Diversification of Paralogous α-Isopropylmalate Synthases by Modulation of Feedback Control and Hetero-Oligomerization in Saccharomyces cerevisiae

Geovani López, a Héctor Quezada, a Mariana Duhne, a James González, a Mijail Lezama, a Mohammed El-Hafidi, b Maritrini Colón, a Ximena Martínez de la Escalera, a Mireille Citlali Flores-Villegas, a Claudio Sczaccchio, c,d Alexander Deluna, a Alicia González a

Departamento de Bioquímica y Biología Estructural, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Mexico City, Mexico; Departamento de Biomedicina Cardiovascular, Instituto Nacional Cardiología Ignacio Chávez, Juan Badiano No. 1, Colonia Sección XVI, Tlalpan, Mexico City, Mexico; Departamento de Microbiología, Imperial College London, South Kensington Campus, London, United Kingdom; Instituto de Génética y Microbiología, CNRS UMR 8621, Université Paris-Sud, Orsay, France; Laboratorio Nacional de Genómica para la Biodiversidad, Centro de Investigación y Estudios Avanzados del IPN, Irapuato, Guanajuato, Mexico.

Production of α-isopropylmalate (α-IPM) is critical for leucine biosynthesis and for the global control of metabolism. The budding yeast Saccharomyces cerevisiae has two paralogous genes, LEU4 and LEU9, that encode α-IPM synthase (α-IPMS) isozymes. Little is known about the biochemical differences between these two α-IPMS isoenzymes. Here, we show that the Leu4 homodimer is a leucine-sensitive isoform, while the Leu9 homodimer is resistant to such feedback inhibition. The leu4Δ mutant, which expresses only the feedback-resistant Leu9 homodimer, grows slowly with either glucose or ethanol and accumulates elevated pools of leucine; this phenotype is alleviated by the addition of leucine. Transformation of the leu4Δ isozyme has a distinct leucine sensitivity behavior. Determination of α-IPMS activity in ethanol-grown cultures showed that α-IPM biosynthesis and growth under these respiratory conditions depend on the feedback-sensitive Leu4 homodimer. We conclude that retention and further diversification of two yeast α-IPMSs have resulted in a specific regulatory system that controls the leucine-α-IPM biosynthetic pathway by selective feedback sensitivity of homomeric and heterodimeric isofoms.

The Leu4 and Leu9 α-isopropylmalate synthases (α-IPMSs), paralogous isozymes from Saccharomyces cerevisiae, catalyze the first committed step of leucine biosynthesis: the synthesis of α-isopropylmalate (α-IPM) from acetyl coenzyme A (acetyl-CoA) and α-ketoisovalerat (α-KIV). This reaction is carried out in the mitochondria (1–8), and α-IPM is then transported from the mitochondria to the cytosol by the yeast oxaloacetate/sulfate carrier Oac1 (9). The concerted action of Leu1 and Leu2 converts α-IPM to α-ketoisocaproate, the immediate precursor of leucine; these reactions are performed in the cytoplasm (8). The last step in leucine biosynthesis is carried out both in the mitochondria and the cytoplasm through the action of the differentially localized Bat1 or Bat2 aminotransferase (10) (Fig. 1). Most of the α-IPMS activity in wild-type S. cerevisiae cells is provided by the mitochondrially localized LEU4-encoded isozyme and not by the Leu4 (Leu4 s) isoform, which naturally lacks the mitochondrial import sequence and is thus localized in the cytosol (1, 2).

Leu4 enzymatic activity is inhibited by leucine and CoA, and the amino acid residues responsible for this property have been identified (7). Although no detailed biochemical characterization of the LEU9-encoded isozyme has been performed, it has been shown that it is less sensitive to leucine inhibition than Leu4 is (3).

It is noteworthy that the leucine biosynthesis intermediate α-IPM plays a dual cellular role. On the one hand, it acts as an intermediate in leucine biosynthesis (5, 6), and on the other, it acts as the coactivator of the Leu3 master regulator (6), which modulates the expression of a number of genes within and beyond amino acid metabolism (6). At low α-IPM concentrations, Leu3 acts as a transcriptional repressor, while at high α-IPM concentrations, it acts as an activator (6). It has been recently found that α-IPM could also have a role in the ability of Leu3 to determine the chronological life span of yeast (11), 12).

A LEU4 deletion (leu4Δ) results in leucine bradytroph on fermentable and nonfermentable carbon sources (5). Conversely, a leu9Δ deletion strain is able to grow without leucine on either glucose or nonfermentable carbon sources at a wild-type rate. This indicates that most of the leucine pool needed for growth with either glucose or ethanol can be supplied through the activity of Leu4. The fact that cells lacking both enzymes are absolute leucine auxotrophs indicates that Leu9 has a role in leucine biosynthesis. However, the expression of LEU9 is low on both fermentable and nonfermentable carbon sources (6). The expression of LEU4 is higher than that of LEU9 on glucose; it is induced in ethanol and is positively regulated by Gcn4 and Leu3–α-IPM (6, 13). Thus, Leu4 seems to be the main enzyme involved in α-IPM biosynthesis.

Gene duplication is a source of new or specialized protein func-

Received 24 February 2015 Accepted 1 April 2015 Accepted manuscript posted online 3 April 2015
Citation López G, Quezada H, Duhne M, González J, Lezama M, El-Hafidi M, Colón M, Martínez de la Escalera X, Flores-Villegas MC, Sczaccchio C, DeLuna A, González A. 2015. Diversification of paralogous α-isopropylmalate synthases by modulation of feedback control and hetero-oligomerization in Saccharomyces cerevisiae. Eukaryot Cell 14:564–577. doi:10.1128/EC.00033-15.
Address correspondence to Geovani López, glopez@email.fc.unam.mx.
Supplemental material for this article may be found at http://dx.doi.org/10.1128/EC.00033-15.
Copyright © 2015, American Society for Microbiology. All Rights Reserved. doi:10.1128/EC.00033-15
S. cerevisiae is able to grow under fermentative conditions by using a variety of carbon sources; interestingly, the WGD event shows a strong correlation with the appearance of fermentative lifestyles in the Saccharomyces lineage (22). Accordingly, it has been suggested that the selective conservation of certain duplicated genes could have enhanced the acquisition of facultative metabolic properties (23). In particular, it has been proposed that retention of GDH1/GDH3 and LYS20/LYS21 has afforded mechanisms allowing α-ketoglutarate (α-KG) utilization without impairing the integrity of the tricarboxylic acid cycle as an energy-providing system. The relative abundance of each one of the Gdh1/Gdh3 and Lys20/Lys21 isoforms under fermentative or respiratory conditions modulates the rate at which α-KG is channeled to glutamate and lysine biosynthesis (18, 19).

Results presented in this paper show that the biochemical diversification of LEU4- and LEU9-encoded α-IPMSs has resulted in the formation of homodimeric and heterodimeric α-IPMS isoforms with specific sensitivity to leucine inhibition, providing a mechanism that controls the flux of intermediates through the leucine and α-IPM biosynthetic pathway. The relative abundance of Leu4 and/or Leu9 monomers could determine the formation of the Leu4, Leu9, or Leu4-Leu9 isoform bearing differential leucine sensitivity, thus providing a mechanism controlling α-IPM biosynthesis and acetyl-CoA utilization under fermentative and respiratory conditions.

