Maintenance of Mitochondrial Morphology Is Linked to Maintenance of the Mitochondrial Genome in *Saccharomyces cerevisiae*

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ABSTRACT

In the yeast *Saccharomyces cerevisiae*, certain mutant alleles of *YME4*, *YME6*, and *MDM10* cause an increased rate of mitochondrial DNA migration to the nucleus, carbon-source-dependent alterations in mitochondrial morphology, and increased rates of mitochondrial DNA loss. While single mutants grow on media requiring mitochondrial respiration, any pairwise combination of these mutations causes a respiratory-deficient phenotype. This double-mutant phenotype allowed cloning of *YME6*, which is identical to *MMM1* and encodes an outer mitochondrial membrane protein essential for maintaining normal mitochondrial morphology. Yeast strains bearing null mutations of *MMM1* have altered mitochondrial morphology and a slow growth rate on all carbon sources and quantitatively lack mitochondrial DNA. Extragenic suppressors of *MMM1* deletion mutants partially restore mitochondrial morphology to the wild-type state and have a corresponding increase in growth rate and mitochondrial DNA stability. A dominant suppressor also suppresses the phenotypes caused by a point mutation in *MMM1*, as well as by specific mutations in *YME4* and *MDM10*.

MITOCHONDRIA are dynamic structures that alter their morphology, cellular location, and metabolism in response to changes in cellular energy and metabolic needs (Hermann and Shaw 1998). Several different methods have been employed to unravel the cellular processes and components involved in the dynamic rearrangements of mitochondrial compartments, including electron and fluorescent microscopy coupled to biochemical and genetic analyses. Several nuclear-encoded gene products involved in mitochondrial dynamics have been identified in the yeast *Saccharomyces cerevisiae* by these methods, including Mdm1p (McConnell and Yaffe 1992), a protein with characteristics typical of intermediate filament proteins, and Mdm10p, Mdm12p, and Mmm1p (Burgess *et al.* 1994; Sogo and Yaffe 1994; Berger *et al.* 1997). This last trio of gene products is proposed to form a complex in the outer membrane of mitochondria and to mediate interactions with the yeast cytoskeleton (Boldogh *et al.* 1998). Recent studies with the *Neurospora crassa* homolog suggest a more general role for Mmm1p in maintaining mitochondrial morphology (Prokisch *et al.* 2000).

We have investigated the dynamic nature of the mitochondrial compartment by employing a genetic screen to identify gene products necessary for the maintenance of mitochondrial compartments and genome integrity in the yeast *S. cerevisiae* (Thorsness and Fox 1990, 1993). The nuclear gene *TRP1*, necessary for tryptophan biosynthesis, was integrated into the yeast mitochondrial genome and the nuclear *TRP1* gene was deleted. Such cells are tryptophan auxotrophs, but become tryptophan prototrophs when DNA containing *TRP1* is transferred from mitochondria to the nucleus. Mutation of nuclear genes can alter the rate at which mitochondrial DNA (mtDNA) is transferred to the nucleus, and these mutations have been named *yme*, for *y*east *m*itochondrial DNA *e*scape. Inactivation of *YME1* leads to a number of phenotypes, including an increased rate of mitochondrial DNA escape compared to wild-type yeast (Thorsness *et al.* 1993) and altered mitochondrial morphology (Campbell *et al.* 1994; Campbell and Thorsness 1998). Mitochondrial compartments in *yme1* cells lack the normal elongated and reticulated structures found in wild-type cells and instead are swollen, punctate structures. Yme1p is a metal- and ATP-dependent protease associated with the inner mitochondrial membrane (Nakai *et al.* 1995; Pearce and Sherman 1995; Leonhard *et al.* 1996; Weber *et al.* 1996). Yeast bearing mutations of *YME2*, which encodes an integral inner mitochondrial membrane protein, have a normal mitochondrial reticulum, a comparatively high rate of mtDNA escape, and, in combination with *yme1*, are deficient for growth on nonfermentable carbon sources (Hanekamp and Thorsness 1996).

Our continued analysis has identified at least three other *yme* genes. Here we report that the *yme4-1*, *mmm1-6*,

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TABLE 1

Yeast strains

Name	Genotype[a]	[R]eference
BFY1	*MAT*α *lys2 ura3-52 leu2-3,112 trp1-Δ1 yme4-1 mdm10-17 [ρ$^+$, TRP1]*	This study
BFY3	*MAT*α *lys2 ura3-52 leu2-3,112 trp1-Δ1 mmm1-6[b] mdm10-17 [ρ$^+$, TRP1]*	This study
CSY7	*MAT*α *lys2 ura3-52 leu2-3,112 trp1-Δ1 dnm1Δ-::kanMX6 [ρ$^+$, TRP1]*	This study
DUY17	*MAT*α *lys2 ura3-52 leu2-3,112 trp1-Δ1 mdm10-17 [ρ$^+$, TRP1]*	This study
IRY2	*MAT*α *lys2 ura3-52 leu2-3,112 trp1-Δ1 YNT61-5 [ρ$^+$, TRP1]*	This study
IRY26	*MAT*α *lys2 ura3-52 leu2-3,112 trp1-Δ1 YNT61-3 [ρ$^+$, TRP1]*	This study
IRY31	*MAT*α *lys2 ura3-52 leu2-3,112 trp1-Δ1 mmm1-Δ1::LEU2 ynt60-1 [ρ$^-$]*	This study
IRY32	*MAT***a** *ade2 ura3-52 leu2-3,112 trp1-Δ1 mmm1-Δ1::LEU2 ynt60-2 [ρ$^-$]*	This study
IRY33	*MAT*α *lys2 ura3-52 leu2-3,112 trp1-Δ1 mmm1-Δ1::LEU2 YNT61-1 [ρ$^+$, TRP1]*	This study
IRY34	*MAT***a** *ade2 ura3-52 leu2-3,112 trp1-Δ1 mmm1-Δ1::LEU2 YNT61-2 [ρ$^+$, TRP1]*	This study
IRY35	*MAT*α *lys2 ura3-52 leu2-3,112 trp1-Δ1 mmm1-Δ1::LEU2 YNT61-3 [ρ$^+$, TRP1]*	This study
IRY36	*MAT***a** *ade2 ura3-52 leu2-3,112 trp1-Δ1 mmm1-Δ1::LEU2 YNT61-4 [ρ$^+$, TRP1]*	This study
IRY37	*MAT*α *lys2 ura3-52 leu2-3,112 trp1-Δ1 mmm1-Δ1::LEU2 YNT61-5 [ρ$^+$, TRP1]*	This study
MDY1	*MAT*α *ade2 ura3-52 leu2-3,112 trp1-Δ1 mdm10-17 [ρ$^+$, TRP1]*	This study
MDY3	*MAT***a** *ade2 ura3-52 leu2-3,112 trp1-Δ1 mdm10-17 [ρ$^+$, TRP1]*	This study
MTY30	*MAT***a** *ade2 ura3-52 leu2-3,112 trp1-Δ1 mmm1-6[b]::URA3 mdm10-17 [ρ$^+$, TRP1]*	This study
PTY33	*MAT***a** *ade2 ura3-52 leu2-3,112 trp1-Δ1 [ρ$^+$, TRP1]*	Thorsness and Fox (1993)
PTY44	*MAT*α *lys2 ura3-52 leu2-3,112 trp1-Δ1 [ρ$^+$, TRP1]*	Thorsness and Fox (1993)
PTY53	*MAT*α *ade2 ura3-52 leu2-3,112 trp1-Δ1 yme4-1 mmm1-6[b] [ρ$^+$, TRP1]*	Thorsness and Fox (1993)
PTY60	*MAT***a** *ade2 ura3-52 leu2-3,112 trp1-Δ1 yme1-Δ1::URA3 [ρ$^+$, TRP1]*	Campbell *et al.* (1994)
PTY67	*MAT***a** *ade2 ura3-52 leu2-3,112 trp1-Δ1 yme4-1 [ρ$^+$, TRP1]*	Shafer *et al.* (1999)
PTY68	*MAT*α *lys2 ura3-52 leu2-3,112 trp1-Δ1 yme4-1 [ρ$^+$, TRP1]*	Thorsness and Fox (1993)
PTY71	*MAT***a** *ade2 ura3-52 leu2-3,112 trp1-Δ1 mmm1-6[b] [ρ$^+$, TRP1]*	This study
PTY72	*MAT*α *lys2 ura3-52 leu2-3,112 trp1-Δ1 mmm1-6[b] [ρ$^+$, TRP1]*	Thorsness and Fox (1993)
PTY104	*MAT*α *lys2 ura3-52 leu2-3,112 trp1-Δ1 yme4-1 mmm1-6[b] [ρ$^+$, TRP1]*	This study
THY14	*MAT***a** *ade2 ura3-52 leu2-3,112 trp1-Δ1 mmm1-6[b]::URA3 [ρ$^+$, TRP1]*	This study
THY18	*MAT***a** *ade2 ura3-52 leu2-3,112 trp1-Δ1 yme2-Δ1 [ρ$^+$, TRP1]*	This study
THY22	*MAT***a** *ade2 ura3-52 leu2-3,112 trp1-Δ1 mmm1-Δ1 [ρ°]*	This study
THY23	*MAT*α *lys2 ura3-52 leu2-3,112 trp1-Δ1 mmm1-Δ1::LEU2 [ρ°]*	This study
THY24	*MAT***a** *ade2 ura3-52 leu2-3,112 trp1-Δ1 mmm1-Δ1::LEU2 [ρ°]*	This study
THY162	*MAT***a** *ade2 ura3-52 leu2-3,112 trp1-Δ1 yme9-Δ1::LEU2 [ρ$^+$, TRP1]*	This study

