Paralogous ALT1 and ALT2 Retention and Diversification Have Generated Catalytically Active and Inactive Aminotransferases in Saccharomyces cerevisiae

Georgina Peñalosa-Ruiz1, Cristina Aranda1, Laura Ongay-Larios2, Maritini Colon1, Hector Quezada3, Alicia Gonzalez1*

1 Departamento de Bioquímica y Biología Estructural, División de Ciencia Básica, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, México City, México, 2 Unidad de Biología Molecular, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, México City, México, 3 Departamento de Bioquímica, Instituto Nacional de Cardiología, México City, México

Abstract

Background: Gene duplication and the subsequent divergence of paralogous pairs play a central role in the evolution of novel gene functions. S. cerevisiae possesses two paralogous genes (ALT1/ALT2) which presumably encode alanine aminotransferases. It has been previously shown that Alt1 encodes an alanine aminotransferase, involved in alanine metabolism; however the physiological role of Alt2 is not known. Here we investigate whether ALT2 encodes an active alanine aminotransferase.

Principal Findings: Our results show that although ALT1 and ALT2 encode 65% identical proteins, only Alt1 displays alanine aminotransferase activity; in contrast ALT2 encodes a catalytically inert protein. ALT1 and ALT2 expression is modulated by Nrg1 and by the intracellular alanine pool. ALT1 is alanine-induced showing a regulatory profile of a gene encoding an enzyme involved in amino acid catabolism, in agreement with the fact that Alt1 is the sole pathway for alanine catabolism present in S. cerevisiae. Conversely, ALT2 expression is alanine-repressed, indicating a role in alanine biosynthesis, although the encoded-protein has no alanine aminotransferase enzymatic activity. In the ancestral-like yeast L. kluyveri, the alanine aminotransferase activity was higher in the presence of alanine than in the presence of ammonium, suggesting that as for ALT1, lkALT1 expression could be alanine-induced. ALT2 retention poses the questions of whether the encoded protein plays a particular function, and if this function was present in the ancestral gene. It could be hypothesized that ALT2 diverged after duplication, through neo-functionalization or that ALT2 function was present in the ancestral gene, with a yet undiscovered function.

Conclusions: ALT1 and ALT2 divergence has resulted in delegation of alanine aminotransferase activity to Alt1. These genes display opposed regulatory profiles: ALT1 is alanine-induced, while ALT2 is alanine repressed. Both genes are negatively regulated by the Nrg1 repressor. Presented results indicate that alanine could act as ALT2 Nrg1-co-repressor.

Introduction

Alanine aminotransferases (ALTs) are pyridoxal phosphate-dependent enzymes, which catalyze the reversible transamination between alanine and 2-oxoglutarate to form pyruvate and glutamate (Figure 1). The presence of at least two alanine aminotransferase isozymes is widely distributed in animals, plants, yeasts and bacteria. In these systems, ALT isozymes have been considered to constitute the main pathway leading to alanine biosynthesis and catabolism [1–6]. Alanine catabolic and anabolic pathways are central in nitrogen and carbon metabolic networks. During growth under glucose-restricted conditions, alanine catabolism constitutes a key pathway for gluconeogenesis, since pyruvate can be readily converted to oxaloacetate through the action of pyruvate carboxylase, leading to glucose production [7]. In addition, it has been shown that in Arabidopsis, pyruvate anaerobic fermentation constitutes a metabolic mechanism that allows adaptation to low oxygen conditions. Under hypoxia/anoxia, pyruvate fermentation determines the synthesis of lactate, ethanol and acetaldehyde, thus favoring the regeneration of NAD+ from NADH. Energy shortage provoked by lower ATP yield due to the inactivation of oxidative phosphorylation is relieved by increasing NAD+ production and glycolytic rate [8–10]. Among other mechanisms that confer tolerance to hypoxia or anoxia, Medicago truncatula seedlings limit the accumulation of lactate to preserve the pH balance. In this regulatory circuit, alanine...
biosynthesis plays a crucial role, since it restricts the utilization of pyruvate to form lactate or ethanol, preventing acidification by lactate and shortage in carbon availability [11–12].

Differences in structure, kinetic parameters, tissue and subcellular distribution and gene regulation of ALT isozymes suggest that the two isoforms could have opposed tendencies for either alanine or pyruvate synthesis in mammals [4]. In fact, it has been found that mitochondrial ALT isozyme (m-ALT) expressed in gluconeogenic tissues, like liver and kidney, is involved in alanine catabolism. In glucogenic tissues like skeletal and cardiac muscle, ALT is cytosolic (c-ALT) and participates in alanine biosynthesis [13]. During starvation c-ALT synthesizes alanine as a consequence of amino acid degradation in glucogenic tissues. It is also known that alanine travels through the bloodstream and in the liver it is transaminated by m-ALT to yield pyruvate, which is converted to glucose [7].

No orthologous counterparts of the bacterial alanine biosynthetic enzymes, alanine dehydrogenase (AlaD) [14–15] or aspartate-4-decarboxylase (AspD) [16] have been found in fungal systems; it was thus considered that in fungi the main biosynthetic pathway leading to alanine production would be constituted by ALT isozymes. Since the action of ALTS is reversible and no other alanine catabolic enzyme has been found in yeast, it was assumed that ALTS would constitute the sole pathway for alanine catabolism. Purification and biochemical characterization of ALTS from Candida maltosa showed that this enzyme has high affinity towards alanine, suggesting that it could play a catabolic function [5]; its role in alanine biosynthesis has not been determined.