MATERIALS AND METHODS

Strains. Table 1 describes the characteristics of the strains used in the present work. The construction of S. cerevisiae strain CLA11-700 (MATa...
**TABLE 1 Strains used in this work**

| Strain     | Relevant genotype | Source or reference |
|------------|-------------------|---------------------|
| BY4741     | MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 LEU4 LEU9 | Open Biosystems     |
| BY4741-700 | MATα his3Δ1 leu2Δ0 met15Δ0 ENO1pr-yeCitrine LEU4 LEU9 | This study          |
| BY4741-701 | MATα his3Δ1 leu2Δ0 met15Δ0 LEU4 yE Citrine | This study          |
| BY4741-702 | MATα leu2Δ0 met15Δ0 LEU4 LEU9-yeCitrine | This study          |
| Y8205     | MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 can1Δ : STE2pr-Sp-his5 lyp1Δ : STE3pr-LEU2 | This study          |
| Y8205-701 | MATα MATα his3Δ1/Δ leu2Δ0 met15Δ0 ura3Δ0 can1Δ : STE2pr-Sp-his5 lyp1Δ : STE3pr-LEU2 | This study          |
| Y8205-702 | MATα MATα MATα his3Δ1/Δ leu2Δ0 met15Δ0 ura3Δ0 can1Δ : STE2pr-Sp-his5 lyp1Δ : STE3pr-LEU2 | This study          |
| Y8205-703 | MATα MATα MATα his3Δ1/Δ leu2Δ0 met15Δ0 ura3Δ0 can1Δ : STE2pr-Sp-his5 lyp1Δ : STE3pr-LEU2 | This study          |
| Y8205-704 | MATα MATα MATα his3Δ1/Δ leu2Δ0 met15Δ0 ura3Δ0 can1Δ : STE2pr-Sp-his5 lyp1Δ : STE3pr-LEU2 | This study          |
| Y37237    | MATα MATα ura3Δ0 ura3Δ0 leu2Δ0 leu9Δ0 his3Δ1/Δ his3Δ1/Δ met15Δ0 MET15 LYS2/lysΔ0 | EUROSCARF collection |
| Y32364    | MATα MATα ura3Δ0 ura3Δ0 leu2Δ0 leu9Δ0 his3Δ1/Δ his3Δ1/Δ met15Δ0 MET15 LYS2/lysΔ0 | EUROSCARF collection |
| S288C     | MATα matα gal12 | Cold Spring Harbor Laboratory |

| Strain     | Relevant genotype | Source or reference |
|------------|-------------------|---------------------|
| CLA11-700 | MATα LEU4 LEU9 leu2Δ0 : LEU2 ura3 | 20                  |
| CLA11-701 | MATα leu2Δ0 : kanMX4 LEU9 leu2Δ0 : LEU2 ura3 | This study          |
| CLA11-701-2 | MATα leu2Δ0 : natMX4 LEU9 leu2Δ0 : LEU2 ura3 | This study          |
| CLA11-702 | MATα LEU4 leu2Δ0 : kanMX4 leu2Δ0 : LEU2 | This study          |
| CLA11-703 | MATα leu2Δ0 : ura3Δ0 leu2Δ0 : kanMX4 leu2Δ0 : LEU2 | This study          |
| CLA11-703-2 | MATα leu2Δ0 : natMX4 ura3Δ0 : kanMX4 leu2Δ0 : LEU2 | This study          |
| CLA11-704 | MATα ENO2pr-LEU4 leu9Δ0 : kanMX4 leu2Δ0 : LEU2 | This study          |
| CLA11-704-1 | MATα ENO2pr-LEU4-yeCitrine leu9Δ0 : kanMX4 leu2Δ0 : LEU2 | This study          |
| CLA11-705 | MATα leu2Δ0 : ura3Δ0 ENO2pr-LEU9 leu2Δ0 : LEU2 | This study          |
| CLA11-705-1 | MATα leu2Δ0 : natMX4 ENO2pr-LEU9 yE Citrine leu2Δ0 : LEU2 | This study          |
| CLA11-706 | MATα ENO2pr-LEU4 ENO2pr-LEU9 leu2Δ0 : LEU2 | This study          |
| CLA11-706-1 | MATα ENO2pr-LEU4-yeCitrine ENO2pr-LEU9 leu2Δ0 : LEU2 | This study          |
| CLA11-706-2 | MATα ENO2pr-LEU4 ENO2pr-LEU9-yeCitrine leu2Δ0 : LEU2 | This study          |

YE44 LEU9 leu2Δ0 : LEU2 ura3Δ0 has been previously described (20). To obtain the leu4Δ mutant strain (CLA11-701) from CLA11-700, LEU4 was replaced by homologous recombination as previously described (24). A module containing the kanMX4 cassette flanked by 915 bp of the LEU4 5′ untranslated region (UTR) (−915 to −1) and 140 bp of the LEU4 3′ UTR (−1861 to +2001) sequences was amplified with deoxyoligonucleotides G9-G10 and G11-G12, respectively, with genomic DNA from strain Y32364 from the EUROSCARF (European Saccharomyces cerevisiae Archive for Functional analysis) collection used as the template (for the deoxyoligonucleotides used in this study, see Table S1 in the supplemental material). Transformsants were selected for G418 resistance (200 mg/liter; Life Technologies) and confirmed by PCR. To obtain a leu9Δ mutant, strain CLA11-700 was transformed with a 2391-bp PCR product containing the kanMX4 cassette flanked by 720 bp of the LEU9 5′ UTR (−720 to −1) and 117 bp of the LEU9 3′ UTR (−1816 to +1933). The module was amplified from genomic DNA of strain Y32364 from the EUROSCARF collection with deoxyoligonucleotides G3 and G4. Transformsants were selected for G418 resistance, generating strain CLA11-702; mutants were PCR verified. A leu4Δ leu9Δ double mutant strain of CLA11-703 was constructed by interrupting LEU4 with URA3 and LEU9 with kanMX4. The URA3 gene was amplified by PCR with deoxyoligonucleotides G5 and G6 on genomic DNA from wild-type strain S288C; once obtained, this PCR module was digested with BamHI, generating cohesive ends, and inserted into a pGEM-T easy vector that contained LEU4 sequence previously linearized with BamHI. The leu4Δ URA3 construct was then PCR amplified with deoxyoligonucleotides G1 and G2, producing a 3.150-bp DNA fragment that was transformed into strain CLA11-702. Transformsants were selected for uracil prototrophy and verified by PCR. The S. cerevisiae leu4Δ leu9Δ double mutant (CLA11-703-2) with nourseothricin and G418 resistance (leu4Δ : natMX4 leu9Δ : kanMX4) was obtained by transforming leu4Δ (CLA11-701) with the clonNAT module obtained by EcoRI digestion of plasmid p4339; this module replaces kanMX4 by homologous recombination (25), generating strain CLA11-701-2 (leu4Δ : natMX4 LEU9 ura3Δ0 leu2Δ0 : LEU2). The LEU9 gene was subsequently replaced by homologous recombination with a module containing the kanMX4 cassette amplified from strain CLA11-702 with deoxyoligonucleotides G7 and G8, generating strain CLA11-703-2. **Construction of strains expressing LEU4 and/or LEU9 from the ENO2 promoter.** To fuse the LEU4 and LEU9 coding sequences to the ENO2 promoter, the LEU4 and LEU9 ORFs were amplified with deoxyoligonucleotide pairs G9-G10 and G11-G12, respectively, with genomic DNA from strain CLA11-700 as the template. The resulting modules were flanked by sequences homologous to the 5′ and 3′ UTRs of ENO2 (50 bp upstream from the start codon and 50 bp downstream of the stop codon). The modules were transformed independently into leu4Δ leu9Δ double mutant strain CLA11-703, generating strains CLA11-704 (MATα ENO2pr-LEU4 leu9Δ : kanMX4 leu2Δ0 : LEU2) and CLA11-705 (MATα leu4Δ : URA3 ENO2pr-LEU9 leu2Δ0 : LEU2) (Table 1). Transformsants were selected for leucine prototrophy and PCR verified. An ENO2pr-LEU4 ENO2pr-LEU9 double mutant was constructed as follows. A PCR module containing ENO2pr-LEU4 was amplified from strain CLA11-704 with deoxyoligonucleotides G13 and G14. This module was used as the template for a second PCR with deoxyoligonucleotides G15 and G16 in order to add flanking regions homologous to the 5′ and 3′ UTRs of the LEU4 gene.

