Secreted 3-Isopropylmalate Methyl Ester Signals Invasive Growth during Amino Acid Starvation in *Saccharomyces cerevisiae*†

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ABSTRACT: The *Saccharomyces cerevisiae* methyltransferase encoded by *TMT1* catalyzes the AdoMet-dependent monomethylation of 3-isopropylmalate, an intermediate of the leucine biosynthetic pathway. The biological significance of methylating 3-isopropylmalate and the relationship between Tmt1 and the leucine biosynthetic pathway is not yet established. We present evidence here showing that methylation of 3-isopropylmalate functions to extracellularly signal yeast to grow invasively. We show that methyl esterification generates 3-isopropylmalate-1-methyl ester. We find that the Tmt1 methyltransferase functions independently of the biosynthetic pathway but is induced when cells are starved for amino acids; the largest induction is observed with the removal of leucine from the media. This amino acid starvation stress response is controlled by the transcriptional activator Gcn4. After methylation, 3-isopropylmalate methyl ester is secreted into the media within 3 h. Thin layer chromatography and gas chromatography mass spectroscopy confirm that the intact molecule is secreted. Finally, we show that purified 3-isopropylmalate methyl ester can enhance the ability of the haploid yeast strain 10560-23C to grow invasively. Our data identifies 3-isopropylmalate methyl ester as an autoinductive molecule that provides a signal to yeast to switch from vegetative to invasive growth in response to amino acid starvation.

Many yeast are dimorphic and can grow either vegetatively or invasively (*1*, *2*). Yeast such as *Saccharomyces cerevisiae* undergo a morphological switch from unicellular growth to filamentous invasive growth when nutrients are limited. The depletion of ammonia, glucose, amino acids, and oxygen, as well as heat stress, can lead to invasive growth (*1*−*5*). During invasive growth, the spherical/ovoid cell shape becomes elongated, cells adhere to each other and surfaces, cells penetrate agar, and cells display uni- and bipolar budding patterns. Adhesion to other cells and to substrate requires the expression of *FLO11*, encoding a surface glycoprotein attached to the cell wall via a glycosyl-phosphatidylinositol linkage (*6*, *7*).

Most systemic fungal infections that afflict humans are caused by dimorphic yeast (*8*). It has been reported that 70% of women experience vaginitis at least once in their life time caused by the major human pathogen, *Candida albicans* (*9*). In addition, 70% of AIDS patients contract oropharyngeal candidiasis, more commonly known as thrush. The ability to grow invasively is an important factor in fungal pathogenesis and virulence, and much effort has been devoted to elucidate its mechanism (*8*). Both the mitogen-activated protein kinase and the cAMP-activated protein kinase A cascades are involved in the internal signaling, although little is known about how signals are relayed extracellularly. Recently, secreted autoinductive molecules have been identified in the signaling of this morphological switch (*10*). These catabolic byproducts of aromatic amino acid degradation, phenylethanol and tryptophanol, function as autoinductive molecules that promote yeast to grow invasively when ammonia levels are depleted. Interestingly, invasive growth brought upon by glucose depletion appears to be independent of these species (*3*). It is currently unknown if other autoinductive molecules exist to signal invasive growth.

Our laboratory has been characterizing the *Saccharomyces cerevisiae* YER175C gene product Tmt1, which encodes a methyltransferase involved in small molecule methylation. Originally, Tmt1 was identified as the yeast homologue of the *Escherichia coli* Tam gene product that catalyzes the AdoMet[1]-dependent monomethylation of *trans*-aconitate, a potentially toxic small molecule (*11*−*13*). Spontaneously formed from the tricarboxylic acid cycle intermediate *cis*-aconitate, *trans*-aconitate is not an intermediate for any known metabolic pathway but is a good inhibitor of at least two tricarboxylic acid cycle enzymes, aconitase and fumarase (*14*−*16*). Both Tam and Tmt1 have been shown to attenuate the inhibitory properties through monomethylation of *trans*-aconitate (*11*). Although both enzymes can function in *trans*-aconitate detoxification, *E. coli* Tam catalyzes the formation of the 6-methyl ester, while yeast Tmt1 generates the 5-methyl ester (*11*).

In *E. coli*, the major endogenous substrate for the methyltransferase is *trans*-aconitate, while the situation in yeast is more complex (*11*). Here, *in vivo* methylation studies

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[1] Abbreviations: AdoMet, *S*-adenosyl-L-methionine; [^{3}H]AdoMet, *S*-adenosyl-[*methyl*-^{3}H]-L-methionine; [^{14}C]AdoMet, *S*-adenosyl-[methyl-^{14}C]-L-methionine; GC/MS, gas chromatography/mass spectrometry; HPLC, high performance liquid chromatography.

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Table 1: Strains and Plasmid List

strain/plasmid	genotype/description	ref
BY4742	MATα, *his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0*	*a*
BY4741	MAT**a**, *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0*	*a*
DDY001	BY4742, Δ*tmt1::*Kan^r	*a*
DDY002	BY4742, Δ*leu1::*Kan^r	*a*
DDY003	*his3Δl, leu2Δ0, ura3Δ0,* Δ*leu4*::Kan^r, Δ*leu9*::Kan^r	this study
DDY004	BY4742, Δ*gcn4::*Kan^r	*a*
DDY005	BY4742, Δ*leu4::*Kan^r	*a*
DDY006	BY4741, Δ*leu9::*Kan^r	*a*
DDY007	10560-23C, Δ*tmt1::*Kan^r	this study
10560-23C	MATα, *ura3-52, his3::hisG, leu2::hisG*	*b*
D273-10B/A1	MΔTα, *met6*	*c*
HCY001	MATα, *his4-159, leu2-3, 112, ura3-52, trp1, can1,* Δ*tmt1::*Kan^r	10
HCY005	HCY001, containing pJK2	10
pJK2	overexpression vector for Yer175c containing Yer175c between EcoRI and HindIII sites in the multicloning site of pRS426	10

[a] Obtained from Open Biosystems, www.openbiosystems.com. [b] Obtained from Dr. Greg Payne, UCLA. [c] Obtained from Dr. Cathy Clarke, UCLA. [d] The mating-type (MATα/a) and the presence of *MET15* and *LYS2* was not determined.

revealed that *trans*-aconitate is the minor substrate, while the major endogenous substrate is 3-isopropylmalate, an intermediate belonging to the leucine biosynthetic pathway (*12*, *17*). Although the tricarboxylic acid cycle and the leucine biosynthetic pathway share similar chemical mechanisms, 3-isoproylmalate and *trans*-aconitate are not corresponding chemical species. The analogous intermediates in each pathway (isopropylfumarate and *trans*-aconitate; isocitrate and 3-isopropylmalate) show very different methyl-accepting activities, suggesting that Tmt1 has a distinct function in each pathway (*12*).

The functional role for the methylation of yeast 3-isopropylmalate is unclear. A phenotype has not been observed in initial experiments comparing strains with intact or disrupted *Tmt1* genes (*12*). However, the parental strains (BY4741, BY4742, and GPY1100α) used in these previous studies are leucine auxotrophs that lack the intact *LEU2* gene product responsible for converting the methyltransferase substrate 3-isopropylmalate to the α-keto acid of leucine and must be supplemented with exogenous leucine for growth (Table 1). In addition, these commercial laboratory yeast strains, derived from the S288C strain, are unable to grow invasively (*18*). The relationship between Tmt1 and the leucine biosynthetic pathway has not been established. Leucine exhibits a feedback regulation on the first step of the pathway at Leu4 that catalyzes the formation of 2-isopropylmalate (Figure 1). A minor amount (20%) of 2-isopropylmalate is produced by a Leu4-redundant protein Leu9, although leucine does not inhibit its activity (*19*). 2-Isopropylmalate functions as a pathway intermediate and a positive regulator of the transcription of the leucine biosynthetic pathway genes when bound to the transcription co-repressor/activator Leu3. Thus, the combination of leucine in the growth media and the absence of Leu2 actively metabolizing 3-isopropylmalate may mask any phenotypic differences between *TMT1*$^+$ and *tmt1*$^-$ mutant strains.

In this work, we utilize a number of yeast strains to show that the Tmt1-dependent methylation of 3-isopropylmalate is not directly related to the leucine biosynthetic pathway. We show that Tmt1 is induced in a Gcn4-dependent manner when cells are starved for amino acids. Under these conditions, the methyl ester product of Tmt1 is rapidly secreted from the cytoplasm where it can function as an autoinductive molecule to signal haploid invasive growth. These results elucidate a new mechanism and pathway for inducing yeast haploid invasive growth.

MATERIALS AND METHODS

Media. Standard media consists of 2% D-galactose, 0.5% ammonium sulfate, 0.17% yeast nitrogen base (lacking amino acids and ammonium sulfate; DIFCO, Lawrence, KS), and 0.069% complete supplement mixture minus leucine (CSM-LEU; Bio 101, San Diego, CA). Where indicated, galactose was replaced by dextrose in some experiments. For histidine-starvation media, CSM-HIS was used in place of CSM-LEU. Standard media was supplemented with leucine at a final concentration of either 0.2 mM or 2 mM. Synthetic complete media consists of 2% D-glucose, 0.5% ammonium sulfate, 0.17% yeast nitrogen base (lacking amino acids and ammonium sulfate), and 0.079% complete supplement mixture (CSM). For synthetic complete dextrose lacking uracil media, CSM-URA was used in place of CSM. All media plates consist of the indicated media with 2% agar (DIFCO). Rich media (YPD) consists of 2% D-glucose, 1% yeast extract (DIFCO), and 2% peptone (DIFCO).

