Mmf1p Couples Amino Acid Metabolism to Mitochondrial DNA Maintenance in *Saccharomyces cerevisiae*

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**ABSTRACT** A variety of metabolic deficiencies and human diseases arise from the disruption of mitochondrial enzymes and/or loss of mitochondrial DNA. Mounting evidence shows that eukaryotes have conserved enzymes that prevent the accumulation of reactive metabolites that cause stress inside the mitochondrion. 2-Aminoacrylate is a reactive enamine generated by pyridoxal 5′-phosphate-dependent α,β-eliminases as an obligatory intermediate in the breakdown of serine. In prokaryotes, members of the broadly conserved RidA family (PF14588) prevent metabolic stress by deaminating 2-aminoacrylate to pyruvate. Here, we demonstrate that unmanaged 2-aminoacrylate accumulation in *Saccharomyces cerevisiae* mitochondria causes transient metabolic stress and the irreversible loss of mitochondrial DNA. The RidA family protein Mmf1p deaminates 2-aminoacrylate, preempting metabolic stress and loss of the mitochondrial genome. Disruption of the mitochondrial pyridoxal 5′-phosphate-dependent serine dehydratases (Ilv1p and Cha1p) prevents 2-aminoacrylate formation, avoiding stress in the absence of Mmf1p. Furthermore, chelation of iron in the growth medium improves maintenance of the mitochondrial genome in yeast challenged with 2-aminoacrylate, suggesting that 2-aminoacrylate-dependent loss of mitochondrial DNA is influenced by disruption of iron homeostasis. Taken together, the data indicate that Mmf1p indirectly contributes to mitochondrial DNA maintenance by preventing 2-aminoacrylate stress derived from mitochondrial amino acid metabolism.

**IMPORTANCE** Deleterious reactive metabolites are produced as a consequence of many intracellular biochemical transformations. Importantly, reactive metabolites that appear short-lived *in vitro* have the potential to persist within intracellular environments, leading to pervasive cell damage and diminished fitness. To overcome metabolite damage, organisms utilize enzymatic reactive-metabolite defense systems to rid the cell of deleterious metabolites. In this report, we describe the importance of the RidA/YER057c/UK114 enamine/imine deaminase family in preventing 2-aminoacrylate stress in yeast. *Saccharomyces cerevisiae* lacking the enamine/imine deaminase Mmf1p was shown to experience pleiotropic growth defects and fails to maintain its mitochondrial genome. Our results provide the first line of evidence that uncontrolled 2-aminoacrylate stress derived from mitochondrial serine metabolism can negatively impact mitochondrial DNA maintenance in eukaryotes.

**KEYWORDS** 2-aminoacrylate, RidA, enamine deaminase, metabolite stress, mitochondrial genome

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imine substrates, to ketoacids (3–8). In cellular metabolism, 2AA is generated by pyridoxal 5’-phosphate (PLP)-dependent α,β-eliminase enzymes as an intermediate in the conversion of amino acids to pyruvate (3, 4, 7). Following release from the enzyme, 2AA can be spontaneously converted to pyruvate by solvent water (7). Although 2AA can covalently modify enzymes in vitro (9–15), the potential for intracellular enamine damage was initially dismissed because of the short (~1.5-s) half-life of 2AA in water (16). However, biochemical and genetic data demonstrate that in the absence of RidA, unbound 2AA persists in vivo and inactivates PLP-dependent enzymes (1, 17, 18). These data indicate that the cellular milieu lacks sufficient free water to rapidly hydrolyze 2AA and provides the framework for phenotypes displayed by ridA mutants (6, 17–20). The prevalence of PLP-dependent enzymes (including α,β-eliminases) involved in basic metabolic pathways, coupled with the broad distribution of RidA homologues, suggests that 2AA stress likely occurs in diverse organisms.

Little is known about eukaryotic Rid proteins, although early reports suggested involvement in a variety of cellular processes mediated by undefined mechanisms (21–30). Interestingly, the mitochondrial RidA protein Mmf1p (mitochondrial matrix factor) influences mitochondrial DNA (mtDNA) stability in Saccharomyces cerevisiae (29, 30). Unlike some mitochondrial DNA maintenance factors (e.g., Abf2p) (31), Mmf1p does not bind and stabilize the mitochondrial nucleoid directly (29). Characterization of enamine deaminase activity in vitro and genetic complementation analyses in vivo suggest that RidA proteins from the three domains of life share a conserved cellular function (7, 28). The biochemical activity of RidA from yeast (e.g., Mmf1p) was not addressed by those previous studies. Here, we demonstrate that 2AA generation provokes the irreversible loss of mtDNA in S. cerevisiae lacking Mmf1p. The data indicate that iron present in the growth medium influences the stability of mtDNA when 2AA stress is encountered. Furthermore, 2AA stress elicits a growth defect that is distinct from the respiratory deficiency caused by mtDNA loss. This report establishes the role of Mmf1p in indirectly stabilizing mtDNA by preventing 2AA stress in a eukaryote and highlights damage that results from reactive metabolite imbalance in mitochondria.

