Evolutionary Diversification of Alanine Transaminases in Yeast: Catabolic Specialization and Biosynthetic Redundancy

Ximena Escalera-Fanjul, Carlos Campero-Basaldua, Maritrini Colón, James González, Dariel Márquez and Alicia González*

Instituto de Fisiología Celular, Departamento de Bioquímica y Biología Estructural, Universidad Nacional Autónoma de México, Mexico City, Mexico

Gene duplication is one of the major evolutionary mechanisms providing raw material for the generation of genes with new or modified functions. The yeast Saccharomyces cerevisiae originated after an allopolyploidization event, which involved mating between two different ancestral yeast species. ScALT1 and ScALT2 codify proteins with 65% identity, which were proposed to be paralogous alanine transaminases. Further analysis of their physiological role showed that while ScALT1 encodes an alanine transaminase which constitutes the main pathway for alanine biosynthesis and the sole pathway for alanine catabolism, ScAlt2 does not display alanine transaminase activity and is not involved in alanine metabolism. Moreover, phylogenetic studies have suggested that ScALT1 and ScALT2 come from each one of the two parental strains which gave rise to the ancestral hybrid. The present work has been aimed to the understanding of the properties of the ancestral type Lacchancea kluyveri LkALT1 and Kluyveromyces lactis KIALT1, alanine transaminases in order to better understand the ScALT1 and ScALT2 evolutionary history. These ancestral -type species were chosen since they harbor ALT1 genes, which are related to ScALT2. Presented results show that, although LkALT1 and KlALT1 constitute ScALT1 orthologous genes, encoding alanine transaminases, both yeasts display LkAlt1 and KIALT1 independent alanine transaminase activity and additional unidentified alanine biosynthetic and catabolic pathway(s). Furthermore, phenotypic analysis of null mutants uncovered the fact that KIALT1 and LkALT1 have an additional role, not related to alanine metabolism but is necessary to achieve wild type growth rate. Our study shows that the ancestral alanine transaminase function has been retained by the ScALT1 encoded enzyme, which has specialized its catabolic character, while losing the alanine independent role observed in the ancestral type enzymes. The fact that ScAlt2 conserves 64% identity with LkAlt1 and 66% with KIALT1, suggests that ScALT2 diversified after the ancestral hybrid was formed. ScALT2 functional diversification resulted in loss of both alanine transaminase activity and the additional alanine-independent LkALT1 function, since ScALT2 did not complement the LkALT1Δ phenotype. It can be concluded that LkALT1 and KIALT1 functional role as alanine transaminases was delegated to ScALT1, while ScALT2 lost this role during diversification.

Keywords: orthologous genes, alanine metabolism, functional diversification, alanine transaminase, transcriptional regulation
INTRODUCTION

Gene duplication plays a central role in evolution, enhancing organism robustness, and providing material for the emergence of new or specialized functions. Different models have been posed to explain which is the possible fate of duplicated genes. The gene-dosage amplification model considers that gene doubling is not followed by functional diversification and that after duplication both gene copies are retained, augmenting the ancestral function, which could represent a selective advantage (Kondrashov et al., 2002; Kondrashov and Kondrashov, 2006; Tang and Amon, 2013). Conversely, three models including functional divergence have been developed; loss of function model sustains that as a result of the accumulation of loss-of-function mutations one gene copy is lost, becoming a pseudogene (Conrad and Antonarakis, 2007). Alternatively, the neofunctionalization model considers that one of the copies conserves the ancestral function, while the other one acquires a new adaptative function by the accumulation of neutral mutations (Ohno, 1970). And finally, the subfunctionalization model proposes that the accumulation of degenerative mutations split or specialize the ancestral function(s) between the two paralogous, therefore both copies are necessary to perform the function which in the ancestor was carried out by a single gene (Hughes, 1994; Innan and Kondrashov, 2010; Sikosek et al., 2012; Espinosa-Cantú et al., 2015).

For many years, it was accepted that the Saccharomyces cerevisiae (S. cerevisiae) lineage arose from a Whole Genome Duplication (WGD), making this yeast an interesting model to study diversification of paralogous genes (Wolfe and Shields, 1997; Kellis et al., 2004). Recently, a phylogenetic study found compelling evidence indicating that S. cerevisiae lineage arose from an interspecies hybridization between one strain related to the Kluyveromyces, Lachancea and Eremothecium (KLE) clade and another one related to Zygosaccharomyces rouxii and Torulaspora delbrueckii (ZT). Although whether the hybrid was the result of the fusion of two diploid cells or two haploid cells that underwent a WGD, is still an open question, both scenarios result in the formation of an allotetraploid with two copies of every gene. After the allotetraploid was formed, intragenic recombinations, full gene conversion, differential gene loss and selection pressures shaped S. cerevisiae genome to the one we observe today, harboring conserved blocks of duplicated genes (Marcet-Houben and Gabaldón, 2015). Thus, the paralogous genes that are observed today can both come from one of the species involved in the formation of the hybrid, or each parologue could have originated from each one of the parental species which constituted the hybrid. In S. cerevisiae ScALT1 and ScALT2 genes are part of the duplicated blocks coming from the hybridization; however, while ScALT1 comes from the ZT parental strain, ScALT2 parental is closer to the KLE clade, as it can be seen in Figure 1 (Huerta-Cepas et al., 2014).