In S. cerevisiae, two paralogous genes ALT1 and ALT2, have been found and it was proposed that these genes encode alanine aminotransferases determining alanine biosynthesis and catabolism. This paralogous pair forms part of a duplicated chromosomal block generated from the Whole Genome Duplication (WGD) event [17,18] (http://www.gen.tcd.ie/~kwolfe/yeast/nova/index.html). An additional inspection using the Yeast Gene Order Browser (http://wolfe.gen.tcd.ie/ygob/) [19] also suggests that ALT1/ALT2 are in a duplicated block. This evidence points to the origin of the ALT1-ALT2 duplicated gene pair as part of the WGD duplication event rather than to an isolated gene duplication phenomenon.

Previous results from our laboratory showed that simultaneous ALT1 and ALT2 impairment did not result in alanine auxotrophy in media containing glucose as sole carbon source, indicating the
existence of an alternative pathway for alanine biosynthesis [20]. Although these results do not discard the possibility that Alt1 and Alt2 could play a role in alanine biosynthesis, the existence of at least one additional pathway must be surmised. Accordingly it was proposed that the pathway could be afforded by glutamine-pyruvate aminotransferase. This enzyme forms part of the α-amidase pathway [21–25], which has been considered to play a biosynthetic role; it cannot provide a catalytic pathway, since the reaction catalyzed by glutamine aminotransferase is irreversible (Figure 1) [24,25]. In regard to the role of Alt1 and Alt2 in alanine catabolism, it was found that alt1Δ mutants do not grow on alanine as sole nitrogen source. Because alt2Δ mutants have no evident phenotype, it was concluded that Alt1 displayed both biosynthetic and catabolic capacities. Accordingly, ALT1 expression was found to be alanine-induced, again suggesting a catabolic character. It is noted that neither ALT2 expression, nor enzymatic activity was detected under the conditions tested [20].

Analysis of metabolic flux in fourteen hemiascomycetous yeasts revealed that most species that diverged after the genome duplication contain both ALT isoforms. As mentioned earlier, both biosynthetic and catabolic roles have been assigned to Alt1, whereas the physiological role of Alt2 has not been ascertained. Earlier divergent species contained a unique alanine aminotransferase, showing high identity with ALT1 of S. cerevisiae [26].

This paper addresses the questions of whether ALT2 encodes an active alanine aminotransferase, and whether Alt1 and Alt2 have diversified their in vivo physiological role. Our results indicate that: i) the ALT2 encoded enzyme does not bear pyruvate-alanine aminotransferase activity. Its retention suggests it could have an alternative function, ii) that ALT1 and ALT2 gene expression profiles have diversified, that iii) as expected for earlier yeast divergent species, kAlt1 has an enzymatic activity profile similar to that observed for Alt1, and iv) that ALT1 and ALT2 gene expression is regulated through differential Nrg1-dependent repression.

Results

Alt1 Constitutes the Main Pathway for Alanine Biosynthesis

Previous work from our laboratory indicated that when glucose is present as sole carbon source, an ALT1-ALT2 independent pathway plays an important role in alanine biosynthesis, since double alt1Δ alt2Δ mutants show wild type growth rate [20]. To analyze Alt1 and/or Alt2 contribution to alanine biosynthesis, single and double mutants were grown on ammonium-glucose with or without alanine (Figure 2A and 2B). It was found that in both conditions, the wild type strain and alt2Δ mutant showed equivalent growth rates, indicating alanine prototrophy. However, the alt1Δ mutant showed a lower growth rate on glucose ammonium than that displayed by the wild type strain (0.31 ± 0.36 h⁻¹; Figure 2C). The alt1Δ alt2Δ double mutant showed lower growth rate than the wild type strain (0.28 ± 0.36 h⁻¹) and than the alt1Δ single mutant (0.28 ± 0.31 h⁻¹; Figure 2C). Single and double mutants grew as well as the wild type strain in the presence of alanine, indicating that alt1Δ and double alt1Δ alt2Δ mutant require alanine to achieve wild type phenotype. Growth rates between the wild type strain and the single alt1Δ mutant, and between the single alt1Δ and double mutant strains were significantly different (P value <0.01). These results suggest that both, Alt1 and Alt2 participate in alanine production.

Alanine pools were determined in the wild type, single mutant and double mutant strains. During early exponential and late exponential growth phases (OD₆₀₀ 0.3, 0.6 and 0.9) Alt1 contributed 75–60% to the alanine pool, whereas the Alt1-Alt2 independent pathway(s) provided only 25–40%. Alt2 contribution seems negligible because no difference in the alanine pool was observed between the WT and the alt2Δ strain. The absence of ALT1 (single alt1Δ and double alt1Δ alt2Δ) evidenced the contribution of the Alt1-Alt2 independent pathway(s). During stationary phase (OD₆₀₀ 2.0) Alt1 afforded 82%, while the Alt1-Alt2-independent pathway(s) contributed 6%. Only under this condition, Alt2 contribution (12%) became significant, since alt1Δ mutant showed higher alanine pool than the double alt1Δ alt2Δ mutant; the positive role of Alt2 was only observed in an alt1Δ genetic background (Figure 2D). The deletion of an enzyme-encoding gene may have other effects on metabolic pathways besides the reduction in the corresponding enzymatic activity [27]. Nevertheless, in the alt1Δ mutant, the alanine pool decreased 30% as compared with the wild type strain, indicating that Alt1 plays an important role in the building up of the alanine reservoir. Moreover, the fact that single alt2Δ mutants showed a growth rate and an alanine pool equivalent to that found in the wild type strain, indicates null Alt2 contribution in an otherwise wild type strain.