RESULTS

Disruption of MMF1 leads to a growth defect and loss of mtDNA. The MMF1 locus of the haploid S. cerevisiae strain YJF153 (32) was replaced with a drug cassette by targeted gene disruption, and the drug marker was resolved to generate mutant strain DMy22 (mmf1Δ). A plasmid expressing MMF1 or an empty vector (pSF-TEF1-G418; Sigma) was introduced into DMy22, the wild-type parent (p+), and a chemically induced cytoplasmic petite (p−) strain derived from the wild-type strain. The resulting strains were assessed for growth on minimal medium containing a fermentable (dextrose [D]) or nonfermentable (glycerol [G]) carbon substrate (Fig. 1A). The mmf1Δ mutant had two significant growth phenotypes: (i) an inability to grow on glycerol and (ii) a reduced ability to grow on dextrose. Plasmid-borne MMF1 failed to restore growth on glycerol, consistent with the irreversible loss or mutation of mtDNA observed in pθ or p− cytoplasmic petites (33) (Fig. 1A). Results of deconvolution microscopy confirmed that the mmf1Δ mutant lacked detectable mtDNA and therefore was likely a pθ− cytoplasmic petite (see below). In contrast, growth of the mmf1Δ mutant on dextrose was restored to a level similar to that of the p− control by providing plasmid-borne MMF1. This indicated that transient metabolic deficiencies distinguishable from the respiratory defect were encountered by strains lacking Mmf1p. The addition of isoleucine and, to a lesser extent, threonine (an isoleucine precursor) restored growth of the pθ mmf1Δ mutant on dextrose (Fig. 1B) but not on glycerol. Together, these data distinguished the reversible and irreversible consequences of the mmf1Δ mutation, and each was considered in turn.

Ilv1p-generated 2-aminoacrylate causes a growth defect in the absence of Mmf1p. The data support a model where both the growth defect and mtDNA loss are consequences of the toxic accumulation of 2AA in mitochondria lacking Mmf1p. The
growth defect of a \( \rho^0 \) \( \text{mmf1}\Delta \) mutant on dextrose is reminiscent of a \( \text{ridA} \) mutant phenotype in \( S. \text{enterica} \) (19). In this case, 2AA generated by serine/threonine dehydratase (IlvA; EC 4.3.1.19) accumulates and compromises growth. Isoleucine allosterically inhibits IlvA, prevents 2AA generation, and restores growth in defined medium. Two nuclearly encoded serine/threonine dehydratases (EC 4.3.1.19) are active in the \( S. \text{cerevisiae} \) mitochondrion. Ilv1p is the biosynthetic serine/threonine dehydratase required for isoleucine biosynthesis (34), and Cha1p is a catabolic dehydratase induced by serine or threonine (35). Much like the bacterial enzyme IlvA, Ilv1p catalyzes the first committed step in isoleucine biosynthesis, is subject to feedback inhibition by isoleucine, and uses serine as a substrate as an alternative to threonine (36) (Fig. 2A). In \textit{vitro}, Ilv1p dehydrated serine and released 2AA, which Mmf1p used as a substrate, leading to an increased rate of pyruvate formation (Fig. 2B). On a per-mole basis, the 2AA-hydrolyzing activity of Mmf1p was indistinguishable from that of the well-characterized RidA enzyme from \( S. \text{enterica} \) (Fig. 2B). These data support the hypothesis that the absence of Mmf1p leads to accumulation of 2AA following serine dehydration.

Taken together, the data favored the scenario depicted in Fig. 2A and suggested that the growth-stimulating role of isoleucine was exerted via the allosteric inhibition of Ilv1p. If true, preventing allosteric inhibition of Ilv1p would abolish the benefit of the presence of isoleucine in cells lacking Mmf1p. To test this hypothesis, an allosterically resistant variant of Ilv1p (Ilv1p\( ^{R416F} \)) was generated using an allosterically resistant variant of \( E. \text{coli} \) IlvA (IlvA\( ^{R362F} \)) as a template (37). In \textit{vitro}, recombinant Ilv1p\( ^{R416F} \) was insensitive to isoleucine concentrations that completely inhibited the wild-type enzyme (Fig. 2C). Importantly, the catalytic efficiency of serine dehydroxylation by the variant enzyme was not significantly different from the efficiency seen with the wild-type enzyme (Fig. 2D). Wild-type \( \text{ILV1} \) was replaced with the full-length allele (\( \text{ILV1-1} \)) encoding Ilv1p\( ^{R416F} \) to generate a strain where 2AA production by Ilv1p could
not be inhibited. Isoleucine failed to completely reverse the growth defect of the mmf1Δ mutant strain expressing Ilv1pR416F in minimal synthetic dextrose (SD) medium (Fig. 2E). Therefore, isoleucine improves growth of the mmf1Δ mutant strain in part through allosteric inhibition of Ilv1p and not by satisfying an isoleucine requirement.

Takentogether,thesedatashowthatIlv1pgenerates2AAfromendogenousserineandthatgrowthislimitedunlessMmf1porisoleucinequenches2AAorinhibitsIlv1pactivity, respectively.