López et al.
The resulting modules contained 50 bp of the upstream sequence of the LEU4 start codon, 742 bp of the 5' UTR of the ENO2 promoter (−742 to −1), the LEU4 ORF (1,860 bp), 381 bp of the 3' UTR of ENO2 (+1315 to +1695), and 50 bp downstream of the LEU4 stop codon. This module was used to transform strain CLA11-705. Transformants were selected for 5-fluoroorotic acid resistance, producing strain CLA11-706 (MATα ENO2pr-Leu4 ENO2pr-Leu9 leu2:LEU2 ura3Δ). Correct insertion of the modules was verified by PCR.

**Construction of yECitrine-tagged mutant S. cerevisiae strains.** Deoxyoligonucleotides were designed on the basis of the ENO1 promoter (G16a, G16b), LEU4, (G17, G18), ENO2 promoter-LEU4 (G17, G21), LEU9 (G19, G20), or ENO2 promoter-LEU9 (G19, G21) carrying 18 bp of yECitrine (forward) or URA3 (reverse) and used to PCR amplify yECitrine and URA3 from plasmid pKT175. The resulting modules were selectively transformed into BY4741, CLA11-704, CLA11-705, and CLA11-706 to generate strains BY4741-700, BY4741-701, BY4741-702, CLA-11-704-1, CLA11-705-1, CLA11-706-1, and CLA11-706-2 (25, 26) and confirmed by PCR.

**Plasmid construction for bimolecular fluorescence complementation (BiFC) assay analysis.** To analyze interactions between the paralogous proteins Leu4 and Leu9, a pair of plasmids encoding either the N- or C-terminal domain of the yECitrine protein were generated with the QuikChange site-directed mutagenesis kit (Stratagene catalog no. 200518). In order to prepare a plasmid coding for the 155 amino acids of the C-terminal domain of the yECitrine protein were generated with the QuikChange site-directed mutagenesis kit (Stratagene catalog no. 200518). In order to prepare a plasmid coding for the 155 amino acids of the yECitrine T-IPMS enzyme assay and protein determination. For α-IPMS determination, strains expressing LEU4 and/or LEU9 from the ENO2 promoter were grown in YP-Gal 2% to an OD600 of ~1.6, and glucose was added to a final concentration of 10%; the cells were allowed to grow for 6 h. Collected cells were washed twice with FACFlow (BD catalog no. 342003) and suspended in this solution to a final OD600 of 0.1, and 10^4 events were recorded. The measurements were performed with a BD FACSCalibur flow cytometer. The fluorescent signal was obtained with a 488-nm excitation and 530-nm band-pass filter photomultiplier. Data were analyzed offline with the Cytlogic software. For further analysis, we obtained the geometric mean of each set of data as a measurement of protein prevalence as previously reported (30).
completion of the reaction of DTNB with the contaminant CoA present in the commercial acetyl-CoA preparation, the assay was started by adding cell extract and the initial reaction rate was obtained from the change in absorbance at 412 nm in a Varian Cary 400 spectrophotometer with a 1-cm path length. 2-Nitro-5-thiobenzoate production was quantified by using an extinction coefficient of 14.15 M⁻¹ cm⁻¹ (31). Specific activity is given in nanomoles of CoA formed per minute per milligram of protein. Protein concentration was determined as described previously (32).

**Leu4 and Leu9 α-IPMS purification.** α-IPMS was purified from extracts prepared from cultures of strains CLA11-704, CLA11-705, and CLA11-706 (Table 1), which overexpress LEU4, LEU9, or both under the control of the ENO2 promoter. Cells were grown in YP-Gal 2% to an OD₆₀₀ of ~1.6, and glucose was added to a final concentration of 10%; samples were collected after 6 h. Yeast mitochondria were isolated as previously described (33). Mitochondria were harvested by centrifugation and washed twice with cold distilled water, after which samples were disrupted with glass beads in phosphate buffer A. The extract obtained was applied to a Q Sepharose column (Sigma Q1126) previously equilibrated with 50 mM phosphate buffer, pH 7.5. Unbound proteins were eluted with 4 column volumes, and α-IPMS was eluted with 50 mM phosphate buffer, pH 7.5, and a linear gradient of 0.0 to 0.5 M NaCl; fractions showing the highest activity were collected and dialyzed with an Amicon centrifugal filter with a 30-kDa molecular mass cutoff in 50 mM phosphate buffer, pH 7.5.

**Molecular mass determination.** The native molecular masses of purified α-IPMS isozymes were determined by running the samples on a Superdex 200 HR 10/30 (Amersham Pharmacia Biotech) column equilibrated with 50 mM phosphate buffer (pH 7.5)–0.15 M NaCl. The column was calibrated with the molecular size markers thyroglobulin (670 kDa), alcohol dehydrogenase (150 kDa), and bovine serum albumin (66 kDa) from Sigma. The flow rate in the column was 0.25 ml/min, and 0.25-ml fractions were collected; the eluted fractions were monitored at 280 nm with a Pharmacia fast protein liquid chromatography system. Blots were scanned with the program ImageQuant 5.2 (Molecular Dynamics).

**Enzyme extraction and analysis.** Cell extracts were prepared from exponentially growing cultures. Samples used for intracellular amino acid determination were treated as previously described (20).