Strains. Table 1 describes the strains used in this work. Strain DDY003 was created by mating DDY005 and DDY006 strains. Diploids were screened by plating the resulting colonies on synthetic complete dextrose lacking methionine and lysine in the CSM. Diploid cells from an overnight culture were washed and incubated in sporulation media (1% potassium acetate, 0.1% yeast extract, 0.05% D-glucose, 60 mg/L of L-leucine, 20 mg/l of L-histidine, and 20 mg/L of uracil) and incubated for 5 days at 30 °C. Sporulated cells were pelleted, resuspended in 70 μL of ST buffer (1 M sorbitol, 50 mM Tris-HCl pH 7.5), treated with 30 μL of zymolase (20 mg/mL in ST, ICN Pharmaceuticals, no. 32092), and incubated at 30 °C for 10 min. The mixture was then diluted in water and placed on ice. Tetrad analysis was conducted using a dissecting microscope. Briefly, zymolase treated cells were aliquoted (30 μL) onto a YPD plate, sporulated cells were disrupted, and spores were separated. PCR using primers for *LEU4* (5′-AAAGCTGAGTTGAATGTGTGTCATA, 5′-AACAATTTCACGGAACATATCACTT) and *LEU9* (5′-TCACTTGTGGCCAAGTAA-GTATGTA, 5′-TTAATGTTAGCTCTTCCTTCTGCAT) was conducted to confirm which spore had the *leu4*

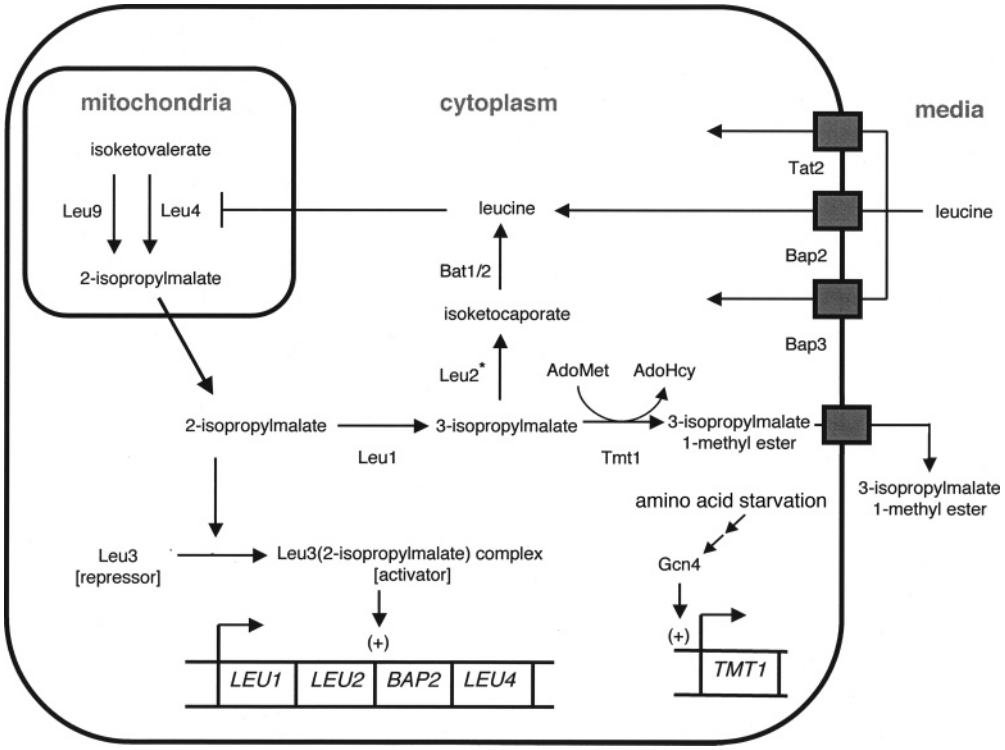

FIGURE 1: 3-Isopropylmalate methyl ester and leucine biosynthesis and regulation in *Saccharomyces cerevisiae*. Isoketovalerate is converted to leucine in four steps catalyzed by Leu4/Leu9, Leu1, Leu2, and Bat1/Bat2 (reviewed in Kohlhaw et al.) (*19*). We show here that the Leu1 product, 3-isopropylmalate, is recognized for methyl esterification at the 1-position by Tmt1, and is secreted into the media. Exogenous leucine, once transported into cells, feedback inhibits Leu4 activity, lowering the concentration of 2-isopropylmalate, a positive regulator of the transcriptional corepressor/activator Leu3 (*19*). Without 2-isopropylmalate bound, Leu3 represses the transcription of the biosynthetic *LEU1, LEU2,* and *LEU4* genes (*19*). We demonstrate in this work that *TMT1* gene expression is controlled by the Gcn4 transcriptional activator in response to amino acid starvation. Many standard yeast strains are defective in the *LEU2* gene (shown with an asterisk here), resulting in a leucine auxotrophy and the accumulation of the 3-isopropylmalate substrate for Tmt1.

leu9 genotype. Strain DDY007 was made by disrupting the *TMT1* gene in the parent strain 10560-23C as described by Cai et al., 2001 (*12*). Briefly, primers (5′-GATGAATAC-CACGACGGAGAAAGGAAATTACTCGTA-GATGTCAGCTG AAGCTTCGTACGC) and (5′-CTTT-AATAAACCAATCTGCTACATCTTCTTTATCTTTGGCAT-AGGCCAC TAGTGGATCTG) were used to generate a 1.6 kb kanamycin-resistance disruption cassette. Parental strain 10560-23C was transformed with the PCR reaction mixture according to Guldener et al. (*20*). Transformed cells were plated on YPD plates containing 300 μg of geneticin disulfate (antibiotic G418, Sigma, no. A8601) and allowed to grow for 3 days. Colonies were restreaked on G418 plates to yield individual colonies. Strains lacking *tmt1*$^-$ were verified by *in vivo* methylation studies as described below.

Growth Conditions and Cell Lysis. Unless otherwise stated, single colonies were seeded into 5 mL of YPD and incubated 18 h at 30 °C with shaking (250 rpm/min). One mL of overnight culture was inoculated in 29 mL of the designated media in 125 mL Erlenmeyer flasks and allowed to grow to 1−1.2 optical density unit with shaking (250 rpm/min) at 30 °C. Cells were separated from the media by centrifugation (15 min at 3440*g*). For GC/MS experiments, cells were inoculated into 1 l of synthetic complete media, allowed to grow until 1 optical density unit at 600 nm at 30 °C with shaking, washed, and then incubated in 100 mL of standard dextrose media. For 3-isopropylmalate methyl ester purification experiments, *TMT1*$^+$ overexpression strain HCY005 or *tmt1*$^-$ strain HCY001 was grown to 1.0 optical density units in 1 l of synthetic complete dextrose lacking uracil or synthetic complete dextrose, respectively. Cells were washed and resuspended in 75 mL of standard dextrose media at 30 °C for 5 h, and then the media was isolated from the cells.

Cells were lysed after washing twice with sterile water and resuspended in 2 pellet volumes (estimated by eye) of 50 mM Tris-HCl, pH 7.0. Cells were disrupted using 1 pellet volume of glass beads (acid-washed, 0.5 mm diameter from Biospec, Inc., Bartlesville, Oklahoma) and 6 cycles of vortexing for 1 min at 20,800*g* × g followed by incubation for 1 min on ice. The crude lysates were centrifuged at 20,800*g* for 20 min at 4 °C, and the supernatant was taken as the lysate. Protein concentration was determined by a Lowry assay after trichloroacetic acid precipitation (*21*).

Chemical Methylation of 3-Isopropylmalate for NMR Analysis. A 5.9 mg amount of (*2R,3S*)-3-isopropylmalate prepared as previously described (*17*) was incubated at room temperature with 60 μL of methanol and 1 μL of concentrated HCl (12 M) for 16 h. The reaction was dried under vacuum and resuspended in 169 μL of 15 mM KPO_4, pH 4.3. An aliquot of the sample (10 μL) was subjected to high performance liquid chromatography (HPLC) on a silica anion-exchange column (Whatman Partisil SAX; 10-μm bead diameter, 250 mm long, 4.6-mm inside diameter) equilibrated and eluted with 15 mM KPO_4, pH 4.3, at 1 mL/min; the absorbance at 214 nm of eluted fractions was measured. The column was equilibrated with 15 mM KPO_4, pH 4.3, at 1 mL/min. Fractions from 8 to 9 min were collected from multiple runs, pooled, and extracted with acidic ethyl acetate as described below. The pooled organic extracts were dried

under vacuum, resuspended in 450 μL of D_2O, and subjected to heteronuclear multiple bond correlation analysis as described previously (*11*).

