Identification of the Gene and Characterization of the Activity of the *trans*-Aconitate Methyltransferase from *Saccharomyces cerevisiae*†

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ABSTRACT: We have identified the yeast open reading frame *YER175c* as the gene encoding the *trans*-aconitate methyltransferase of *Saccharomyces cerevisiae*. Extracts of a yeast strain with a disrupted *YER175c* gene demonstrate a complete loss of activity toward the methyl-accepting substrates *trans*-aconitate, *cis*-aconitate, DL-isocitrate, and citrate. Reintroduction of the *YER175c* gene on a plasmid results in an overexpression of the activity toward each of these methyl-accepting substrates. We now designate this gene *TMT1* for *t*rans-aconitate methyltransferase. We examined the methyl-accepting substrate specificity of this enzyme in extracts from overproducing cells. We found that *trans*-aconitate was the best substrate with a K_m of 0.66 mM. Other substrates were recognized much more poorly, including *cis*-aconitate with a K_m of 74 mM and the decarboxylation product itaconate with a K_m of 44 mM. The ratio of the maximal velocity to the K_m of these substrates was only 0.24% and 0.9% that of *trans*-aconitate; for other substrates including citrate and other tricarboxylate and dicarboxylate derivatives, this ratio ranged from 0.0003% to 0.062% that of *trans*-aconitate. We then asked if any of these compounds were present endogenously in yeast extracts. We were able to identify *trans*-aconitate 5-methyl ester as well as additional unidentified radiolabeled products when *S*-adenosyl-L-[*methyl*-^{3}H]methionine was mixed with *TMT1*$^+$ extracts (but not with *tmt1*$^-$ extracts), suggesting that there may be additional substrates for this enzyme. We showed that the product 5-methyl ester of *trans*-aconitate is not readily metabolized in yeast extracts. Finally, we demonstrated that the activity of the yeast *trans*-aconitate methyltransferase is localized in the cytosol and increases markedly as cells undergo the metabolic transition at the diauxic shift.

With the completion of the genome sequences of a number of organisms, we have been interested in identifying novel types of methyltransferases that may function in new metabolic pathways. We recently identified a *trans*-aconitate methyltransferase (Tam) in *Escherichia coli* that catalyzes the formation of the 6-methyl ester of *trans*-aconitate (*1*, *2*). While the enzyme also methylates *cis*-aconitate, isocitrate, and citrate at lower velocities and affinities compared to those of *trans*-aconitate, it has little or no activity with succinate, fumarate, malate, oxalacetate, or tricarballylate as methyl-accepting substrates. *trans*-Aconitate methyl ester is formed in extracts of wild-type cells incubated with *S*-adenosyl-L-methionine, and the free acid appears to be the major endogenous substrate for the Tam methyltransferase (*1*). *trans*-Aconitate can form spontaneously from the citric acid cycle intermediate *cis*-aconitate, but it does not play any known role in the normal metabolism of this organism.

Deletion mutants of *E. coli* lacking this methyltransferase appear to grow normally on a variety of media, and the survival of parent and mutant strains is similar in stationary phase and under heat shock, osmotic stress, and oxidative stress (*1*). The enzyme activity is induced in early stationary phase under the control of the RpoS sigma factor for the core polymerase, suggesting that the ability to methylate *trans*-aconitate may be most important when cell division is limited and *trans*-aconitate might accumulate (*1*). Because *trans*-aconitate is a known inhibitor of at least two citric acid cycle enzymes, aconitase and fumarase, we hypothesized that a possible function of the methyltransferase might be in a detoxification pathway (*1*). This hypothesis has been recently supported by data showing that the inhibition of *E. coli* aconitase by *trans*-aconitate is almost entirely relieved by its enzymatic methylation to give the 6-methyl ester (*2*).

Although a number of prokaryotic gene homologues have been found for the *E. coli* enzyme, no eukaryotic homologues have been detected (*1*). However, we were able to detect significant *trans*-aconitate methyltransferase activity in extracts of the budding yeast *Saccharomyces cerevisiae* (*1*). We further demonstrated that the methylation product of the yeast activity, the 5-methyl ester of *trans*-aconitate, is different from the 6-methyl ester formed by the *E. coli* enzyme (*2*). This result suggests that the yeast *trans*-aconitate methyltransferase may be distinct from the *E. coli* enzyme. Consistent with this idea, there was no apparent homologue of *E. coli* Tam in the encoded sequences of complete yeast genome searched by ungapped BLAST (*1*). To identify the gene encoding this activity, we took advantage of the systematic search for *S*-adenosyl-L-methionine-dependent methyltransferases in yeast using the common short-sequence motifs present in a variety of these enzymes (*3*). Here, 26

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putative methyltransferases identified have been designated *F1*−*F26*. In preliminary work, extracts prepared from null mutants of six of these genes (*F1, F3, F7, F8, F10,* and *F12*) were shown to have normal levels of *trans*-aconitate methyltransferase activity, indicating that none of them appears to encode the yeast *trans*-aconitate methyltransferase activity (*4*).

In this report, we have now been able to identify the gene for the *trans*-aconitate methyltransferase as the putative methyltransferase *F9* gene and designate it *TMT1*. Since yeast TMT1 catalyzes methyl esterification on a different carboxyl group in *trans*-aconitate from the *E. coli* enzyme, we were interested in further characterizing the substrate specificity of the yeast *trans*-aconitate methyltransferase, as well as its intracellular localization and activity as cells enter stationary phase. We found that the yeast enzyme methylates isocitrate and citrate more poorly than the *E. coli* enzyme, while it methylates itaconate, a decarboxylated form of *cis*- and *trans*-aconitate that is a very poor substrate for the *E. coli* enzyme. While we do show that *trans*-aconitate is an endogenous substrate for this enzyme in yeast extracts, we also find products distinct from *trans*-aconitate and itaconate methyl esters, suggesting that additional cellular substrates are recognized by this methyltransferase.

EXPERIMENTAL PROCEDURES

Media. Rich medium (YPD) consisted of 2% D-glucose, 1% yeast extract (DIFCO), and 2% peptone (DIFCO). Synthetic medium consisted of 2% D-glucose, 0.5% ammonium sulfate, 0.17% yeast nitrogen base (DIFCO, yeast nitrogen base without amino acids and ammonium sulfate), and either 0.077% CSM (complete supplement mixture for SD) or CSM-URA (complete supplement mixture without uracil for SD-URA), both obtained from BIO 101, Inc.

Preparation of Yeast and Escherichia coli Cytosolic Extracts for Methyltransferase Assay. Yeast cells were cultured in 5 mL of YPD at 30 °C and collected at an optical density of 9 at 600 nm by centrifugation at 1500*g* for 5 min. Cells were washed with 1 mL of sterile water twice. Cells were resuspended in 2 volumes of 50 mM Tris-HCl (pH 7.5) (relative to the volume of the cell pellet) and mixed with 2.5 volumes of glass beads (acid-washed, 0.5 mm diameter from Biospec Products, Inc.). Cells were disrupted by vortexing for seven cycles of 1 min followed by cooling on a water−ice bath for 1 min. Samples were then centrifuged at 10000*g* at 4 °C for 10 min, and the supernatant was saved as the cytosolic extract. Protein concentrations were determined by Lowry assay after precipitation with trichloroacetic acid (*5*). For the large-scale preparation of the overproduced enzyme, volumes were increased proportionally.

E. coli cytosolic extracts were prepared from BL21(DE3) cells containing the vector pHC107 as described previously (*1*). This vector results in a 630-fold overexpression of the *E. coli* Tam methyltransferase.

trans-Aconitate Methyltransferase Assay. The assay mixture included 2 μM [^{14}C]AdoMet[1] [diluted from a stock of 80 μM [^{14}C]AdoMet in dilute sulfuric acid (pH 2.5−3.5), specific radioactivity 53 mCi/mmol, Amersham Pharmacia Biotech], methyl-accepting substrates in a final concentration of 0.3 M NaHEPES buffer adjusted to pH 7.0 with NaOH, and enzyme. In some cases where indicated, 0.1 M NaHEPES buffer at pH 7.5 was used. Reactions mixtures were set up in 1.5 mL polypropylene microcentrifuge tubes with 30 μL of the methyl-accepting substrate at the bottom of the tube and 5 μL drops containing [^{14}C]AdoMet and enzyme pipetted along the upper wall of the tube so that they did not mix with each other or the material at the bottom of the tube. Assays were initiated by spinning the tube at 700 rpm for 5 s to mix the reactants, and the tubes were then placed in a 37 °C water bath for times ranging from 10 min to 1 h. This procedure avoids aerosol formation that can occur with vortexing small volumes. Concentrations of enzyme and reaction times were chosen so that the reactions proceeded under initial velocity conditions. Reactions were stopped by placing the tubes on dry ice for 10 min. Individually, each tube was then partially melted by submerging the tube into a warm water bath at 70° C for about 5 s. The reaction was quenched by the addition of 40 μL of 2 N NaOH at room temperature, and the tube then was vortexed at top speed for approximately 4 s until the mixture fully thawed. Immediately, 60 μL of the solution was pipetted onto an accordion-pleated 1.5 × 8 cm piece of thick filter paper (Bio-Rad 165-0962), and the paper was placed into the neck of a 20 mL scintillation vial containing 5 mL of Safety-Solve scintillation fluid (Research Products International Corp.), then capped, and incubated at room temperature for 2 h. The amount of volatile radioactivity (as [^{14}C]methanol from the hydrolysis of the product methyl ester) was determined by liquid scintillation counting in a Beckman LS6500 counter after the removal of the filter paper. A background value consisting of no substrate added was subtracted in each case. Assays were performed in triplicate unless otherwise indicated.

