam18Δ1–60 cells. MJD2 was placed under the control of the strong, constitutive GPD promoter, which resulted in overexpression of MJD2 on the order of 10-fold (fig. 7C). Mdj2 was unable to compensate for the anaerobic function of the deleted IMS domain of Pam18, even when overexpressed. In fact, overexpression of Mdj2 was deleterious for cell growth of both wild-type and pam18Δ1–60 cells, under both aerobic and anaerobic conditions.

We also tested the affect of overexpression of Pam18Δ1–60. Contrary to the result with Mdj2, 3-fold overexpression of Pam18Δ1–60 partially suppressed the anaerobic growth defect caused by the lack of the IMS domain at 18 °C but only slightly improved growth at 37 °C (fig. 8, and data not shown). Together our results indicate that Mdj2 does not play a critical role under anaerobic conditions, whereas the IMS domain of Pam18 is important under this...
condition. However, the defect caused by the absence of the IMS domain can be partially overcome by overexpression of the variant lacking this domain.

**Discussion**

The results from our evolutionary analyses indicated that duplication of an ancestral mitochondrial J-protein gene occurred prior to the divergence of the subphylum *Saccharomyces*, where one duplicate gave rise to the rapidly evolving *MDJ2* and the other give rise to *PAM18*, which subsequently gained a novel N-terminal IMS domain. Although a specific function of *Mdj2* is yet to be identified, the work reported within demonstrated that the IMS domain of *Pam18* is advantageous in the absence of oxygen.

**Evolution of *PAM18* and *MDJ2* Gene Duplicates**

Our analyses indicated that the emergence of the *PAM18* and *MDJ2* gene pair and the acquisition of the IMS domain of *Pam18* corresponded to an ancient gene duplication event. BLAST analyses clearly revealed two paralogs in all *Saccharomyces*, whereas all other fungi harbored only a single copy homolog. Phylogenetic reconstruction strongly supported two monophyletic clades of paralogs, each originating basal to the strongly supported two monophyletic clades of paralogs, all of *Pam18* corresponded to an ancient gene duplication event. Our analyses indicated that the emergence of the evolution of *MDJ2* and the deletion of the gene encoding another mitochondrial J-protein, *Mdj1*, a soluble matrix J-protein involved in protein folding (Westermann and Neupert 1997). However, perhaps due to differences in strain background, we could not reproduce this reported slight temperature sensitivity of *mdj1*-Δ *mdj2*-Δ cells relative to *mdj1*-Δ cells (data not shown). But, this lack of an identifiable phenotype does not rule out the possibility that *Mdj2* plays a significant role in the wild under conditions not typically used in the laboratory. Perhaps, it forms a low-abundance specialized translocation apparatus needed for efficient translocation of a subset of proteins under specific conditions. It is also plausible that only minor functional changes have evolved, and the observed increased rate of evolution is simply a by-product of a reduced level of gene expression and resultant relaxed negative selection pressures.

The mechanism by which one duplicate acquired the IMS domain after the *PAM18/MDJ2* gene duplication event is a matter of conjecture. Two extreme possibilities come to mind. First, the IMS domain might have been acquired by domain shuffling from a preexisting gene (Babushok et al. 2007). Arguing against this idea, our BLAST analysis, using the amino acid sequence of *S. cerevisiae* *Pam18* IMS domain as a query, revealed no region of significant similarity in other ORFs of the *S. cerevisiae* genome. Alternatively, a mutation(s) activating an in-frame AUG, or an ancestrally cryptic, start codon upstream from the original start codon of the ancestral gene might have been the instigating event for acquisition of the N-terminal extension forming the *Pam18* IMS domain. One piece of evidence consistent with such a scenario is the conserved methionine residue near the beginning of the transmembrane domain, present in all but one of the fungal homologs analyzed. Regardless of the mechanism, the ability to unambiguously colocalize the duplication event and origination of the novel IMS domain in its entirety, to such a short branch of the fungal tree, leads us to hypothesize that the duplication event itself was somehow key in the origin of the functionally novel IMS domain. Perhaps, the presence of the duplicate gene provisionally maintained essential mitochondrial import function while a specialized function of the novel domain evolved.