In Vitro Enzymatic Assay of Tmt1. Reaction mixtures consist of 10 μL of 0.4 M sodium HEPES buffer, pH 7.0, 16 μL of water, 4 μL of 10 mM *trans*-aconitate, 5 μL of [^{14}C]AdoMet, and 5 μL of lysates (5−10 mg/mL) diluted 100-fold in a final volume of 40 μL. The reaction was allowed to proceed for 10 min at 30 °C and then placed on ice and quenched with 40 μL of 2 N NaOH. A 60 μL aliquot of each quenched reaction mixture was spotted on a thick accordion-pleated 1.5 × 8 cm filter paper (BioRad, no. 165−0962) which is placed in the neck of a 20 mL scintillation vial containing 5 mL of scintillation fluid (Safety Solve, Research Products International Corp., Mount Prospect, Illinois) and capped. Vials were incubated at room temperature for 2 h to allow radioactive methanol formed by base hydrolysis of [^{14}C]methyl esters to diffuse into the scintillation fluid. The filter papers were discarded and amount of radioactivity in the fluor was determined by liquid scintillation counting using a Beckman LS6500 instrument.

Amino Acid Analysis. Amino acids were detected by fluorescence after *o*-phthaldialdehyde derivatization (*22*). Briefly, reagent (20 μL), made from 90 μL 0.4 M potassium borate pH 10.3, 10 μL methanol, 0.4 μL 2-mercaptoenthanol, 0.4 mg of *o*-phthaldialdehyde, was mixed with aliquots (5 μL) of media or cell lysate. Samples (15 μL for media and 10 μL cell lysates) were immediately subjected to HPLC C-18 reverse phase analysis at 37 °C with an elution rate of 1 mL/min using the following gradient: 0:100:0, 1:90:10, 20:80:20, 30:60:40, 40:50:50, 50:35:65, 52:0:100 (time: % buffer A: % buffer B). Buffer A consists of 895 mL of 0.1 M sodium acetate pH 7.2, 10 mL of tetrahydrofuran, and 95 mL of methanol, and buffer B consists of methanol. Quantification of leucine was determined by comparing the peak areas from the sample to the peak areas from amino acid standard H (Pierce, no. 20088). The concentration of leucine corrected for dilution during cell lysate preparation by measuring the protein concentration and with the assumption that the protein concentration in intact cells is 200 mg/mL.

Quantitation of ^{3}H-Methyl Esters and ^{3}H-Methanol in Cell Lysates and Media. [^{3}H]Methanol formation was measured by spotting 10 μL from *in vivo* [^{3}H]AdoMet-labeled cell lysates or media onto an accordion-pleated filter paper for volatile radioactivity assay as described above. For quantification of [^{3}H]methyl ester formation, 10 μL of *in vivo* radiolabeled lysates or media were mixed with 30 μL of water followed by the addition of 40 μL of 2 N NaOH. A 60 μL amount of the reaction mixture was spotted on filter paper and analyzed as described above.

Acidic Organic Extraction of Cell Lysates and Media for TLC and GC/MS Analysis. Yeast cell lysates and media samples were acidified to pH 0 using concentrated HCl or H_2SO_4. An equal volume of ethyl acetate was added to the solution and vortexed, and the phases were separated using a tabletop centrifuge at 20,800*g* for 1 min or a separatory funnel for volumes larger than 1 mL. The organic layer was isolated, and the aqueous phase was extracted two additional times. All organic extracts were combined and dried under vacuum. Samples were resuspended with indicated solvent.

Analysis of 3-Isopropylmalate Methyl Ester by TLC. A standard of 3-isopropylmalate-1-[^{14}C]methyl ester was prepared enzymatically from a reaction mixture containing 10 μL of 0.4 M sodium HEPES buffer, pH 7.0, 20 μL of 100 mM (*2R,3S*)-3-isopropylmalate (*17*), 5 μL of [^{14}C]AdoMet (0.455 mM, 53 mCi/mmol, Amersham Biosciences, Piscataway, NJ), and 5 μL of a diluted lysate (0.005 mg protein/mL) from the Tmt1 overexpressing strain HCY005. The reaction was allowed to proceed for 1 h at 30 °C. 3-Isopropylmalate-1-[^{14}C]methyl ester was isolated after acidic ethyl acetate extraction as previously described (*17*). This standard was mixed with an acidic ethyl acetate extraction of ^{3}H-labeled media and was spotted and chromatographed on a 20 cm silica gel coated TLC plate (Whatman PE SIL G, 250 μm layer). The mobile phase consisted of acetic acid/methanol/chloroform in a ratio of 0.5/10/89.5 (v/v/v). The TLC plate was dried under an air stream and then cut into 3 mm slices. The silica from each slice was scraped off and transferred to a 1.5 mL microcentrifuge tube filled with 300 μL of water. The samples were place under vigorous shaking conditions for 3 h and then transferred to a 20 mL vial containing with 5 mL of scintillation fluid (Safety Solve) for quantification of total radioactivity.

GC/MS Analysis of Yeast Metabolites. Dried samples from acidic ethyl acetate extractions of lysates and media were resuspended in 300 μL of ethyl acetate and transferred to 6 × 50 mm glass tube and dried again under vacuum. Samples were resuspended in 300 μL of benzene and dried once more to remove any traces of water. Samples were converted to the trimethylsilane derivatives as previously described (*17*). Aliquots of the reaction mixture (1 μL) were injected 1:100 split mode onto a medium polarity bonded phase fused silica capillary column (30 m × 0.32 mm inside diameter, 5% phenyl and 95% methylpolysiloxane, HP-5ms, Agilent Technologies) on an Agilent Technologies 6890 gas chromatograph with helium as the carrier gas at 1.2 mL/min constant flow. The column effluent was directed into the electron (EI, 70 eV, 180 °C) ionization source of a repetitively scanning (50−800, 2 scans/s, positive ion mode) quadrapole mass spectrometer (Agilent, Santa Clara, California). The GC injector port and the GC/MS transfer line were maintained at 250 °C, and the GC oven was held at 50 °C for 3 min following injection and then increased linearly at 20 °C to a plateau of 300 °C. Samples were quantitated using a standard curve of 2-isopropylmalate.

Purification of 3-Isopropylmalate Methyl Ester for Invasive Growth Assays. Media from 5 h incubations of *TMT1*$^+$ overexpression strain HCY005 or *tmt1*$^-$ strain HCY001 were adjusted to pH 0 using concentrated H_2SO_4 and then extracted with acidic ethyl acetate as described above. The organic phase was dried under vacuum, resuspended in 500 μL of 250 μL of ethyl acetate, mixed with 250 μL of *n*-hexane, and applied to a silica column (3 g of silica) equilibrated with 1:1 ethyl acetate and hexane. The column was eluted with 75 mL of 1:1 ethyl acetate:hexane, and 3 mL fractions were collected. Each fraction was dried under vacuum, and a portion was subjected to GC/MS analysis as described above to identify the 3-isopropylmalate methyl ester-containing fractions. Overnight yeast cultures were spotted on synthetic complete dextrose plates that had been partially dried for 3 days in a 30 °C incubator. Designated positions on the plate were first spotted with 3 μL of the indicated

amount of purified 3-isopropylmalate methyl ester-containing fractions from extracted media or sterile water. After 25 min to allow the absorption into the plate, 5 μL of yeast cultures were then applied to the same position on the plate. Plates were incubated at 30 °C for the indicated number of days and then digitally photographed with a Canon S50 camera through the eyepiece of a 20× dissecting microscope. Cells were then washed on the plates under a gentle stream of deionized water at room temperature as described by Reynolds et al. (*6*). Plates were rephotographed as above. The amount of invasive growth was scored on a scale of zero (no invasive growth) to four (maximal invasive growth) by eleven independent observers in a blind survey of unlabeled individual photographs. We found very little variation between the observers; the standard deviation shown is that from four separate experiments.

RESULTS

Tmt1 Methyltransferase Esterifies 3-Isopropylmalate at the 1-Carboxylate. 3-Isopropylmalate is the major endogenous substrate for Tmt1; enzyme activity results in its monomethylation (*17*). To determine which of the two carboxylate groups are methylated, we chemically methylated 3-isopropylmalate and then separated the two monomethyl esters by HPLC. These species eluted at 8−9 min and 10−11 min and were identified by mass spectrometry and one-dimensional NMR. We found that the chemically methylated species eluting at 8−9 min corresponds to the Tmt1 biological methyl ester by its coelution with the radiolabeled enzymatic product. We isolated the 8−9 min chemically methylated methyl ester from multiple chromatographic runs and analyzed it by a two-dimensional heteronuclear multiple bond correlation NMR experiment to determine the methylation site (Figure 2). This experiment correlates the ^{1}H proton spectra with natural occurring ^{13}C carbon spectra, which can be used to elucidate the molecular connectivity between atoms two to three bond lengths apart. Peaks in the proton spectra were identified by their splitting patterns and downfield shifts. The 4-carboxylate peak in the ^{13}C spectra was identified by its correlation with the proton on C5. This assignment allowed us to identify the methyl ester group with the 1-carboxylate. Thus, Tmt1 catalyzes the formation of 3-isopropylmalate-1-methyl ester. This result is consistent with the model obtained with kinetic studies suggesting that the 1-methylation site is structurally analogous to the known 5-methylation site of the minor enzymatic product *trans*-aconitate (*17*).