trans-Aconitic acid, *cis*-aconitic acid, (2*R*,3*S*)-isocitric acid [*threo*-D_s(+)-isocitric acid, monopotassium salt, approximately 99%], DL-isocitric acid [*threo*-D_sL_s-isocitric acid, trisodium salt, 93−99%], citric acid (tripotassium salt), tricarballylic acid, (±)-methylsuccinic acid, itaconic acid, citraconic acid, mesaconic acid, and L-malic acid (95−100%) were Sigma products; oxalacetic acid and succinic acid (99.9%) were products of Fisher Scientific, and *trans*-glutaconic acid (90%) was an Aldrich product. All substrates were dissolved in 0.4 M NaHEPES buffer, pH 7.0, and the pH was readjusted to 7.0 with either NaOH or HCl.

Yeast Chromosomal Disruption of YER175c. The disruption of yeast open reading frame *YER175c* was carried out using a one-step gene replacement method (*6*). The disruption cassette was generated by PCR with primers YER175c-KO-5 (5′-GATGAATACCACGACGGAGAAAGGAAATTACT-CGTAGATGTcagctgaagcttcgtacgc-3′) and YER175c-KO-3 (5′-CTTTAATAAACCAATCTGCTACATCTTCTTTATC-TTTGgcataggccactagtggatctg-3′). In the KO-5 primer, the bases in upper case letters are from +7 to +47 from the start codon, while the bases in upper case letters in the KO-3 primer correspond to the reverse complement of bases +730 to +693. In both primers, the bases in lower case letters correspond to the left and right of the flanking regions of the KanMX module in the plasmid pUG6A (*6*). The KanMX module was amplified from pUG6A with a 50 μL PCR

[1] Abbreviations: AdoMet, *S*-adenosyl-L-methionine; [^{3}H]AdoMet, *S*-adenosyl-L-[*methyl*-^{3}H]methionine; [^{14}C]AdoMet, *S*-adenosyl-L-[*methyl*-^{14}C]methionine; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate.

reaction containing 5 μL of 10× Promega buffer, 2.5 μL of 50 mM $MgCl_2$, 1 μL of 20 mM dNTP, 1 μL of 20 μM each of the primers, 15 ng of pUG6A, and 0.4 unit of Taq. The PCR conditions were 95 °C for 30 s, 55 °C for 30 s, 72 °C for 2.5 min (30 cycles). A single band about 1.6 kb was cut and purified from an agarose gel with GeneClean II (Bio101, Inc.). Six micrograms of disruption cassette in 10 μL of water was used to transform yeast strain GPY1100α as described by Guldener et al. (*6*). After 36 h of incubation at 30 °C on a YPD plate containing 50 μg/mL geneticin disulfate (antibiotic G418; Sigma), microcolonies were replica-plated onto a fresh geneticin-containing plate. Colonies observed after overnight incubation were restreaked twice before the disruption was confirmed by PCR analysis using primers YER175c-KO-5 (described above) and YER175c-OE-3 (5′-TCGACCCTTTTGCCAAGTTTG-3′, corresponding to bases +819 to +799 from the start codon). The resulting 1.7 kb fragment is consistent with a deletion between bases +47 and +693 and the insertion here of the geneticin marker into the *YER175c* gene. The resulting strain is designated HCY001 (Table 1).

Overexpression of the YER175c Gene Product in Yeast. The *YER175c* gene was PCR-amplified from colonies of GPY1100α using the primers F9-EcoRI.F (5′-ttttgaattc-TCATGACTCACAGTA-3′; the bases in upper case letters correspond to nucleotides −254 to −240 from the translational start site with the bases in lower case letters added to create an *Eco*RI site) and F9-T7.R (5′-AATCAAGCTTC-CTGGCAGTAACAGA-3′; corresponding to the reverse complement of bases +1019 to +995 with the underlined bases changed to create a *Hin*dIII site; the stop codon is located at bases +817 to +819). The 1283 bp PCR fragment was cleaved with *Eco*RI and *Hin*dIII and the large fragment purified as described above. This fragment was then cloned into the corresponding sites in the multicloning site of the pRS316 or pRS426 vectors (*7*) to generate pJK1 and pJK2. DNA sequence analysis using both oligonucleotides described above as primers showed that no mutations were introduced during the cloning procedure. The plasmids were then transformed into HCY001 to give HCY003 and HCY005 (Table 1). The vectors pRS316 and pRS426 were also transformed into HCY001 to give HCY002 and HCY004 and were used as negative controls.

In Vitro Labeling of the Endogenous Substrate(s) of the Yeast trans-Aconitate Methyltransferase. Cytosolic extracts from the parent strain (GPY1100α) and the knock-out strain (HCY001) were prepared as described above and used for in vitro labeling. Twenty-five microliters of *S*-adenosyl-L-[*methyl*-^{3}H]methionine ([^{3}H]AdoMet, 13.0 μM, in dilute hydrochloric acid/ethanol 9:1 (pH 2.0−2.5), 77.0 Ci/mmol, Amersham Pharmacia Biotech) and 20 μL of 0.4 M Na-HEPES, pH 7.5, were added to 50 μL of the cytosolic fractions containing 0.85 mg of total protein, and the reactions were incubated at 37 °C for 2 h. The reaction mixtures were then acidified with 20 μL of 10 N sulfuric acid, and 200 μL of ether was used to extract the product(s) 3 times. The ether phases were combined, air-dried, and dissolved into 200 μL of 60 mM potassium phosphate, pH 4.5.

An itaconate methyl ester standard was made in a reaction containing 5 μL of 0.02 M sodium itaconate, pH 7.5, 10 μL of 400 mM NaHEPES, pH 7.5, 1 μL of [^{14}C]AdoMet or 5 μL of [^{3}H]AdoMet, 5 μL of extracts of strain GPY1100α containing 60 μg of protein, and water to a total volume of 40 μL. The sample was incubated at 37 °C for 2 h before it was ether-extracted and purified as by anion-exchange chromatography on a SAX column as described (*1*). The peak fraction at 5 min was used for further analysis.

Turnover Analysis of trans-Aconitate 5-Methyl Ester in Yeast. The purified yeast methylation product of *trans*-aconitate labeled with [^{14}C]AdoMet was made as described (*2*). Two microliters of the purified methylated *trans*-aconitate (1200 cpm) was incubated in 23 μL of 50 mM Tris-HCl, pH 7.5, with or without 5 μL of the yeast cytosolic extract (8.4 mg/mL) at 37 °C for 2 h. Five microliters of 50 mM Tris-HCl, pH 7.5, was used to replace the crude extract as a control. To the sample was then added 40 μL of water or 40 μL of 2 N NaOH, and 60 μL of the mixture was immediately spotted on the folded filter paper and incubated in a capped scintillation vial with 5 mL of the scintillation fluid. The volatile radioactivity was counted after 3 h incubation at room temperature. When esterase was used, 2 μL of methylation product was added either to 50 μL of 100 mM potassium phosphate, pH 7.5, or to 50 μL of the same buffer containing pig liver esterase (Sigma product E3128, 150 units/mg of protein) at a concentration of either 10 or 50 μg/mL. The sample was incubated at 37 °C for 2 h before being quenched with 52 μL of water or 2 N NaOH, and the volatile radioactivity was detected as described above.