Cha1p increases 2-aminoacrylate stress when exogenous serine is present. The Δ-mmf1Δ mutant characterized as described above was constructed on rich (yeast extract-peptone-dextrose [YPD]) medium containing isoleucine, so feedback inhibition prevented Ilv1p from generating significant 2AA. Therefore, if 2AA stress were to contribute to mtDNA loss, an additional enzyme would be required to generate 2AA in

![Image of Ilv1p-mediated pyruvate formation](image-url)
the presence of isoleucine. The catabolic Ser/Thr dehydratase Cha1p is a logical source of 2AA, since
CHA1 expression is induced by serine, which is present in YPD, and since the enzyme is insensitive to regulation by isoleucine (35). Growth analysis in SD medium plus isoleucine, with or without serine, confirmed that Cha1p contributed to 2AA stress in the absence of Mmf1p (Fig. 3A). Both the ρ0 mmf1Δ and ρ0 mmf1Δ cha1Δ mutants grew in medium with isoleucine, but the addition of serine compromised growth only of the ρ0 mmf1Δ mutant, indicating CHA1 is required for sensitivity to exogenous serine. These data indicate that 2AA is produced following induction of Cha1p by exogenous serine and that Ilv1p is inhibited by isoleucine via feedback inhibition. The increased level of 2AA compromised growth in the absence of MMF1. Unlike the results seen with a ridΔ mutant in S. enterica (38), the growth defect of the ρ0 mmf1Δ mutant was not reversed by the addition of common nutritional supplements (i.e., amino acids or vitamins). This result suggests that the cellular deficiencies underpinning the 2AA-dependent growth defect of mmf1Δ mutant strains in minimal medium are more complex than those caused by a single compromised enzyme.

Disruption of serine dehydratase-dependent 2-aminoacrylate production preserves mtDNA in the absence of MMF1. Despite the connection between Mmf1p and 2AA, it remained possible that the irreversible loss of mtDNA in an mmf1Δ mutant strain was unrelated to 2AA accumulation. However, data from order-dependent genetic manipulations and growth analyses demonstrate that 2AA stress specifically caused mtDNA loss (Fig. 3B). First, CHA1 was disrupted in a ρ+ MMF1 background. Second, MMF1 was disrupted in the cha1Δ strain whereas Ilv1p was inhibited by isoleucine in

![Graph](https://example.com/graph.png)

**FIG 3** Cha1p contributes to 2AA generation when serine is provided exogenously. (A) Disruption of CHA1 prevents conversion of exogenous serine (5 mM) to 2AA, thereby alleviating exogenous serine sensitivity in a ρ0 mmf1Δ cha1Δ background. Data are displayed as averages and standard deviations of results from three independent experiments. (B) Preventing 2AA production by simultaneously inhibiting the activity of Ilv1p (with isoleucine) and deleting CHA1 renders Mmf1p nonessential for mtDNA maintenance despite the presence of serine in the growth medium. All mutants were constructed in the order indicated by the genotype and selected on YPD medium prior to streaking on the displayed YPD or YPG plates. The mmf1Δ cha1Δ strain (DMy17) was constructed independently from the cha1Δ mmf1Δ strain (DMy20), and the order in which the mutations were introduced differed as indicated in the text. Growth was recorded after 48 h at 30°C.
the YPD-based selection medium. This resulted in a \( \rho^+ \) cha1Δ mmf1Δ double mutant with unique properties. Significantly, introducing the genetic lesions in this order preempted the production of 2AA and resulted in a strain that respirated glycerol (Fig. 3B) and maintained its mtDNA (Fig. 4A). Therefore, preventing both Ilv1p and Cha1p serine dehydratase activity renders Mmf1p nonessential for mtDNA maintenance. The inversely constructed mmf1Δ CHA1 strain lost the ability to respire glycerol, and subsequent disruption of CHA1 in the absence of MMF1 did not restore growth on glycerol (Fig. 3B). These results demonstrate that disruption of MMF1 prior to CHA1 leads to permanent loss of mtDNA, a conclusion supported by mitochondrial staining of the \( \rho^+ \) mmf1Δ cha1Δ strain (Fig. 4A). Thus, identical genotypes constructed in opposing series result in dramatically different outcomes with regard to mtDNA stability. These data support the conclusion that the preemptive disruption of CHA1, coupled with isoleucine-mediated inhibition of Ilv1p, blocks 2AA production in the mitochondrial matrix and bypasses the need for Mmf1p to maintain the mitochondrial genome.

The \( \rho^+ \) cha1Δ mmf1Δ mutant maintained wild-type growth indefinitely when propagated on medium containing isoleucine. However, when the inhibition of Ilv1p

![Figure 4](mbio.asm.org)
was lifted by removing isoleucine, a growth defect of the \(p^+\) cha1Δ mmf1Δ mutant strain (\(\mu = 0.18 \pm 0.03\)) relative to the \(p^+\) wild-type control (\(\mu = 0.45 \pm 0.01\)) was detected (Fig. 4B). Isoleucine supplementation restored the growth rate (\(\mu\)) of the \(p^+\) cha1Δ mmf1Δ strain (\(\mu = 0.39 \pm 0.04\)) to a level similar to that of the \(p^+\) wild-type control (\(\mu = 0.48 \pm 0.04\)). A parallel result was observed with the respiration-deficient \(p^0\) mmf1Δ cha1Δ mutant strain where the addition of isoleucine increased the growth rate to the level of the \(p^+\) wild-type control (\(\mu = 0.26\)) (Fig. 4B). These data show that the \(p^+\) cha1Δ mmf1Δ mutant is susceptible to 2AA stress and experiences diminished growth when cultured on minimal medium. However, the respiratory capacity of the \(p^+\) cha1Δ mmf1Δ mutant was maintained indefinitely during growth on minimal medium (see Fig. S1 in the supplemental material). This result suggests that mtDNA is stable in spite of the growth defect given the moderate level of 2AA stress generated by Ilv1p on minimal medium. Enhancing flux through Ilv1p to increase 2AA stress severely diminished the ability of the \(p^+\) cha1Δ mmf1Δ mutant to respire glycerol after growth on minimal medium supplemented with 5 mM serine (Fig. S1). Taken together, these data indicate that the absence of Mmf1p, Ilv1p acts on endogenous serine to generate moderate 2AA stress that elicits a minor and reversible growth defect akin to the bacterial paradigm (5). However, exogenous serine stimulates production (via Ilv1p and/or Cha1p) of sufficient 2AA to cause irreversible loss of the mitochondrial genome.