Tmt1 Activity is Independent of the Leucine Biosynthetic Pathway. Since the major substrate of the Tmt1 methyltransferase, 3-isopropylmalate, is an intermediate of the leucine biosynthetic pathway, we first asked if this reaction participates in the regulation of leucine synthesis. Since leucine feedback inhibits the production of 3-isopropylmalate, we compared the growth of strains with and without the *TMT1* gene that also lacked the Leu2 enzyme that converts 3-isopropylmalate to isoketocaporate (Figure 1). The *TMT1*$^{+}$*leu2*$^{-}$ parental strain BY4742 and the *tmt1*$^{-}$*leu2*$^{-}$ strain DDY001 were grown in a standard galactose media supplemented with either 2.0 mM (high) or 0.2 mM (low) leucine (Figure 3). Galactose was used as a carbon source because a 10-fold enhancement in leucine import is achieved with galactose compared to glucose (*23*). At high leucine levels, no difference in growth was found between strains (Figure 3). We observed, however, that both strains grew at a slower rate and to a lower optical density under low leucine levels but again saw no difference between the strains (Figure 3). Here, cells underwent further growth after additional leucine supplementation, indicating that leucine was the limiting factor. The absence of any *TMT1*-dependent growth phenotype suggests that the methyltransferase may not be a part of the leucine regulatory system.

We then asked if the activity of the Tmt1 methyltransferase was affected by changes in leucine metabolism. Since leucine negatively regulates the leucine biosynthetic pathway and subsequently the production of 3-isopropylmalate, we hypothesized that Tmt1 should be more active under limiting leucine conditions where the substrate is more abundant. To test this hypothesis, BY4742 cells were harvested at early log phase from cultures grown in high and low leucine media, and Tmt1 activity was quantified (Table 2). We found that Tmt1 activity was increased about 2-fold when cells were grown in standard galactose media supplemented with 0.2 mM leucine than when supplemented with 2.0 mM leucine. A similar increase was found when glucose was used as a carbon source instead of galactose (data not shown). The difference in Tmt1 activity between cells grown in low and high leucine increased as cells reached mid log phase (data not shown). The increased Tmt1 activity could either reflect the lower leucine levels or be in response to the increase of leucine precursors. To answer this question, methyltransferase activity was assayed for yeast strains lacking various leucine biosynthetic genes that are unable to accumulate pathway intermediates (Table 2). All strains were found to display similar basal levels of Tmt1 activity when grown in 2.0 mM leucine and to have increased activity when grown in 0.2 mM leucine. The parental BY4742 and *leu1*$^{-}$ DDY002 strains had 2-fold increased activity while the *leu4*$^{-}$ *leu9*$^{-}$ DDY003 strain had about 5-fold more activity in the low leucine growth conditions. DDY003 exhibited a slower growth compared to the other strains in low leucine-containing media but not in the high leucine containing media (data not shown). The absence of 2-isopropylmalate, both an intermediate and positive transcriptional activator, in DDY003 may result in a lower leucine uptake by reducing the expression of the major leucine permease Bap2 (Figure 1) (*24*). These results suggest that the increased Tmt1 activity may occur in response to limiting exogenous leucine levels and may be independent of the concentration of the leucine biosynthetic intermediates.

We next asked to what extent was the expression of Tmt1 dependent upon cells sensing the leucine levels in the cytoplasm, in the exogenous media, or in a mixture of both. A leucine autotrophic yeast strain (D273-10b) was cultured in standard galactose media supplemented with either 2.0 mM leucine, 0.2 mM leucine, or unsupplemented and assayed for Tmt1 activity. Interestingly, the Tmt1 activity remained at the basal level regardless of the amount of leucine present in the growth media in cells with an intact leucine biosynthetic pathway (Table 2). Tmt1 appears not to be induced during *de novo* leucine biosynthesis, suggesting that the methyltransferase may function outside of the biosynthetic pathway.

Tmt1 Expression Is Induced by Amino Acid Starvation in a Gcn4-Dependent Reaction. To further examine the in-

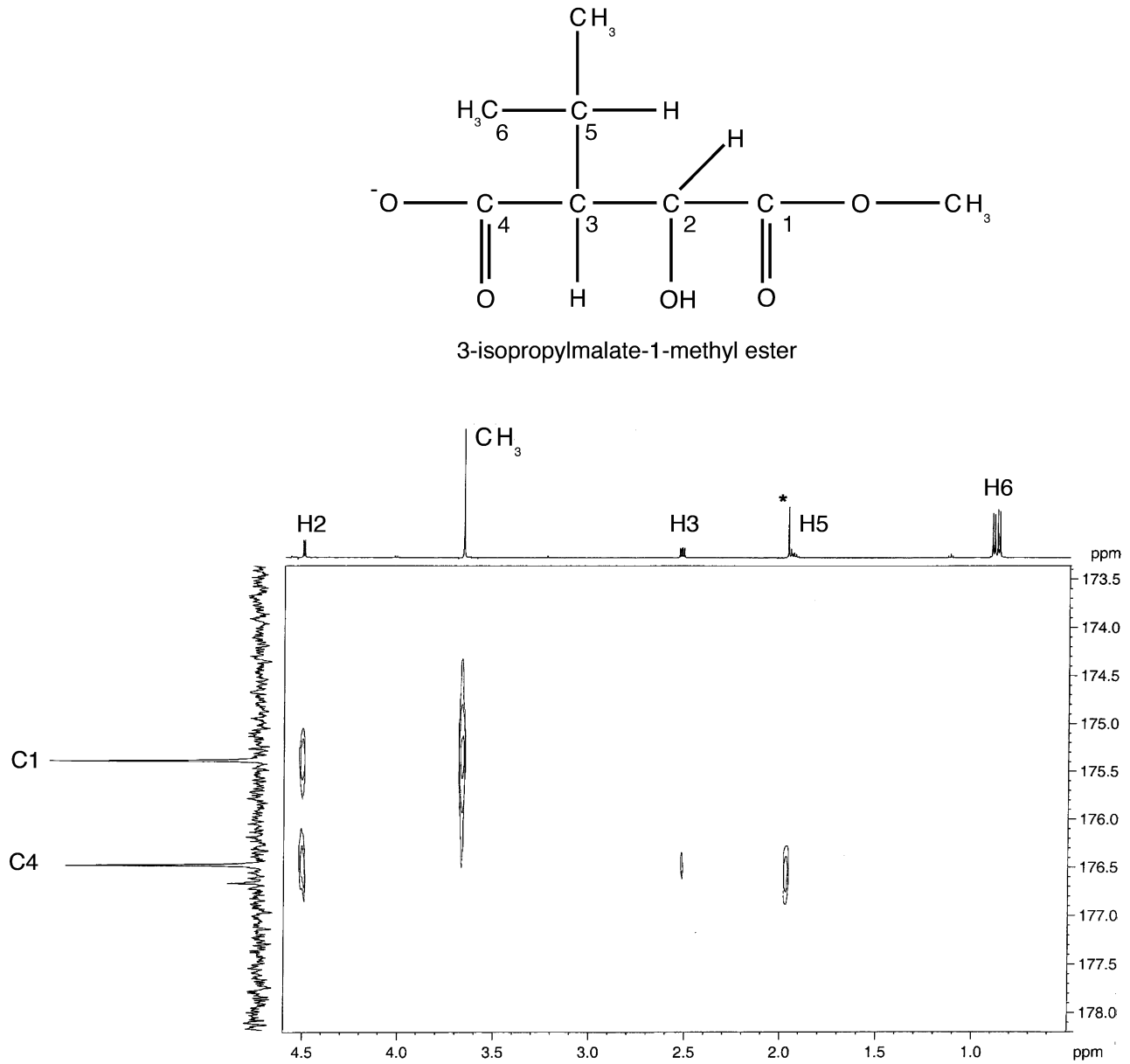

FIGURE 2: Tmt1 catalyzes the methyl esterification of 3-isopropylmalate at the 1-carboxylate group. The two monomethyl esters of 3-isopropylmalate were synthesized chemically as described in the "Materials and Methods" section. Using heteronuclear multiple bond correlation NMR analysis as previously described (*11*), we analyzed the synthetic monomethyl ester that coeluted at 8−9 min on HPLC with the biological product of the Tmt1 methyltransferase. The structure of 3-isopropylmalate-1-methyl ester is shown above the two-dimensional NMR spectra. Correlations of the ^{1}H proton spectra (top) and the ^{13}C carbon spectra (left side) demonstrate that the 1-carboxylate group rather than the 4-carboxylate group is modified by the Tmt1 enzymatic methylation reaction. The asterisk (*) denotes a contaminating peak found in the 3-isopropylmalate-1-methyl ester standard.

creased Tmt1 activity in *leu2*$^{-}$ cells (BY4742 background) grown under limiting leucine, we designed conditions to test how strains respond to rapidly removing leucine from the media in a "switch" experiment. Here, cells were grown in standard galactose media containing 2.0 mM leucine to log phase (1.0−1.2 optical density units), and then the media was replaced with standard galactose media (no leucine) for an additional 2 h before being assayed for methyltransferase activity. Under these conditions, we observed in the *leu2*$^{-}$ strain a 2-fold higher Tmt1 activity than seen previously in cells grown in the low 0.2 mM leucine (Table 2). Strains DDY002 and DDY003, with additional disruptions to the leucine biosynthetic pathways, behaved similarly to the parental BY4742. The addition of the protein synthesis inhibitor cycloheximide to the switch media, however, prevented all increases in Tmt1 activity over the basal level, suggesting that the increase in activity occurs at the level of protein synthesis. Interestingly, leucine autotrophic cells (D273-10b) subjected to the "switch" experiment showed a lesser increase in Tmt1 activity. Additionally, Tmt1 activity then decreased when D273-10b cells were incubated for an additional 4 h, whereas the activity continued to increase in the *leu2*$^{-}$ strains (data not shown). These results indicate that Tmt1 is induced in response to limiting extracellular leucine levels, but the induction can be negated if leucine can be synthesized.