### RESULTS

**leu4Δ mutants accumulate larger leucine pools than the wild-type strain.** To analyze the physiological role of Leu4 and Leu9, single and double LEU4 and LEU9 null mutants were constructed. As previously reported (5), the leu4Δ leuΔ double mutant has a leucine auxotrophic phenotype when grown in either glucose- or ethanol-containing medium, while the leu9Δ single mutant displayed a wild-type phenotype with both carbon sources (Fig. 2A and B). Conversely, the leu4Δ single mutant is a partial leucine auxotroph with either glucose or ethanol (Fig. 2A and B; see Table S2 in the supplemental material), indicating that Leu9 cannot fully compensate for the absence of Leu4. Addition of leucine to the growth medium restores the leu4Δ single and leu4Δ leu9Δ double mutants to the wild-type growth rate with either glucose or ethanol (Fig. 2A and B; see Table S2). The leu4Δ mutant strain showed a 30-h lag phase when grown with ethanol as the sole carbon source, compared to the 10-h lag phase of the wild-type or leu9Δ mutant strain grown under identical conditions (Fig. 2B). After the lag phase, the growth rate of this mutant strain with ethanol was half of that of the wild-type (Fig. 2B; see Table S2). No lag phase was observed when glucose was used as a carbon source, although the growth rate was 2-fold lower than that of the wild type (Fig. 2A; see Table S2). Transformation with a centromeric plasmid carrying the LEU4 gene fully complemented the leu4Δ leu9Δ leucine auxotrophy and the leu4Δ leu9Δ bradytrophic phenotype with either glucose or ethanol (Fig. 2C and D; see Table S2). As expected, LEU9 did not complement the leu4Δ LEU9 bradytrophic phenotype, while the leu4Δ leu9Δ double mutant, harbor-
FIG 2 Growth curves of *S. cerevisiae* LEU4 and LEU9 single and double mutants. Growth curves of LEU4 LEU9, leu4Δ LEU9, LEU4 leu9Δ, and leu4Δ leu9Δ mutant strains in MM with 2% glucose (A) or 2% ethanol (B) as a carbon source with or without (w/o) leucine, as shown. (C) Growth of the LEU4 LEU9/pRS416, leu4Δ leu9Δ/pRS416, leu4Δ leu9Δ/pRS416-LEU4, and leu4Δ leu9Δ pRS416-LEU9 mutant strains cultivated on 2% glucose or ethanol. (D) Growth of the LEU4 LEU9/pRS416, leu4Δ LEU9/pRS416, leu4Δ LEU9/pRS416-LEU4, and leu4Δ LEU9/pRS416-LEU9 mutant strains cultivated on 2% glucose or ethanol. The values presented are the means of at least four experiments ± the standard deviations.
ing LEU9 on a centromeric plasmid, displayed the leu4 Δ LEU9 bradytrophic phenotype (Fig. 2C and D; see Table S2).

To further analyze the phenotype of the wild-type and mutant strains, intracellular pools of leucine were determined during exponential growth. The leucine pools of wild-type and leu9Δ mutant cultures grown with glucose or ethanol were similar (Fig. 3), confirming that Leu9 plays a marginal role in leucine biosynthesis in the presence of Leu4. Intriguingly, the intracellular leucine pools of the leu4 Δ strain were two or four times as high as those of the wild-type strain grown with glucose or ethanol, respectively. This indicates that the leucine bradytrophic phenotype displayed by the leu4Δ mutant is not due to leucine depletion. Complementation with a centromeric plasmid harboring LEU4 (pRS416-LEU4) restored leucine pools to wild-type levels (Fig. 3).

These counterintuitive results suggest that the leucine-bradytrophic phenotype of the leu4Δ mutant is a consequence of the enhanced flux of intermediates through the leucine biosynthesis pathway, probably resulting in decreased acetyl-CoA provision to synthesize crucial intermediates whose depletion could hamper cell growth. These phenotypes also suggest that Leu4, but not Leu9, is feedback inhibited by leucine.

Leu4 and Leu9 α-IPMSs show different sensitivities to leucine inhibition. It has been previously reported that α-IPMS is sensitive to leucine feedback inhibition (3). The gene deletion phenotype results presented above suggested different behaviors of the paralogous proteins with respect to feedback inhibition. To investigate the leucine sensitivity of each isozyme, the half-maximal inhibitory concentrations (IC50s) for the Leu4 and Leu9 isoforms were determined in cell extracts obtained from cultures of the wild-type and leu9Δ and leu4Δ single mutants grown exponentially on glucose plus ammonium sulfate. We found that the IC50 for Leu9 was 1.10 mM of leucine, while that for Leu4 was 0.03 mM; thus, a 36-fold sensitivity difference between the two isozymes exists. This result suggests that the insensitivity of the Leu9 isozyme to leucine feedback inhibition is the cause of the leucine bradytrophic phenotype observed in a leu4Δ mutant strain, which can be relieved if leucine is added to the culture medium, so that the leucine intracellular pool reaches Leu9 inhibitory concentrations, establishing feedback control (Fig. 2A and B).

Leu4 is the predominant α-IPMS isoform in either glucose- or ethanol-grown cultures. Determination of α-IPMS activity in glucose-grown cultures indicated that in the wild-type strain, the LEU4-encoded isozyme contributes 80 to 85% of the total α-IPMS enzymatic activity while Leu9 contributes only 15 to 20% of the activity. In a leu4Δ mutant strain, Leu9 represented 25 to 30% of the total activity present in the wild-type strain (Fig. 4A). Under respiratory conditions, with ethanol provided as the sole carbon source, Leu4-dependent enzymatic activity was 4-fold higher than...
that determined on glucose while Leu9 activity was similar to that found in glucose, representing 3.6% of the wild-type activity (Fig. 4A). These results indicate that Leu4 is the main activity present in both glucose- and ethanol-grown cultures. To further analyze the relative prevalence of Leu4 and Leu9, the amount of Leu4 or Leu9 protein present in the wild-type in either glucose or ethanol was determined by flow cytometric analysis of independent strains harboring yECitrine-tagged LEU4 or yECitrine-tagged LEU9 (BY4741-701 and BY4741-702, respectively, in Table 1). For each strain grown with glucose or ethanol as a carbon source, 1 × 10⁴ events were recorded as described in Materials and Methods. The geometric mean of each set of data was obtained, and it was considered a measurement of protein prevalence (30). In both glucose- and ethanol-grown cultures, the Leu4 fluorescent signal level was higher than that of Leu9 and was considered 100%; Leu9 corresponded to 20% of the fluorescent signal in glucose and 3% of that in ethanol.

To analyze whether enzyme activity correlates with LEU4 and LEU9 expression, Northern analysis was carried out with total RNA samples obtained from wild-type cultures grown with either glucose or ethanol. It was found that LEU4 displayed higher expression than LEU9 in samples obtained from cultures grown with either one of the two carbon sources (Fig. 4B), in agreement with the α-IPMS enzymatic activity determination (Fig. 4A) and flow cytometry analysis. LEU9 expression and α-IPMS activity were enhanced 3- to 5-fold in a leu4Δ mutant background, indicating that, as has been previously observed for other paralogous pairs, deletion of one of the paralogs causes the upregulation of its counterpart (36).

These results indicate that LEU4 and LEU9 transcriptional regulation determines the levels of Leu4- and Leu9-dependent α-IPMS activity and that Leu4 negatively modulates LEU9 expression.

Considering that, as will be shown below, Leu4 and Leu9 α-IPMS are dimeric isoforms and that the formation of heterodimeric isoforms could result in the presence of a Leu4-Leu9 isozyme with intermediate leucine sensitivity, we analyzed the interaction of Leu4 and Leu9.