ScALT1 and ScALT2 are annotated as putative alanine transaminases, however, previous studies of alanine metabolism in S. cerevisiae and ScAlt1 and ScAlt2 physiological roles, showed that when this yeast is grown on glucose and ammonium sulfate as carbon and nitrogen sources, (biosynthetic conditions), alanine is mainly synthesized by the ScALT1-encoded alanine transaminase, while the ScALT2-encoded protein does not contribute to alanine biosynthesis (Peñalosa-Ruiz et al., 2012). Furthermore, ScAlt1Δ mutants are not alanine auxotrophs, indicating the existence of a hitherto undescribed alanine biosynthetic pathway, that could be constituted by the previously described glutamine transaminase, which has been proposed to give rise to alanine and α-ketoglutaramate, since it uses pyruvate and glutamine as preferred substrates (Soberón and González, 1987), providing an alternative alanine biosynthetic pathway and supporting the observation of a wild type growth phenotype of the Scalt1Δ mutant. Worth mentioning is the fact that the glutamine transaminase could not provide an alanine catabolic pathway, since α-ketoglutarate is spontaneously cyclized and used by the ω-amidase to produce α-ketoglutarate (Soberón and González, 1987). Accordingly, it was found that the ScAlt1Δ mutant strain does not grow in the presence of alanine as sole nitrogen source, demonstrating that the ScAlt1 protein constitutes the only pathway for alanine catabolism, and that ScAlt2 does not play a role in either alanine biosynthesis or catabolism. In addition, it was found that ScAlt2 was devoid of alanine transaminase activity, and consequently, ScAlt2 function remains to be discovered (Peñalosa-Ruiz et al., 2012).

The physiological roles and the evolutionary histories of ScALT1 and ScALT2 are appealing, since these genes are inferred to descend from each of the different parental strains involved in the formation of the hybrid that gave rise to the S. cerevisiae lineage, and consequently, it is not possible to tell if at the moment of the hybridization, these two genes, ScALT1 and ScALT2, had the same function or had already functionally diverged. Worth of mention is the fact that, we have previously shown that ScAlt2 does not bear alanine transaminase activity and it might have acquired by either subfunctionalization or neofunctionalization a distinct physiological role. To better understand how ScALT1 and ScALT2 diverged, and the putative ancestral function of ScALT2 we decided to study the functional role of presumed alanine aminotransferases from pre-WGD yeasts that share a closest ancestor with the parental lineage which carried ScALT2. As such this could be regarded as a proxy to “ancestral type” ScALT2. For this, Lachancea kluyveri (LkALT1) and Kluyveromices lactis (KlALT1), were selected for functional characterization of their encoded enzymes. LkALT1 and KlALT1 were identified by sequence identity, synteny and phylogenetic analysis as the sole orthologous genes of both ScALT1 and ScALT2 present in L. kluyveri and K. lactis, respectively. Both species belong to the so called KLE clade, which is the closest one to ScALT2 parental strain, and share a more distant common ancestor with the ZT clade, from which ScALT1 was originated (Marcet-Houben and Gabaldón, 2015).

Results presented in this paper indicate: (i) LkALT1 and KlALT1 are functionally equivalent to ScALT1 functional orthologous genes, encoding alanine transaminase with a characteristic Ping Pong kinetic mechanism, (ii) while for S. cerevisiae alanine catabolism is strictly ScAlt1-dependent, in L. kluyveri and K. lactis additional unidentified pathways contribute to both alanine biosynthesis and catabolism, (iii) LkAlt1Δ and KlAlt1Δ mutants retain alanine transaminase...
activity indicating the existence of a second alanine transaminase encoding gene in \(L.\ kluyveri\) and \(K.\ lactis\). (iv) \(Lk\text{-}\text{Alt}1\) and \(Kl\text{-}\text{Alt}1\) enzymatic activity profile, respectively, follow \(Lk\text{ALT}1\) and \(Kl\text{ALT}1\) expression profile, and (v) that as shown by reciprocal complementation tests, \(Lk\text{-}\text{Alt}1\) and \(Kl\text{-}\text{Alt}1\) have a yet unknown alanine-independent alternative function which neither \(Sc\text{ALT}1\) nor \(Sc\text{ALT}2\) can complement.

### MATERIALS AND METHODS

#### Strains

Table 1 describes the characteristics of the strains used in the present work. All strains constructed for this study were derivatives of \(CLA1-2\) (ura3 leu2::LEU2), \(Lk156-1\) (ura3) or \(KI155\) (ade2 his3 ura3) for \(S.\ cerevisiae\), \(L.\ kluyveri\), and \(K.\ lactis\), respectively. Mutant strains as \(Scalt1\Delta\) (\(CLA1-2\)-1), \(Scalt2\Delta\) (\(CLA1-2\)-2), \(Scalt1\Delta Scalt2\Delta\) (\(CLA1-2\)-D) (Quezada et al., 2008; Garcia-Campusano, 2009). The \(L.\ kluyveri\) \(Lkl\text{-}\text{Alt}1\) (\(Lk156-2\)) mutant strain was kindly provided by Dr. Lina Riego, and was obtained by replacing the ORF of \(Lk\text{ALT}1\) with the selectable marker \(kan\text{MX}4\). The \(Lk\text{ALT}1\) gene was replaced by homologous recombination using a module containing the \(kan\text{MX}4\) cassette (1469 bp) flanked by 1146 bp of 5\(^\prime\) UTR (−1181 to −34) and 1207 bp of 3\(^\prime\) UTR (+1604 to +2811)