Importantly, we found that the induction of Tmt1 is not exclusive to limiting exogenous leucine. The "switch" experiment was repeated with the BY4742 strain, which is also a histidine auxotroph, where the "switch" media

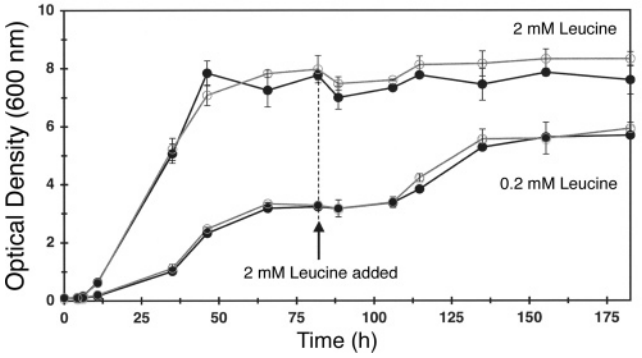

FIGURE 3: The Tmt1 methyltransferase does not affect the growth of leucine auxotropic yeast strain under limiting or nonlimiting conditions. Growth curves at 30 °C are shown for the parental strain BY4742 (*leu2*$^-$ *TMT1*$^+$; filled circles, black lines) and the DDY001 *tmt1*$^-$ mutant strain (*leu2*$^-$ *tmt1*$^-$*;* open circles, gray lines) in standard galactose media supplemented with either 0.2 mM or 2 mM leucine. Cultures were initially inoculated with YPD overnight cultures at an optical density of 0.01 at 600 nm. Data points are averages from three independent cultures grown in parallel. At 82 h after inoculation, all cultures were supplemented with additional leucine (2 mM).

contained 2.0 mM leucine but where the normally present histidine was absent. In this histidine starvation media, Tmt1 activity increased more than 2-fold above that of the basal activity (Table 2). Because Tmt1 activity was not induced in the absence of protein synthesis, we asked whether Tmt1 may be transcriptionally activated when amino acids are limiting. Analysis of the promoter region of the *TMT1* gene revealed a consensus Gcn4 binding site 192 base pairs upstream of the translational start site (*25*). Gcn4 is a transcriptional factor that is activated under conditions of amino acid starvation (*26*, *27*). This finding suggested that the increase in Tmt1 activity observed with leucine or histidine starvation may be mediated by Gcn4. We directly tested this hypothesis by measuring Tmt1 activity in the *gcn4*$^-$ yeast strain in a BY4742 background (DDY004). Here, we observed only basal levels of Tmt1 activity regardless of the leucine concentration in the media (Table 2). Additionally, Tmt1 activity in DDY004 did not increase when cells were subjected to the "switch" experiment (Table 2). We conclude that the induction of Tmt1 activity is dependent upon Gcn4, linking methyltransferase activity and 3-isopropylmalate methyl ester formation to an amino acid starvation stress response.

We asked whether the activation of Tmt1 by Gcn4 was determined by amino acid levels in the media, or in cells, or in some combination of both. We measured the concentration of leucine in the media (extracellular) and lysates (intracellular) in several yeast strains under the starvation conditions used previously (Figure 4). We found that there was little correlation of intracellular leucine levels with Tmt1 activation. For example, when we compared the high Tmt1-inducing BY4742 cells after the "switch" experiment with the moderate Tmt1-inducing BY4742 cells grown in 0.2 mM leucine, we actually found more intracellular leucine in the former case (10.5 mM) than the latter case (5.6 mM). On the other hand, Tmt1 induction was inversely correlated with the extracellular leucine concentrations. For example, the high Tmt1-inducing conditions described above resulted in a media concentration of only 0.03 mM leucine whereas the moderate inducting conditions resulted in a media concentration of 0.14 mM, and the no induction level of 0.40 mM (Figure 4). Thus, it appears that yeast are able to sense the concentration of extracellular leucine and induce the expression of Tmt1 via Gcn4.

The 3-Isopropylmalate Methyl Ester Product of Tmt1 Is Secreted into the Media. While it is clear that the Tmt1 methyltransferase is induced in amino acid starvation, the biological significance of this induction is unknown. We previously postulated that Tmt1 methylation may function in detoxification or as a branch step to a novel metabolic pathway (*17*). To test these hypotheses, we wanted to determine the metabolic fate of the 3-isopropylmalate methyl ester product. Yeast cells (*TMT1*$^+$ or *tmt1*$^-$) were subjected to the "switch" experiment to induce Tmt1 as shown in Table 2 and were then radiolabeled with [^{3}H]AdoMet to follow the methyl group in the lysate and media directly after the labeling and after further incubation. We quantified total ^{3}H-methyl esters as base-labile volatile radioactivity and the ester hydrolysis product [^{3}H]methanol as volatile radioactivity in these samples (Figure 5). In the *TMT1*$^+$ strain, we found significant methyl ester radioactivity at time zero in the lysate along with a smaller amount in the media. However, after 18 h, there was little methyl ester radioactivity remaining in the lysate, but there was a large amount present in the media that could quantitatively account for all of the initial methyl ester radioactivity in the lysate and media. Control experiments with the *tmt1*$^-$ strain demonstrated negligible amounts of radioactivity in any of the samples, suggesting that all methyl ester radioactivity was a product of Tmt1. We found little or no [^{3}H]methanol production in any of the samples, indicating that the methyl ester linkage is stable. We have ruled out the possibility that the presence of the ^{3}H-methyl ester in media is the result of autolysis since the majority of cells from the parental BY4742 and *tmt1*$^-$ strains remain viable on YPD plates (data not shown). Similar results were obtained when using a leucine autotrophic strain (data not shown). Taken together, these results suggest that a methyl ester or methyl esters dependent upon Tmt1 activity is secreted from cells.

To determine the time course for the secretion of the Tmt1-dependent base-labile metabolite, methyl esters were quantified in the cell lysate and in the media as a function of incubation time after radiolabeling (Figure 6). Within 1 h after *in vivo* radiolabeling cells, the majority of base-labile radioactivity is secreted from the cytoplasm. A similar trend was observed when resuspending *in vivo* radiolabeled cells into synthetic complete dextrose media instead of the standard dextrose media. We find that the radioactive methyl ester is fated for secretion whether in standard dextrose media (no leucine) or synthetic complete dextrose media (with leucine) (Figure 6).

The secreted ^{3}H-methyl ester radioactivity measured in Figures 5 and 6 could include 3-isopropylmalate methyl ester as well as metabolites where the methyl ester linkage is preserved. To identify the secreted molecule(s), media from [^{3}H]AdoMet labeled cells incubated for 18 h was extracted with acidic ethyl acetate, mixed with a 3-isopropylmalate [^{14}C]methyl ester standard, and subjected to thin layer chromatography (Figure 7). We found an exact concordance of the ^{3}H and ^{14}C radioactivity in the chromatograph, suggesting that the secreted product is indeed the unmodified methyl ester of 3-isopropylmalate. We confirmed this

Table 2: *trans*-Aconitate Methyltransferase (Tmt1) Activity in Yeast. Tmt1 Activity Was Assayed in Cell Lysates Using 1 mM *trans*-Aconitate as the Substrate

		growth conditions (standard galactose media without leucine)					
					2 mM leucine, then switched to standard galactose media (2 h)		
strain	relevant genotype	0.2 mM leucine	2.0 mM leucine	0 mM leucine	without cycloheximide	with cycloheximide[d]	2 mM leucine, then switched to histidine starvation media (2 h)
BY4742	*leu2*$^-$	346[b] ± 21[c]	186 ± 27		779 ± 52	151 ± 69	451 ± 26
DDY002	*leu1*$^-$ *leu2*$^-$	369 ± 12	180 ± 29		714 ± 37		
DDY003	*leu4*$^-$ *leu9*$^-$ *leu2*$^-$	1028 ± 43	222 ± 14		748 ± 64		
DDY004	*gcn4*$^-$ *leu2*$^-$	168 ± 5	174 ± 9		168 ± 5		
D273-10B	wild type[a]	181 ± 14	127 ± 40	168 ± 21	326 ± 17		

[a] Intact leucine biosynthetic pathway. [b] Methyltransferase activity is reported as the average specific activity (pmol/min/mg of protein). [c] Standard deviations were determined from reactions performed in triplicate. [d] The final concentration of cycloheximide used equaled 100 μg/mL.