**Leu4 and Leu9 monomers interact in vivo.** Subcellular localization analysis showed that Leu4 and Leu9 are localized in the mitochondria (Fig. 5, rows 1 and 2). We did not detect the minor Leu4 cytosolic isoform (Leu4s in Fig. 1) that has been previously reported (1), probably because of a fluorescence signal below the limit of detection. Next, we investigated the association between paralogous proteins Leu4 and Leu9 by BiFC (37–39). To this aim, LEU4- and LEU9-encoded proteins were selectively tagged with either the amino or the carboxy half of the fluorescent yECitrine protein (BY8205-701 and BY8205-702 in Table 1). A strong fluorescence signal was observed in strains expressing these constructs, indicating an interaction between Leu4 and Leu9 (Fig. 5, rows 3 and 4). As a positive control, the interaction between the monomers constituting the Bat1 dimeric branched-chain aminotransferase (10) was used (BY8205-703 in Table 1) (Fig. 5, row 5).

As a negative control, we monitored the interaction between Bat1 tagged at the carboxy terminus with the amino half of yECitrine and the BAT1 promoter sequence fused to the 17 amino acids that constitute the Bat1 mitochondrial presequence tagged at the carboxy terminus with the carboxy half of yECitrine (BAT1pr-Bat1-17aa-yC) (BY8205-704 Table 1) (Fig. 5, row 6). These results indicate that Leu4 and Leu9 can form hetero-oligomeric isozymes in vivo.

**Purification of α-IPMS isozymes indicates the formation of a hetero-oligomeric isozyme.** To purify the Leu4, Leu9, and Leu4-Leu9 isoforms, we constructed strains expressing LEU4, LEU9, or both paralogs under the control of the ENO2 promoter, which results in high expression on glucose as the sole carbon source (40). Northern blot analysis of LEU4 and LEU9 expression driven by the ENO2 promoter showed that the expression of both genes was higher than that observed with the endogenous promoter (Fig. 6A). Accordingly, the α-IPMS activity in crude extracts of cultures of the ENO2pr-LEU4 leu9Δ (CLA11-704) and leu4Δ ENO2pr-LEU9 (CLA11-705) mutant strains was 20- and 50-fold higher, respectively, than when LEU4 or LEU9 was expressed from its endogenous promoter (Fig. 6B). Furthermore, flow cytometric analysis of independent strains expressing ENO2pr-LEU4- yECitrine or ENO2pr-LEU9-yECitrine showed that both paralogs were expressed in nearly equimolecular amounts. The Leu4 fluorescent signal level was higher than that of Leu9 and was considered 100%, while Leu9 fluorescence corresponded to 75% of this value.

α-IPMS from cultures of the ENO2pr-LEU4 leu9Δ (CLA11-704), leu4Δ ENO2pr-LEU9 (CLA11-705), and ENO2pr-LEU4 ENO2pr-LEU9 (CLA11-706) mutant strains were purified 30-, 26-, and 17-fold, respectively (see Table S3 in the supplemental material). Denaturing SDS-PAGE showed a molecular mass of about 68 kDa (Fig. 7A), which was expected for the monomeric form. The isozymes purified from the strain overexpressing both Leu4 and Leu9 also showed a 68-kDa monomeric subunit in denaturing gels. The oligomeric organization of α-IPMS isoforms from S. cerevisiae was analyzed by gel filtration with a Superdex 200 column as the matrix. The native molecular size was estimated to be 130 to 150 kDa, which is compatible with a dimeric organization (1, 41).

To analyze whether simultaneous expression of LEU4 and LEU9 from the ENO2 promoter resulted in the formation of Leu4-Leu9 heterodimeric isozymes, the ENO2pr-LEU4-yECitrine leu9Δ (CLA11-704-1), leu4Δ ENO2pr-LEU9-yECitrine (CLA11-705-1), ENO2pr-eno4-yECitrine ENO2pr-LEU9 (CLA11-706-1), and ENO2pr-LEU4 ENO2pr-LEU9-yECitrine (CLA11-706-2) mutant strains were grown with 10% glucose as described in Materials and Methods. Crude extracts were prepared and electrophoresed in a native agarose gel (34). Distinct single bands composed of either Leu4-yECitrine or Leu9-yECitrine homodimers, respectively, were observed (lanes 2 and 5 of Fig. 7B). Differences in mobility are due to the different isoelectric points of Leu4 (5.8) and Leu9 (6.7). Leu4 and Leu9 form only dimers, and larger multimers were not observed. Lanes 3 and 4 show single bands whose positions in the gel indicate that they were composed of Leu4-yECitrine-Leu9 or Leu4-Leu9-yECitrine, respectively. These results show that when LEU4 and LEU9 are expressed simultaneously, only Leu4-Leu9 heterodimers were formed, precluding the formation of Leu4 and Leu9 homodimers. Accordingly, analysis of the leucine sensitivity of α-IPMS isoforms present in crude extracts obtained from the ENO2pr-LEU4 leu9Δ (CLA11-704), leu4Δ ENO2pr-LEU9 (CLA11-705), and ENO2pr-LEU4 ENO2pr-LEU9 (CLA11-706) mutant strains showed that for Leu4 (Fig. 7C, I), Leu9 (Fig. 7C, II), and Leu4-Leu9 (Fig. 7C, III), a monophasic curve was found, indicating a single component that displays differential leucine sensitivity. This further indicated that these preparations contained a single isoform. As expected, the preparation containing a mixture of identical amounts of Leu4 and Leu9 showed...
biphasic behavior (Fig. 7C, IV). These results show that the enzyme purified from the ENO2pr-LEU4 ENO2pr-LEU9 mutant strain corresponded to a single isoform with specific leucine sensitivity. As expected, the IC50 of the enzyme present in an extract prepared from glucose-ammonium-grown cultures of the ENO2pr-LEU4 ENO2pr-LEU9 mutant strain displayed an IC50 of 0.16 mM of leucine. This value did not correspond to the average sensitivity of Leu4 and Leu9 that would be expected if the independent Leu4 and Leu9 isozymes were present in equimolar amounts, which would correspond to a presumed IC50 of 0.56, again indicating that when LEU4 and LEU9 are expressed simultaneously, only the hetero-oligomeric isoform is formed.

To analyze the biochemical properties of the homo- and heterodimeric isoforms, a kinetic analysis of the purified enzymes was carried out.

Each one of the homo- and heterodimeric α-IPMS isoforms has a specific leucine sensitivity. In order to address the question of whether the Leu4, Leu9, and Leu4-Leu9 isozymes had acquired differential catalytic capacities, affinities for substrates, or leucine sensitivity, kinetic analyses of the purified Leu4 and Leu9 homodimers and the Leu4-Leu9 heterodimer were carried out. In the absence of leucine, the three enzymes showed hyperbolic kinetics (see Fig. S1 in the supplemental material), similar affinities for substrates (Ka and kb in Table 2), and similar turnover numbers (catalytic events that occur per unit of time, kcat in Table 2).