ALT1 and ALT2 are Differentially Expressed Under Biosynthetic or Catabolic Conditions

Northern blot analyses were carried out in samples obtained throughout different growth phases attained on glucose-ammonium (Figure 3A). ALT1 expression reached its highest peak during late exponential phase (OD₆₀₀ 0.9) which was followed by a decrease in expression during stationary phase. The increase in ALT1 expression is consistent with the higher contribution of Alt1 to the alanine pool during stationary phase OD₆₀₀ 2.0 (Figure 2D). Conversely, low expression of ALT1 observed at OD₆₀₀ 0.3 and 0.6 reflects the low Alt1 contribution to the alanine pool at OD₆₀₀ 0.9 (Figure 2D). ALT1 and ALT2 expression patterns on glucose-ammonium (Figure 3A) suggest that the endogenous alanine pool could simultaneously determine ALT1 induction and ALT2 repression. ALT2 expression was only observed during early exponential phase, indicating that Alt2 could contribute to alanine biosynthesis during early exponential growth; however, alt2Δ mutant shows alanine pools similar to those of the wild type strain.

ALT1 and ALT2 expression was monitored on samples obtained from glucose-alanine grown cultures (Figure 3B). ALT1 mRNA induction was observed throughout the exponential phase. In the pre-stationary and stationary phases relatively low ALT1 levels were detected. In contrast, ALT2 expression was repressed throughout the different growth phases, suggesting Alt2 role in alanine biosynthesis. These results confirm previous observations indicating that ALT1 expression pattern is characteristic of a gene encoding a catabolic enzyme [20]. Accordingly, an alt1Δ ALT2 mutant is unable to grow on alanine as sole nitrogen source [20], showing that Alt2 cannot complement Alt1 catabolic role. To further analyze this issue, cultures were grown on 7 mM proline as sole nitrogen source to mid-exponential phase; the culture was then divided: half of it was allowed to continue growth on proline, 7 mM alanine was added to the rest of the culture. Aliquots for RNA extraction were taken at different OD₆₀₀ nm and Northern analysis was performed. Figure 4A shows that after alanine addition, ALT1 expression increased, while ALT2 was repressed, indicating that alanine influences ALT1 and ALT2 expression profile. Furthermore, when Northern analysis was performed on total RNA samples obtained from cultures grown on two different alanine concentrations, ALT1-dependent induction was transiently observed when 3 mM alanine was added (OD₆₀₀ nm 0.3–0.6).
However, when 7 mM alanine was present in the medium, \( ALT1 \) induced expression was observed for a longer period (OD\(_{600}\) nm 0.3–0.9; Figure 4B), suggesting that differential alanine exhaustion from the media affected \( ALT1 \) induction. As expected, \( ALT2 \) expression showed a reciprocal behavior (Figure 4B).

Alt1 and Alt2 Protein Concentration is Consistent with \( ALT1 \) and \( ALT2 \) Expression Profile

Immunoblots were carried out with \( ALT1\)-TAP and \( ALT2\)-TAP derivatives as described in methods. Both, Alt1 and Alt2 protein concentrations were consistent with mRNA expression profiles. In glucose-ammonium cultures, increased \( ALT1 \) transcription resulted in higher Alt1 concentration, whereas Alt2 was only detected during the early exponential growth phase (Figure 3C and 3D). As expected, in extracts obtained from glucose-alanine cultures, Alt2 was not detected, whereas Alt1 was present throughout the various growth phases (Figure 3E and 3F). Figure S1A and S1B shows that \( ALT1\)-TAP and \( ALT2\)-TAP displayed a similar expression profile to that observed in the CLA1 strain collection, shown in Figure 3A and B.

Nrg1 Determines \( ALT1 \) and \( ALT2 \) Expression Repression

The overall results indicate \( ALT1 \) and \( ALT2 \) display differential expression patterns on glucose with either ammonium or alanine as nitrogen sources: alanine induces \( ALT1 \) expression and represses that of \( ALT2 \). Additionally it was found that \( ALT1 \) expression is repressed during pre-stationary and stationary growth phases.