#### TABLE 1

| Strain | Relevant genotype | Reference |
|--------|-------------------|-----------|
| \(CLA1-2\) | \(MATa Scalt1\ \text{Scalt2}\ \text{ura3 leu2::LEU2}\) | Quezada et al., 2008 |
| \(CLA1-2\)-1 | \(MATa Scalt1\Delta::kanMX4 Scalt2\text{ura3 leu2::LEU2}\) | Garcia-Campusano, 2009 |
| \(CLA1-2\)-2 | \(MATa Scalt1\ \text{Scalt2}\Delta::kanMX4 \text{ura3 leu2::LEU2}\) | Garcia-Campusano, 2009 |
| \(CLA1-2\)-D | \(MATa Scalt1\Delta::kanMX4 Scalt2\Delta::natMX4 \text{ura3 leu2::LEU2}\) | Garcia-Campusano, 2009 |
| \(LK156-1\) | \(Mata Lk\text{ALT}1\ \text{ura3}\) | Montalvo-Arredondo et al., 2015 |
| \(LK156-2\) | \(Mata Lkl\text{Alt}1\Delta::kanMX4 \text{ura3}\) | This study |
| \(KI155\) | \(Mata ade2 his3 ura3\) | Colón et al., 2011 |
| \(KI155-2\) | \(Mata Kl\text{Alt}1\Delta::kanMX4 ade2 his3 ura3\) | This study |
sequences of \textit{LkALT1}. This module (3608 bp) was amplified by overlapped extension PCR with deoxyoligonucleotides X7 and X8 (-1007 to +2061) using a template built up by three independent modules: (i) the \textit{LkALT1} 5’UTR amplified using the X1 and X2 deoxyoligonucleotides and genomic DNA from strain \textit{Lk156-1} as a template, (ii) the \textit{kanMX4} module which was amplified from the pFA6a plasmid using deoxyoligonucleotides X3 and X4, and (iii) the \textit{LkALT1} 3’UTR amplified using deoxyoligonucleotides X5 and X6 and genomic DNA from strain \textit{Lk156-1} as a template. The PCR product was transformed into the \textit{Lk156-1} strain. Transformants were selected for G418 resistance (200 µg ml⁻¹). Transformation of \textit{Kl155} was carried out using the NEBuilder HiFi DNA Assembly Master Mix; to clone the \textit{ScALT1} gene into \textit{YEpKD352} plasmid, a 2619 bp region between -937 bp upstream from the start codon and +137 bp downstream from the stop codon was amplified with deoxyoligonucleotides X23 and X24 using genomic DNA from the \textit{S. cerevisiae} (CLA1-2) wild type strain as a template, after PCR amplification, and plasmid digestion with EcoRI and \textit{XhoI}, cloning was carried out using the NEBuilder HiFi DNA Assembly Master Mix; for the \textit{ScALT2} gene, a 2626 bp region between -996 bp upstream from the start codon and +105 bp downstream from the stop codon was amplified with deoxyoligonucleotides X27 and X28 using genomic DNA from the \textit{S. cerevisiae} (CLA1-2) WT strain as a template; for the \textit{LkALT1} gene, a 2611 bp region between -920 bp upstream to the start codon and +281 bp downstream from the stop codon was amplified with deoxyoligonucleotides X29 and X30 using genomic DNA from the \textit{L. kluyveri} (\textit{Lk156-1}) wild type strain as a template; and for \textit{KlALT1}, a 2619 bp region between -937 bp upstream from the start codon and +95 bp downstream to the stop codon was amplified with deoxyoligonucleotides X31 and X32 using genomic DNA from the \textit{K. lactis} (\textit{Kl155}) wild type strain as a template. The PCR products and pRS416 plasmid were digested with restriction enzymes and ligated after gel purification.

**Metabolite Extraction and Analysis**

Cell extracts were prepared from exponentially growing cultures. Samples used for intracellular amino acid determination were deproteinized with perchloric acid. Intracellular alanine was determined by HPLC with an Ultrasphere ODS C18 column (Beckman Coulter) with ortho-phthalaldehyde (OPA) derivatization and a mobile phase consisting of a 30 min gradient from 10 to 75% methanol and 25 % 0.1 M potassium acetate buffer (pH 5.5) flowing at 1.5 ml min⁻¹. Quantification was carried out with a fluorescent detector (Quezada et al., 2008).

**Cell Extract Preparation and Alanine Transaminase Enzymatic Assay**

Yeast cells were harvested by centrifugation, and washed with cold water. Pellets were suspended in cold extraction buffer (50 mM HEPES, 1 mM PMSF, 1 mM EDTA, 1 mM DTT). For \textit{KlALT1} extraction buffer was supplemented with complete, mini protease inhibitor cocktail, by Roche. Cells were mechanically disrupted with glass beads. The resulting extract was centrifuged to eliminate cellular debris (5,000 rpm, 4°C, 15 min). The supernatant was recovered, and pyridoxal-5-phosphate was added to a 100 µM final concentration. Protein was measured by the method of Lowry (Lowry et al., 1951), using bovine serum albumin as a standard. Enzymatic assay was a modified version from (Graßl and Supp, 1985). The reaction mixture contained reaction buffer (pH 7.0, 100 mM Tris-HCl, 400 mM alanine, 24 mM...
α-ketoglutarate, 300 μM NADH, 40 μM pyridoxal-5-phosphate and 5 U/mL of lactate dehydrogenase). As control, assays were performed without alanine. To determine specific activity the slope obtained from this negative control was subtracted to obtained with the complete assay. All assays were performed at 340 nm, 25°C in a Varian Cary 50 spectrophotometer.