Fractionation of Mitochondria and Cytoplasm from Yeast. An overnight culture (5 mL) of yeast strain GPY1100α (Table 1) was diluted into 500 mL of fresh YPD media in a 2 L flask and incubated with shaking at 30 °C for 24 h. Cells were collected at an optical density of 9 at 600 nm and washed twice with 100 mL of water. Cells were lysed, and cytosolic (S2) and mitochondrial (M) fractions were isolated by the method of Glick and Pon (*8*). The mitochondrial fraction was further purified by the linear Nycodenz gradient method (*8*). The mitochondrial fraction at 16% Nycodenz density was collected, and the Nycodenz was removed by spinning down the mitochondria at 12000*g* for 10 min and suspending the pellet in 1 mL of 0.6 M sorbitol, 20 mM K-HEPES, pH 7.4. Immediately before analyses, 10% Triton X-100 (w/v) was added to the mitochondrial preparation to a final concentration of 1% and mixed well, and the sample was incubated at room temperature for 5 min. The Triton-treated mitochondria were then centrifuged at 12000*g* at 4 °C for 10 min, and the supernatant (M1) was then used as the mitochondrial fraction.

Enzyme Analyses and Western Analysis of the Cytosolic and Mitochondrial Fractions. The protein concentration and the *trans*-aconitate methyltransferase activity of the mitochondrial and cytosolic fractions were determined as described above. Alcohol dehydrogenase activity was measured by mixing 0.5 mL of 50 mM potassium phosphate buffer, pH 7.5, 0.17 mL of 2 M ethanol, 0.33 mL of 0.025 M NAD^+ (Sigma Chemical Co.), and 10 μL of cell fractions (*9*). The reaction was carried out at room temperature, and the change of absorbance at 340 nm was monitored for 3 min. For the Western analysis, samples (10 μg of protein) from each fraction were separated on a 12% SDS−polyacrylamide gel. Proteins were transferred from the gel to a piece of nitrocellulose transfer membrane (WP4HYB0010, Micron Separations Inc., Westborough, MA). The membrane was

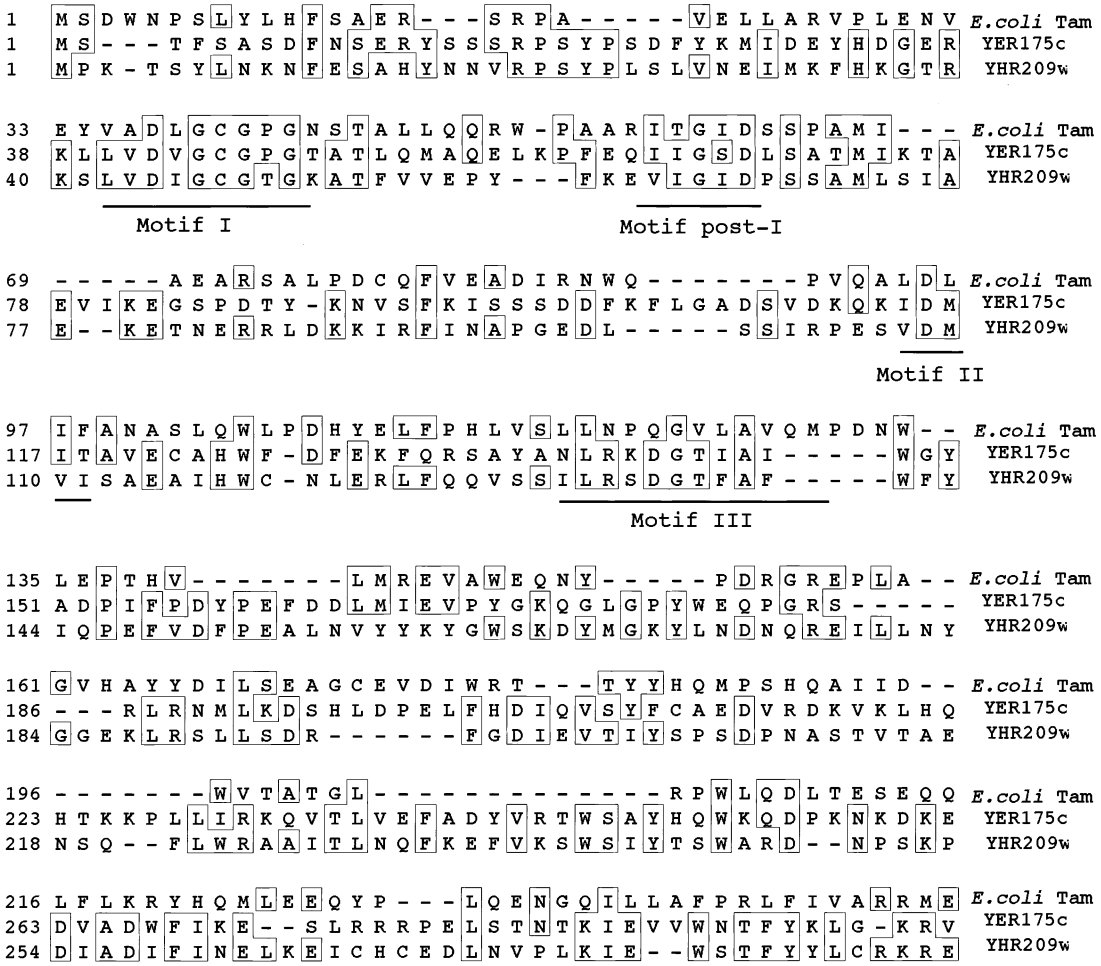

FIGURE 1: Protein sequence comparison between the products of the *E. coli trans*-aconitate methyltransferase gene, the yeast *YER175c* (*TMT1*) gene, and the yeast *YHR209w* gene. The protein sequences are aligned using the Clustal method in the MegAlign program (DNAStar). Residues that are present in two of the three sequences are boxed. The methyltransferase motifs in the *E. coli trans*-aconitate methyltransferase are underlined.

blotted overnight with milk-containing blotting buffer and probed with Hsp60 antibody as described by Poon et al. (*10*), followed by detection of the interaction between Hsp60 and its antibody by chemiluminescence (ECL Western blotting analysis system, Amersham Pharmacia Biotech, RPN 2108).

RESULTS

Identification of the Yeast Gene Encoding the trans-Aconitate Methyltransferase. We previously detected *trans*-aconitate methyltransferase activity in extracts of *S. cerevisiae* at levels comparable to those found in *E. coli* extracts (*1*). However, we were unable to find an apparent homologue of *E. coli trans*-aconitate methyltransferase *tam* gene in the complete yeast genome database using a BLAST 1.4 search algorithm. We have now researched the yeast genome database using the gapped alignments of the BLAST 2.0 algorithm (*11*). The top three matches to the *E. coli tam* product displayed marginally significant *E* (expect) scores of 0.001, 0.019, and 0.043 and corresponded to previously identified putative methyltransferases F7 (Ycr047c), F10 (YHr209w), and F9 (Yer175c)(*3*). Because normal levels of *trans*-aconitate methyltransferase activity had previously been found in a *Ycr047c* null mutant (*4*), we focused our efforts on the *YER175c* and the *YHR209w* products. The amino acid sequences of these products are very similar to each other (expect value is 1e-33). In Figure 1, we align these sequences with the *E. coli tam* gene product and show that all three proteins include the conserved methyltransferase motifs (*3*).

We then measured *trans*-aconitate methyltransferase activity in extracts of yeast strains where the *YER175c* and *YHR209w* genes were disrupted, resulting in null mutants (Tables 1 and 2). We found that the null mutant of *YHR209w* contains the same level of *trans*-aconitate methyltransferase activity as its parent strain (Table 2), confirming the result obtained with a different knockout strain (*4*). On the other hand, no methyltransferase activity was found in extracts of a mutant strain with a disrupted *YER175c* gene (Table 2). When assayed with alternative substrates, normal levels of methyltransferase activity were found toward *cis*-aconitate, DL-isocitrate, and citrate in extracts of the *YHR209w* mutant, but no activity was found in extracts of the *YER175c* mutant (data not shown). These results point to the *YER175c* gene as a prime candidate for encoding the yeast *trans*-aconitate methyltransferase, and suggest that a single gene product is responsible for the methylation of all of these substrates. We found that the *YER175c* mutant grows normally in YPD media and in synthetic media containing glucose, glycerol, ethanol, or acetate as carbon sources.

Table 1: Strains and Plasmids Used

strain/plasmid	genotype/description	ref
	Strains	
GPY1100α	MATα, *leu*2-3, 112, *ura*3-52, *his*4-159, *trp*1, *can*1	*a*
HCY001	GPY1100α, Yer175c (Δ16−231)::Kan^r	this study
BY4741	MAT**a**, *his*3Δ1, *leu*2Δ0, *met*15Δ0, *ura*3Δ0	*b*
2903	BY4741, ΔYhr209w::Kan^r	*b*
HCY002	HCY001 carrying pRS316	this study
HCY003	HCY001 carrying pJK1	this study
HCY004	HCY001 carrying pRS426	this study
HCY005	HCY001 carrying pJK2	this study
	Plasmids	
pJK1	overexpression vector for Yer175c, containing Yer175c between *Eco*RI and *Hin*dIII sites in multicloning site of pRS316	this study
pJK2	overexpression vector for Yer175c, containing Yer175c between *Eco*RI and *Hin*dIII sites in multicloning site of pRS426	this study

[a] Obtained from Dr. Greg Payne, UCLA. [b] Obtained from the Saccharomyces Genome Deletion Project, www-sequence.stanford.edu/group/yeast_deletion_project/deletions3.html.