Multiple sequence alignment of \( ALT1 \) and \( ALT2 \) promoters present in four \textit{Saccharomyces} sensu stricto, identified a consensus binding sequence for the \( NRG1 \)-encoded repressor in each promoter (Figures S2, S3 and S4). To examine if Nrg1 was involved in repression, \( nrg1\Delta \) mutants were constructed and Northern blot analysis was carried out. It was found that both, \( ALT1 \) and \( ALT2 \) expression was Nrg1-repressed (Figure 5A and 5B). To determine Nrg1 occupancy of the \( ALT1 \) and \( ALT2 \) promoters, chromatin immunoprecipitation (ChIP) was performed in extracts obtained from glucose-alanine grown cultures. Nrg1 was bound to the \( ALT1 \) promoter at OD\(_{600}\)nm 0.3, when expression was induced, and at OD\(_{600}\)nm 1.8 when repression was observed; this suggests that Nrg1 is permanently bound to the \( ALT1 \) promoter. Nrg1 was bound to the \( ALT2 \) promoter in both exponential and stationary growth phases (Figure 5C and 5D).
Phenotypic analysis of null nrg1D mutants showed that on glucose-ammonium, these strains displayed a lower growth rate, compared with the NRG1 wild type strain (0.11 vs 0.20 h^{-1}). The effect on growth rate observed in the nrg1D mutants cannot be solely attributed to changes in ALT1 and ALT2 expression, since Nrg1 is a global transcriptional modulator which mediates glucose repression and negatively regulates a variety of processes [28]. On glucose-alanine, the wild type strain showed a growth rate of

Figure 3. ALT1 and ALT2 display differential gene expression pattern, which is consistent with Alt1-Alt2 intracellular concentration. Northern blot of total RNA prepared from wild type strain grown on either glucose-ammonium (A) or glucose-alanine (B). Samples were collected from various OD_{600} as stated. Representative results from three experiments are shown. Cell-free extracts from ALT1-TAP (C and E) or ALT2-TAP (D and F) tagged strains grown on glucose-ammonium (C and D) or glucose-alanine (E and F) were prepared and subjected to immunoblot analysis using anti-TAP polyclonal antibody. As a loading control, each nitrocellulose membrane was also subjected to immunoblot analysis using anti-Lys20/Lys21 antibody. All lanes were loaded with 100 μg of protein. An ALT1-TAP sample was included as a loading control in panel F. Results are representative of three independent experiments.

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Figure 4. Alanine determines ALT1 expression induction and ALT2 expression repression. Northern blot of total RNA obtained from wild type strains grown on 7 mM proline with or without alanine (A) or on 3 mM or 7 mM alanine (B), as nitrogen sources.

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0.15 h⁻¹, while the ng1Δ mutant strain had a growth rate of 0.05 h⁻¹. The single and double alt1Δ and alt1Δ ng1Δ mutants were unable to grow on alanine as sole nitrogen source, showing that even under derepressive conditions, Alt2 was unable to complement the lack of Alt1 (data not shown).

**ALT2-encoded Protein does not Display Alanine Aminotransferase Activity**

To increase ALT2 expression, ALT2 promoter was substituted by the bacterial TET promoter in the R1158-3 alt1Δ ALT2 background, generating alt1Δ tet07-ALT2 strain. Figure 6A shows that when RNA was prepared from cultures grown on glucose-ammonium, transcription from the TET promoter increased ALT2 expression. C-tagged strains ALT1 tetO7- ALT2-yECitrine and ALT1- ALT2- yECitrine were constructed; Alt2 was immunodetected in both strains. As expected, Alt2 concentration was higher in the tetO7- ALT2- yECitrine mutant than in the ALT1- ALT2- yECitrine control strain, thus indicating that a relatively high ALT2 expression was accompanied by higher protein synthesis (Figure 6B and 6C).

Alt2 enzymatic activity was assayed in alt1Δ tet07-ALT2, and alt1Δ ng1Δ extracts of cultures grown on ammonium-glucose to an OD₆₀₀nm of 1.0. No activity was detected, suggesting that Alt2 was completely devoid of alanine aminotransferase enzymatic capacity. Furthermore alt1Δ tet07-ALT2 did not grow on glucose alanine, showing that ALT2-encoded protein was unable to complement Alt1 lack. Background activity values from alt1Δ mutants (Table 1), cannot be attributed to alanine aminotransferase, since the observed activity is practically identical, regardless of whether it was assayed in a strain displaying wild type ALT2 levels (alt1Δ ALT2 0.019 μmol/min⁻¹ mg of protein⁻¹) or an ALT2 overexpressing mutant (alt1Δ tet07-ALT2 0.022 μmol/min⁻¹ mg of protein⁻¹). During stationary phase (OD₆₀₀nm 2.0), and in the absence of Alt1, Alt2 apparently exerted a small positive effect over alanine intracellular pool (Figure 2D). The fact that an alt1Δ alt2Δ mutant showed a lower alanine pool than that of the alt1Δ strain indicates that Alt2 could play a role as a positive modulator of the enzyme(s) contributing the alternative pathway(s) of alanine biosynthesis. However a direct role of Alt2 in alanine biosynthesis can be excluded.

A phylogenetic study of ALT1-ALT2 in pre- and post-WGD yeasts, showed that the ancestral like yeasts (L. kluyveri, Kluyveromyces lactis and Kluyveromyces waltii) constitute a group which originated both ALT1 and ALT2, supporting the WGD origin of these genes. Figure 7 shows that ALT2 and Saccharomyces "sensu strico" orthologues form a subtree with longer branches than ALT1 orthologues, which are clustered in a different subtree. This result suggests that Alt2 has diverged as consequence of a high
substitution rate whereas Alt1 has undergone few modifications.