[a] All strains are isogenic and were derived from D273-10B. The mitochondrial genotype is bracketed.
[b] The *mmm1-6* allele was previously named *yme6-1*.

and *mdm10-17* mutations, in addition to increasing the rate of mtDNA escape to the nucleus, alter mitochondrial morphology in a carbon-source-specific fashion, with a coincident destabilization of the mitochondrial genome. Genetic and microscopy-based analyses lead us to propose an important role for the corresponding gene products in both the maintenance of mitochondrial morphology and the maintenance of mtDNA.

MATERIALS AND METHODS

Strains and media: The genotypes of the *S. cerevisiae* strains used in this study are listed in Table 1 and are isogenic to D273-10B. *Escherichia coli* strains employed for the preparation and manipulation of plasmid DNA were DH5α F$^-$, GM2163 (New England Biolabs, Beverly, MA) and XL1-Blue (Stratagene, La Jolla, CA). *E. coli* strains carrying plasmids were grown in Luria broth (Sambrook *et al.* 1989) supplemented with 125 μg/ml of ampicillin. Yeast were grown in complete dextrose medium (YPD), complete ethanol/glycerol medium (YPEG), or synthetic dextrose medium (SD) with nutrients as indicated (Thorsness and Fox 1993). Geneticin-resistant growth was assayed on YPD containing 300 μg/ml geneticin (Sigma, St. Louis).

Isolation of *MMM1*: Colonies from a ρ$^+$ *yme4-1 mmm1-6* double-mutant strain (PTY53) were grown in YPD broth and transformed with a yeast genomic DNA library (Rose *et al.* 1987). Transformants were selected on SD medium lacking uracil and then replica plated onto YPEG medium to select for respiratory-competent colonies. Plasmid DNA from potential candidates was isolated, amplified in *E. coli*, and introduced into *yme4-1 mmm1-6* yeast (PTY53) to verify complementation of the respiratory-growth defect and also into *yme4-1* (PTY68) and *mmm1-6* (PTY72) yeast strains to test for complementation of the high rate of mtDNA escape phenotype.

Plasmid and strain constructions: An *mmm1* minimal complementing construct, designated pRSMY6, was generated by subcloning a 2.1-kb *Bcl*I sequence from a DNA library clone, pH 1, into the *Bam*HI sites of the *CEN/ARS* yeast shuttle vector pRS316 (Sikorski and Hieter 1989). The same insert DNA was cloned into the integration vector pRS306, generating pRSIY6, which was used to establish linkage between *MMM1* and the cloned DNA sequence. pRSIY6 was also used to generate a *URA3*-marked *mmm1-6* allele. The plasmid was linearized within the *MMM1* coding sequences via digestion with *Nru*I and transformed into *mmm1-6* yeast (PTY72). Ura$^+$ trans-

formants were mated to wild type (PTY33), sporulated, and dissected to generate the strain THY14, in which a high rate of mtDNA escape was linked to *URA3* (*mmm1-6::URA3*).

Null alleles of *MMM1* were constructed by subcloning a 2.1-kb *Xho*I/*Xba*I fragment from pRSIY6 into Bluescript KS$^+$ (Stratagene). The ensuing construct, pBSY6, was digested with *Nru*I and *Msc*I to remove 787 bp of coding DNA sequences from *MMM1*. A *URA3*-disrupted *mmm1* allele was constructed by inserting a 3.8-kb *Bam*HI/*Xba*I fragment containing a *hisG::URA3::hisG* cassette from pBS31 (Alani *et al.* 1987) into the *Nru*I/*Msc*I sites of the pBSY6 vector backbone. The resulting construct, pBY6HUH, was digested with *Eco*RI and *Sac*II to release the 5.1-kb insert sequence and transformed into PTY33xPTY44 diploid yeast. An unmarked *mmm1* null allele was generated by repeatedly streaking a *hisG::URA3::hisG*-disrupted *mmm1* transformant onto 5-fluoroorotic acid (5-FOA) to select for uracil auxotrophs. A *LEU2*-disrupted *mmm1* allele was constructed by inserting a 2.2-kb *Sma*I fragment containing a *LEU2* cassette from pPHB6 into the *Nru*I/*Msc*I sites of pBSY6 to create pBY6LEU. The 3.4-kb insert sequence was released via *Sma*I/*Not*I digestion and transformed into haploid (PTY44) and diploid (PTY33xPTY44) yeast. Successful disruption of *MMM1* in yeast with all three alleles was verified by PCR using the forward primer YME6-F2 (5′-GCG GAT CCT TGG CAA TTA TCA CAC AA-3′) and the reverse primer YME6-R1 (5′-GCG GAT CCT GCA ATT GTA CGC GGG CAT-3′).