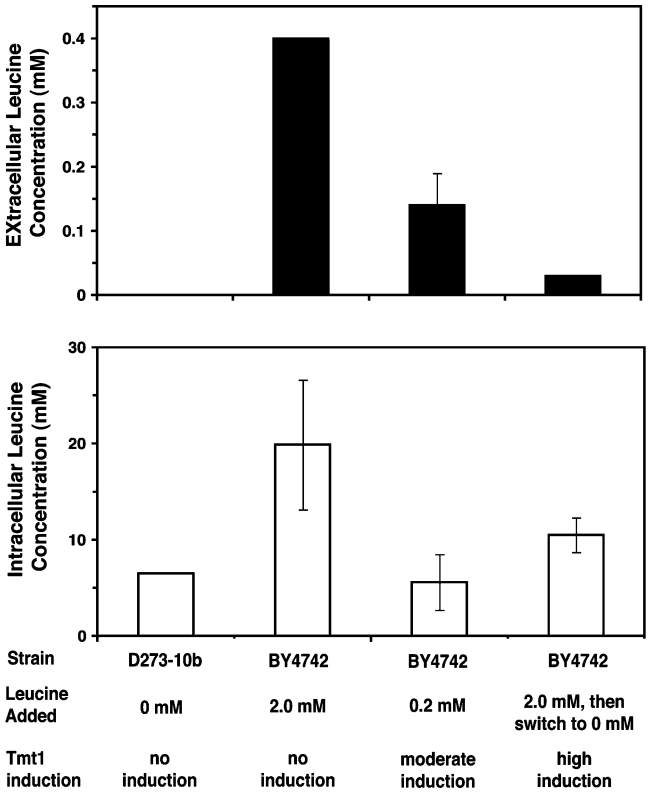

FIGURE 4: Comparison of Tmt1 induction with extracellular and intracellular leucine concentrations in yeast strains. The levels of leucine in the media and in cell lysates was measured by amino acid analysis as described in the Methods and Materials. Results are shown for BY4742 (*leu2*$^-$) strains grown in 2 mM leucine (no induction), in 0.2 mM leucine (moderate induction), and for cells switched from 2 mM leucine to no leucine (high induction), as well as for the D273-10B wild type cells grown in the absence of leucine (no induction).

identification by gas chromatography/mass spectrometry. Figure 8A depicts the chromatograph from both *TMT1*$^+$ and *tmt1*$^-$ acid extracted media samples, and a peak unique to the *TMT1*$^+$ media sample was detected eluting at 10.42 min. A similar result was found when the lysate samples were analyzed, although the relative abundance of the peak was dramatically lower (Figure 8B). The mass spectrum corresponding to the 10.40−10.44 min region for both *TMT1*$^+$ and *tmt1*$^-$ acidic ethyl acetate extracted media samples is shown in Figure 8C. The *TMT1*$^+$ sample contains ions that are consistent with the previously observed fragmentation pattern of 3-isopropylmalate methyl ester (*17*), and these ions

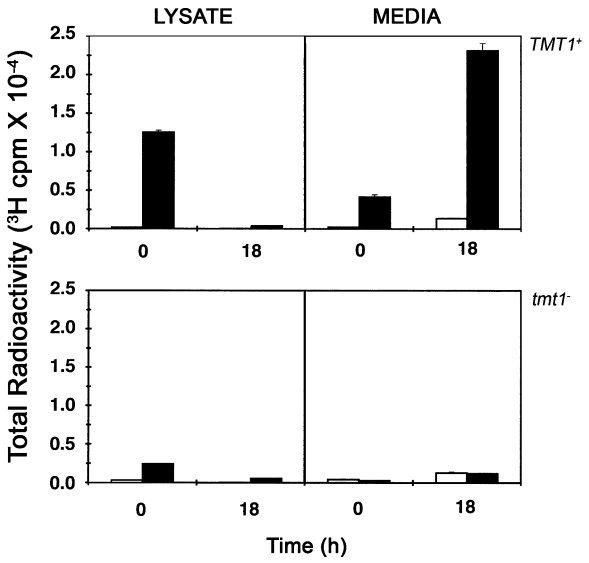

FIGURE 5: Fate of the biosynthetic 3-isopropylmalate methyl ester. Yeast cells (BY4742; *leu2*$^-$ *TMT1*$^+$ and DDY001; *leu2*$^-$ *tmt1*$^-$) were grown at 30 °C to log phase (1.0−1.2 optical density at 600 nm) in 29 mL of standard galactose media supplemented with 2 mM leucine from a 1 mL YPD overnight culture. Cells were washed and resuspended in 30 mL of standard galactose media (without leucine) and incubated for an additional 2 h. Cells were collected from 14 optical density units of the culture, centrifuged, and resuspended in 2 mL of standard galactose media and then mixed with 100 μL of *S*-adenosyl-[*methyl*-^{3}H]-L-methionine ([^{3}H]AdoMet 12.2 μM, 82 Ci/mmol, in dilute HCl/ethanol 9:1 (pH 2.0−2.5), Amersham Biosciences Piscataway, NJ) and incubated for 15 min. The radioactive media was immediately separated from the cells by centrifugation, and the cells were then washed three times and resuspended in 2 mL of standard galactose media. For the 0 h sample, 1 mL of the resuspended cells was centrifuged to separate cells and media. Cell lysates were prepared as described in Materials and Methods. Volatile radioactivity (as [^{3}H]methanol) was measured in 10 μL of cell lysates and media fractions with and without base treatment to hydrolyze methyl esters by a vapor phase assay. The remaining 1 mL of resuspended cells was incubated in standard galactose media for an additional 18 h before the analysis of radioactivity as above. The total amount of volatile radioactivity (white bars) and base-labile radioactivity (black bars) is shown. The value for volatile radioactivity in the 18 h lysate samples is less than the width of the line.

are absent in the *tmt1*$^-$ sample. These results prove that the secreted molecule is 3-isopropylmalate methyl ester.

The secretion of the 3-isopropylmalate methyl ester led us to ask if yeast use Tmt1 to regulate the cellular level of 3-isopropylmalate as a potentially toxic metabolic intermediate (*28*). If this were true, we would expect a pathway similar

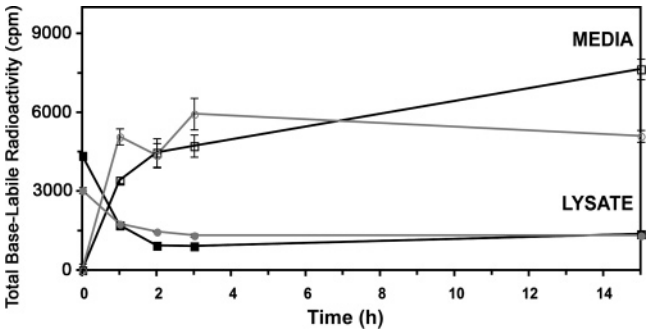

FIGURE 6: Effect of exogenous leucine on the secretion of 3-isopropylmalate methyl ester from yeast cells. Cells (BY4742) were grown at 30 °C to log phase in synthetic complete media to log phase (1.0−1.2 O.D. at 600 nm), washed, resuspended in standard dextrose media, incubated for 1 h at 30 °C, and then *in vivo* radiolabeled with [^{3}H]AdoMet for 15 min as described in the Figure 4 caption but using 50 optical density units of cells. Cells were washed two times in standard dextrose media to remove [^{3}H]-AdoMet. One aliquot of cells was then resuspended in standard dextrose media (no leucine) and one aliquot in synthetic complete media (0.8 mM leucine), and both were incubated at 30 °C for 15 h. At various time points, volatile and base-labile radioactivity was measured in triplicate in the media (open symbols) and in cell lysates (closed symbols) as described in the Materials and Methods. Results are shown for cells incubated without leucine (circles, gray lines) and with leucine (squares, black lines).

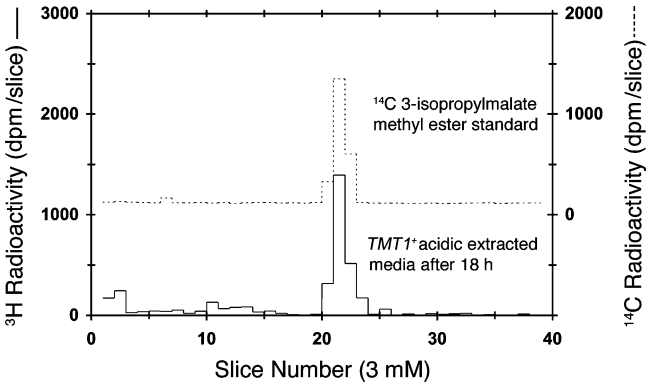

FIGURE 7: 3-Isopropylmalate methyl ester is secreted from yeast cells and is stable in the media. Thin layer chromatography was performed as described in Materials and Methods on organic acid-extracted media from the 18 h sample of *in vivo* [^{3}H]AdoMet-labeled *TMT1*$^+$ yeast cells described in Figure 4. This sample was mixed with a 3-isopropylmalate methyl ester standard prepared by incubation of Tmt1 with [^{14}C]AdoMet and 3-isopropylmalate as described in Materials and Methods. Sections of the TLC plate were counted on a Beckman LS6500 counter under the ^{3}H−^{14}C dual label program to obtain the dpm of each label.

to the **a**-mating factor secretion where the unmethylated peptide is largely retained within cells (*29*). We thus asked if unmodified 3-isopropylmalate was retained in cells or was also secreted to the media. We examined GC/MS chromatographs of the media samples for ions that would result from the fragmentation of 3-isopropylmalate but not its methyl ester. We found daughter ions specific for 3-isopropylmalate at 10.90−11.00 min in both *TMT1*$^+$ and *tmt1*$^-$ media samples (Figure 8A, B), indicating that methylation is not a requirement for excretion. Further scrutiny of the mass spectra of this region revealed the presence of 2-isopropylmalate, the metabolic precursor of 3-isopropylmalate, as well. We showed that a standard of 3-isopropylmalate elutes from the GC/MS at 10.93 min, while a standard of 2-isopropylmalate elutes at 10.97 min; these compounds could be differentiated by their unique fragmentation signatures (data not shown). For both the *TMT1*$^+$ and *tmt1*$^-$ samples, the major peak corresponds to 2-isopropylmalate; 3-isopropylmalate is present at lower levels (Figure 8A,B). Thus 3-isopropylmalate methyl ester, 3-isopropylmalate, and 2-isopropylmalate can all be secreted from the cell during amino acid starvation. These results suggest that Tmt1 is not functioning in a detoxification pathway because 3-isopropylmalate does not accumulate in the cytoplasm to any significant extent, even in the *tmt1*$^-$ cells. Furthermore, 2-isopropylmalate, the precursor of 3-isopropylmalate, accounts for the bulk of the secretion of these compounds. We conclude that 3-isopropylmalate methyl ester may have an independent function in the media.