Experimental data were fitted to equation 1, which corresponds to a compulsory ordered bi-bi reaction under steady-state treatment (42) (Fig. 8A to F; Table 2). Although the overall kinetic properties were similar, a small difference based on the Kia constant, which reflects the acetyl-CoA dissociation constant of the free enzyme, was noted; the Leu9 homodimer showed a slightly lower affinity for this substrate than the other two isozymes did (Table 2). In contrast, in the presence of leucine, dramatic differences were observed. Leucine behaved as a mixed inhibitor, decreasing both the apparent affinity for the substrates and the apparent maximum enzyme reaction rate (Vmax). Experimental data...
were fitted to equation 2 when the acetyl-CoA concentration was varied and to equation 3 when the variable substrate was α-KIV (42) (Fig. 8A to F; Table 2) (see the text and equations in Materials and Methods). This model predicts that leucine can bind either to the free enzymes or to the substrate-bound enzymes; the former process is reflected in the $K_{ii}$ constant, whereas the latter is reflected in the $K_{ii}$ value (Table 2, equations 2 and 3), whose numerical values are inversely related to the affinity for leucine. Interestingly, the Leu9 homodimer was resistant to leucine inhibition, while the heterodimer was sensitive (Table 2). Since the intracellular concentration of acetyl-CoA has been estimated to be 0.23 mM (43), which is saturating for the three isozymes, it could be considered that the predominant form is the enzyme bound to acetyl-CoA for the three isoforms. If we assume that the concentration of α-KIV is also saturating, the physiologically relevant inhibition constant would be $K_{ii}$ (Table 2). The Leu9 homomeric enzyme showed a $K_{ii}$ value that was 24-fold higher than that displayed by Leu4. If the α-KIV concentration was not saturating, the competitive component (reflected in $K_{is}$) would also be important. In such a scenario, the value corresponding to the Leu9 homomeric enzyme is also much higher than that of the Leu4 isozyme, indicating that the Leu9 homodimer was not the best-suited isoform to sense the leucine concentration and determine flux control through the biosynthetic pathway. It is worth mentioning that the hetero-oligomeric form showed lower sensitivity to leucine than the Leu4 homodimer did, suggesting that whenever the heterodimer was present, its lower threshold of feedback inhibition would allow the formation of larger intracellular leucine pools than when the Leu4 homodimer was the predominant isoform.

**DISCUSSION**

This study addressed the question of whether the formation of hetero-oligomeric paralogous isozymes, with emerging biochemical properties, could form part of the strategies determining the retention and functional diversification of paralogous genes. To this end, we studied the S. cerevisiae LEU4- and LEU9-encoded dimeric α-IPMS paralogous isozymes. The results presented show that Leu4 and Leu9 have diverged in their biochemical properties, giving rise to leucine-sensitive and -resistant isoforms organizing in vivo homo- and heterodimeric isozymes, each with a peculiar leucine sensitivity profile. Leu4 and Leu9 leucine sensitivity diversification could contribute to a refined negative feedback regulation of the leucine and α-IM biosynthetic pathway.

Divergent LEU4 and LEU9 expression profiles, the leucine sensitivity of encoded α-IPMS, heterodimeric organization, and subcellular localization have contributed to the functional diversification of LEU4 and LEU9. The intracellular amino acid concentration is determined by the combined action of amino acid uptake, de novo synthesis, and recycling. De novo synthesis is controlled mainly by feedback regulation, adjusting the flow of intermediates and preventing depletion of metabolites, which simultaneously feed other pathways. It could thus be considered that the alternative formation of various isozymes with peculiar sensitivity to feedback control could provide a robust system, selectively determining the accumulation of fitting amino acid levels appropriate to contrasting physiological conditions such as glucose versus ethanol. Formation of paralogous isozymes with different enzymatic capacities had already been observed, and it had been proposed that the various enzymatic properties were due to the existence of multiple homo- and hetero-oligomeric isoforms. Accordingly, it was considered that the NADP-Gdh1-Gdh3 isozyme activity could provide the pacemaker mechanism, ensuring optimum glutamate biosynthesis and operation of the energy-yielding metabolism under either fermentative or respiratory conditions (18, 19). The study presented here analyzed the existence and role of hetero-oligomeric organization in LEU4 and LEU9 functional diversification. We show that the Leu4 homodimer is the predominant isozyme present in either glucose- or ethanol-grown cultures. The fact that LEU4 expression is increased 3-fold in ethanol-grown cultures while that of LEU9 is almost nil allows Leu4 homodimer formation and prevents Leu9 isozyme formation. This supports feedback control acting on the Leu4 leucine-sensitive isozyme, preventing leucine and α-IPM oversynthesis and mediating a more balanced distribution of pyruvate and acetyl-CoA to intermediary biosynthesis and energy-yielding metabolism, particularly under respiratory conditions.
Accordingly, in a leu4Δ LEU9 mutant, the action of the leu-
cine-resistant Leu9 homodimer leads to increased leucine biosyn-
thesis (Fig. 3) and growth impairment (Fig. 2A and B), most pos-
sibly as a result of impaired energy and substrate production or an
increased α-IPM pool, which could counteract the regulatory role
of Leu3 (6). The fact that this growth impairment is reversed when
leucine is added to the growth medium (Fig. 2A and B) indicates
that leucine addition results in Leu9 inhibition, recovering flux
control and wild-type flow of substrates through this pathway.

The observation that transformation with plasmids harboring
LEU4 restores wild-type growth and a wild-type leucine pool
indicates that Leu4 plays two roles in metabolism, α-IPM and leu-
cine biosynthesis and control of Leu9 abundance, through LEU9
expression control and the formation of Leu4-Leu9 hetero-oli-
gomers. leu4Δ leu9Δ double mutants are absolute leucine auxo-
trophs, which implies that Leu9 contributes to wild-type levels
of intracellular leucine biosynthesis. In addition, Leu9 is required
to organize hetero-oligomeric isoforms with intermediate leucine
sensitivity. Worth mentioning is the fact that no particular phys-
iological condition under which maintaining a high Leu9-depen-
dent leucine pool level could be physiologically relevant has been found, and thus, a particular role for the leucine-resistant isoform in a leu4Δ genetic background remains to be determined.

**Organization of the Leu4-Leu9 heteroisozyme is favored over that of homo-oligomeric isoforms.** Using differentially γECitrine-tagged LEU4 and LEU9 mutants, we showed that when LEU4 and LEU9 are expressed in equimolecular amounts, the formation of Leu4-Leu9 hetero-oligomers is preferred to that of Leu4 and Leu9 homo-oligomers (Fig. 7B). These observations suggest that there is higher affinity between different monomers than between monomers encoded by the same gene. Although there is previous evidence indicating that the formation of hybrid proteins could be preferred to that of homomeric isozymes (44), the physiological importance of this phenomenon has not been addressed.

It could be proposed that the preference for hybrid organization could have a key evolutionary significance, allowing the formation of best-fitted isozymes. Our results indicate that the $K_{ii}$ of the hetero-oligomeric enzyme is similar to the previously calculated

### FIG 8
The Leu4-Leu9 heterodimer shows a leucine sensitivity intermediate between those of the Leu4-sensitive and Leu9-resistant isoforms. Saturation curves at different leucine concentrations of the Leu4 (A, D) and Leu9 (B, E) homodimers and the Leu4-Leu9 heterodimer (C, F). Continuous lines represent the global fit to equation 2 or 3.