Table 2: *trans*-Aconitate Methyltransferase Activities in Yer175c and Yhr209w Null Mutants[a]

strain	genotype	methyltransferase activity [pmol min^{-1} (mg of protein)$^{-1}$]
GPY1100α	*Yer175c*$^+$, *Yhr209*$^+$	412.5 ± 9.0
HCY001	*Yer175c*$^-$, *Yhr209*$^+$	−0.2 ± 0.1
BY4741	*Yer175c*$^+$, *Yhr209*$^+$	203.9 ± 4.0
2903	*Yer175c*$^+$, *Yhr209*$^-$	209.5 ± 8.6

[a] Cytosolic extracts from each of the strains were prepared and assayed for *trans*-aconitate-dependent activity as described under Experimental Procedures at pH 7.5 using 1 mM *trans*-aconitate. Results are expressed with the standard deviations obtained from three parallel experiments.

Overexpression of the Yer175c Gene Product Results in the Overexpression of trans-Aconitate Methyltransferase Activity in Yeast. To confirm that *YER175c* is the gene encoding *trans*-aconitate methyltransferase in yeast, we cloned it into a centrimeric pRS316 plasmid and a multicopy pRS426 yeast shuttle vector as described under Experimental Procedures. The resulting plasmids, designated pJK1 and pJK2, were then introduced into HCY001, the null mutant of *YER175c*, to generate yeast strains HCY003 and HCY005 (Table 1). We found that both plasmids restored the *trans*-aconitate methyltransferase activity in the HCY001 mutant strain which otherwise lacks the activity (Table 3). The *trans*-aconitate methyltransferase activity was found to be overexpressed by 6.9-fold in the HCY003 strain with the pJK1 centrimeric plasmid and by 33.9-fold in the HCY005 strain with the pJK2 multicopy plasmid, each compared to the specific activity in the GPY1100α parent strain (Table 3). Extracts from strains HCY002 or HCY004 carrying only the vector showed no methyltransferase activity (Table 3). These results demonstrate that the *YER175c* gene does in fact encode a yeast version of the *trans*-aconitate methyltransferase, and the gene has now been designated *TMT1*. Of the 320 residues aligned in Figure 1, only 43 (13%) are identical in the yeast *TMT1* and the *E. coli tam* gene products.

Table 3: Overexpression of Yer175c in Yeast[a]

strains	*trans*-aconitate methyltransferase activity [pmol min^{-1} (mg of protein)$^{-1}$]	fold-induction
GPY1100α	218.3	1.0
HCY001	0.1	0.0005
HCY002	−0.3	0
HCY003	1496.3	6.9
HCY004	0.7	0.003
HCY005	7409.6	33.9

[a] An aliquot of an overnight culture of the cells (20 μL) from HCY002, HCY003, HCY004, and HCY005 in SD-URA was diluted into 20 mL of fresh SD-URA medium, incubated with shaking at 30 °C, and cultured to an optical density at 600 nm of 3.5−4. Cells of GPY1100α were grown by the same method, but in SD medium. Cytosolic extracts were then prepared and assayed at pH 7.5 for *trans*-aconitate methyltransferase activity as described under Experimental Procedures in the presence of 1 mM *trans*-aconitate. The values are averages of two experiments.

To date we have found no clear homologues of the *TMT1* gene in other organisms. From a BLASTP 2.1.2 search of the GenBank nonredundant database (version of 16 January 2001), the best matches were the *S. cerevisiae YHR209w* gene product (expect value 1e-33), a hypothetical protein of *Pseudomonas aeruginosa* (expect value 1e-12), and an embryonic abundant-like protein from *Arabidposis thaliana* (expect value 4e-12). The closest match in the completed genome of the fission yeast *Schizosaccharomyces pombe* had an expect value of 5e-6. These results indicate that the yeast *trans*-aconitate methyltransferase may be a relatively poorly distributed species, or that it has rapidly evolved in other organisms.

Yeast YER175c trans-Aconitate Methyltransferase Exhibits a Distinct Methyl-Accepting Substrate Specificity from the E. coli Enzyme. Both yeast and *E. coli trans*-aconitate methyltransferases catalyze the monoesterification of *trans*-aconitate, but form the 5-methyl ester and 6-methyl ester, respectively (*2*). To investigate the specificity of the yeast methyltransferase, we first did kinetic analysis with the known substrates of the *E. coli* enzyme, including *trans*-aconitate, *cis*-aconitate, (2*R*,3*S*)-isocitrate, DL-isocitrate, and citrate. We used a cell extract of the overproducing yeast strain HCY005 as the source for the yeast *trans*-aconitate methyltransferase (Table 1), and an extract of *E. coli* strain BL21(DE3) cells containing the plasmid pHC107 as a source for the *E. coli* enzyme (*1*). We found that the methyl-accepting substrates with the highest apparent affinities gave data consistent with simple Michaelis−Menten kinetics, and substrates with the lowest affinities demonstrated a linear increase in enzyme velocity with concentration (Figure 2).

We confirmed that the best of these methyl-accepting substrates for both the yeast enzyme and the *E. coli* enzyme is indeed *trans*-aconitate with K_m values of 0.66 and 0.52 mM, respectively (Figure 2, Table 4). Both enzymes also displayed similar kinetic values for *cis*-aconitate, although this substrate was recognized with about 100-fold weaker affinity and 4−5-fold lower velocity for each enzyme than for *trans*-aconitate (Figure 2, Table 4). While some of the activity seen with *cis*-aconitate can result from a minor contamination of this compound with *trans*-aconitate, purification of the *cis*-aconitate preparation by anion-exchange chromatography to remove the *trans*-isomer results in