Exogenous 3-Isopropylmalate Methyl Ester Enhances Invasive Growth. Recently, phenylethanol and tryptophanol, byproducts of phenylalanine and tryptophan degradation, have been identified as secreted autoinductive molecules that signal yeast invasive growth when cells are cultured in low-ammonia media (*10*). We hypothesized that secreted 3-isopropylmalate methyl ester may function in a similar manner when cells are starved for amino acids. We note that yeast starved for histidine or tryptophan with antimetabolites are induced to undergo invasive growth in a Gcn4-dependent manner (*4*). We thus measured invasive growth in response to exogenous 3-isopropylmalate methyl ester. Here, we used BY4742-derived strains as controls because they poorly express Flo11, a surface adhesion protein required for invasive growth (*7*). We then constructed *tmt1*$^-$ mutants in the 10560-23C yeast strain derived from the Σ1278 background which has been shown to grow well invasively (*30*). To test the signaling properties of 3-isopropylmalate methyl ester, we spotted cells with water or with partially purified media extracts from the Tmt1 overexpressing strain HCY005 (containing abundant 3-isopropylmalate methyl ester) or from the *tmt1*$^-$ strain HCY001 (lacking the methyl ester) on synthetic complete dextrose plates as described in Materials and Methods, and invasive growth was assayed over time. These plates contain sufficient amounts of glucose and ammonia to lower the background of invasive growth that occurs from other pathways. Figure 9A demonstrates the invasive growth of the yeast 10560-23C strain (*TMT1*$^+$) and the lack of invasive growth in the BY4742 strain (*TMT1*$^+$) after 3 days of incubation. Figure 9B shows the invasive growth after 2−4 days of incubation for the parental yeast 10560-23C strain (*TMT1*$^+$) and the isogenic yeast DDY007 *tmt1*- strain. Both strains are capable of growing invasively, but increased agar penetration and cell-to-cell adhesion (clumping) is observed on day 3 when the methyl ester is present (Figure 9B, C; compare the overexpressed *TMT1*$^+$ extract with the *tmt1*$^-$ extract or with H_2O). The amount of invasive growth on day 3 is greater in cells lacking Tmt1 than in cells that have the methyltransferase (Figure 9B, C; compare *TMT1*$^+$ set to *tmt1*$^-$). We expected that *TMT1*$^+$ cells would show a less dramatic effect because the endogenous methyl ester can be produced and secreted. We quantitated the invasiveness as shown in Figure 9C, confirming the autoinductive properties of 3-isopropylmalate methyl ester. These results show that presence of exogenous 3-isopropylmalate methyl ester can enhance the ability of yeast cells to grow invasively. The fact that all of the cells eventually became invasive (day 4) reflects that multiple pathways can independently signal invasive growth (*1*, *2*).

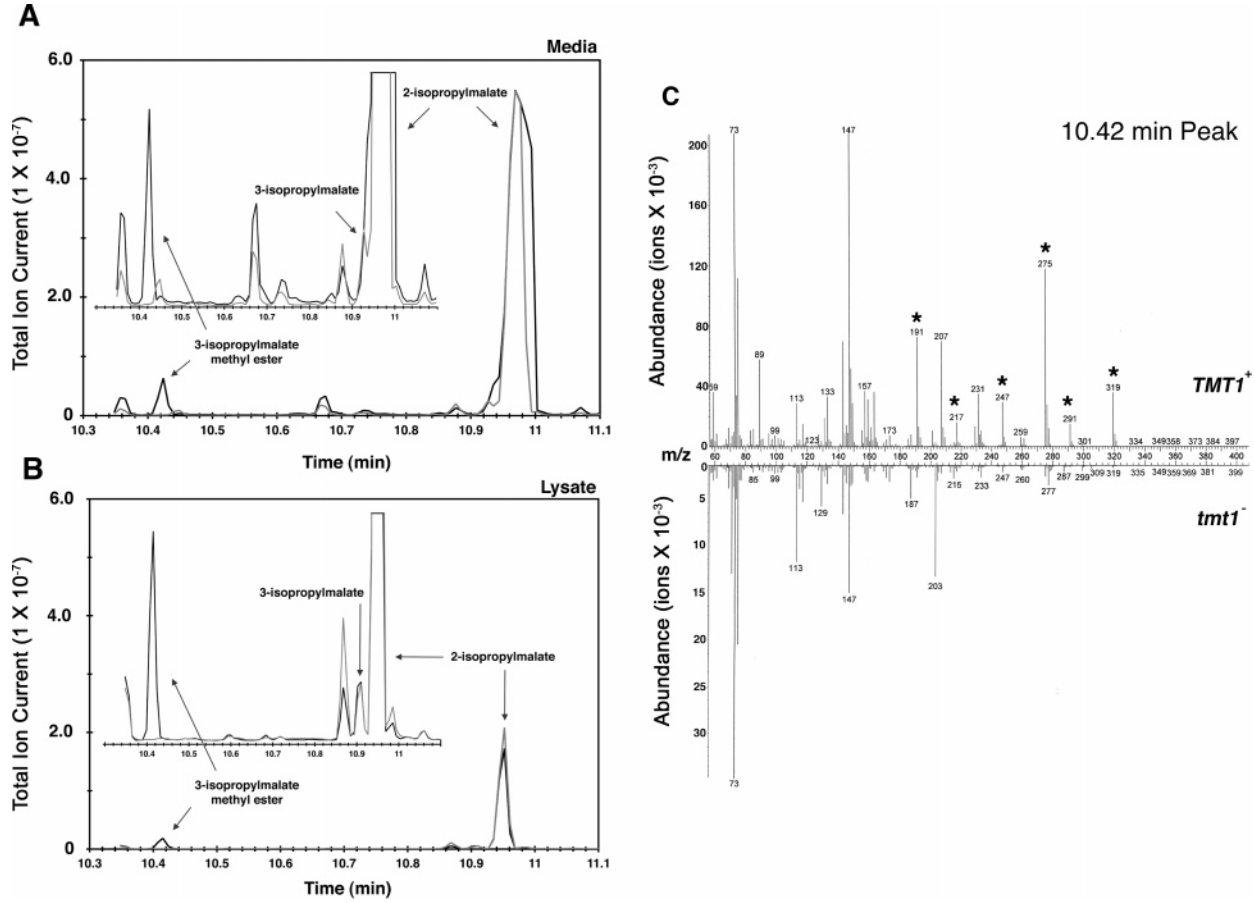

FIGURE 8: Identification of 3-isopropylmalate methyl ester by GC/MS as a Tmt1-dependent secretion product in yeast. Yeast cells (BY4742; *leu2*$^-$ *TMT1*$^+$ and DDY001; *leu2*$^-$ *tmt1*$^-$) were grown at 30 °C to log phase (1.0−1.2 optical density at 600 nm) in 1 of synthetic complete dextrose media and were then washed and resuspended in 100 mL of standard dextrose media for 4 h at 30 °C. The media and cell pellets were separated by centrifugation and then extracted with acidic ethyl acetate as described in Materials and Methods. The organic extracts were dried, derivatized, and subjected to GC/MS analysis as described in Materials and Methods. A portion of the total ion current is shown for the samples from the media in panel A and from the cell lysates in panel B. Data from the Tmt1$^+$ extracts are shown by the black lines; data from the Tmt1$^-$ extracts are shown by the gray lines. The elution positions of 3-isopropylmalate methyl ester, 3-isopropylmalate, and 2-isopropylmalate are indicated with arrows. The insets show an expanded total ion current axis. In panel C, we compare the mass spectrum of the 3-isopropylmalate methyl ester peak at 10.42 min for the *TMT1*$^+$ and *tmt1*$^-$ samples. Asterisks (*) denote ion fragments originating from 3-isopropylmalate methyl ester.