Cloning and Expression
The LkALT1 and KIALTI genes were PCR amplified using the deoxyoligonucleotides pairs X33/X34 and X35/X36, respectively. To amplify LkALT1 genomic DNA of the Lk156-I WT strain was used as template. Alternatively, to amplify KIALTI genomic DNA of the KI155 wild type strain genomic DNA was used as template. Obtained PCR product and the pET-28a(+) plasmid were Nhel/Xhol digested for LkALT1 cloning and Ndel/XhoI digested for KIALTI cloning. After gel purification plasmids and inserts were ligated. Ligations were transformed into the DH5α E. coli strain. After plasmid purification, correct cloning was verified by sequencing. For heterologous expression, the Rosetta2 (DE3) E. coli strain was transformed. Selected clones were grown in LB medium supplemented with 50 µg/ml kanamycin and 70 µg ml−1 chloramphenicol, cells were incubated at 37°C with shaking (250 rpm). When the cultures reached an ODabsmax 0.75 the expression of the proteins was induced with 100 µmol/L of IPTG (Iso-Propil-Tio-Galactoside), incubated over night at 30°C with shaking (250 rpm). IPTG induced cells were harvested by centrifugation at 3750 rpm for 30 min.

Rosetta 2(DE3) Whole Cell Soluble Protein Extract
Cells were thawed and resuspended in 4 ml of lysis buffer per 100 ml of culture (10 mmol/L imidazol, 50 mmol/L NaCl, and 10 mmol/L Tris-HCI pH8.0). Protein extracts were obtained by sonication (Ultrasonic Processor Model: VCX 130) with a tip sonicator keeping the tubes on ice; five cycles (60% amplitude, one second on and one second off for 1 min) with 1 min of incubation on ice between each cycle. After centrifugation at 17000 rpm for 10 min at 4°C, the supernatant was recovered, and pyridoxal-5-phosphate was added to a 100 μM final concentration.

Affinity Chromatography
To purify alanine transaminase isozymes, supernatant obtained as stated above, was loaded on an equilibrated nickel column (Ni-NTA Agarose 100, Thermo Fisher Scientific), which was then washed 30 times with 30 mmol/L imidazol. The protein was eluted with 200 mmol/L imidazol and 100 μM final concentration of pyridoxal-5-phosphate was added. Protein homogeneity was verified by polyacrylamide gel electrophoresis 10% (SDS-PAGE) stained with Coomassie Blue (Supplementary Figure S1).

Enzyme Kinetics and Analysis of Kinetic Data
Initial velocity measurements were performed by varying both substrates simultaneously (for LkALT1 alanine from 1.00 to 20.00 mM and α-ketoglutarate from 0.05 to 1.50 mM; for KIALTI alanine from 4.00 to 80.00 mM and α-ketoglutarate from 0.25 to 5.00 mM), and the results where globally fitted to the equation for an enzyme with a ping-pong mechanism (Segel, 1975) with the Prism software GraphPad Prism 6.00 (Software Inc.).

Northern Blot Analysis
Northern blot analysis was carried out as previously described by Struhl and Davis (1981). Total yeast RNA was prepared from 100 ml cultures grown to indicated OD600 nm in MM with 2% glucose, ammonium sulfate, or alanine as a nitrogen source. PCR products were used as probes. For LkALT1, a 400 bp product was amplified with deoxyoligonucleotides X37 and X38; for KIALTI, a 897 bp PCR product was amplified with deoxyoligonucleotides X39 and X40; Lk18s and KI18s were used as internal loading standards. Both Lk18s and KI18s were amplified with deoxyoligonucleotides X41 and X42 a product of 476 bp and 477 bp was amplified for LkALT1 and KIALTI, respectively.