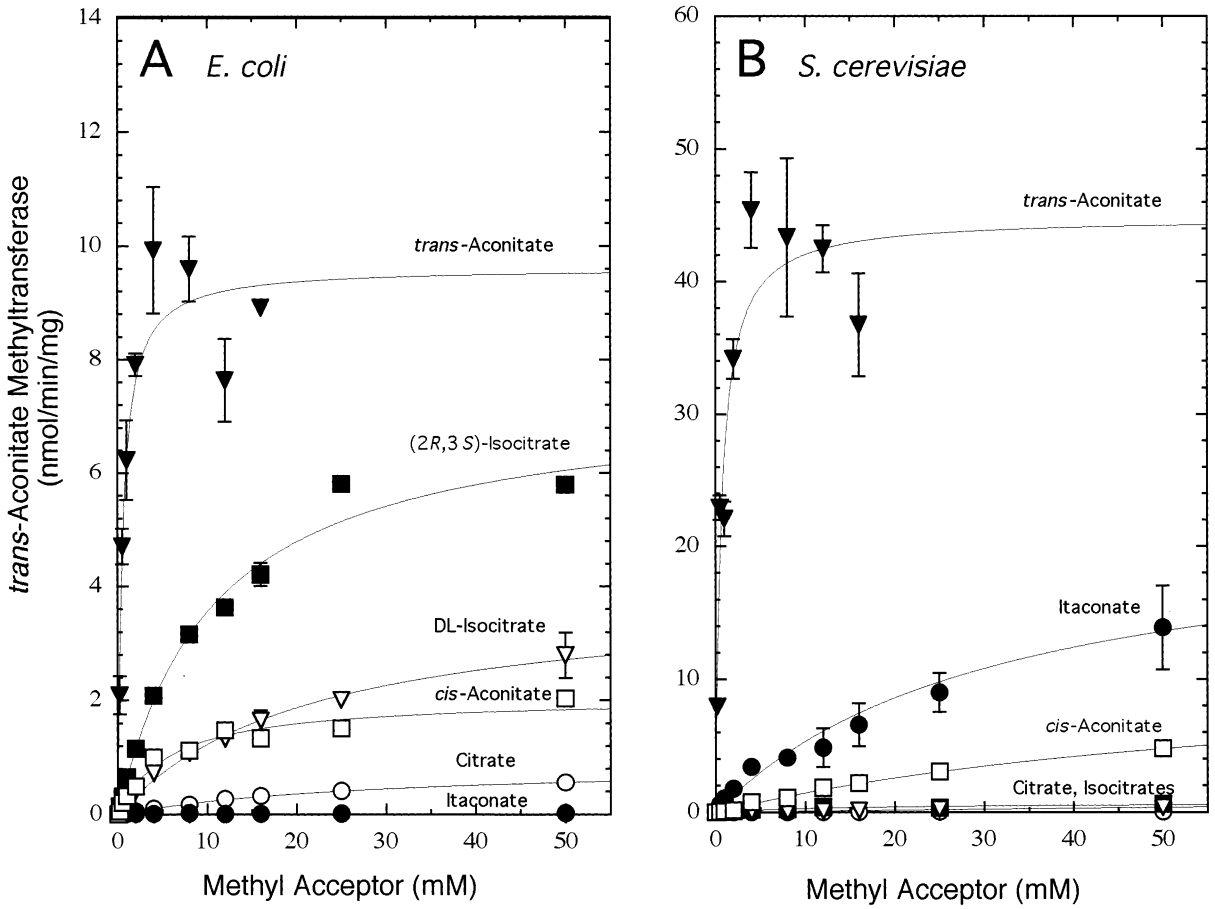

FIGURE 2: Methyl-accepting substrate specificity of the yeast and *E. coli trans*-aconitate methyltransferases. Methyltransferase activity was determined as described under Experimental Procedures using extracts of a yeast *TMT1* overproducing strain and an *E. coli* Tam overproducing strain as described in Table 4. Assays were performed in triplicate, and the standard deviation is shown for all points where it was greater than the width of the plot symbol. These data were fitted to Michaelis−Menten curves as described in Table 4. Reaction times varied for each substrate, and between enzymes [for *E. coli* enzyme, *trans*-aconitate, *cis*-aconitate, itaconate, citrate, (2*R*,3*S*)-isocitrate, and DL-isocitrate reacted for 10 min while oxalacetate reacted for 1 h; for yeast enzyme, *trans*-aconitate and itaconate reacted for 10 min whereas *cis*-aconitate, citrate, (2*R*,3*S*)-isocitrate, DL-isocitrate, and oxalacetate were allowed to react for 1 h]. All reactions were incubated at 37° C and maintained a pH value of 7.0. [*trans*-Aconitate is represented by closed triangles, *cis*-aconitate by open squares, itaconate by closed circles, DL-isocitrate by open triangles, (2*R*,3*S*)-isocitrate by closed squares, and citrate by open circles.]

material that is still recognized by both the yeast enzyme (data not shown) and the *E. coli* enzyme (*1*). On the other hand, (2*R*,3*S*)-isocitrate, DL-isocitrate, and citrate, which are marginally good substrates for the *E. coli* enzyme, with K_m values in the 10−45 mM range, are much poorer substrates for the yeast enzyme. Here, we could not measure a K_m value (Figure 2), and the relative V_{max}/K_m values are 100−200-fold less than that of the *E. coli* enzyme (Table 4, Figure 3). For the *E. coli* enzyme, we found that the activity with (2*R*,3*S*)-isocitrate was almost exactly twice that of the racemic mixture DL-isocitrate, suggesting that only the former compound is recognized by this enzyme. These results indicate that the yeast enzyme is somewhat more specific than the *E. coli* enzyme, particularly in its very poor recognition of citrate and isocitrate.

We then assayed these enzyme preparations with a much wider variety of dicarboxylic acids and tricarboxylic acids structurally related to *trans*-aconitate (Table 4, Figure 3). In this group of compounds, we did find one species that was a better substrate for the yeast enzyme than the *E. coli* enzyme. This is itaconate, a decarboxylation product of aconitate, that demonstrated a K_m for the yeast enzyme of 44 mM and a maximal velocity of almost 60% that of *trans*-aconitate (Figure 2). Only a barely detectable methylation activity for itaconate was observed for the *E. coli* enzyme with a relative V_{max}/K_m value about 1000 times lower than that of the yeast enzyme (Figures 2 and 3). All of the other substrates test either were found to not be substrates or were very poor substrates, with relative values of the V_{max}/K_m no more than 0.06% that of *trans*-aconitate for the yeast enzyme and no more than 0.003% for the *E. coli* enzyme (Figure 3, Table 4). These results suggest that while the yeast enzyme is not absolutely specific for its *trans*-aconitate substrate, it certainly recognizes it better than any of the other tested substrates. This is also the case for the *E. coli* enzyme.

Endogenous Substrates of Yeast trans-Aconitate Methyltransferase. In *E. coli*, we showed that *trans*-aconitate is an endogenous substrate of the methyltransferase by comparing the methylation products from in vitro labeled cytosolic extracts and enzymatically methylated *trans*-aconitate standard (*1*). Here, we took the same approach to examine the endogenous substrates of the yeast *trans*-aconitate methyltransferase. We incubated crude extracts of the wild-type parent GPY1100α strain (*TMT1*$^+$) and the mutant HCY001 strain (*tmt1*$^-$) with [^{3}H]AdoMet in the absence of any exogenous methyl acceptors. We analyzed the extracts for

Table 4: Substrate Specificity of the *E. coli* and *S. cerevisiae trans*-Aconitate Methyltransferases[a]

	E. coli Tam enzyme			*S. cerevisiae* TMT1 enzyme		
substrate	K_m (mM)	V_{max} [nmol min^{-1} (mg of protein)$^{-1}$]	V_{max}/K_m	K_m (mM)	V_{max} [nmol min^{-1} (mg of protein)$^{-1}$]	V_{max}/K_m
trans-aconitate	0.52 ± 0.04	9.6 ± 0.6	18.5	0.66 ± 0.06	44.8 ± 3.8	67.9
cis-aconitate	36 ± 13	2.1 ± 0.1	0.06	74 ± 22	12.1 ± 3.1	0.16
itaconate			0.0002 ± 0.0001	44 ± 12	26.9 ± 9.1	0.61
(2*R*,3*S*)-isocitrate	10.4 ± 0.1	7.2 ± 0.4	0.69			0.012 ± 0.004
DL-isocitrate	23.1 ± 4.5	4.1 ± 0.8	0.18			0.0077 ± 0.0017
citrate	45 ± 14	1.2 ± 0.3	0.03			0.0006 ± 0.0002
methylsuccinate			0.0001 ± 0.00001			0.042 ± 0.008
succinate			0.0003 ± 0.0001			0.015 ± 0.002
mesaconate			0[b]			0.011 ± 0.005
tricarballylate			0.0003 ± 0.0001			0.006 ± 0.002
L-malate			0.0005 ± 0.0001			0.004 ± 0.001
citraconate			0[b]			0.002 ± 0.0008
oxaloacetate			0[b]			0.0007 ± 0.0002
trans-glutaconate			0[b]			0.0002 ± 0.0001

[a] Methyltransferase activity was measured as described under Experimental Procedures to obtain initial velocity data. The enzyme sources were overproduced extracts of the *E. coli tam* gene expressed in *E. coli* (0.36 μg of protein) and the *S. cerevisiae TMT1* gene expressed in *S. cerevisiae* (0.027 μg of protein). Assays were with *cis*-aconitate, itaconate, (2*R*,3*S*)-isocitrate, DL-isocitrate, citrate, and oxaloacetate at concentrations from 0.2 to 50 mM for 10−60 min and with *trans*-aconitate at concentrations of 0.2−16 mM for 10 min. Mesaconate, citraconate, *trans*-glutaconate, succinate, methylsuccinate, tricarballylate, and malate were assayed at 4, 16, and 50 mM for 1 h. K_m and V_{max} values (with standard deviation values) were determined by averaging the values obtained by fitting the data to the Michaelis−Menten equation using the curve-fitting program at www.biomechanic.org. in three separate experiments. V_{max}/K_m values were determined by dividing the maximum velocity by the K_m for substrates that were fit to a Michaels−Menten curve. For the substrates that could not be fit to a Michaelis−Menten curve, a linear best-fit curve was used to determine the slope of the velocity/substrate concentration curve in three separate experiments. This value equals the V_{max}/K_m value when the assumption is made that $K_m \gg [S]$ in the Michaelis−Menten equation: $v = V_{max}[S]/(K_m + [S])$. The standard deviation of these values is also given. All stock substrate solutions were made by dissolving the substrate in water; then the pH value was adjusted with either NaOH or HCl to a final value of 7.0. Both enzyme and [^{14}C]AdoMet solutions were diluted in 0.4 M NaHEPES buffer at pH 7.0. [b] No activity detected.