The *mdm10-17* mutant allele was generated by replacing the chromosomal *MDM10* locus with the full-length *MDM10* open reading frame (ORF) fused in frame at the 3′-end to an ORF encoding an epitope tag (Longtine *et al.* 1998). This tag, three tandem repeats of the influenza virus hemagglutinin epitope (3HA), increases the theoretical size of Mdm10p by ~8 kD. The oligonucleotides used to generate this PCR construct were F1-MDM10 (5′-TTT CCC GGC AAA GTT TGG CAT ACA ATT CCA GTA CTC CAC ACG GAT CCC CGG GTT AAT TAA-3′) and R1-MDM10 (5′-GAC ACA AAA GAT AAG GTG TTC GGT TAC TTT ATT CTG CTT TGA ATT CGA GCT CGT TTA AAC-3′). The PCR product, which also contained a geneticin resistance gene, was used to transform PTY44 to geneticin resistance, and the resistant colonies were screened by PCR to verify the chromosomal structure at the *MDM10* locus.

PCR amplification of *mmm1-6* and characterization of the PCR-generated allele: Genomic DNA was isolated from an *mmm1-6* mutant strain (PTY72). PCR primers YME6-F2 and YME6-R1 were employed to PCR amplify the mutant *mmm1-6* allele in 10 independent reactions using *Taq*-DNA polymerase. PCR products were pooled and sent to the Iowa State Sequencing Facility (Ames, IA) for automated sequencing. The *mmm1-6* allele was also amplified with *Pfu*-DNA polymerase using the same primers, digested with *Bam*HI, and cloned into the *Bam*HI site of the *LEU2*-marked shuttle vector pRS315. The resulting construct, pRSY6-1, was transformed into an *mmm1* deletion strain (THY22) carrying a complementing *MMM1* gene on a *URA3/CEN* plasmid (pRSMY6) to ensure maintenance of a ρ^+ mitochondrial genome. Transformants were selected and purified on SD medium lacking leucine. Leu$^+$ transformants were streaked on 5-FOA to select against pRSMY6, and Leu$^+$ ura$^-$ transformants were tested for mtDNA escape.

Determination of ρ^-/ρ° frequencies: Respiratory-competent cells of wild-type and mutant strains were grown in YPEG broth overnight, diluted into YPD, and incubated until a Klett of 100 (~1×10^7 cells/ml) was reached. Serial dilutions were prepared and cell suspensions were spread onto YPD plates at a 1:50,000 dilution. After outgrowth at 30° for 2–3 days, colonies were overlaid with tetrazolium as described (Donnini *et al.* 1990) and respiring (red) and nonrespiring (white) colonies were scored. In parallel, serial platings of mutant strains carrying an *ade2* mutation were performed. Colony color on YPD medium was utilized to score respiratory competence (red colonies) or incompetence (white colonies) of emerging colonies.

Microscopic analysis of mitochondrial morphology: To visualize the mitochondrial compartment, yeast were transformed with a plasmid that expressed the green fluorescent protein targeted to the mitochondrial matrix (Otsuga *et al.* 1998). Cells were grown overnight on the appropriate solid medium (YPD or YPEG), mounted on a glass slide, and observed with a confocal fluorescence microscope. To assess changes in mitochondrial morphology in response to changes in carbon source, cells were grown on solid YPD overnight and then shifted to YPEG. Alternately, cells were first grown on solid YPEG overnight and then shifted to YPD. In both cases, cells were stained with DiOC6 (Koning *et al.* 1993) immediately, and at various time points after the carbon source shift, and observed on a fluorescence microscope.

Identification and analysis of suppressors of the petite phenotype of *mmm1Δ*: Eighteen single colonies of an *mmm1-Δ1::LEU2* strain (THY23 or THY24) were grown overnight at 30° in 2 ml SD broth without leucine. Serial dilutions were prepared and plated onto SD plates without leucine. Fast-growing colonies were isolated, colony purified on the same medium, and backcrossed with an *mmm1-Δ1* deletion of the opposite mating type to determine whether the suppressing mutation was dominant or recessive. Suppressor strains were also backcrossed with wild type (PTY33 and PTY44) to analyze complementation and linkage groups and to screen for collateral phenotypes of the suppressing mutations. Strains that carried *YNT61-3* (IRY26) and *YNT61-5* (IRY2) in a wild-type background were used to test whether these mutations suppress the phenotypes of *mmm1-6*, *mdm10-17*, and *yme4-1* mutants. Specifically, IRY26 and IRY2 were crossed to THY14 (*mmm1-6::URA3*), to MDY3 (*mdm10-17*), and to PTY67 (*yme4-1*). Diploids were sporulated, tetrads were dissected, and the resulting spores were analyzed for mtDNA escape and mitochondrial morphology. IRY26 and IRY2 were also crossed to MTY30 (*mmm1-6::URA3 mdm10-17*). Diploids were sporulated, tetrads dissected, and the resulting spores were assayed for growth on YPEG, for mtDNA escape, and for mitochondrial morphology.

RESULTS

Phenotypes of *yme4-1*, *mmm1-6*, and *mdm10-17* and double-mutant strains: Mutant yeast strains bearing the *yme4-1* and *mmm1-6* alleles were generated via chemical mutagenesis and isolated on the basis of their high rate of mtDNA escape to the nucleus (Thorsness and Fox 1993). The *mmm1-6* allele was originally designated *yme6-1* (Thorsness and Fox 1993). However, we found that *YME6* was allelic with *MMM1* (see below) and adopted that nomenclature. The *mdm10-17* mutant allele was generated by replacing the chromosomal *MDM10* locus with the full-length *MDM10* open reading frame fused in frame at the 3′-end to an ORF encoding an epitope tag (Longtine *et al.* 1998). This tag, three tandem repeats of 3HA, increases the theoretical size of Mdm10p by ~8 kD.