**Availability of iron influences mtDNA stability in the absence of Mmf1p.** Given the precedents seen in bacterial systems, we theorized that the phenotypes observed for mmf1Δ mutants are caused by 2AA inactivation of PLP-dependent enzymes in the yeast mitochondrion. Several PLP-dependent enzymes in the yeast mitochondrion influence iron homeostasis (39, 40), and the disruption of iron homeostasis can lead to mitochondrial iron accumulation, enhanced oxidative stress, and diminished mtDNA stability (41, 42). Therefore, the impact of iron on 2AA-dependent loss of mtDNA was assessed. A \(p^+\) mmf1Δ (DMy41) mutant was made on yeast extract-peptone-glycerol (YPG) medium as described previously (29). Subsequent transfer of the \(p^+\) mmf1Δ mutant to YPD medium resulted in the loss of respiratory capacity (Fig. 5). However, transfer of the \(p^+\) mmf1Δ mutant to YPD medium containing the iron chelator bathophenanthrolinedisulfonic acid (BPS) preserved respiratory capacity (Fig. 5). These data indicate that the loss of mtDNA caused by 2AA stress is contingent on the presence of iron in the growth medium. Sequestration of iron in the growth medium using BPS prevented 2AA-dependent loss of mtDNA, perhaps reflecting that 2AA stress induces accumulation of iron in the mitochondria, leading to damage and eventual loss of mtDNA. These data offer a starting point for future characterization of targets modified by 2AA in the yeast mitochondrion.

**DISCUSSION**

Our work shows that accumulation of 2AA causes metabolic stress and loss of mtDNA in \(S.\ cerevisiae\). Furthermore, Mmf1p prevents 2AA accumulation in the \(S.\ cerevi-
siae mitochondrion. Two consequences of 2AA accumulation in the mitochondrion were identified: the irreversible loss of mtDNA giving rise to respiration-deficient ρ0 cytoplasmic petites and a transient growth defect on fermentable carbon substrates. The latter phenotype is reminiscent of the growth defects caused by 2AA stress in S. enterica (6, 19) and other organisms (8, 28). PLP-dependent enzymes are the only targets of 2AA damage characterized to date (43), suggesting that the mmf1Δ strain growth defect is due to inhibition of one or more (of 10 possible) target PLP enzymes in the mitochondrion (44) (Fig. 6). 2AA tested with a bacterial system is not mutagenic in vivo (see Table S2 in the supplemental material), making it unlikely that direct DNA damage by 2AA causes the loss of mtDNA. We suggest that loss of mtDNA is caused by the stress that 2AA exerts on the mitochondrial metabolic network. Specifically, we favor a model in which 2AA damages multiple mitochondrial PLP-dependent enzymes, ultimately leading to destabilization and loss of the mitochondrial genome (Fig. 6). The independent disruption of mitochondrial PLP-dependent enzymes involved in heme biosynthesis (Hem1p; EC 2.3.1.37), iron-sulfur cluster biogenesis (Nfs1p; EC 2.8.1.7), one-carbon metabolism (Shm1p; EC 2.1.2.1), and aspartate metabolism (Aat1p; EC 2.6.1.1) has been reported to influence mtDNA stability to various degrees (40, 45–47). Notably, Hem1p and Nfs1p are directly involved in iron metabolism; damage to these enzymes caused by 2AA may underlie the iron sensitivity of mmf1Δ mutants. The iron sensitivity of the mmf1Δ mutant is reminiscent of that of yeast lacking the frataxin homologue YFH1 (41, 42), consistent with mitochondrial iron accumulation giving rise to oxidative stress that damages mtDNA. Given the variety of PLP-dependent enzymes that individually impact iron homeostasis and mtDNA stability, it is reasonable to conjecture that the presence of a combination of partially defective enzymes could result in mtDNA loss. Further understanding of how 2AA leads to loss of mtDNA may uncover a novel stress response pathway in mitochondria.
Our work identified a lethal consequence of uncontrolled reactive metabolite accumulation in the mitochondrion. Synthesis of 2AA is unavoidable in PLP-dependent serine dehydration, resulting in the need for RidA proteins (e.g., Mmf1p) to prevent accumulation of this reactive metabolite. Strikingly, when S. cerevisiae is exposed to serine, CHA1 and MMF1 are the two most highly expressed genes (48), suggesting that these enzymes act in concert to safely reduce serine levels in the mitochondrion. Interestingly, Cha1p is reported to be a component of the mitochondrial nucleoid in S. cerevisiae (49), perhaps reflecting an added benefit of Cha1p-dependent physical stabilization of the nucleoid during periods of elevated serine catabolism and 2AA production. The Mmf1p homologue in humans, UK114 (PF01042), is variably described as a tumor antigen, calpain activator, or translation inhibitor in diverse animal cell types (21, 26, 50–52). Importantly, UK114 can substitute for Mmf1p to maintain the mitochondrial genome in S. cerevisiae (29), which argues in favor of an evolutionarily conserved biological function. PLP-dependent serine dehydratases are broadly distributed among eukaryotes, which emphasizes the breadth of 2AA stress (53–55). Furthermore, many cell types, including cancer cells and neurons, require high serine levels to promote growth and proliferation (56–58), predisposing certain cell types to high concentrations of a known 2AA precursor. Our work provides a framework for understanding the physiological role of Mmf1p and other eukaryotic RidA proteins, in addition to dissecting the mechanism by which 2AA stress causes loss of mtDNA.