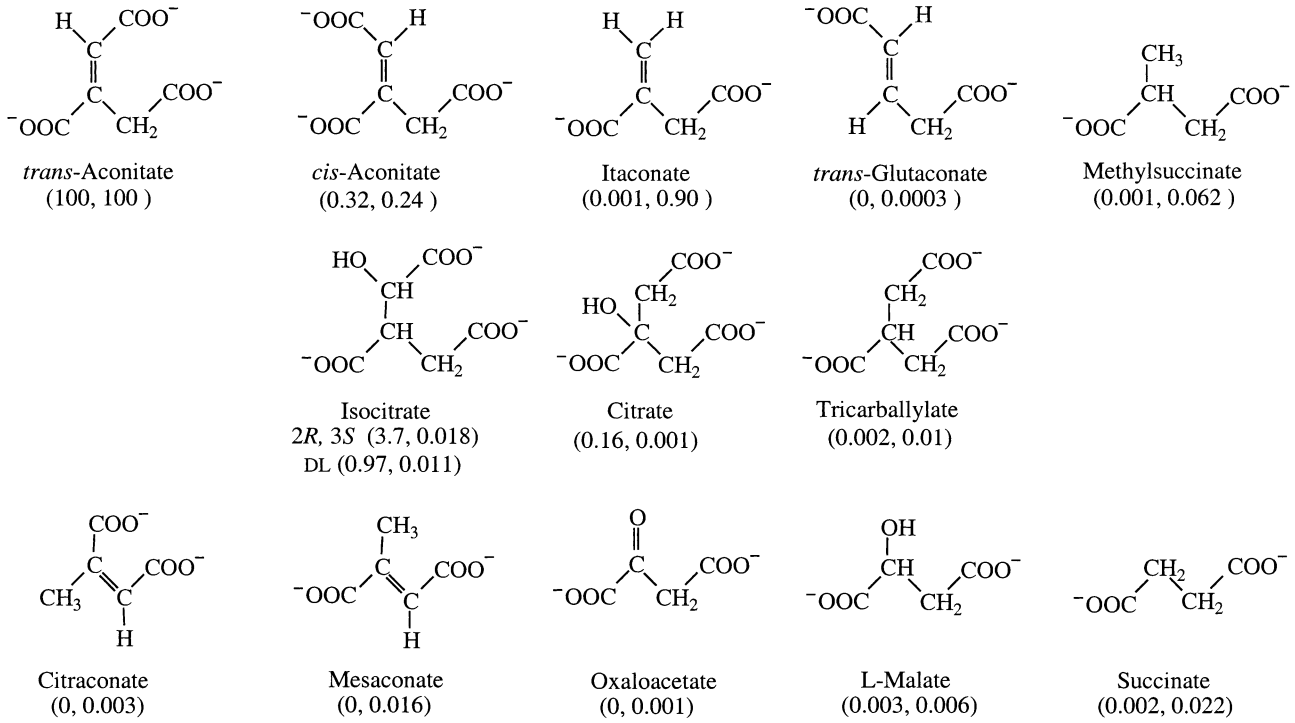

FIGURE 3: Recognition of di- and tricarboxylic acids as substrates for the *trans*-aconitate methyltransferase of *E. coli* (Tam) and *S. cerevisiae* (TMT1). The numbers below each structure give values of the V_{max}/K_m (Table 4) for the Tam and TMT1 enzymes, respectively, expressed as a percentage of that of *trans*-aconitate.

radioactivity in compounds present in the parent but not in the mutant strain lacking the *trans*-aconitate methyltransferase by chromatographing the acidic ether-soluble fractions from both strains on an anion-exchange HPLC column. We found a peak of radioactivity that comigrated with a synthetic standard of the 5-methyl ester of *trans*-aconitate in the wild-type extract but no radioactivity at that position in the extract from the methyltransferase-deficient mutant. All of this radioactivity could be converted to a volatile form after base-hydrolysis, consistent with a methyl group in an ester linkage and confirming the presence of *trans*-aconitate in yeast (Figure 4). However, we found that the bulk of the methyl ester radioactivity from the wild-type extract eluted in the early fractions of the column. No methyl ester radioactivity

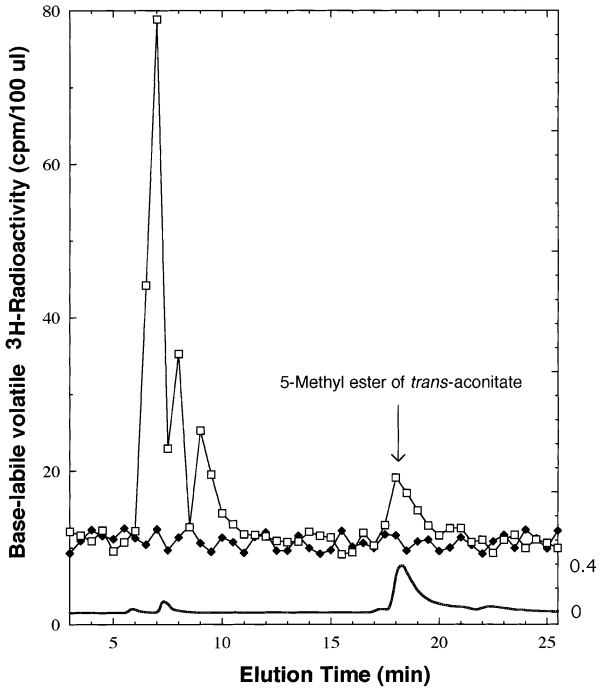

FIGURE 4: Detection of the endogenous substrates for yeast *trans*-aconitate methyltransferase in crude extracts. Cytosolic extracts (15 μL, 25 μg of protein) from the parent strain (GPY1100α) and the *TMT1* knock-out strain (HCY001) were mixed with 5 μL of 2.6 μM [^{3}H]AdoMet and 20 μL of 0.4 M NaHEPES, pH 7, and incubated at 37 °C for 2 h as described under Experimental Procedures. The reaction mixtures were then acidified and ether-extracted as described. After mixing with a synthetic standard of the 5-methyl ester of *trans*-aconitate (*2*), 100 μL of the ether phase dissolved in 60 mM potassium phosphate, pH 4.5, was loaded onto an SAX anion-exchange column and eluted with 60 mM potassium phosphate at 1 mL/min. Fractions (1 mL) were collected, and 100 μL of each fraction was counted directly to obtain the total radioactivity, or quenched with 100 μL of 2 N NaOH and processed for the vapor-phase diffusion assay as described under Experimental Procedures to obtain the base-labile volatile radioactivity and quantitate methyl esters. With the exception of a small increase in radioactivity in the fraction eluting at 6 min, the total radioactivity was found to be similar to the base-labile volatile radioactivity; therefore, only the base-labile volatile radioactivity is shown in open squares for the parent and in closed diamonds for *TMT1* knockout cell extracts. The position of the synthetic 5-methyl ester of *trans*-aconitate is shown by the absorbance trace at 214 nm (solid line).

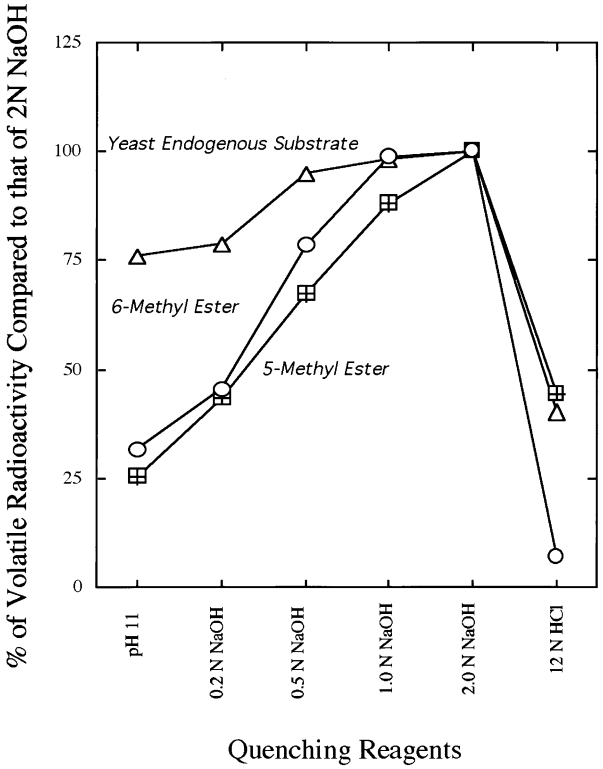

FIGURE 5: Comparison of the base- and acid-lability of yeast and *E. coli* methylation products of *trans*-aconitate and the methylated yeast endogenous substrate. The yeast methylation product of *trans*-aconitate (5-methyl ester) was prepared as described in Cai et al. (*2*), the yeast methylation product of the endogenous substrate was prepared as described under Experimental Procedures, and the *E. coli* methylation product of *trans*-aconitate (6-methyl ester) was prepared as described (*1*). All the products were purified on the SAX anion-exchange HPLC column. An aliquot (20 μL) of the fraction that contained the highest radioactivity was mixed with 20 μL of 2 M Na_2CO_3, pH 12, to result in a final pH of 11, or with 50 μL of the indicated reagents and incubated for 3 h at room temperature. The volatile radioactivity was detected the same way as described in the methyltransferase activity assay. The values are averages of two independent experiments.

was detected from the methyltransferase-deficient extracts, suggesting that these products were also made by the *trans*-aconitate methyltransferase (Figure 4).