To further analyze this possibility, alanine aminotransferase activity was determined in extracts obtained from cultures of the *L. kluyveri* wild type strain grown on glucose + ammonium or on glucose + ammonium + alanine. It was found that, as well as for the *S. cerevisiae* ALT1-encoded isozyme [20], LkAlt1 alanine aminotransferase specific activity increased almost ten-fold in the presence of alanine (0.450 ± 0.007 μmol/min mg of protein), compared with the activity found on ammonium (0.049 ± 0.009 μmol/min mg of protein). These results indicate that the single orthologue present in *L. kluyveri* encodes an active alanine aminotransferase with an activity profile similar to that observed for Alt1. ALT1 and ALT2 have diverged, and since ALT2 branch is longer it could be considered that the encoded-enzyme has evolved a new function (Figure 7) [29–31]. However a physiological analysis would be necessary to test this possibility.

To determine whether loss of Alt2 aminotransferase activity could be due to mutations in catalytic amino acids, a multiple alignment of the Alt proteins from *S. cerevisiae* and post WGD yeasts, was performed. It was found that the catalytic residues...
involved in cofactor and substrate binding have been conserved in all cases [32]. (Figure S5). These results suggest that lack of Alt2 activity could be due to mutations affecting oligomeric organization or other amino acids playing a critical role in enzyme structure and catalytic capacity.

**Discussion**

This paper addresses the roles of Alt1 and Alt2 in alanine metabolism in *S. cerevisiae*. The results show i) that in glucose-grown cells, Alt1 is instrumental in alanine catabolism, and contributes from 60 to 80% of the alanine intracellular pool and ii) that Alt2 does not display alanine aminotransferase activity and does not complement Alt1 lack. These data indicate that the biosynthetic and catabolic capacity displayed by alanine aminotransferases has been delegated to Alt1, and that *ALT2*-encoded protein does not participate in alanine metabolism, thus explaining Alt2 incapacity to complement Alt1 lack. The existence of a strictly biosynthetic pathway would have left *ALT2* free to evolve new functions, while *ALT1* retention would be favored, since it constitutes the sole pathway for alanine catabolism. It has been proposed that alanine biosynthesis could be achieved through the action of the glutamine aminotransferase, an irreversible pathway, unable to participate in alanine catabolism [20,24,25] (Figure 1).

Nrg1 Determines Negative *ALT1* and *ALT2* Transcriptional Regulation

*ALT2* expression is repressed in the presence of alanine, suggesting that this amino acid could act as Nrg1 co-repressor. After alanine exhaustion, Nrg1 determines *ALT1* repression, suggesting that for this gene, the co-repressor could be a product

![Figure 7. Evolutionary relationships of alanine aminotransferases present in post-WGD yeasts.](https://www.plosone.org/figure/Evolutionary-relationships-of-alanine-aminotransferases-present-in-post-WGD-yeasts.288539e822.png)

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**Table 1.** Alanine aminotransferase specific activity in extracts prepared from glucose-NH₄ grown cultures.

| Strains                  | Specific activity |
|--------------------------|-------------------|
| CLA1-2 (ALT1 ALT2)       | 0.104±0.030       |
| CLA1-2-1 (alt1Δ ALT2)    | 0.011±0.003       |
| CLA1-2-2 (ALT1 alt2Δ)    | 0.117±0.030       |
| CLA1-2-D (alt1Δ-alt2Δ)   | 0.023±0.000       |
| CLA 603 (alt1Δ-ALT2 nrg1Δ) | 0.015±0.004     |
| R1158 (ALT1 ALT2)        | 0.130±0.060       |
| R1158-3 (alt1Δ ALT2)     | 0.019±0.008       |
| R1158-4 (ALT1 tetO-ALT2) | 0.110±0.040       |
| R1158-5 (alt1Δ tetO-ALT2)| 0.022±0.003       |

Specific activity is expressed as μmol/min⁻¹ mg of protein⁻¹. Values are presented as mean from at least three measurements ± S. D. doi:10.1371/journal.pone.0045702.t001
of alanine catabolism. This system could function as a genetic switch, ensuring that gene activity is turned off when the end product is available. The negative regulation of the genes involved in alanine metabolism could be determined by Nrg1-containing complexes, which could alternatively determine repression of either ALT1 or ALT2 depending on the intracellular content of either alanine or a product of its catabolism. In fact, arginine metabolism constitutes an example in which small substrate molecules serve as signals directly influencing transcription [33]. In the presence of arginine, the ArgII-Argl-Mcm1 heteromeric complex binds to its cognate site in the pertinent promoters repressing expression of biosynthetic genes, and preventing binding of the CARGR repressor, thus allowing expression of CargA and CargB catabolic genes [34]. This system facilitates biosynthesis repression and catabolism induction in the presence of arginine.

Since Alt1 has retained both the biosynthetic and catabolic character of alanine aminotransferase, it could be suspected that Alt1 could catalyze futile cycles. However the fact that ALT1 expression is activated in the presence of alanine and repressed after alanine consumption indicates that the combined action of induction-repression mechanisms could contribute to the avoidance of such cycles. For example, in the case of the Bat1 and Bat2 branched chain aminotransferases, functional divergence through differential gene expression, has resulted in the asymmetric distribution of the biosynthetic and catabolic character between Bat1 and Bat2 [35]. The independent regulation of each gene, selectively determines the presence of the pertinent isozymes under either biosynthetic or catabolic conditions [35].

Why has ALT2 been Retained?