MATERIALS AND METHODS

Strains, media, and chemicals. Saccharomyces cerevisiae strain YJF153 (MATa HO::dsdAMX4) was derived from an oak tree isolate (YPS163) and provided by Justin Fay (Washington University) (32). Rich medium (YP) consisted of 20 g/liter peptone (Fisher Scientific) and 10 g/liter yeast extract (TECTON). Minimal medium (S medium) contained 1.7 g/liter yeast nitrogen base without amino acids or nitrogen (Sunrise Science; catalog no. 1500-100) and 5 g/liter ammonium sulfate. Either dextrose (D; 20 g/liter) or glycerol (G; 30 g/liter) was provided as the sole carbon source. Solid medium was made by adding 20 g/liter Difco agar (TECTON). Antibiotics used for deletion marker selection were added at the following final concentrations: 400 mg/ml Geneticin (G418; Gold Biotechnology), 200 μg/ml hygromycin B (Gold Biotechnology), and 100 μg/ml nourseothricin sulfate (cloNAT; Gold Biotechnology). A lower concentration of Geneticin (200 μg/ml) was used for maintenance of strains with confirmed G418 resistance. Isolucine or threonine was added to minimal growth medium at a final concentration of 1 mM.

Escherichia coli strain BL21-AI was used for recombinant protein overproduction. Standard E. coli growth medium (LB broth) consisted of 10 g/liter tryptone, 5 g/liter yeast extract, and 10 g/liter NaCl. Superbroth containing tryptone (32 g/liter), yeast extract (20 g/liter), sodium chloride (5 g/liter), and sodium hydroxide (0.2 g/liter) was used when high cell densities were required for protein overproduction. Ampicillin (150 μg/ml) was added to the growth medium as needed. Reagents and chemicals were purchased from Sigma-Aldrich unless otherwise specified.

Genetic techniques and growth methods. Gene disruptions in S. cerevisiae were made following the standard gene replacement method described by Hegemann and Heick (59), resulting in the strains listed in Table 1 in the supplemental material. Disruption cassettes were amplified using the appropriate primers and plasmid templates listed in Table S1. Purified DNA (1 μg) was transformed into S. cerevisiae by incubating cells suspended in a mixture of 33% polyethylene glycol 3350 (PEG 3350), 100 mM lithium acetate, and 0.28 mg/ml salmon sperm DNA at 30°C for 30 min followed by 30 min of heat shock at 42°C. The transformed cells were recovered in rich medium containing dextrose (YPD) for 1 h at 30°C and were subsequently plated on solid YPD containing the relevant selection agent. Colonies that arose after 2 to 3 days of incubation were transferred to selective medium, and individual colonies were screened via PCR for the appropriate genetic recombinants. (Additional details of the methods used in the study are provided in Text 1 in the supplemental material.)

For growth analyses, yeast strains were revived from −80°C freezer stocks and streaked for isolation on YPD. Single colonies were inoculated into 2-ml YPD cultures and incubated at 30°C with shaking (200 rpm) overnight. Turbid cultures were (i) 10-fold serially diluted with NaCl for spot plating (10 μl) on solid medium or (ii) inoculated (1%) into 5 ml of SD medium to monitor growth over time on the basis of the change in optical density at 600 nm (OD600). Growth curves were plotted as averages and standard deviations of results from three independent cultures using GraphPad Prism 7.0. Specific growth rates (μ) were calculated on the basis of the equation ln(X(t))/t, where X represents OD600, X₀ is the initial OD600 of the linear growth period monitored, and t is time in hours.

Molecular techniques. Plasmids were constructed using standard molecular techniques. DNA was amplified using Q5 DNA polymerase (New England Biolabs) with primers purchased from Eton Bioscience Inc. (Research Triangle Park, NC). Plasmids were isolated using a Wizard Plus SV miniprep kit (Promega), and PCR products were purified using an E.Z.N.A. DNA isolation kit (Omega BioTek). Restriction endonucleases used for molecular cloning were purchased from New England Biolabs. T4 ligase (Thermo Scientific) was used to ligate inserts to vectors. The plasmids and primers used are listed in Table S1.
make pDM1469. All constructs were transformed into DH5α (Eton Bioscience Inc.). Integration of the appropriate allele in DMy43 was confirmed by sequence analysis.