### TABLE 2
| Isozyme(s)       | $k_{cat}$ (s$^{-1}$) | $K_a$ ± SE | $K_{ia}$ ± SE | $K_b$ ± SE | $K_{is}$ ± SE | $K_{ii}$ ± SE |
|------------------|---------------------|-----------|--------------|-----------|--------------|--------------|
| Leu4             | 13.79               | 0.014 ± 0.002 | 0.047 ± 0.017 | 0.011 ± 0.002 | 0.048 ± 0.009 | 0.226 ± 0.045 |
| Leu9             | 7.28                | 0.009 ± 0.003 | 0.207 ± 0.078 | 0.009 ± 0.002 | 1.379 ± 0.202 | 5.448 ± 0.668 |
| Leu4-Leu9        | 10.91               | 0.013 ± 0.002 | 0.047 ± 0.016 | 0.014 ± 0.003 | 0.207 ± 0.025 | 0.703 ± 0.047 |

*Values represent the fitted kinetic parameters ± the standard errors. For all of the fitting analyses, the $R^2$ value was higher than 0.981. Similar $K_{is}$ and $K_{ii}$ values were obtained with equations 2 and 3 as described in Materials and Methods.*
average leucine intracellular pool (0.60 mM) (43, 45); this kinetic parameter could make it suitable to control leucine biosynthesis and intermediary flux through the pathway.

Although it has been recognized that protein oligomerization plays a key role in the regulation of proteins, conferring several functional advantages, such as improved stability, increased enzymatic activity, and selective sensitivity to inhibitors (46–48), the role of oligomerization leading to functional diversification has not been analyzed previously. The formation of hetero-oligomeric isoforms could be a common theme in the mechanisms leading to the retention and subfunctionalization of duplicated genes. Worth mentioning is the fact that previous work from other laboratories had considered the formation of hybrid paralogous isoforms between the ENO1- and ENO2-encoded dimeric enolases (40, 49). However, it was not established whether a hetero-oligomeric isoform was formed in vivo (49).

It can be concluded that the retention of paralogous gene pairs can result in the functional diversification of each of the two isoforms, followed by the in vivo formation of hybrid hetero-oligomeric proteins with peculiar kinetic properties that are built up of monomers encoded by each of the genes retained. For the genes whose products are involved in amino acid biosynthesis, the constitution of hetero-oligomers with specific feedback sensitivity can have a crucial function in metabolic homeostasis.

ACKNOWLEDGMENTS

We thank Juan Pablo Pardo for illuminating discussions during this course of work and Rocio Romualdo Martinez for helpful secretarial assistance.

This study was funded by Dirección General de Asuntos del Personal Académico, UNAM, grant IN204412 (http://dgapa.unam.mx); Instituto de Ciencia y Tecnología del Distrito Federal, México, grant PIFUTP08-1654 (http://www.icyt.df.gob.mx), Consejo Nacional de Ciencia y Tecnología grant 152448, and grant NoCRP/MEX10-03 from the International Center for Genetic Engineering and Biotechnology. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. Geovani López had a CONACYT doctoral fellowship, and data in this work are part of his doctoral dissertation in the Posgrado en Ciencias Bioquímicas at the Universidad Nacional Autónoma de México.

This paper is dedicated to Armando Gomez Puyou as a tribute to his lifetime generous dedication to science and teaching.

REFERENCES

1. Beltzer JP, Morris SR, Kohlhaw GB. 1988. Yeast LEU4 encodes mitochondrial and nonmitochondrial forms of alpha-isopropylmalylate synthase. J Biol Chem 263:368–374.
2. Casalone E, Barberio C, Cavaliere D, Polisini M. 2000. Identification of functional analysis of the genes encoding a- and c-isopropylmalylate synthase II (LEU9) in Saccharomyces cerevisiae. Yeast 16:539–545. http://dx.doi.org/10.1002/(SICI)1096-6453(20000416)16:6<539::AID-YEAS37>3.0.CO;2-K.
3. Cavaliere D, Casalone E, Benoni D, Fia G, Polisini M, Barberio C. 1999. Trifluoroleucine resistance and regulation of alpha-isopropylmalylate synthase in yeast. Mol Gen Genet 261:152–160. http://dx.doi.org/10.1007/s004380050952.
4. Chang L, Cuminham TS, Gatzeck PR, Chen W, Kohlhaw GB. 1984. Cloning and characterization of yeast LEU4, one of the two genes responsible for a-isopropylmalylate synthase. Genetics 108:91–106.
5. Chang LF, Gatzeck PR, Kohlhaw GB. 1985. Total deletion of yeast LEU4: further evidence for a second alpha-isopropylmalylate synthase and evidence for tight LEU4-MET4 linkage. Gene 33:333–339. http://dx.doi.org/10.1016/0378-1119(85)90241-0.
6. Kohlhaw GB. 2003. Leucine biosynthesis in fungi: entering metabolism through the back door. Microbiol Mol Biol Rev 67:1–15. http://dx.doi.org/10.1182/MMBR.67.1.1-15.2003.
7. Roeder PR, Kohlhaw GB. 1980. Alpha-isopropylmalylate synthase from yeast. A zinc metalloenzyme. Biochim Biophys Acta 613:482–487. http://dx.doi.org/10.1016/0005-2744(80)90103-5.
8. Ryan E, Tracy JW, Kohlhaw GB. 1973. Subcellular localization of the leucine biosynthetic enzymes in yeast. J Bacteriol 116:222–225.
9. Marobbio CMT, Giannuzzi G, Paradies E, Pierri CL, Palmieri F. 2008. a-Isopropylmalate, a leucine biosynthesis intermediate in yeast, is transported by the mitochondrial oxaloacetate carrier. J Biol Chem 283:28445–28453. http://dx.doi.org/10.1074/jbc.M804637200.
10. Colón M, Hernández F, López K, Quezada H, González J, López G, Aranda C, González A. 2011. Saccharomyces cerevisiae Bat1 and Bat2 aminotransferases have functionally diverged from the ancestral-like Kluyveromyces lactis orthologous enzyme. PLoS One 6:e16099. http://dx.doi.org/10.1371/journal.pone.0016099.
11. Alvers AL, Fishwick LK, Wood MS, Hu D, Chung HS, Dunn WA, Jr, Aris JP. 2009. Autophagy and amino acid homeostasis are required for chronological longevity in Saccharomyces cerevisiae. Aging Cell 8:335–369. http://dx.doi.org/10.1111/j.1474-9726.2009.00469.x.
12. Garay R, Campos SE, González de la Cruz J, Gaspar AP, Jinich A, DeLuna A. 2014. High-resolution profiling of stationary-phase survival reveals yeast longevity factors and their genetic interactions. PLoS Genet 10:e1004168. http://dx.doi.org/10.1371/journal.pgen.1004168.
13. Peters MH, Beltzer JP, Kohlhaw GB. 1990. Expression of the yeast LEU4 gene is subject to four different modes of control. Arch Biochem Biophys 276:294–298. http://dx.doi.org/10.1016/0003-9861(90)90041-V.
14. Ohno S. 1970. Evolution by gene duplication. Springer Verlag. New York, NY.
15. Force A, Lynch M, Pickett FB, Amores A, Yan YL, Postlethwait J. 1999. Preservation of duplicate genes by complementary, degenerative mutations. Genetics 151:1531–1545.
16. Xing RY, Whitman WB. 1991. Characterization of enzymes of the branched chain amino acid biosynthetic pathway in Methanococcus spp. J Bacteriol 173:2086–2092.
17. Bello-M, Birren BW, Lander ES. 2004. Proof and evolutionary analysis of ancient genome duplication in the yeast Saccharomyces cerevisiae. Nature 428:617–624. http://dx.doi.org/10.1038/nature02424.
18. DeLuna A, Avendaño A, Riego L, González A. 2001. NADP-glutamate dehydrogenase isoenzymes of Saccharomyces cerevisiae. Purification, kinetic properties and physiological roles. J Biol Chem 276:43775–43783. http://dx.doi.org/10.1074/jbc.M107986200.
19. Avendaño A, Riego L, DeLuna A, Aranda C, Romero G, Ishaïa C, Vázquez-Acvedo M, Rodarte B, Recillas-Targa F, Valenzuela L, González S, González A. 2005. Swi/SNF-GCN5-dependent chromatin remodelling determines induced expression of GDF13, one of the paralogous genes responsible for ammonium assimilation and glutamate biosynthesis in Saccharomyces cerevisiae. Mol Microbiol 57:291–305. http://dx.doi.org/10.1111/j.1365-2958.2005.04689.x.
20. Quezada H, Aranda C, DeLuna A, Hernández H, Calcagno M, Marin-Hernández A, González A. 2008. Specialization of the paralogue LYS21 determines lysine biosynthesis under respiratory metabolism in Saccharomyces cerevisiae. Microbiology 154:1656 –1667. http://dx.doi.org/10.1099/mic.0.2008/0017103-0.
21. Marques AC, Vinckenbosch N, Brawand D, Kaessmann H. 2008. Functional diversification of duplicate genes through subcellular adaptation of encoded proteins. Genome Biol 9:R54. http://dx.doi.org/10.1186/gb-2008-9-4-r54.
22. Merico A, Sulo P, Piskur J, Compagno C. 2007. Fermentative lifestyle in yeasts belonging to the Saccharomyces complex. FEMS J 274:976–989. http://dx.doi.org/10.1111/j.1469-2668.2007.05645.x.
23. Piskur J. 2001. Origin of the duplicated regions in the yeast genomes. Trends Genet 17:302–303. http://dx.doi.org/10.1016/S0168-9525(01)02308-3.
24. Wach A, Brachat A, Alberti-Segui C, Rebischung O, Philippens P. 1997. Three new dominant drug resistant cassettes for gene disruption in Saccharomyces cerevisiae. Yeast 13:1065–1075.
25. Longtine MS, McKenzie A, Demarini DJ, Shah NG, Wach A, Brachat A, Philippens P, Pringle JR. 1998. Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. Yeast 14:953–966.
26. Sheff MA, Thorn KS. 2004. Optimized cassettes for fluorescent protein
tagging in *Saccharomyces cerevisiae*. Yeast 21:661–670. http://dx.doi.org/10.1002/yea.1130.