Nucleosome Scanning Assay (NuSA)
The nucleosome scanning assay was made to see the chromatin organization LkALT1 and KIALTI promoter, and the procedure to the study of the positioning of nucleosomes on promoters was made as described by Infante et al. (2011). When the cultures reached an OD600 nm of 0.6 at, genetic DNA was obtained of KI155, and Lk156-I strains grown on MM with 40 mM ammonium sulfate or 7 mM alanine as nitrogen source and 2% glucose. Cells were treated with formaldehyde (1% final concentration) for 20 min at 37°C and then glycine (125 mmol/L final concentration) for 5 min at 37°C. Formaldehyde-treated cells were harvested by centrifugation, washed with Tris-buffered saline, and then incubated in Buffer Z2 (1 mol/L Sorbitol, 50 mmol/L Tris-Cl at pH 7.4, 10 mmol/L β-mercaptoethanol) containing 2.5 mg of zymolase 20T for 20 min at 30°C on rocker platform. Spheroplast were pelleted by centrifugation at 3000g, and resuspended in 1.5 ml of NPS buffer (0.5 mmol/L Spermidine, 0.075% NP-40, 50 mmol/L NaCl, 10 mmol/L Tris pH 7.4, 5 mmol/L MgCl2, 1 mmol/L CaCl2, 1 mmol/L β-mercaptoethanol). Samples were divided into three 500 μl aliquots that were then digested with 22.5 U of MNase (Nulease S7 from Roche) at 50 min at 37°C. Digestions were stopped with 12 μl of Stop buffer (50 mmol/L EDTA and 1% SDS) and were treated with 100 μg of proteinase K at 65°C over night.
DNA was extracted twice by phenol/chloroform and precipitated with 20 μl of 5 mol/L NaCl and equal volume of isopropanol for 30 min at −20°C. Precipitates were resuspended in 40 μl of TE and incubated with 20 μg RNase A for 1 h at 37°C. DNA digestions were separated by gel electrophoresis from a 1.5% agarose gel. Monosomal bands (150 bp) were cut and purified by Wizard SV Gel Clean-Up System Kit (Promega, REF A9282). DNA samples were diluted 1:30 and used in quantitative polymerase chain reactions (qPCR) to quantify the relative MNase protection of each LkALT1 and KIALTI template. qPCR analysis was performed using a Corbett Life Science Rotor Gene 6000 machine. The detection dye used was SYBR Green (2× KAPA SYBR FAST qBioline and Platinum SYBR Green from Invitrogen). Real-time PCR was carried out as follows: 94°C for
5 min (1 cycle), 94° for 15 s, 58° for 20 s, and 72° for 20 s (35 cycles). Relative protection was calculated as a ratio to the control LkVCXI, and KlVCXI template found within a well-positioned nucleosome in +250 bp of the ORFs. The PCR primers amplify from around -950 to +250 bp (Supplementary Tables S2, S3) of LkALT1 and KIALT1 locus whose coordinates are given relative to the ATG (+1).

**Statistical Analysis**

All the experiments were repeated two or three times. Data were analyzed statistically using unpaired t-test with Welch’s correction using Prism version 7.0c computer software. P values of 0.1 or less were considered statistically significant.

**RESULTS**

*L. kluvyeri* LkALT1 and *K. lactis* KIALT1 Encoded Enzymes Contribute to Alanine Metabolism

To address whether *L. kluvyeri* LkALT1 and *K. lactis* KIALT1 preformed similar physiological roles to that of ScAlt1, an analysis of its contribution to alanine metabolism was assessed by phenotypic analysis, through the obtention and phenotypic analysis of pertinent null mutants (Figure 2A). In a biosynthetic medium, with ammonium as nitrogen source, Lkalt1Δ displayed a 20% lower growth rate than the one observed for the wild type strain, surprisingly, alanine addition to the medium did not restore wild type growth rate. In a catabolic condition, when alanine was provided as sole nitrogen source, Lkalt1Δ growth rate was near half to that observed for the wild type strain; such growth reduction was not alleviated when ammonium sulfate was added to the growth medium, indicating that the role played by LkAlt1 is not fully restored neither with alanine nor with ammonium sulfate, this observation will be discussed further. Conversely, in a biosynthetic medium *K. lactis* Klalt1Δ null mutant displayed wild type phenotype, and the braditrophic phenotype observed on alanine as sole nitrogen source was fully restored in the presence of both ammonium sulfate and alanine. These results indicate that, as well as for ScALT1, the orthologous genes LkALT1 and KIALT1 are involved in alanine catabolism. Nevertheless, unlike in *S. cerevisiae*, in *L. kluvyeri* and *K. lactis* there is an alternative pathway to catabolize alanine. The growth rate displayed by Lkalt1Δ and Klalt1Δ in a biosynthetic medium suggests that as well as for *S. cerevisiae*, there is an independent pathway(s) for alanine biosynthesis, hampering the possibility to observe LkALT1 and KIALT1 contribution to alanine biosynthesis by growth rate analysis.

To further analyze the contribution of LkALT1 and KIALT1 to alanine biosynthesis, intracellular concentrations of this amino acid were determined in wild type and single mutants Scalt1Δ, Lkalt1Δ and Klalt1Δ during early exponential growth phase (OD600 nm 0.3–0.6) on biosynthetic medium. To estimate alanine intracellular concentrations, a cell volume of 42 fl was considered for the three strains under study (Jørgensen et al., 2002; Klis et al., 2014). As reported earlier, ScAlt1 contributed with 75–60% of the alanine pool, whereas the ScAlt1-independent pathway only afforded 25–40% (Peñalosa-Ruiz et al., 2012). In Lkalt1Δ and Klalt1Δ strains a decrease of 42 and 70% in the intracellular alanine concentration, as compared to that found in the pertinent wild type strains, was observed (Figure 2B). In summary, presented results established that LkALT1 and KIALT1 are involved in alanine biosynthesis and catabolism, and that as well as for *S. cerevisiae*, there are alternative pathway(s) involved in alanine biosynthesis, which respectively provide 58 and 30% of the alanine pool in *L. kluvyeri* and *K. lactis*, respectively. In regard to alanine catabolism, Lkalt1Δ and Klalt1Δ achieve 50%
growth rate as compared with the one achieved in the wild type strain, indicating that as opposed to what has been observed in *S. cerevisiae*, in *L. kluyveri* and *K. lactis*, there are additional alanine catabolic pathways.