Yeast strains bearing a *yme4-1*, *mmm1-6*, or *mdm10-17* mutant allele exhibit a high rate of mtDNA escape to the nucleus when compared to an isogenic wild-type

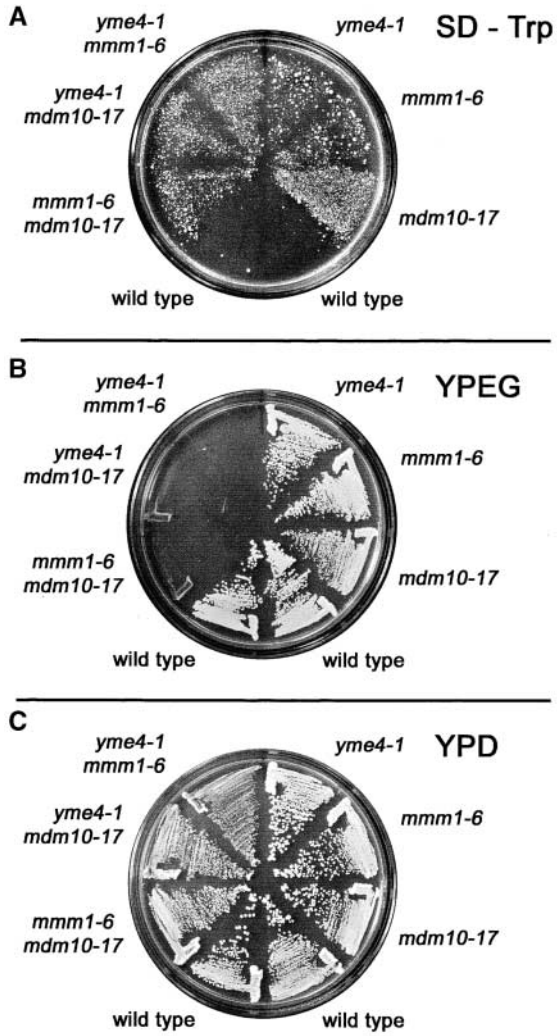

FIGURE 1.—Mitochondrial DNA escape and growth phenotypes of yeast-bearing *yme4-1*, *mmm1-6*, and *mdm10-17* alleles. (A) Relative rates of mtDNA escape to the nucleus. Yeast strains with the indicated mutant loci were grown as patches on complete glucose media at 30° for 2 days and then replica plated to minimal glucose media lacking tryptophan (SD-Trp) and incubated for 5 days at 30°. The number of papillae growing in each sector reflects the rate of transfer of mtDNA to the nucleus for that strain. (B) Growth on nonfermentable carbon sources. Yeast strains with the indicated mutant loci were streaked onto YPEG and incubated for 5 days at 30°. (C) Growth on fermentable carbon source. Yeast strains with the indicated mutant loci were streaked onto YPD and incubated for 3 days at 30°. Strains: *yme4-1*, PTY68; *mmm1-6*, PTY72; *mdm10-17*, DUY17; wild type, PTY44; *mmm1-6 mdm10-17*, BFY3; *yme4-1 mdm10-17*, BFY1; *yme4-1 mmm1-6*, PTY104.

parent (Figure 1A). While these single-mutant strains grow at approximately the same rate as wild-type strains on nonfermentable media, all double mutants grow very slowly on a nonfermentable carbon source (Figure 1B) and grow noticeably more slowly than wild type on fermentable media (Figure 1C). Additionally, all three single mutants have a high rate of formation of ρ^- and ρ° cells when grown on a nonfermentable carbon source (Figure 2A). This is particularly striking because yeast require an intact mitochondrial genome (ρ^+) to grow on nonfermentable carbon sources. However, growth of these mutant strains on permissive complete glucose media did not significantly alter the rate of ρ^-/ρ° formation compared to wild type (Figure 2A). This rate is ~10-fold lower when mutants are grown on glucose than when they are grown on nonfermentable media. Strains bearing any pair of mutant alleles experience a very high rate of ρ^-/ρ° formation (~90%) when grown on glucose. Deletion of *DNM1*, a gene required for the fission of mitochondrial compartments (BLEAZARD *et al.* 1999; SESAKI and JENSEN 1999), causes a similarly high rate of ρ^-/ρ° formation during growth on ethanol/glycerol media (Figure 2A). In contrast, inactivation of *YME9* led both to an increase in the escape of mtDNA to the nucleus (data not shown) and to a high rate of ρ^-/ρ° formation when cells were cultured on complete glucose media, but not when cultured on ethanol/glycerol media (Figure 2A). An increase in the rate of escape of mtDNA to the nucleus does not, by itself, increase the rate of ρ^-/ρ° formation, since other mutations that increase the rate of mtDNA escape to the nucleus, *YME1* and *YME2* (HANEKAMP and THORSNESS 1996; THORSNESS and FOX 1993; THORSNESS *et al.* 1993), do not increase the rate of ρ^-/ρ° formation. When compared to wild type, the percentage of cytoplasmic petites (ρ^- and ρ° cells) that are ρ° is as much as 4-fold higher in yeast mutants that have an increased rate of mtDNA escape to the nucleus, regardless of their propensity to become cytoplasmic petites (Figure 2B).

The single-mutant *yme4-1*, *mmm1-6*, and *mdm10-17* strains also display severe mitochondrial morphology defects on nonfermentable carbon sources, but not on glucose media. Wild-type, *yme4-1*, *mmm1-6*, and *mdm10-17* yeast grown on glucose media exhibit well-defined reticulated mitochondrial networks (Figure 3). This mitochondrial morphology does not change substantially when wild-type yeast are grown on a nonfermentable carbon source. In contrast, *yme4-1*, *mmm1-6*, and *mdm10-17* strains accumulate intensively stained larger aggregates of mitochondria when grown on ethanol/glycerol media (Figure 3). At least for *mdm10-17* strains, these morphological defects appear to accumulate as cells divide, since changes in mitochondrial morphology are not observed until several hours after cells are shifted from glucose to ethanol/glycerol media. In contrast, restoration of the normal reticulated network occurs more rapidly, initiating within 2 hr of shifting cells from ethanol/glycerol to glucose media (data not shown). Severe mitochondrial morphology defects are also apparent in *yme4-1 mmm1-6*, *yme4-1 mdm10-17*, and *mmm1-6 mdm10-17* double mutants when grown on complete glucose media (Figure 3).

Cloning, identification, and analysis of *MMM1*: The growth defect of *yme4-1 mmm1-6* strains on nonfermentable carbon sources allowed isolation of DNA sequences