27. Tong AH, Lesage G, Bader GD, Ding H, Xu H, Xin X, Young J, Berriz GF, Bro-lk RL, Chang M, Chen Y, Cheng H, Chua G, Friesen H, Goldberg DS, Haynes J, Humphries C, He G, Hussein S, Ke L, Kro-gan N, Li Z, Levinson JN, Lu H, Ménard P, Munyana C, Parsons AB, Ryan O, Tonikian R, Roberts T, Sidic AM, Shapiro J, Sheikh B, Suter B, Wong SL, Zhang LV, Zhu H, Burd CG, Munro S, Sander C, Rine J, Greenblatt J, Peter M, Bretscher A, Bell G, Roth FP, Brown GW, Andrews B, Bussey H, Boone C. 2004. Global mapping of the yeast genetic interaction network. Science 303:808–813. http://dx.doi.org/10.1126/science.1091317.

28. Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

29. Ito H, Fukuda Y, Murata K, Kimura A. 1983. Transformation of intact cells treated with alkali cations. J Bacteriol 153:163–168.

30. Kojima T, Karasawa S, Miyawaki A, Tsumuraya T, Fujii I. 2011. Novel screening system for protein-protein interactions by bimolecular fluorescence. J Biosci Bioeng 111:397–401. http://dx.doi.org/10.1016/j.jbiosc.2010.12.013.

31. Boldogh IR, Pon LA. 1997. Directed protein reassembly: application to the green fluorescent protein. J Biol Chem 272:265–275.

32. DeLuna A, Springer M, Kirschner MW, Kishony R. 2010. Need-based up-regulation of protein levels in response to deletion of their duplicate genes. PLoS Biol 8:e1000347. http://dx.doi.org/10.1371/journal.pbio.1000347.

33. Ghosh I, Hamilton AD, Regan L. 2000. Antiparallel leucine zipper directed protein reassembly: application to the green fluorescent protein. J Am Chem Soc 122:5658–5659. http://dx.doi.org/10.1021/ja994421w.

34. Ali MH, Imperiali B. 2005. Protein oligomerization: how and why. Bioorg Med Chem 13:5013–5020. http://dx.doi.org/10.1016/j.bmc.2005.05.037.

35. Matthews JM, Sunde M. 2012. Dimers, oligomers, everywhere. Adv Exp Med Biol 747:1–18. http://dx.doi.org/10.1007/978-1-4614-3229-6_1.

36. Marananayagam NJ, Sunde M, Matthews JM. 2004. The power of two: protein dimerization in biology. Trends Biochem Sci 29:618–625. http://dx.doi.org/10.1016/j.tibs.2004.09.006.

37. Kerppola TK. 2006. Design and implementation of bimolecular fluorescence complementation (BiFC) assays for the visualization of protein interactions in living cells. Nat Protoc 1:1278–1286. http://dx.doi.org/10.1038/nprot.2006.201.

38. Sung MK, Huh WK. 2007. Bimolecular fluorescence complementation analysis system for in vivo detection of protein-protein interaction in *Saccharomyces cerevisiae*. Yeast 24:767–775. http://dx.doi.org/10.1002/yea.1504.

39. Entian KD, Meurer B, Kohler H, Mann KH, Mecke D. 1987. Studies on the regulation of enolases and compartmentation of cytosolic enzymes in *Saccharomyces cerevisiae*. Biochim Biophys Acta 925:214–221. http://dx.doi.org/10.1016/0304-4157(87)90006-7.

40. Tracy JW, Kohlihaw GB. 1977. Evidence for two distinct CoA binding sites on yeast alpha-isopropylmalate synthase. J Biol Chem 252:4085–4091.

41. Segel IH. 1993. Enzyme kinetics: behavior and analysis of Rapid equilibrium and steady state enzyme systems, p 274–298. John Wiley & Sons, Inc., New York, NY.

42. Zomosa-Signoret V, Martínez-Martínez E, Garza-Ramos G, Pérez-Montfort R, Tuena De Gómez-Puyou M, Gómez-Puyou A. 2011. The Lys20 homocitrate synthase isoform exerts most of the flux control over the lysine synthesis pathway in *Saccharomyces cerevisiae*. Mol Microbiol 82:578–590. http://dx.doi.org/10.1111/j.1365-2958.2011.07832.x.

43. Kerppola TK, Yoshizawa K, Ohsumi Y, Anraku Y. 2007. Design and implementation of bimolecular fluorescence complementation (BiFC) assays for the visualization of protein interactions in living cells. Nat Protoc 1:1278–1286. http://dx.doi.org/10.1038/nprot.2006.201.

44. McAlister I, Holland MJ. 1982. Targeted deletion of a yeast enolase structural gene. Identification and isolation of yeast enolase isoymes. J Biol Chem 122:7181–7188.