Constructs for protein overproduction were made using pET20b (Novagen) as the vector backbone. Aliles were amplified using high-fidelity Q5 polymerase (NEB) and primer sets designed to amplify the full-length allele of the gene of interest and introduce NdeI or XhoI restriction sites. The amplified alleles were purified (Zymo) and ligated into pET20b following restriction enzyme digestion of the insertion and vector, (ilv1_NdeI_F_truncated_pET20, ilv1_Not1_R_pET20) lacking the N-terminal mitochondrial targeting sequence, and transformed into BL21-AI strain containing pDM1463. An overnight culture of DM15531 grown in 10 ml of superbroth containing ampicillin was inoculated into 2 liters of superbroth with ampicillin. Cultures were grown for 3 h at 37°C with aeration (200 rpm) until an OD650 of 0.5 was reached. Fresh arabinose was added to reach a final concentration of 0.02%, and cultures were shifted to 30°C and incubated for an additional 16 h while being shaken. Cells were harvested by centrifugation and resuspended in binding buffer consisting of 50 mM Tris-HCl (pH 8), 200 mM sodium chloride, 10 mM imidazole, 1 mM TCEP [Tris(2-carboxyethyl)phosphine], and 10% glycerol. Lysozyme (1 mg/ml), phenylmethylsulfonyl fluoride (100 g/ml), and DNase (25 g/ml) were added to the cell suspension, and the reaction mixture was incubated on ice for 1 h. Cells were lysed using a Constant Systems Limited One Shot (United Kingdom) system by passing cells through the disruptor one time with the pressure set to 21,000 lb/in². Following lysis, the extract was clarified, filtered, and injected into a HiTrap high-performance (HP) Ni-Sepharose column (5 ml). The column was washed with five column volumes of binding buffer with 40 mM imidazole added. Mmf1p was eluted by increasing the concentration of imidazole from 40 to 300 mM over 10 column volumes, and 3-ml fractions were collected and analyzed by SDS-PAGE to determine protein purity. Fractions containing pure (>99%) protein were pooled and concentrated by centrifugation with a 4,000-molecular-weight-cutoff filter unit (Millipore). The concentrated protein sample was transferred to storage buffer containing 10 mM HEPES and 10% glycerol using a PD-10 desalting column (GE Healthcare). Protein yield as determined using the bicinchoninic acid (BCA) assay (Pierce) was approximately 14 mg/ml. Protein aliquots were frozen in liquid nitrogen and stored at −80°C.

### TABLE 1 Strain list

| Strain | Relevant genotype |
|--------|-------------------|
| YJF153a | MATa HO::dsdAMX4 |
| DMy13 | mmf1::kanMX (µP) |
| DMy16 | cha1::phoMX |
| DMy17 | mmf1::kanMX cha1::phoMX (µP) |
| DMy18 | ilv1::natMX |
| DMy20 | cha1::phoMX mmf1::kanMX |
| DMy23 | YJF153 (µP) |
| DMy21 | mmf1::phoMX-loxP (µP) |
| DMy22 | mmf1Δ0 (µP) |
| DMy31 | YJF153/pDM1481 |
| DMy32 | YJF153/pSF-empty |
| DMy33 | mmf1Δ0 (µP)/pDM1481 |
| DMy34 | mmf1Δ0 (µP)/pSF-empty |
| DMy35 | YJF153 (µP)/pDM1481 |
| DMy36 | YJF153 (µP)/pSF-empty |
| DMy41 | mmf1::loxP-kanMX-loxP (µP*) |
| DMy42 | ilv1Δ0::ilv1-1 |
| DMy46 | ilv1Δ0::ilv1-1 mmf1::kanMX |
| DM15531 | E. coli BL21-AI/pDM1463 |
| DM15533 | E. coli BL21-AI/pDM1467 |
| DM15910 | E. coli BL21-AI/pDM1536 |

aAll yeast strains were constructed in a YJF153 strain background.

bAllele ILV1-1 encodes an Ilv1pR416F feedback-resistant variant.

Plasmids pUG6, pUG74, and pUG75 were used as templates for drug cassette amplification (EUROSCARF). Plasmid pFA6a-kanMX was provided by David Garfinkel (University of Georgia). pSF episomal shuttle vector (Sigma-Aldrich; catalog no. OGS542) containing a TEF1 promoter, TPI1 terminator, and Geneticin (yeast)/ampicillin (bacteria) resistance markers was used to express MMF1 in S. cerevisiae (pDM1481); full-length MMF1 was PCR amplified for cloning using mmf1_pSF_NcoI_F and mmf1_pSF_XbaI_R. Constructs for protein overproduction were made using pET20b (Novagen) as the vector backbone. Primers were designed to amplify MMF1 (mmf1_Ndel_F_truncated_pET20, mmf1_Xhol_R) and ILV1 (ilv1_Ndel_F_truncated_pET20, ilv1_Not1_R_pET20) lacking the N-terminal mitochondrial targeting sequences and ligated into pET20b following restriction enzyme digestion of the insertion and vector, forming pDM1463 and pDM1467, respectively. The full-length allele of ILV1 was cloned into pET20b to make pDM1469. All constructs were transformed into DH5α following ligation and selected on LB medium containing the appropriate drug. Plasmid insertions were confirmed by sequence analysis performed at Eton Bioscience Inc. Constructs pDM1467 and pDM1469 were used as templates for site-directed mutagenesis to generate pDM1536 and pDM1540, respectively. Site-directed mutants of ILV1 were made by amplifying pDM1467 and pDM1469 with Q5 polymerase and primer ilv1_R416F, changing the codon for arginine-416 (AGA) to phenylalanine-416 (TTC), generating allele ILV1-1. Following transformation into DH5α, site-directed mutants were confirmed by Sanger sequencing (Eton Bioscience). Plasmid pDM1540 served as a template to amplify the ILV1-1 allele for integration into the chromosome at the ilv1::natMX locus of DMy18. Replacement of the natMX drug cassette in DMy18 with the feedback-resistant ILV1-1 allele restored isoleucine prototrophy to DMy43 and abolished nourseothricin sulfite resistance. Integration of the appropriate allele in DMy43 was confirmed by sequence analysis (Eton Bioscience Inc.).