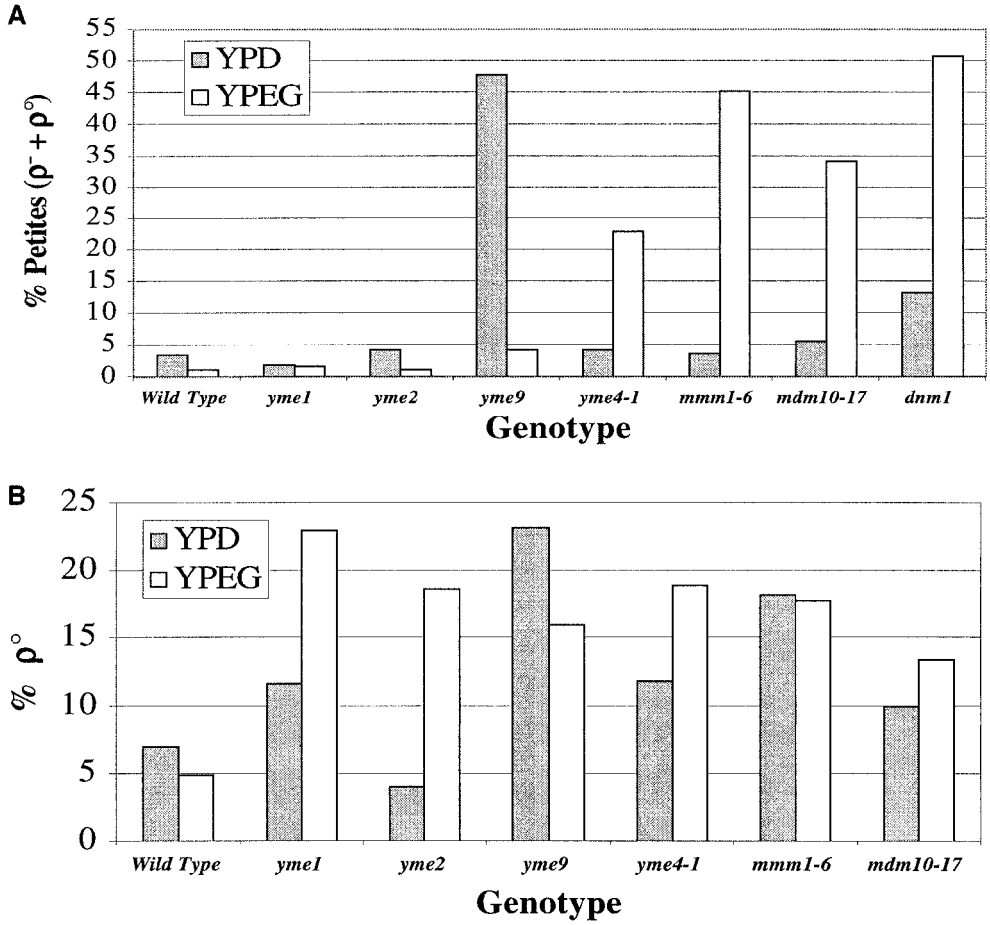

FIGURE 2.—Cytoplasmic petite formation in yeast bearing mutations in genes that affect mitochondrial morphology. (A) Percentage of cytoplasmic petites in culture. Colonies were grown from single cells on the media indicated, and the proportion of cytoplasmic petite cells ($\rho^- + \rho^\circ$) was determined as described in MATERIALS AND METHODS. At least 10 independent colonies were surveyed for cells grown on YPD and YPEG. At least 100 cells from each colony were assessed for ρ^+ or ρ^-/ρ° character. (B) Percentage of ρ° cells in a population of cytoplasmic petites ($\rho^- + \rho^\circ$). Cytoplasmic petites, arising independently from ρ^+ cells, were assessed for the presence (ρ^-) or absence (ρ°) of mtDNA by staining cells with DAPI and observing them with a fluorescence microscope. The presence of brightly staining, extranuclear foci in an isolate was scored as ρ^- and its absence as ρ°. Each value on the graph represents the percentage of ρ° isolates found in 100 independently isolated cytoplasmic petites. Strains: wild type, PTY33; *yme1*Δ, PTY60; *yme2*Δ, THY18; *yme9*Δ, THY162; *yme4-1*, PTY67; *mmm1-6*, PTY71; *mdm10-17*, MDY1; *dnm1*Δ, CSY7.

that complement that phenotype (data not shown). All complementing clones were derived from the same genetic locus and rescued the synthetic *pet*$^-$ phenotype of the double mutant and the high rate of mtDNA escape phenotype of *mmm1-6* strains but not of *yme4-1* strains. Linkage analysis of a clone of this locus confirmed that the mtDNA escape phenotype observed in *mmm1-6* mutants was linked to the cloned DNA. DNA sequence analysis of the minimal complementing DNA fragment revealed that it encodes *MMM1*, a gene previously demonstrated to have a role in the maintenance of mitochondrial morphology and segregation of mitochondria into daughter cells (BURGESS *et al.* 1994).

Null mutants of *MMM1* (*mmm1*Δ) were constructed and found to be respiratory incompetent and to grow very slowly even on complete glucose media, suggesting that the *mmm1-6* allele is a missense mutation. These phenotypes are complemented by a plasmid carrying *MMM1* (data not shown). While *mmm1-6* strains display elevated levels of mtDNA escape compared to wild type, *mmm1*Δ strains show no mtDNA escape (data not shown). The complete absence of mtDNA escape suggested that *mmm1*Δ strains are quantitatively ρ°. Several observations supported this hypothesis. First, diploids derived from a cross between a ρ° wild-type strain and an *mmm1*Δ strain are unable to respire. Second, *mmm1*Δ spores dissected from an *MMM1*x*mmm1*Δ heterozygous diploid do not grow on nonfermentable media. Finally, *mmm1*Δ spores germinated on glucose do not have mtDNA [assessed by 4′,6-diamidino-2-phenylindole (DAPI) staining and fluorescent microscopy; data not shown]. Hence, we conclude that *mmm1*Δ strains are completely devoid of mtDNA, consistent with the observed rapid loss of mitochondrial DNA from yeast bearing a temperature-sensitive allele of *MMM1* when the strain is cultured at the restrictive temperature (HOBBS *et al.* 2001).

Strains deleted for *MMM1* form giant mitochondria that are preferentially localized at the cell periphery (BURGESS *et al.* 1994). We reproduced these observations using confocal microscopy of *mmm1*Δ strains carrying mitochondrially localized green fluorescent protein (Figure 3). Comparison of the mitochondrial morphology of *mmm1*Δ strains with the double-mutant

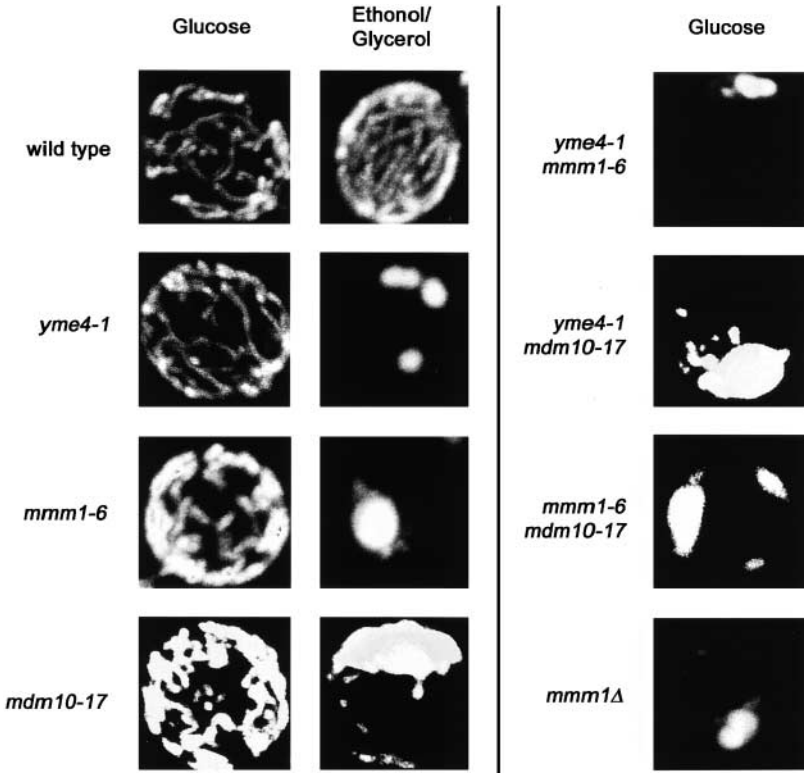

FIGURE 3.—Mitochondrial morphology of yeast-bearing *yme4-1*, *mmm1-6*, and *mdm10-17* alleles. Yeast expressing green fluorescent protein targeted to the mitochondrial matrix were cultured on complete glucose or complete ethanol/glycerol media overnight. Cells were then mounted on a glass slide and observed with a confocal fluorescence microscope. The images shown are projections of, on average, 10 optical sections and are of a single yeast. Strains: wild type, PTY44; *yme4-1*, PTY68; *mmm1-6*, PTY72; *mdm10-17*, DUY17; *yme4-1 mmm1-6*, PTY104; *yme4-1 mdm10-17*, BFY1; *mmm1-6 mdm10-17*, BFY3; *mmm1*Δ, THY23.

strains *yme4-1 mmm1-6*, *yme4-1 mdm10-17*, and *mmm1-6 mdm10-17* and the single-mutant strains *mmm1-6*, *yme4-1*, and *mdm10-17* grown on nonfermentable carbon sources revealed similar defects (Figure 3).