**The requirement of the *Pam18* IMS Domain under Anaerobic Conditions**

Results reported here reveal the importance of the IMS domain of *Pam18* for growth in the absence of oxygen. Mutations in genes encoding a variety of other components in the import apparatus had either little or no affect on growth in the absence, compared with the presence, of oxygen. In addition, another mutation in the region encoding the matrix domain of *Pam18* grew as well anaerobically, as aerobically. Thus, we conclude that the IMS domain carries...
out a function particularly required under anaerobic conditions and does not simply reflect a need for a more efficient function of the import apparatus.

A clue to the possible function of IMS domain of Pam18 comes from studies with aerobically grown cells, as this domain is able to interact with Tim17, one of the two proteins that make up the core translocon through which the translocating polypeptides transit (Chacinska et al. 2005; D’Silva et al. 2008). Given what is known about the mitochondrial protein translocation system and the differences in structure between mitochondria of anaerobically and aerobically grown cells, we can speculate as to the possible reasons that the Pam18 IMS domain is required under these particular environmental conditions. First, it has been reported that under aerobic conditions, Pam18 associates, perhaps directly, with the respiratory chain by binding to Complex III/IV in the inner membrane (van der Laan et al. 2006; Wiedemann et al. 2007), leading to the idea that this interaction either promotes the assembly of the translocation complex or stabilizes Pam18’s association with it (van der Laan et al. 2010). However, yeast cells do not possess a functional respiratory chain under anaerobic conditions (Groot et al. 1971; Rosenfeld et al. 2004). In addition, the lipid composition of the mitochondrial inner membrane is critical for maintaining the stability of the translocon complex and thus efficiency of the import process. For example, disruption of the synthesis of cardiolipin, a major component of the inner membrane, results in impairment of mitochondrial protein import (jiang et al. 2000) and destabilization of the import apparatus of the inner membrane (Kutik et al. 2008; Tamura et al. 2009) as well as other multimeric protein complexes in the inner membrane (Pfeiffer et al. 2003). Mitochondria isolated from anaerobically grown cells have a lower percentage of cardiolipin in their mitochondrial membranes than do mitochondria from aerobically grown cells (Paltauf and Schatz 1969), thus potentially leaving multimeric complexes, such as the translocon, more prone to dissociation. These observations raise the intriguing possibility that the IMS domain of Pam18 may be critical in the absence of oxygen to stabilize the association of the import motor with the translocon or to precisely position it relative to other components of the motor itself.

Regardless of the reason for the requirement of IMS domain of Pam18 when oxygen is eliminated from the environment, it is intriguing that comparative genomic analyses have previously identified several other differences in mitochondrial related functions between Saccharomyces and Pezizomycotina. For example, Saccharomyces, unlike the Pezizomycotina, exhibit unusually rapidly evolving mitochondrial ribosomal proteins and excess novel gene clusters localized to the mitochondria (Arvas et al. 2007) as well as multiple differences in the presence and absence of various subunits of oxidative phosphorylation complexes I–V (Lavin et al. 2008). In addition, Saccharomyces, but not the Pezizomycotina, have lost the ability of mitochondria to carry out fatty acid degradation via beta oxidation (Cornell et al. 2007; Shen and Burger 2009), whereas on the other hand, a regulatory mechanism causing rapid degradation of mRNA for proteins imported to the mitochondria via unique 3’ untranslated region binding sites is found only within the Saccharomyces (jiang et al. 2010). Ultimately these functional differences, along with the acquisition of the IMS domain of Pam18, may all play a role in the molecular diversification that underlies the overall differences in facultative anaerobic growth derived within the Saccharomyces and potentially the fermentative capabilities of the more derived Saccharomyces.

**Supplementary Materials**

Supplementary table S1 and supplementary figures S1 and S2 are available at Molecular Biology and Evolution online (http://www.mbe.oxfordjournals.org).

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**References**