**Purification of Mmf1p**

Mmf1p21-147, lacking the N-terminal amino acids required for mitochondrial localization, was purified from an E. coli BL21-AI strain containing pDM1463. An overnight culture of DMy15531 grown in 10 ml of superbroth containing ampicillin was inoculated into 2 liters of superbroth with ampicillin. Cultures were grown for 3 h at 37°C with aeration (200 rpm) until an OD650 of 0.5 was reached. Fresh arabinose was added to reach a final concentration of 0.02%, and cultures were shifted to 30°C and incubated for an additional 16 h while being shaken. Cells were harvested by centrifugation and resuspended in binding buffer consisting of 50 mM Tris-HCl (pH 8), 200 mM sodium chloride, 10 mM imidazole, 1 mM TCEP [Tris(2-carboxyethyl)phosphine], and 10% glycerol. Lysozyme (1 mg/ml), phenylmethylsulfonyl fluoride (100 g/ml), and DNase (25 g/ml) were added to the cell suspension, and the reaction mixture was incubated on ice for 1 h. Cells were lysed using a Constant Systems Limited One Shot (United Kingdom) system by passing cells through the disruptor one time with the pressure set to 21,000 lb/in². Following lysis, the extract was clarified, filtered, and injected into a HiTrap high-performance (HP) Ni-Sepharose column (5 ml). The column was washed with five column volumes of binding buffer with 40 mM imidazole added. Mmf1p was eluted by increasing the concentration of imidazole from 40 to 300 mM over 10 column volumes, and 3-ml fractions were collected and analyzed by SDS-PAGE to determine protein purity. Fractions containing pure (>99%) protein were pooled and concentrated by centrifugation with a 4,000-molecular-weight-cutoff filter unit (Millipore). The concentrated protein sample was transferred to storage buffer containing 10 mM HEPES and 10% glycerol using a PD-10 desalting column (GE Healthcare). Protein yield as determined using the bicinchoninic acid (BCA) assay (Pierce) was approximately 14 mg/ml. Protein aliquots were frozen in liquid nitrogen and stored at −80°C.
Purification of Ilv1p(46-576) and Ilv1p(46-576)R416F. Plasmids encoding His-tagged versions of Ilv1p(46-576) (pDM1467) and Ilv1p(46-576)R416F (pDM1536) were transformed into E. coli BL21-AI for protein purification. The resulting strains were inoculated into 10 ml of superbroth containing ampicillin and grown overnight at 37°C. Overnight cultures were subcultured into 2 liters of superbroth containing ampicillin. The resulting strains were inoculated into 10 ml of superbroth containing ampicillin and grown overnight at 37°C until an OD 600 of 0.7 was reached. Arabinose (0.02%) was added to induce expression, and cultures were shifted to 30°C for 16 h. Cells were harvested at 4°C by centrifugation (15 min at 8,000 × g) and resuspended in binding buffer containing 50 mM potassium phosphate (pH 8), 500 mM sodium chloride, 10 mM imidazole, 1 mM TCEP, 10 μM PLP, and 10% glycerol. Lysozyme (1 mg/ml), phenylmethylsulfonyl fluoride (100 g/ml), and DNase (25 g/ml) were added to each cell suspension, which then sat on ice for 1 h. Cells were mechanically lysed using a French pressure cell (5 passes at 10,342 kPa). Each resulting lysate was clarified by centrifugation (45 min at 48,000 × g) and filtered through a membrane with 0.45-μm pores. Filtered lysates were loaded onto HisTrap HP Ni-Sepharose-columns (5 ml), and the columns were washed with five column volumes of binding buffer containing 40 mM imidazole. Protein was eluted by increasing the concentration of imidazole in the elution buffer from 40 to 300 mM over 10 column volumes. Purified protein was concentrated by centrifugation with a 10,000 molecular-weight cutoff filter unit (Millipore), and the buffer was replaced with 50 mM Tris-HCl (pH 7.5) containing 10 μM PLP and 10% glycerol using a PD-10 desalting column (GE Healthcare). Protein recovery as determined by the BCA assay (Pierce) was approximately 10.8 mg/ml for Ilv1p(46-576) and 13 mg/ml for Ilv1p(46-576)R416F. Protein aliquots were frozen in liquid nitrogen and stored at −80°C.