**LkALT1 and KIAL1 Are the Functional Orthologs of ScALT1**

Above presented results indicate that ScALT1, LkALT1, and KIAL1 have close physiological roles. To analyze whether the three enzymes have an orthologous function, growth rate analysis of clones obtained from reciprocal complementations was performed (Figure 3). In a catabolic condition, where alanine was present as sole nitrogen source, transformation of an *Scalt1Δ* mutant with plasmids carrying either ScALT1, LkALT1, or KIAL1 restored wild type growth (Figure 3A). Under the same culture conditions, LkALT1 mutant strain was only partially complemented with plasmids carrying either ScALT1 or KIAL1, while it was fully complemented when transformed with *LkALT1* (Figure 3B). When a *Klalt1Δ* mutant strain was transformed with plasmids harboring either ScALT1, LkALT1, or KIAL1, wild type growth was attained (Figure 3C). As expected, ScALT2 was unable to complement neither one of the three mutants tested (Figures 3A–C). This result indicates that LkALT1 and KIAL1 catabolic character has been maintained by ScALT1, conversely ScALT2 has completely lost the capacity to fulfill ScALT1, LkALT1, or KIAL1 catabolic role.

As shown in Figure 2 the only mutant with a decreased growth rate in a biosynthetic condition was *Lkalt1Δ*. When it was transformed with plasmids harboring either *LkALT1* or *KIAL1* wild type growth rate was attained, but neither ScALT1 nor ScALT2 were able to restore the growth rate observed in the wild type transformed with an empty plasmid (Figure 3B). Scalt1Δ and Klalt1Δ mutant strains, did not show a growth defect in the presence of ammonium sulfate as sole nitrogen source. In order to analyze their biosynthetic role, alanine pools were determined in extracts prepared from the mutant strains transformed with plasmids bearing the pertinent genes, as controls wild type and mutants transformed with empty plasmids were used (Figure 4), all extracts were prepared from exponential growth phase (OD$_{600}$ nm 0.3–0.6). Both ScALT1 and LkALT1 were able to restore alanine intracellular wild type concentrations in Scalt1Δ, Lkalt1Δ and Klalt1Δ mutant strains (Figures 4A–C). Lkalt1Δ and Klalt1Δ transformed with *KlALT1* displayed a wild type alanine intracellular concentration (Figures 4B,C). Nevertheless, KIAL1 failed to reestablish the wild type alanine concentration of the Scalt1Δ mutant (Figure 4A). The inability of KIAL1 to restore Scalt1Δ alanine pool when grown under biosynthetic conditions could be due to peculiar kinetic properties of KIAL1-encoded protein and/or to a low transcriptional rate of KIAL1. As expected, all mutant strains transformed with plasmids harboring ScALT2 presented an equivalent alanine intracellular concentration to that found in the negative controls.

These results, show that ScALT1, LkALT1, and KIAL1 can fully or partially substitute each other when alanine or ammonium sulfate is present as sole nitrogen source, confirming that ScALT1 has retained enough ancestral characteristics to conclude that LkALT1 and KIAL1 are the functional orthologs of ScALT1 with respect to its performance in both alanine biosynthesis or catabolism, conversely, ScALT2 has lost its role in alanine metabolism.

It is important to highlight the observation that in a medium provided with ammonium sulfate as sole nitrogen source *Lkalt1Δ* with or without an empty plasmid displayed a growth defect (Figures 2, 3B) and wild type growth phenotype was not attained when neither alanine was added to the medium (Figure 2) nor when *Lkalt1Δ* was transformed with a plasmid harboring *ScALT1* (Figure 3B). Although, *Lkalt1Δ* transformed with *ScALT1*, displayed wild type alanine intracellular concentration (Figure 4B), indicating that *Lkalt1Δ* growth defect in a biosynthetic condition was not due to a lower alanine intracellular concentration. Taken together these observations provide strong experimental evidence to sustain that *LkALT1* has a yet unidentified alternative function that is also present in *KIAL1*, since plasmids carrying *KIAL1* not only restored Lkalt1Δ alanine pool (Figure 4B), but also complemented the growth phenotype under the biosynthetic condition tested (Figure 3B). Worth of mention is the fact that in *K. lactis*, KIAL1 alternative function does not result in any apparent phenotype when Klalt1Δ mutant is grown on either ammonium sulfate or alanine as nitrogen sources. The observation that KIAL1 complements Lkalt1Δ decreased growth on ammonium sulfate and alanine uncovers the fact that KIAL1 harbors an equivalent function to that observed in LkAlt1. Furthermore, both ScALT1 and ScALT2 were unable to reestablish wild type growth rate of Lkalt1Δ strain in a medium with ammonium sulfate and alanine as nitrogen sources (Figure 3B), indicating that neither ScALT1 nor ScALT2 can substitute the supplementary function present in LkALT1 (Figures 2A, 3B).