To analyze the nature of the major endogenous product eluting at 7 min on the anion-exchange column, we tested its relative stability under acidic and basic conditions. We measured volatile radioactivity (as methanol) resulting from its methyl ester hydrolysis, as well as that from standards of the 5-methyl ester and 6-methyl ester of *trans*-aconitate. The methylated major endogenous yeast product was found to be more base-labile than the 5-methyl and 6-methyl esters of *trans*-aconitate, releasing 75% of the radioactivity at pH 11 under conditions that only result in the release of 25−35% of the radioactivity from the standards (Figure 5). Under acidic conditions, the major methylated endogenous product and the 5-methyl ester of *trans*-aconitate were found to be more labile than the 6-methyl ester of *trans*-aconitate (Figure 5).

Because we identified itaconate as the next best substrate for the yeast methyltransferase (Table 4), we then asked whether the major endogenous product might represent its methyl ester product. We compared the elution position of the methylated yeast endogenous substrate with an enzymatically methylated itaconate standard in chromatography experiments. We first analyzed the two products by anion-exchange HPLC under conditions previously used to separate *cis*- and *trans*-aconitate (*1*). We found that the two products both eluted at 5 min. Since the peak fractions are very close to the void volume of the column (3.5 min), we decreased the concentration of the elution buffer from 60 to 25 mM, and co-chromatographed the methylated endogenous substrate (labeled with ^{3}H) with the methylated itaconate standard (labeled with ^{14}C). Under these conditions, both species eluted later and showed a difference in elution time, with the methylated endogenous substrate eluting at 7.3 min and the methylated itaconate standard eluting at 6.5 min (Figure 6A). To determine whether an isotopic effect on the elution position may occur (*12*), we showed the methylated itaconate standard labeled either with ^{3}H or with ^{14}C co-chromatographed (Figure 6B). To further confirm that the major endogenous yeast product was not the itaconate methyl

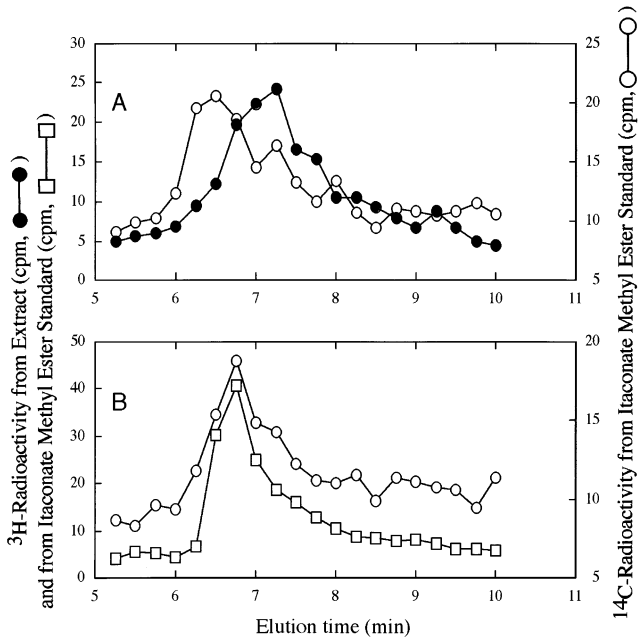

FIGURE 6: Separation of the methylation product of itaconate and the endogenous yeast substrate using SAX anion-exchange chromatography. (A) 20 μL of the peak fraction (5.5 min) from the methylated endogenous substrate and the peak fraction (5.5 min) of the methylated itaconate were mixed and rechromatographed on the SAX anion-exchange column run under the same conditions. Fractions of 0.25 mL were collected and used to obtain the total radioactivity. The ^{3}H-radioactivity from the cell extracts is shown in closed circles, and the ^{14}C-radioactivity from the itaconate methyl ester is shown in open circles. (B) 20 μL of the peak fraction (5.5 min) of the methylated itaconate (either with [^{3}H]AdoMet or with [^{14}C]AdoMet) was mixed and rechromatographed on the SAX anion-exchange column run under the same conditions. The ^{3}H-radioactivity from the itaconate methyl ester is shown in open squares, and the ^{14}C-radioactivity from the itaconate methyl ester is shown in open circles.

ester, we analyzed the products on reverse-phase HPLC and found that the methylated endogenous substrate eluted at 22.8 min whereas the methylated itaconate eluted at 21.5 min (data not shown).

Hydrolysis of the 5-Methyl Ester of trans-Aconitate in Yeast Extracts. We then asked whether *trans*-aconitate 5-methyl ester might be further metabolized in this organism. We incubated enzymatically radiolabeled *trans*-aconitate methyl ester with cytosolic extracts of yeast cells. We compared the base-labile radioactivity present with or without incubation, and found no evidence for hydrolysis of the methyl ester by the extract (data not shown). We also incubated the yeast product with pig liver esterase, a nonspecific esterase (*13*), and also did not observe any release of the methyl ester group. Finally, we showed that incubation with yeast extracts did not change the elution position of *trans*-aconitate [^{3}H]methyl ester upon anion-exchange rechromatography (data not shown). These results indicate that this product may be relatively stable in yeast cells, and may not be further metabolized.

Yeast trans-Aconitate Methyltransferase Is Located in the Cytoplasm. All known substrates of *trans*-aconitate methyltransferase are related to intermediates of the tricarboxylic acid cycle, which is known to occur mainly in the mitochondrion, although most of the cycle enzymes also are found in the cell cytosol (*14*). We thus wanted to determine the intracellular location of the *trans*-aconitate methyltransferase. We isolated yeast cytosolic and mitochondrial fractions from wild-type cells grown in rich media. We demonstrated that the cytosolic fractions are mostly free of mitochondrial matrix contents by Western blot analysis with antibodies to Hsp60, a protein that is only found in the yeast mitochondrial matrix (ref *10*; data not shown). We also showed that the mitochondrial preparation was almost free of cytosolic contents by measuring the activity of alcohol dehydrogenase, an enzyme found only in the cytosol of yeast (ref *15*; Table 5). We found that the specific activity and the total activity of *trans*-aconitate methyltransferase in the mitochondrial fraction were only 5% and 0.05% of those in the cytosolic fraction, respectively. For comparison, the corresponding values for alcohol dehydrogenase activity were 13% and 0.1%. We conclude that yeast *trans*-aconitate methyltransferase is localized in the cytosol, and is not present to any major extent in the mitochondria.

trans-Aconitate-Dependent Methyltransferase Activity Increases after Yeast Cells Undergo Diauxic Shift and Enter Stationary Phase. To determine if the expression of the yeast enzyme is regulated during various stages of cell growth, we assayed extracts using *trans*-aconitate as a substrate (Figure 7). We found that the specific activity of the methyltransferase increases dramatically after cells pass the diauxic shift, but then decreases when cells enter early stationary phase at 2−5 days in culture. The results observed here are consistent with microarray measurements of the relative levels of mRNA for *TMT1* (*YER175c*) at the diauxic shift (*16*). Here, the 2-fold increase in mRNA levels reflects the 2−3-fold increases in enzyme activity observed between cells at 1−7.5 h and from 10 to 13 h (Figure 7).

DISCUSSION

Role of the trans-Aconitate Methyltransferase in Yeast. We show here that the previously identified putative yeast methyltransferase F9 corresponds to a *trans*-aconitate methyltransferase. Deletion mutants in the *TMT1* (*YER175c*) gene encoding this protein have no obvious phenotype. An analysis of possible alternative substrates indicated that only the decarboxylation product of *cis*- and *trans*-aconitate, itaconate, is recognized with any affinity by this enzyme and even in this case the value of its V_{max}/K_m is only 0.9% that of *trans*-aconitate. This result suggests that *trans*-aconitate, which has been shown to form spontaneously from the citric acid cycle intermediate *cis*-aconitate (*17*−*19*), would be an endogenous substrate for this enzyme, and this hypothesis has been confirmed by the detection of its 5-methyl ester product in *TMT1*$^+$ but not *TMT1*$^-$ cell extracts.