**Ilv1p generation of pyruvate assays.** Ilv1p(46-576) serine dehydratase activity was assayed in the presence or absence of Mnf1p or RiddA from *S. enterica* as previously described (7). The activity of purified RiddA was previously confirmed (3). Reaction mixtures (300 μl) consisted of 50 mM N-cyclohexyl-2-aminoethanesulfonic acid (pH 7.5), 0.6 μM Ilv1p(46-576), and Mnf1p (9.5 μM) or RiddA (1.3 μM). Experiments were performed in triplicate, and the results were measured using a 96-well quartz plate and a SpectraMax M2 (Molecular Devices) microplate reader. Reactions were initiated by adding l-serine and monitored continuously at 230 nm for 120 s. Initial rates were determined on the basis of the increase in A 230 corresponding to pyruvate production. A standard curve of pyruvate concentrations relative to A 230 was generated and used to calculate reaction rates, reported as micromoles of pyruvate produced per minute. The data, reported as averages and standard deviations of results from three independent experiments, were fitted with curves on the basis of the Michaelis-Menten equation using GraphPad Prism 7.0. The procedure described above was used to compare the levels of Ilv1p(46-576) and Ilv1p(46-576)R416F catalytic efficiency, with the sole change being that 50 mM potassium phosphate (pH 8) was used instead of 50 mM CHES (pH 9.5).

**Inhibition of Ilv1p variants by isoleucine assays.** The sensitivity of Ilv1p(46-576) and Ilv1p(46-576)R416F to allosteric regulation by isoleucine was determined in vitro. Reaction mixtures (300 μl) consisted of 50 mM potassium phosphate (pH 8) and 0.6 μM Ilv1p(46-576), or 0.6 μM Ilv1p(46-576)R416F. Isoleucine was added to assays at a final concentration of 3.3 mM. Experiments were performed in triplicate, and the results were measured using a 96-well quartz plate and a SpectraMax M2 (Molecular Devices) microplate reader. Reactions were initiated by adding 120 mM l-serine and monitored continuously at 230 nm for 120 s. Initial rates were determined on the basis of the increase in A 230 corresponding to pyruvate formation. A standard curve of pyruvate concentrations relative to A 230 was generated and used to calculate reaction rates, reported as micromoles of pyruvate produced per minute. The reaction rates for a single concentration of serine added are reported as averages and standard deviations of results from three independent experiments.

**Microscopy.** Strains were grown in YPD to full density overnight and then diluted to an OD 600 of 0.1 in complete synthetic dextrose medium (Sunrise Science; catalog no. 1001-010) the following morning. Cultures were then grown at 30°C and 200 rpm for 4 h. Cells were harvested by centrifugation (2 min at 2,000 × g) and resuspended in mounting medium consisting of 10 mM HEPES (pH 7.4) and 5% dextrose. Cells were again pelleted and washed in an equal volume of mounting medium. The mitochondrial matrix was stained with 1 μM rhodamine B (Molecular Probes) for 20 min, followed by 3 min of staining with 10 μM SYTO 18 (Molecular Probes) to detect mitochondrial DNA. Cells were pelleted and resuspended in fresh mounting medium, to which 2 μl of ProLong Live Antifade reagent (Thermo Fisher) was added. Tubes containing cells and antifade reagent were transferred to a sealed box at 4°C overnight. The next morning, 20 μl of cell suspension was mounted on a glass slide and imaged using a DeltaVision microscope system (GE Life Sciences) equipped with an Olympus IX-71 inverted microscope and a xenon arc lamp as the illumination source for exciting the fluorophores. Rhodamine B was visualized with excitation at 555 nm and emission at 507 nm. SYTO 18 was visualized with excitation at 490 nm and emission at 507 nm. Figures were generated by merging Z stacks of rhodamine B and SYTO 18 images, and the resulting merged images were deconvoluted using a conservative ratio and 15 processing cycles and automated softWoRx 6.5.2 (GE image acquisition software). A quick projection was generated, and the resulting images were exported to Adobe Illustrator 21.0.2. Images were cropped and resized without adjusting features related to contrast or brightness. Cell borders were generated in Adobe Illustrator by outlining each cell boundary as determined from a white light snapshot.

**Impact of iron on respiratory capacity.** The *MMF1* locus was disrupted by transforming a pRS wild-type strain with a drug resistance cassette (loxP-kanMX-loxP) and recombinants were selected on YPG medium containing 280 μg/ml Geneticin. Subsequent propagation of the pRS *mmf1Δ* (DMy41) mutant was done on YPG medium to preserve the mitochondrial genome. The pRS *mmf1Δ* mutant and a wild-type control were revived from freezer stocks on solid YPG medium and grown overnight at 30°C. Single colonies were picked and streaked onto solid YPD medium or solid YPD medium containing...
10 μM bathophenanthroline disulfonic acid (BPS). Cultures were incubated at 30°C for 48 h, and then three representative colonies were picked from each preculture and streaked to YPG and YPD plates to assess respiratory capacity. Plates were incubated 48 h at 30°C, and respiration proficiency was assessed on the basis of the ability of strains to grow on glycerol (YPG)-containing medium.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mBio.00084-18.

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