**ScALT1, LkALT1, and KIAL1 Exhibit Alanine Transaminase Activities**

To compare the ScALT1, LkALT1, and KIAL1 transaminase activities displayed during biosynthetic and catabolic conditions, extracts obtained during exponential phase (OD$_{600}$ nm 0.4–0.6) from *S. cerevisiae*, *L. kluyveri*, and *K. lactis* wild type strains, grown in glucose-ammonium sulfate and glucose-alanine, were tested for alanine transaminase activity. Extracts obtained from ScALT1, LkALT1, and KIAL1 mutant strains were used as negative controls (Table 2). In agreement with a previous report from our group (Peñalosa-Ruiz et al., 2012), ScALT1 and LkALT1 alanine transaminase specific activity increased approximately 10-fold in cultures provided with alanine as sole nitrogen source as compared to those grown on ammonium sulfate, suggesting alanine could play and activator role. In *K. lactis* alanine transaminase activity was equivalent in samples prepared from cultures grown on either alanine or ammonium sulfate as nitrogen sources. Unexpectedly, alanine transaminase activity was detected in extracts of the mutant strains Lkalt1Δ and Klalt1Δ grown in either ammonium or alanine as sole nitrogen
FIGURE 3 | ScALT1, LkALT1, and KlALT1 can substitute each other under catabolic conditions. Reciprocal complementation test was performed using glucose as carbon source and as sole nitrogen source ammonium sulfate, alanine or ammonium sulfate plus alanine. Specific growth rate was determined during exponential phase for (A) S. cerevisiae, (B) L. kluyveri, and (C) K. lactis pertinent transformed cells. For ectopic expression, the plasmid pRS416 was used for S. cerevisiae and L. kluyveri and the YEpKD352 for K. lactis. Values are presented as means ± SD from three independent experiments. NG, no growth. The asterisks indicate significantly different (∗p < 0.1; †p < 0.05; ‡p < 0.01) compared with the wt control. NG, no growth.
three yeasts under study and that encoded proteins display alanine transaminase activity in the second alanine transaminase in *L. kluyveri* (*LkALT1*, *ScALT1*, *LkALT1* can be thus concluded that than the observed in the wild type (0.024 vs. 0.036 $\mu$mol min$^{-1}$ mg protein$^{-1}$), suggesting the existence of an additional alanine transaminase. Similarly, the specific activity detected in extracts from the mutant *Klalt1Δ* coming from ammonium or alanine was smaller than the observed in the wild type (0.024 vs. 0.036 $\mu$mol min$^{-1}$ mg protein$^{-1}$), again suggesting the existence of a second alanine transaminase in *L. kluyveri* and *K. lactis*. It can be thus concluded that *ScALT1*, *LkALT1*, and *KIALT1* encoded proteins display alanine transaminase activity in the three yeasts under study and that *L. kluyveri* and *K. lactis* alanine transaminase activity is not exclusively encoded by *LkALT1* and *KIALT1*.

**TABLE 2** | Alanine transaminase specific activity in extracts prepared from glucose–ammonium sulfate or glucose–alanine grown cultures.

| Glucose                | NH$_4$ | L-alanine |
|------------------------|--------|-----------|
| *ScALT1*               | 0.093 ± 0.001 | 0.821 ± 0.093 |
| *Scalt1Δ*              | 0.018 ± 0.001 | NG         |
| *LkALT1*               | 0.259 ± 0.014 | 2.771 ± 0.399 |
| *LkALT1Δ*              | 0.289 ± 0.142 | 0.120 ± 0.031 |
| *KIALT1*               | 0.036 ± 0.003 | 0.036 ± 0.003 |
| *Klalt1Δ*              | 0.024 ± 0.003 | 0.024 ± 0.002 |

*Specific activity is expressed as µmol min$^{-1}$ mg of protein$^{-1}$. No growth (NG). Values are presented as mean from three mesurments ± SD. NG, no growth.*

**Determination of Alanine Transaminase Activity in Complemented Strains**

To confirm the orthologous function of the three studied enzymes, alanine transaminase activity was also tested in *Scalt1Δ*, *Klalt1Δ* and *Klalt1Δ* mutants transformed with plasmids carrying either *ScALT1*, *ScALT2*, *LkALT1*, or *KIALT1* in either glucose ammonium sulfate or glucose alanine, as controls wild type and mutants transformed with empty plasmids were used (Figure 5). In many cases, it was observed that extracts from the mutant strains transformed with plasmids harboring either *ScALT1*, *LkALT1* or *KIALT1* exhibited higher specific activity, than the one observed in the wild type strains in each one of the conditions tested (Figures 5A, C, D, F). These results could be attributed to both a difference in the transcriptional regulation of the heterologous enzymes or to the plasmid copy number, since YEpK352 is a high copy number plasmid and pRS416 is a centromeric (CEN) plasmid, of which one or two copies are maintained per cell (Sikorski and Hieter, 1989), thus, increased enzymatic activity can be expected. As expected the *Scalt1Δ* mutants transformed with plasmids carrying either *ScALT1* or *LkALT1* displayed higher specific activity than the wild type strain in extracts from either glucose-ammonium or glucose-alanine (Figures 5A, D). As in the wild type strain, the specific activity of these transformed strains was higher in extracts coming from alanine supplemented medium than from ammonium sulfate. The specific activity of extracts from *Scalt1Δ* transformed with *KIALT1* were not detectable in neither one of the conditions tested (Figures 5A, D). However, as described earlier, plasmids harboring *KIALT1* were able to functionally substitute *ScALT1* in medium with alanine as sole nitrogen source (Figure 3A), suggesting that lack of alanine transaminase activity in the extracts prepared from this strain grown on either glucose-ammonium sulfate or glucose-alanine was due to an *in vitro* artifact which prevents detection of activity in these extracts. When grown in a medium with ammonium sulfate as sources (Table 2). When the extracts came from ammonium sulfate the activity displayed by the mutant *Lkalt1Δ* was similar to that observed in the *L. kluyveri* wild type strain. However, in extracts prepared from cells grown on alanine as sole nitrogen source, mutant strain *Lkalt1Δ* activity was 20 times lower than that observed in the wild type strain (0.12 vs. 2.77 $\mu$mol min$^{-1}$ mg protein$^{-1}$), although it was only 2.5-fold lower to that observed in extracts from ammonium sulfate (0.12 vs. 0.28 $\mu$mol min$^{-1}$ mg protein$^{-1}$), suggesting the existence of an additional alanine transaminase protein.