The difference in phenotypes observed in *mmm1*Δ and *mmm1-6* strains indicated that the *mmm1-6* mutation is not simply a null allele of *MMM1* but rather is a mutation that alters the *MMM1* gene product to affect both mtDNA maintenance and mitochondrial morphology. The *mmm1-6* allele was recovered from the chromosome and its DNA sequence change determined. The *mmm1-6* mutation changes codon 215 from GAG (Glu) to AAG (Lys). This residue is an aspartate in the *MMM1* homologs found in *Schizosaccharomyces pombe* and *N. crassa* (PROKISCH *et al.* 2000), which conserves the negative charge of wild-type *MMM1* from *S. cerevisiae*. To test whether we had identified the mutation that is responsible for the mtDNA escape phenotype, a PCR-amplified *mmm1-6* mutant allele (pRSY6-1) was introduced into an *mmm1* deletion strain. Subsequent mtDNA escape assays showed that the introduced *mmm1-6* allele promoted a high rate of mtDNA escape while complementing the slow-growth phenotype of the *mmm1*Δ allele (data not shown).

Suppression of *mmm1*Δ: *mmm1*Δ strains grow slowly on glucose media, and faster-growing segregants are readily identified (Figure 4A). Seven independent *mmm1*Δ strains that grew at a faster rate on glucose media than did the parent *mmm1*Δ strain were isolated. Five strains had dominant suppressing mutations and two bore recessive suppressing mutations, as assessed via mating with an *mmm1*Δ strain of the opposite mating type. Each suppressed strain was also crossed to an isogenic wild-type strain and sporulated, tetrads were dissected, and spores were analyzed for the presence of *mmm1*Δ and the extragenic suppressor. In all of the suppressed strains, the suppressing mutation segregated as a single genetic locus, since one-half of all spores with the *mmm1*Δ allele were suppressed. The different suppressors were mated to each other and subjected to tetrad analysis. The five dominant suppressors mapped to the same genetic locus, *YNT61*. The two recessive suppressors were alleles of a second locus, *YNT60*. No *YNT60* or *YNT61* allele caused identifiable defects in cell growth, mtDNA escape, or mitochondrial morphology when isolated away from the *mmm1*Δ mutation (data not shown).

The original suppressed *mmm1*Δ isolates were ρ° and unable to respire. When mtDNA was introduced via a cross with a ρ^{+} strain, *mmm1*Δ strains bearing *YNT61* mutations respired (Figure 4B). Escape of mtDNA to the nucleus was higher in *YNT61 mmm1*Δ strains than in wild-type or *mmm1-6* yeast (Figure 4C). Furthermore, suppressing mutations in *YNT61* partially restored normal mitochondrial morphologies in an *mmm1*Δ background (Figure 5).

Two *YNT61* alleles, *YNT61-3* and *YNT61-5*, were tested for their ability to suppress the phenotypes of the *mmm1-6*, *mdm10-17*, and *yme4-1* mutations. In a cross between a *YNT61-3* strain and an *mdm10-17* strain, the high rate of mtDNA escape phenotype of the *mdm10-17* mutation is suppressed in one-half of the *mdm10-17* spores (Figure 6A). The mitochondrial morphology defect is par-

tially suppressed in those *mdm10-17* spores predicted to carry *YNT61-3* on the basis of suppression of mtDNA escape (data not shown). Similarly, *YNT61-3* suppressed the high rate of mtDNA escape of the *mmm1-6* mutation (Figure 6A) and partially suppressed the high rate of mtDNA of the *yme4-1* mutation (data not shown). The mitochondrial morphology defect of the *mmm1-6* and *yme4-1* mutants is less penetrating than that of the *mdm10-17* mutation, and so it was difficult to assess the effect of *YNT61-3* on mitochondrial morphology in these mutants. Finally, the *YNT61-3* mutation suppressed the YPEG growth and mitochondrial morphology defects of the *mmm1-6 mdm10-17* double mutant (Figure 6B and data not shown). One-half of the double-mutant spores from a cross of *YNT61-3* to *mmm1-6 mdm10-17* were suppressed. *YNT61-3* does not, however, significantly suppress the high rate of mtDNA escape of the *mmm1-6 mdm10-17* double mutant (data not shown). *YNT61-5* also suppressed the *mdm10-17*, *mmm1-6*, and *yme4-1* mutant phenotypes, although not as strongly as *YNT61-3* did (data not shown).

Two recessive suppressors, *ynt60-1* and *ynt60-2*, did not rescue the respiratory growth defect associated with *mmm1*Δ, even after introduction of a ρ^+ mitochondrial genome via mating (Figure 4B). The mitochondrial genome was apparently converted to ρ^- in *ynt60 mmm1*Δ mutants. DAPI staining demonstrated the presence of mtDNA, but the suppressed strains were unable to utilize nonfermentable carbon sources when mated to a wild-type ρ° strain. Mitochondrial morphology was, however, partially repaired in these recessive mutants (Figure 5). Because these recessive suppressors weakly suppressed only the *mmm1*Δ deletion phenotypes, their effect on other *yme* genes and alleles was not assessed.