We have previously postulated a role of this enzyme in the detoxification of *trans*-aconitate, which has been shown to be a good inhibitor of mammalian aconitase and fumarase activities (*18*−*22*). We recently demonstrated that *trans*-aconitate is also an inhibitor of yeast aconitase and that its methyl-esterification results in the nearly complete abrogation of this inhibitory activity (*2*). Although this result is consistent with the detoxification hypothesis, we still need to determine whether *trans*-aconitate does in fact accumulate to levels that are inhibitory to citric acid cycle enzymes, and if so whether the methyltransferase has sufficient activity to effectively convert it to the noninhibitory methyl ester. The

Table 5: Localization of *trans*-Aconitate Methyltransferase in Yeast[a]

fraction	total protein (mg)	*trans*-aconitate specific methyltransferase activity (TMT1) (pmol min^{-1} mg^{-1})	total TMT1 activity (pmol/min)	alcohol dehydrogenase activity (ADH) (μmol min^{-1} mg^{-1})	total ADH activity (μmol/min)
S1	292	357	104284	2.76	806
S2	280	327	91644	2.93	821
M1	2.52	16.5	42	0.38	0.9

[a] Yeast crude lysate was centrifuged at 1500*g* for 10 min, and the supernatant (S1) was fractionated to give the cytosolic fraction (S2) and the mitochondrial (M) fraction, as described under Experimental Procedures. The mitochondrial fraction was further purified to yield M1. The *trans*-aconitate methyltransferase activity (at 1 mM *trans*-aconitate) and alcohol dehydrogenase activity and the total protein of each fraction were measured as described under Experimental Procedures. The values are averages from two parallel experiments.

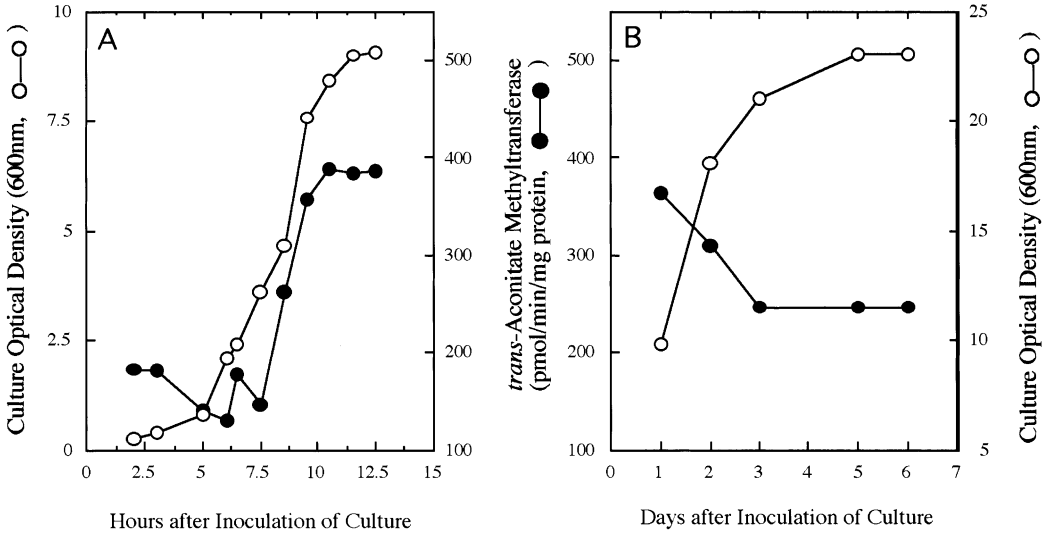

FIGURE 7: Expression of *trans*-aconitate methyltransferase during growth of yeast. An overnight culture of yeast strain GPY1100α was diluted to OD 0.09 into 250 mL of fresh YPD media in a 2 L flask (A) or into 25 mL of fresh YPD media in a 250 mL flask (B), and incubated with shaking at 30 °C. At various time points, the optical density of the culture was measure at 600 nm (A and B, open circles), and 5 mL (A) or 1 mL (B) aliquots of the culture were centrifuged at 10000*g* for 1 min. The crude extracts prepared from these cells were assayed for protein concentration and *trans*-aconitate methyltransferase activity (using 1 mM *trans*-aconitate as a methyl acceptor and assaying for 30 min at pH 7.5) as described under Experimental Procedures (A and B, closed circles). Assays were done in duplicate, and average values are shown.

fate of the endogenously produced methyl ester of *trans*-aconitate is unclear. We have shown here that this product appears to be stable in yeast extracts and does not appear to be significantly hydrolyzed.

Substrate Specificities of the Yeast and E. coli trans-Aconitate Methyltransferases. What are the structural requirements for effective catalysis of various methyl-accepting substrates of these enzymes? For the *E. coli* enzyme, the saturated framework of *trans*-aconitate, tricarballylate, is recognized with a catalytic efficiency (V_{max}/K_m) of only 0.002% that of *trans*-aconitate (Figure 3). The configuration around the double bond also appears to be important; *cis*-aconitate has a catalytic efficiency of less than 0.32% that of *trans*-aconitate. Interestingly, the stereospecific addition of a hydroxyl group to carbon 2 to give (2*R*,3*S*)-isocitrate results in a methyl acceptor with a catalytic efficiency of about 4% that of *trans*-aconitate, while the addition of a hydroxyl group to carbon 3 to give citrate results in a 0.16% catalytic efficiency. The (2*S*,3*R*)-enantiomer of isocitrate appears to be very poorly recognized by this enzyme. All other modifications tested are recognized with a catalytic efficiency less than 0.003% that of *trans*-aconitate.

The yeast enzyme displays a partially overlapping substrate specificity with the *E. coli* enzyme. Here *cis*-aconitate is also recognized, although also with much poorer catalytic efficiency—less than 0.24%. But very sparse activity (V_{max}/K_m values of 0.001−0.018% that of *trans*-aconitate) is seen with isocitrate or citrate (Figure 3). Interestingly, the decarboxylated form of aconitate, itaconate, is recognized by the yeast enzyme with a catalytic efficiency of 0.9% that of *trans*-aconitate. As shown in Figure 3, the three best substrates thus all contain a double bond between carbons 2 and 3 and a carboxyl group at position 6, and all contain the methylene-linked carboxyl group that is the site of methylation (*2*).

Endogenous Products of the Yeast Methyltransferase. In addition to the 5-methyl ester of *trans*-aconitate, we have also detected endogenous products in yeast extracts that migrate as less acidic species on anion-exchange chromatography. These products are distinct from the 1- and 6-methyl esters of *trans*-aconitate and from the enzymatically produced methyl ester of itaconate. Initial characterization of the major product showed that its ester bond is more labile to both base and acid treatment than that of the 6-methyl ester of *trans*-aconitate, but its structure remains to be determined. However, some properties of this molecule have been revealed. First, it has at least one carboxyl group that is methylated by yeast enzyme. Second, this molecule appears to have one free carboxyl group after the methylation, because it is ether-extractable when acidified, and it

elutes at a position on the anion exchange column consistent with monocarboxylic acids. Third, it appears to be more hydrophobic than *trans*-aconitate and itaconate methyl esters.

Localization of the Yeast trans-Aconitate Methyltransferase. trans-Aconitate can be spontaneously formed from *cis*-aconitate, and *cis*-aconitate is an intermediate of the tricarboxylic acid cycle. One might therefore expect that the yeast *trans*-aconitate methyltransferase should be localized in mitochondria where the cycle functions. However, we show here that the yeast *trans*-aconitate methyltransferase is localized in the cytosol. Some enzymes of the tricarboxylic acid cycle do occur in yeast cytosol, including aconitase (reviewed in *14*, *25*). The cytosolic aconitase activity has been associated with a family of iron regulatory proteins which share sequence similarity with aconitases and show aconitase activity when iron is bound (*26*). These proteins could potentially produce *cis*-aconitate which could serve as a precursor to the spontaneous formation of *trans*-aconitate. These results may also point to a cytosolic localization of the unidentified endogenous substrates for this methyltransferase.

Function of the Product of the Homologous S. cerevisiae YHR209w Gene? The function of the protein YHR209w has not been established. It contains the known methyltransferase motifs, indicating it is very likely to be methyltransferase (Figure 1). It shares much better sequence similarity with YER175c than with any other protein in the GenBank protein database, suggesting it is somehow related to the yeast *trans*-aconitate methyltransferase. The *Yhr209w* gene is apparently transcribed (*16*) and shows a similar regulation pattern as that of the *TMT1* gene (ref *16*; the Yeast Proteome Database, www.proteome.com). However, it seems not to contribute to any of the TMT1 activities. Although loss of the *YHR209w* gene does not affect *trans*-aconitate methyltransferase activity in yeast extracts, it is still possible that this gene does encode a *trans*-aconitate methyltransferase, but one that expresses a very low activity form of the enzyme so that its loss is not detectable in extracts containing the *YER175c* product. Finally, yeast two-hybrid analysis suggests a physical linkage of the YHR209w product to the yeast *HSH49* gene product (*27*), an essential splicing factor (*28*), although the significance of this observation is unclear.

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