Although Alt2 shows no alanine aminotransferase activity, its retention as well as its characteristic expression profile, suggest that Alt2 could have originally played a biosynthetic role. This could have been lost as consequence of mutation accumulation in the coding region; however it is noteworthy that the promoter has retained the capacity to respond to alanine concentration. Hence Alt2 could be a monitor of alanine biosynthesis by modulating other gene products. Alternatively, it could be considered that ALT2 plays an additional physiological role not related to alanine metabolism, as it has been shown for the S. cerevisiae Lys20 paralogous enzyme. In addition to the role in lysine biosynthesis through homocitrate synthase activity (HCS) [36] Lys20 is also involved in the process of DNA damage repair [37]. Whether Lys20 paralogous couple (Lys21) is also a bifunctional protein remains to be established. Nonetheless the case of Lys20-Lys21 illustrates that retention of bifunctional duplicated genes could involve diversification of the two functions carried out by the paralogous proteins. Alt2 forms synthetic lethals with various and unique partners [38,39] which suggest that it could have alternative roles not related to that of alanine aminotransferase [38,39]. It could also be considered that some of ALT2 unique interactors (UBP15) could form part of a regulatory circuit influencing alanine metabolism or a more general adaptation response [40]. In this regard, the fact that ALT2 expression is determined through an Nrg-dependent regulation may indicate that the encoded products could play a role in the adaptation to environmental stress [41].

Concluding Remarks

Retained duplicate genes either provide an increased dosage of the same product; or they undergo a process of subfunctionalization or neofunctionalization, during which both copies of the gene loose a subset of their ancestral functions, and acquire new properties. In previous studies, we showed that subfunctionalization led to the retention of the genes encoding NADPH glutamate dehydrogenase (GDH1-GDH3), and homocitrate synthase (LDH20-LDH21). In both cases, subfunctionalization resulted in the biochemical specialization of the encoded enzymes that led to balanced α-ketoacid utilization during growth on non-fermentable carbon sources, and the acquisition of facultative metabolism.

Materials and Methods

Strains

Table 2 describes the characteristics of the strains used in the present work. Construction of strain CLA1-2 (ura3 leu2::LEU2) and its alt1Δ and alt2Δ derivatives has been previously described [20]. To obtain CLA1-2-1, CLA603 and R1158 alt1Δ derivatives, the pertinent strains were transformed with the 2612 bp PCR product containing the kanMX4 cassette and ALT1 upstream (756 bp) and downstream (266 bp) nucleotide sequences amplified from the genomic DNA of CLA1-2 strain, using deoxyoligonucleotides ALT1F and ALT1R (Table 3). The isogenic CLA1-602 nag1Δ:kanMX was obtained from strain CLA1 by gene replacement [45]. A PCR-generated kanMX4 module was prepared with plasmid pFA6a [43], using oligonucleotides NRG1F and NRG1R (Table 3).

Strain R1158 was obtained from Open Biosystems. R1158-1 derivative, ALT2-yECitrine was constructed by transforming strain R1158 with the yEGCitrine::HIS5 cassette at ALT2 C-terminus, generating strain R1158-1 (ALT2 ALT2-yECitrine::HIS5). A pair of deoxyoligonucleotides (ALT2F1 and ALT2R1) was designed based on the ALT2 carboxy-terminal coding sequence, and that of the pKT175 yECitrine::HIS5 multiple
Table 2. Strains used in this study.

| Strain          | Relevant genotype                                                                 | Source       |
|-----------------|-----------------------------------------------------------------------------------|--------------|
| CLA1-2          | MATs ALT1 ALT2 ura3 leu3::LEU2                                                    | [36]         |
| CLA1-2-1        | MATs alt1△:kanMX4 ALT2 ura3 leu2::LEU2                                            | [20]         |
| CLA1-2-2        | MATs ALT1 alt2△:kanMX4 ura3 leu3::LEU2                                            | [20]         |
| CLA1-2-D        | MATs alt1△:kanMX4 ALT2::natMX ura3 leu2::LEU2                                    | [20]         |
| CLA602-1        | MATs ALT1 ALT2 ura3 leu3 nagI△:natMX4                                             | This study   |
| CLA603-1        | MATs ALT2 ura3 leu3 alt1△ kan nagI△:natMX4                                        | This study   |
| CLA1            | MATs ura3 leu2                                                                    | [36]         |
| R1158-ALT1+ALT2 | MATa his3-1 leu2-0 met15-0 URA3::CMV-tTA ALT1 ALT2                               | Open Biosystems |
| R1158-1 ALT1+ALT2+yECitrine | MATa his3-1 leu2-0 met15-0 URA3::CMV-tTA ALT1+ALT2-yECitrine::HIS5 | This study   |
| R1158-2 ALT1 tetO7+ALT2+yECitrine | MATa his3-1 leu2-0 met15-0 URA3::CMV-tTA ALT1 tetO7::kanMX4+ALT2-yECitrine::HIS5 | This study   |
| R1158-3 ALT1+ALT2 | MATa his3-1 leu2-0 met15-0 URA3::CMV-tTA ALT1△:kanMX4 ALT2                      | This study   |
| R1158-4 ALT1+ALT2 | MATa his3-1 leu2-0 met15-0 URA3::CMV-tTA ALT1△:kanMX4 ALT2          | This study   |
| R1158-5 ALT1+ALT2 | MATa his3-1 leu2-0 met15-0 URA3::CMV-tTA ALT1△:kanMX4 tetO7::natMX+ALT2       | This study   |
| L. kluyveri GRY1175 | MATa ura3                                                                 | [49]         |