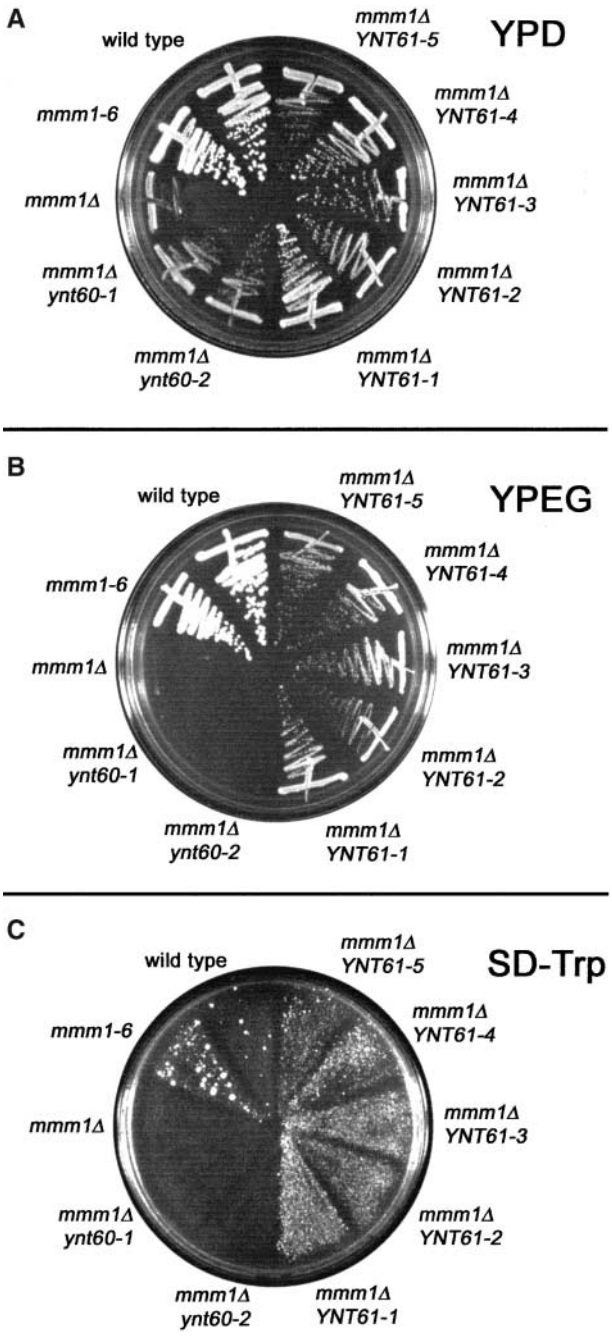

FIGURE 4.—Extragenic suppressors of *mmm1*Δ. (A) Yeast with the indicated mutant loci were streaked onto YPD and incubated at 30° for 3 days. (B) Yeast with the indicated mutant loci were streaked onto YPEG and incubated at 30° for 5 days. (C) Yeast with the indicated mutant loci were grown as patches on complete glucose media at 30° for 2 days, then replica plated to minimal glucose media lacking tryptophan (SD-Trp), and incubated at 30° for 5 days. The number of papillae in each sector reflects the rate of mtDNA escape to the nucleus for that strain of yeast. Strains: wild type, PTY44; *mmm1-6*, PTY72; *mmm1*Δ, THY23; *mmm1*Δ *ynt60-1*, IRY31; *mmm1*Δ *ynt60-2*, IRY32; *mmm1*Δ *YNT61-1*, IRY33; *mmm1*Δ *YNT61-2*, IRY34; *mmm1*Δ *YNT61-3*, IRY35; *mmm1*Δ *YNT61-4*, IRY36; *mmm1*Δ *YNT61-5*, IRY37.

DISCUSSION

The identical phenotypes of *yme4-1*, *mmm1-6*, and *mdm10-17* mutants with respect to carbon-source-dependent alteration of mitochondrial morphology (Figure 3) and mtDNA stability (Figures 1 and 2) lead us to propose that Yme4p, Mmm1p, and Mdm10p function together to maintain proper mitochondrial structure and to assure transmission of mtDNA to daughter cells in the yeast *S. cerevisiae*. Additionally, the synthetic phenotypes of double-mutant strains, which approximate the phenotypes found in *MMM1* or *MDM10* deletion strains (BURGESS *et al.* 1994; SOGO and YAFFE 1994), indicate close cooperation of these gene products in these processes. Finally, the ability of *YNT61* to suppress defects in all three genes (Figure 6) also suggests cooperation among Yme4p, Mmm1p, and Mdm10p. Unfortunately, the nature of the protein encoded by *YME4* remains elusive, as the gene has been refractory to cloning efforts. A powerful genetic screen exists for the isolation of *YME4* DNA by complementation of the respiratory-

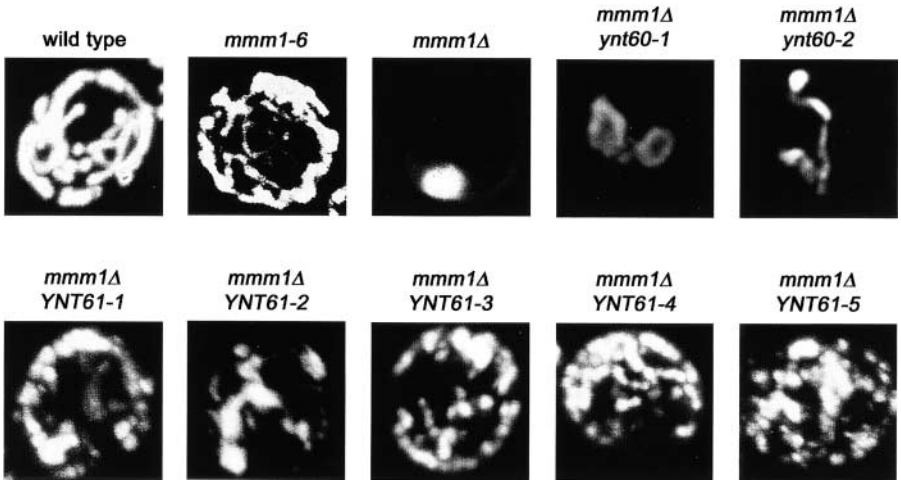

FIGURE 5.—Mitochondrial morphology of *mmm1*Δ yeast bearing suppressing mutations. Yeast were transformed with a plasmid that expressed green fluorescent protein targeted to the mitochondrial matrix. Cells were cultured on complete glucose media overnight, then mounted on a glass slide and observed with a confocal fluorescence microscope. The images shown are projections of, on average, 10 optical sections and are of a single yeast. Strains: wild type, PTY44; *mmm1-6*, PTY72; *mmm1*Δ, THY23; *mmm1*Δ *ynt60-1*, IRY31; *mmm1*Δ *ynt60-2*, IRY32; *mmm1*Δ *YNT61-1*, IRY33; *mmm1*Δ *YNT61-2*, IRY34; *mmm1*Δ *YNT61-3*, IRY35; *mmm1*Δ *YNT61-4*, IRY36; *mmm1*Δ *YNT61-5*, IRY37.

defective phenotype of *yme4-1 mmm1-6* or *yme4-1 mdm10-17* strains. However, we have been unable to isolate *YME4* from several different libraries, while repeatedly isolating *MMM1* and *MDM10*. We do know that *yme4-1* is unlinked to *MMM1*, *MDM10*, or *MDM12* or to any loci reported to have genetic interactions with these genes.