DISCUSSION

Here we present evidence that the yeast Tmt1 methyltransferase is activated in a Gcn4-dependent manner when cells are starved for amino acids. We also show that the 3-isopropylmalate methyl ester product of this enzyme is secreted and functions to signal yeast cells to grow invasively. Invasive growth has been observed to occur when amino acid starvation is induced in yeast cells (*4*), but the mechanism of this induction was not elucidated. When the media is abundant with amino acids or when the cell is capable of synthesizing them, 3-isopropylmalate methyl ester production can be repressed and vegetative growth enhanced. Repression can be achieved by inhibiting production of the 3-isopropylmalate substrate through leucine feedback-inhibition (*19*) and by inactivation of the Gcn4 transcription factor in the presence of amino acids (*26*, *27*) (Figure 1). A limited amount of exogenous amino acids coupled with the inability to synthesize them leads to derepression of the leucine biosynthetic pathway and the Gcn2-dependent activation of Gcn4 (*26*, *27*), which subsequently induces Tmt1. The newly produced 3-isopropylmalate is methylated by Tmt1, and the resulting methyl ester is transported to the media within hours. It is clear that there are multiple pathways leading to invasive growth, of which one participant is the Tmt1 pathway described here. For example, cells lacking the Tmt1 methyltransferase are still able to become invasive, although more time is required. Our model demonstrates one way that yeast can respond to changes in environmental amino acids levels to institute a growth program that is most suited for survival.

In yeast cells with a leucine auxotrophic background, the largest inductions of Tmt1 were observed when leucine was rapidly removed from the media or when leucine import was affected. These results are consistent with previously observed Tmt1 mRNA transcript expression levels determined in genome-wide microarray experiments when yeast cells are starved for other amino acids. The mRNA level of Tmt1 is induced 16-fold in a *his3*$^-$ mutant (*31*) and 7-fold when wild type cells were treated with the His3 inhibitor 3-aminotriazole, a molecule known to induce an amino acid starvation stress response controlled by Gcn4 (*26*). Braus and co-workers treated yeast cells with 3-aminotriazole and showed a link between amino acid starvation and invasive growth (*4*). Interestingly, cells exposed to the DNA damaging agent methyl methanesulfonate results in a 24.5-fold induction of Tmt1 mRNA levels (*32*). Methyl methanesulfonate

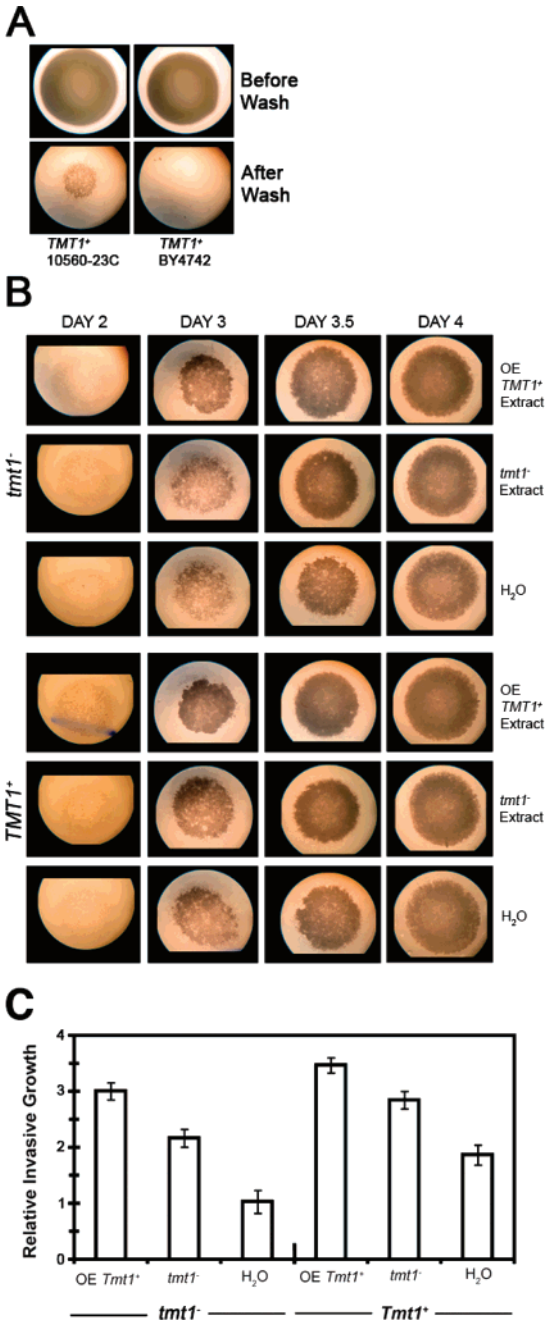

FIGURE 9: 3-Isopropylmalate methyl ester signals yeast to grow invasively. (A) Yeast strains 10560-23C and BY4742 were plated for the invasive growth assay as described in Materials and Methods and grown for 3 days at 30 °C. Photographs of the colony are shown before and after the washing step. (B) Invasive yeast colonies (10560-23C, *TMT1*$^+$; DDY007, *tmt1*$^-$) are shown after 2 to 4 days of incubation at 30 °C of growth. Colonies were spotted either with water, or purified fractions of organic extracts of the media of HCY005 yeast cells overexpressing Tmt1 (OE *TMT1*$^+$) or of HCY001 cells lacking Tmt1 (*tmt1*$^-$). The former extracts contain 3-isopropylmalate methyl ester while the latter lack it (Figure 7). (C) Quantitation of the invasive assay after 3 days of growth at 30 °C. Data is shown for four independent experiments ± the standard deviation as described in Materials and Methods. Significant differences were observed for *tmt1*$^-$ cells on the overexpressed *TMT1*$^+$ extract versus *tmt1*$^-$ extract or water (p values = 0.0018 and 0.000007, respectively), as well as for the wild type *TMT1*$^+$ cells (p values = 0.0074 and 0.00016). Significant differences were also found between the *TMT1*$^+$ and *tmt1*$^-$ cells when either overexpressed extract, mutant extract, or water were spotted to the plates (p values = 0.014, 0.0065, 0.0015, respectively).

has been suggested to activate Gcn4 by an unknown mechanism (*27*).

The identification of the autoinductive properties of 3-isopropylmalate methyl ester adds a new dimension to our knowledge of yeast extracellular signaling leading to invasive growth. Invasive growth has been linked to the expression of Flo11, shown to be required for cell-to-cell and cell-to-substrate adhesion (*6*, *7*). During amino acid starvation, the expression of Flo11 is signaled through Tpk2, the catalytic subunit of protein kinase A (*30*) and Flo8 (*4*). How 3-isopropylmalate methyl ester and the other autoinductive molecules relay this information to the cell remains unknown. It is also unknown if the methyl ester signal is attenuated simply by dilution or if slow degradation occurs. Finally, more effort is required to identify receptors involved in binding these molecules. Interestingly, haploid yeast cells that lack Ssy1, an amino acid sensor belonging to the Ssy1-Ptr3-Ssy5 complex involved in the sensing of exogenous amino acids, grow constitutively invasively (*1*). Ssy1 senses the extracellular concentration of amino acids which ultimately results in the induction of specific amino acid permeases through the proteolytic activation of the transcription factors Stp1 and Stp2 (*33*). The largest induction of permeases occurs in the presence of extracellular leucine (*34*). This result suggests that extracellular leucine may play a special role in signaling amino acid starvation, and that Ssy1 may interact not only with leucine but with its structural homologue 3-isopropylmalate methyl ester, with these ligands exerting opposite effects.

Does 3-isopropylmalate methyl ester function more generally in nature? Tmt1 activity has been detected in extracts of bacteria and yeast but not in nematodes or mice (*13*). BLAST-P searches of Tmt1 from *S. cerevisiae* against the Genbank nonredundant database using a cutoff expect value of 2×10^{-22} (corresponding to at 33% identity in 185 of the 299 residues) revealed similar proteins in fifteen fungal ascomycetes and basidomycetes species. These species include both pathogens such as *Candida albicans* (expect value 1×10^{-43}) and *Cryptococcus neoformans* (2×10^{-23}) as well as nonpathogenic species such as *Neurospora crassa* (1×10^{-26}). However, these matches also include the *S. cerevisiae* YHR209W species (expect value 2×10^{-31}) which does not appear to be a 3-isopropylmalate methyltransferase because the methyl ester is absent in *TMT1* null mutants. Thus, these homologues may represent methyltransferases catalyzing distinct reactions from Tmt1, and the formation of 3-isopropylmalate methyl ester may be limited to *S. cerevisiae* and perhaps only very similar species. Interestingly, it has been shown that tyrosol does not affect invasive growth induced by ammonia starvation in *S. cerevisiae* (*10*), while it does induce invasive growth in the pathogenic yeast *C. albicans* (*35*). Thus, there may be considerable species specificity in the molecules that lead to fungal invasive growth. These results suggest that the enzymes that catalyze the formation of these molecules may be therapeutic targets for antifungal drug development.

Finally, we note that the widespread usage of "well behaved" laboratory yeast strains may have limited our understanding of processes such as invasive growth. For example, the BY4741 and BY4742 strains that are the parent strains for the *Saccharomyces* Genome Deletion Consortium (www.yeastgenome.org) appear to have a mutation in the

FLO8 gene that prevents invasive growth (*18*). The widespread use of common genetic markers such as the genes required for amino acid and nucleotide biosynthesis further complicates the analysis of pathways that are dependent upon their intermediates such as the Tmt1 pathway described in this work. On the other hand, the presence of the *leu2* mutation in strains used in our initial Tmt1 studies allowed the accumulation of the 3-isopropylmalate substrate and the identification of role of the methyl ester product in invasive growth.

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