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cloning site (Table 3). To construct strain R1158-2 tetO7::kanMX4-ALT2-yECitrine, the chromosomal endogenous ALT2 promoter of strain R1158-1 was replaced with a TET-titratable promoter (tetO7); the tetO7 module was amplified from plasmid pCM325, which contains KanR as selectable marker [46]. A pair of deoxyoligonucleotides (ALT2-F2 and ALT2-R2) was designed based on the ALT2 promoter sequence and that of the pCM325 multiple cloning site (Table 3). 2.2 Kbp PCR fragments were transformed into the R1158-1 yeast strain as previously reported [47]. The resulting strain tetO7::kanMX4-ALT2 was transformed with the yECitrine::HIS5 cassette, generating the tetO7::kanMX4-ALT2-yECitrine::HIS5 strain. The yECitrine cassette was amplified from plasmid pKT175-HIS5. The selection marker URA3 from pKT175 was replaced by HIS5 from the pKT101 plasmid. Correct module integration was PCR and sequencing confirmed.

Strain R1158-3 (alt1△ ALT2) was constructed by transforming strain R1158 with the 2612 bp PCR product containing the kanMX4 cassette and ALT1 upstream (756 bp) and downstream (263 bp) nucleotide sequences amplified from the genomic DNA of the R1158 strain, using deoxyoligonucleotides ALT1F and ALT1R (Table 3). Three sets of deoxyoligonucleotides were used to PCR-amplify fragments that were used as probes for sequential hybridization of ALT1 (1410 bp), ALT2 (1335 bp) and SCR1 (155 bp; Table 3). Blots were scanned using the program ImageQuant 5.2 Molecular Dynamics.

Gel Electrophoresis and Immunoblotting
Protein extracts obtained from R1158-1 ALT1 ALT2-yECitrine and R1158-2 ALT1 tetO7ALT2-yECitrine ALT1-TAP, ALT2-TAP were subjected to SDS-PAGE on 10% slab gels and transferred to nitrocellulose membranes. Incubation with antibodies was carried out as described previously [51]. For the TAP-tagged strains, anti-TAP rabbit polyclonal antibody (CAB 1001; OPEN Biosystems) was used at a 1:5,000 dilution. Anti-Rabbit HRP antibody (Santa Cruz Biotechnology Inc.) was diluted 1:10,000. For the yECitrine-tagged strains, anti-GFP mouse Roche monoclonal antibody (Cat. No. 11 814 460 001) was used at a 1:5,000 dilution. Peroxidase-conjugated anti-mouse antibodies were diluted 1:10,000. Immunoblot signaling was optimized by analyzing a number of combinations of antigen and antibody concentrations in the linear range of detectability. As a loading control all membranes were

Growth Conditions
Strains were routinely grown on MM containing salts, trace elements and vitamins following the formula of yeast nitrogen base (Difco). Filter sterilized glucose (2% w/v) was used as carbon source, and 40 mM ammonium sulphate or 0.1% (w/v) alanine were used as the nitrogen source. Amino acids needed to satisfy auxotrophic requirements were added at 0.01% (w/v). Cells were incubated at 30°C with shaking (250 r.p.m.).

L. kluyveri strains were grown in SC medium (containing 0.67% (w/v) yeast nitrogen base without amino acids and nitrogen source; Difco) and 2% (w/v) glucose) with 0.2% (w/v) NH2SO4 (SC+ammonium) or 0.1% (w/v) L-alanine (SC+alanine) as nitrogen sources. Uracile (20 mg/l) was added when required.

Northern Blot Analysis
Northern analysis was carried out as previously described [50]. Total yeast RNA was prepared from 100 ml aliquots of cultures with the stated OD600 of MM with 40 mM (NH4)2SO4 or 0.1% alanine as nitrogen source. Amino acids needed to satisfy auxotrophic requirements were added at 0.01% (w/v). Cells were incubated at 30°C with shaking (250 r.p.m.).
immunoblotted using a 1:7,500 dilution of mouse anti-Lys20/ Lys21 antibody. Anti mouse HRP-conjugated antibody was diluted 1:12,500.

Chromatin Immunoprecipitation (ChIP)

Formaldehyde cross-linking and immunoprecipitations were carried out by the procedure described by Hecht et al. (1995) [52]. Yeast cells (150 ml of OD_{600} 0.3 and 2.0 from glucose-alanine grown cultures) were incubated on ice for 15 min and cross-linked with 1% formaldehyde for 60 min at room temperature. After addition of 125 mM glycine and incubation for 5 min with gentle agitation, cells were harvested and washed with saline Tris-buffer. Pelleted cells were suspended in lysis buffer (140 mM NaCl, 1 mM EDTA, 50 mM HEPES/KPH, 1% Triton X-100, 0.1% sodium deoxycholate) and incubated with proteinase K (Roche) followed by phenol/chloroform/isoamyl alcohol extraction, precipitated and suspended in 1 X TE. The primer sets used for PCR analysis are listed in Table 3.