Mmm1p has an important role in the maintenance of mtDNA (HOBBS *et al.* 2001). It is localized in the outer membrane in coordination with a subset of mtDNA nucleoids in the mitochondrial matrix. Additionally, the presence and nature of mtDNA is strictly correlated with changes in mitochondrial morphology when yeast bearing a temperature-sensitive allele of *MMM1* are shifted to the restrictive condition (HOBBS *et al.* 2001). Our work supports these observations and extends the role for the simultaneous maintenance of mitochondrial morphology and DNA to the gene products of *YME4* and *MDM10*. The specific mutations in *MMM1*, *YME4*, and *MDM10* described in this work cause destabilization of the mitochondrial genome, increasing both the frequency at which mtDNA sequences are transferred to the nucleus (Figure 1) and the frequency of ρ^-/ρ° formation (Figure 2). The destabilization of mtDNA in these mutants parallels the appearance of mitochondrial morphology defects (Figure 3). The carbon-source-dependent acquisition of defects in both mitochondrial morphology and mtDNA stability in *yme4-1*, *mmm1-6*, and *mdm10-17* single mutants is particularly striking. One hypothesis to explain this observation is that in these mutants the increased mitochondrial activity required by respiration induces morphological changes that lead to mtDNA instability.

YNT61, isolated as a dominant suppressor of the *mmm1*Δ slow-growth phenotype (Figure 4), alters the aberrant mitochondrial morphology of *mmm1*Δ strains (Figure 5) as well as of *mdm10-17* and *mmm1-6 mdm10-17* strains. Suppressing mutations in *YNT61* allow mitochondrial morphology that approximates that observed in wild-type cells. This suppressor also allows the maintenance of intact mitochondrial genomes in *mmm1*Δ strains (Figure 4B), suppresses the high rate of mtDNA escape in *mmm1-6*, *mdm10-17*, and *yme4-1* mutants (Figure 6A), and suppresses the respiratory growth defect of *mmm1-6 mdm10-17* double mutants (Figure 6B). Interestingly, *dnm1*Δ strains, which develop a giant mitochondrial reticulum (BLEAZARD *et al.* 1999; SESAKI and JENSEN 1999), also experience genomic instability when cultured on nonfermentable carbon sources (Figure 2A). Strains that lack Fzo1p, an outer mitochondrial membrane protein required for fusion of mitochondrial compartments, have small, fragmented mitochondrial compartments and are cytoplasmic petites (RAPAPORT *et al.* 1998). Collectively, these mutations demonstrate the relationship between the maintenance of wild-type (proper) mitochondrial morphology and mitochondrial genome integrity.

The transmission of mtDNA to subsequent generations is a complex process, employing numerous biochemical and structural components (BERGER and YAFFE 2000). At the grossest level, transmission of mtDNA to daughter cells requires replication of DNA and inheritance of mtDNA during bud growth and cytokinesis. The formation of aberrant mitochondrial structures in *mmm1*, *mdm10*, and *yme4* strains (Figure 3), which are consequently inefficiently transmitted to daughter cells (BURGESS *et al.* 1994; SOGO and YAFFE 1994), may directly interfere with mitochondrial DNA inheritance. Alternatively, the outer membrane proteins Mmm1p and Mdm10p, and by extension Yme4p, may be indirectly involved in the synthesis of mtDNA. Their role in maintaining proper mitochondrial morphology may be necessary for the process of replication.

The slow-growth phenotype of *mmm1*Δ, *yme4-1 mmm1-6*, *yme4-1 mdm10-17*, and *mmm1-6 mdm10-17* strains on complete glucose media (Figure 1) is likely a direct result of their aberrant mitochondrial morphology (Figure 3). Inheritance of mitochondrial compartments is an important step in bud growth, and cytokinesis is delayed until mitochondrial compartments take up residence in the bud (MCCONNELL *et al.* 1990). Sup-

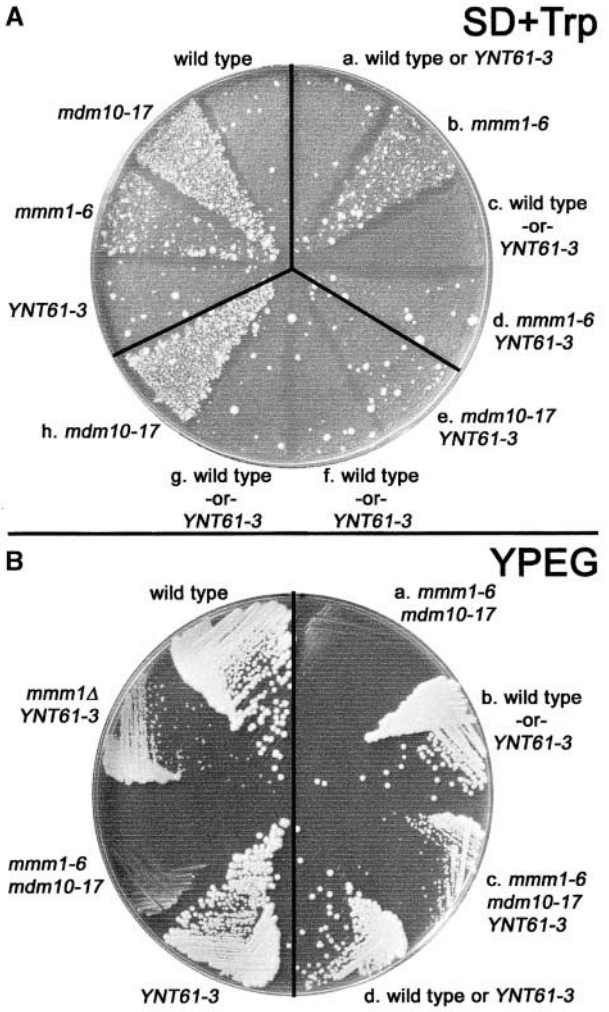

FIGURE 6.—Suppression of *mmm1-6*, *mdm10-17*, and *mmm1-6 mdm10-17* phenotypes by *YNT61-3*. (A) Relative rates of mtDNA escape to the nucleus. Yeast strains with the indicated mutant loci were grown as patches on complete ethanol/glycerol media at 30° for 2 days and then replica plated to minimal glucose media lacking tryptophan (SD-Trp) and incubated for 5 days at 30°. The number of papillae growing in each sector reflects the rate of transfer of mtDNA to the nucleus for that strain. Spores a–d are a tetratype tetrad from a cross between IRY26 and THY14. Spores e–h are a tetratype tetrad from a cross between IRY26 and MDY3. In each tetrad, the *YNT61-3* allele is present in one of the two spores showing wild-type mtDNA escape, but its location is hidden because it has no observable effect in the absence of the *mmm1-6* or *mdm10-17* mutation. (B) Growth on nonfermentable carbon sources. Yeast strains with the indicated mutant loci were streaked on YPEG and incubated for 5 days at 30°. Spores a–d are one tetrad from a cross between IRY26 and MTY30. The *YNT61-3* allele is present in one of the two spores showing wild-type growth, but its location is hidden because it has no observable effect in the absence of the *mmm1-6* and *mdm10-17* mutations. Strains: wild type, PTY44; *mdm10-17,* MDY3; *mmm1-6*, THY14; *YNT61-3,* IRY26; *mmm1-6 mdm10-17,* MTY30; *YNT61-3,* IRY35.

pressors of the slow-growth phenotype increased the growth rate of *mmm1*Δ yeast to an extent roughly equivalent to the increase in the distribution of mitochondrial compartments throughout the cell (Figure 5). These extragenic suppressors of *mmm1*Δ are likely to define additional factors that control the structure of mitochondria and, ultimately, the inheritance of mtDNA.

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