### Table 3. Oligonucleotides used for strain construction.

| Primer | Oligonucleotide sequence | Application |
|--------|--------------------------|-------------|
| ALT1F  | 5'-TCTTGCACGTTGCGATCTCTCG-3' | ALT1 null mutant |
| ALT1R  | 5'-GATACGGTGGGTGCTCAAGACG-3' | ALT1 null mutant |
| NRG1F  | 5'-ATGTTTACAACCTATAACTAATGATG-3' | NRG1 null mutant |
| NRG1R  | 5'-TTATGTGCTCCTTTTCAATGTGTTGTCAGAACGATTATCATGATG-3' | NRG1 null mutant |
| ALT2F1 | 5'-TTCGAGTGGAGAAAGTTCTCAATGAGATTTCCACACACGTCTTTCTG-3' | ALT2-yeCitrine |
| ALT2R1 | 5'-ATTAGTCATAAAATAGGAAAGGCTATACATACATTGTTGGTGTGTCATTGT-3' | ALT2-yeCitrine |
| ALT2F2 | 5'-CTATGTGGTTCTACTAATGCTGCTTGCGGTGCTGCAACGTGCTCTCACTGATTAGCAG-GG-3' | tetO7ALT2 |
| ALT2R2 | 5'-AAATCTCCITTGGTGACAACACCCCTTTCAATCTCTTGTTGCTGATGTCCTCGTACTGACGCTATTG-3' | tetO7ALT2 |
| NRG1F  | 5'-GATAATTGCTGTCACATTAGAAGACATATTAGCAGA-3' | NRG1-myc^{23} |
| NRG1R  | 5'-ATAGTTGACCTGCTGAGAAACCCGATATACCGTCAATTAGAAGATCTGATGCTAAGGCCACAGGTAACCGGTTGAGTTTACAA-3' | NRG1-myc^{13} |
| ALT1Fw | 5'-AGACCCCCGCTTCACAGAGACATGC-3' | ALT1 Northern probe |
| ALT1Rv | 5'-GGAGCTTCCTGAACTGCTGAA-3' | ALT1 Northern probe |
| ALT2Fw | 5'-GACACACCAACAGATTTGGTGAAG-3' | ALT2 Northern probe |
| ALT2Rv | 5'-GCCGTCCTTGCAAAGAGCTT-3' | ALT2 Northern probe |
| SCR1Fw | 5'-GGAGTTTTATCCAGGGTCAGCAGAAGG-3' | SCR1 Northern probe |
| SCR1Rv | 5'-TTTAAATTTGCGTGCACTCGGTTTTA-3' | SCR1 Northern probe |

Lower-case lettering indicates sequence of the multiple cloning site of plasmid pFA6a and pyridoxal-5-phosphate was added to a 100 μM final concentration. Enzymatic assay was a modified version from García-Campusano [20]. The reaction mixture contained reaction buffer (pH 8 50 mM Tris-HCl, 4 mM MgCl₂, 150 mM KCl) 400 mM alanine, 24 mM α-ketoglutarate, 250 μM NADH, 40 μM pyridoxal 5-phosphate and 5 U/mL of lactate dehydrogenase. For Alt2 activity assays were performed at various combinations of pH (5–9), alanine (100–500 mM) and α-ketoglutarate (1.5–24 mM) concentrations, extracts were also dialyzed and no activity was detected. As control assays were performed without alanine. To determine specific activity the slope obtained from this negative control was subtracted to the complete assay. All assays were performed at 340 nm, 30°C in a Varian Cary 50 spectrophotometer.

**Metabolite Extraction and Analysis**

Cell-free extracts and samples for intracellular amino acid determination were prepared as previously described [36].

**Phylogenetic Analyses of Alanine Aminotransferases**

The evolutionary history of ALT1 and ALT2 in post-WGD yeasts was inferred using the Maximum-Likelihood method based on the JTT matrix-based model [29]. The tree with the highest log likelihood (−6922.511) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 14 amino acid sequences. The analysis involved 17 amino acid sequences. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA5 [30,31].
Amino-acid and Promoter Multiple Alignment

Promoter sequences were obtained from http://www.broad.mit.edu/annotation/fungi/comp_yeasts/downloads.html. Amino-acid sequences were obtained from http://www.broadinstitute.org/regev/orthogroups/. Multiple sequence alignment was performed using Clustal W.

Supporting Information

Figure S1 ALT1 and ALT2 display differential gene expression pattern. Northern blot of total RNA prepared from ALT1-TAP and ALT2-TAP strains grown on either glucose-ammonium (A) or glucose-alanine (B). Samples were collected from various OD_600 as stated. Representative results from three experiments are shown.

(TIF)

Figure S2 The ALT1 and ALT2 promoters have consensus Nrg1-binding sites. Full promoters are depicted as rectangles, presumed binding sites for Nrg1, depicted as bars. Conserved binding sites are shaded in black while non-conserved site is depicted as white symbol, according to the multiple alignment of three yeast species performed for each promoter region (Figs S3 and S4). Binding sites are numbered starting from the most 5′ promoter region (Figs S3 and S4). Multiple alignment is depicted as bars. Conserved binding sites are shaded in black rectangles with

Figure S3 ALT1 promoter complete sequence. Multiple alignment of three yeast species shows a single conserved Nrg1 presumed binding site.

(TIF)

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