**FIGURE 4** | Reciprocal complementation test under biosynthetic conditions. Intracellular concentration of alanine from (A) *S. cerevisiae*, (B) *L. kluyveri* and (C) *K. lactis* transformed strains was obtained from glucose ammonium grown cell extracts. After cells were grown and harvested during exponential phase, cell free extracts were prepared and alanine pools were determined. Values are presented as means ± SD from two-three independent experiments. The asterisks indicates significantly different (*p < 0.1; **p < 0.05; ***p < 0.01) compared with the wt control.
sole nitrogen source, extracts from Lkalt1Δ transformed with an empty plasmid, presented equivalent alanine transaminase specific activity to that found in extracts of its corresponding wild type strain (Figure 5B). As observed before, in L. kluyveri alanine transaminase activity was not exclusively displayed by LkAlt1 (Table 2). Thus, the activity measured under this condition, in all the Lkalt1Δ transformed strains, is the result of the combined activities of the heterologous enzyme and the alternative alanine transaminase. Even though this other transaminase activity is also present in extracts of Lkalt1Δ grown in alanine, its contribution is minor, not allowing growth of Lkalt1Δ carrying pRS416 empty plasmid. In this medium extracts from Lkalt1Δ transformed with LkALT1 presented an activity similar to that detected in extracts obtained from the wild type strain, while those obtained from Lkalt1Δ transformed with ScALT1 or KlALT1, presented a four times lower activity than the one observed in the wild type strain (Figure 5E), consistent with the partial complementation phenotype of these strains (Figure 3B). The activity in extracts obtained from glucose–ammonium sulfate or glucose–alanine of Klalt1Δ transformed with ScALT1, LkALT1, or KlALT1 was higher than in the wild type. All the mutants transformed with plasmids harboring ScALT2 did not display activity as well as the negative controls (Figures 5A–F), indicating ScALT2 incapacity to complement ScALT1, LkALT1, and KlALT1 (Figures 3, 4). This result was expected since we had previously observed that even when ScALT2 is over-expressed using a TET promoter, higher production of ScAlt2 protein does not lead to detectable activity (Peñalosa-Ruiz et al., 2012). Increased activity of some samples, due to plasmid copy number, as compared to their respective wild type strain was not followed by an increased alanine pool

**FIGURE 5** Alanine transaminase specific activity in extracts prepared from complemented strains. Cellular extracts from (A) S. cerevisiae, (B) L. kluyveri, and (C) K. lactis in glucose–ammonium sulfate or (D–F) in glucose–alanine grown cultures. Specific activity is expressed as µmol min⁻¹ mg of protein⁻¹, values are presented as means ± SD from three independent experiments. NG, no growth; ND, not detected. The asterisks indicate significantly different (*p < 0.1; **p < 0.05; ***p < 0.01) compared with the wt control.
or higher growth rate (Figures 3, 4), indicating that an increase in the enzyme concentration, does not result in higher alanine production, suggesting that in vivo these enzymes, work far from their Vmax, so increased enzyme concentration does not result in a higher flux through this enzymes.

**LkAlt1 and KlAlt1 Display Similar Kinetic Parameters and a Ping Pong Mechanism**

To investigate LkAlt1 and KlAlt1 kinetic properties, His-tagged enzymes were purified to electrophoretic homogeneity after heterologous over-expression in E. coli. Denaturing SDS-PAGE showed a molecular mass of about 50 kDa for the two enzymes, in agreement with the value deduced from their amino acid sequences, which were 61.16 and 59.23 kDa for LkAlt1 and KlAlt1, respectively (Supplementary Figure S1). The oligomeric structures revealed by light scattering of the purified samples (LkAlt1 100.1 ± 10.8 kDa and KlAlt1 135.9 ± 25.8 kDa) indicate that the active form of both enzymes is an homodimer. Enzymatic activity of the pure enzymes was measured in Tris-HCl buffer at pH values ranging from 5.5 to 8.5, maximum activity was obtained around pH 7 for the two enzymes. It is worth mentioning that LkAlt1 was sensitive to acidic pH while KlAlt1 was sensitive to basic pH (Supplementary Figure S2).

Initial velocity measurements were made at different α-ketoglutarate and alanine concentrations. The resulting double reciprocal plot pattern obtained for both enzymes, corresponded to that expected for enzymes with a Ping-Pong mechanism (Figures 6A, B). Thus, the data were globally fitted to a Ping-Pong equation (Figures 6C, D). The resulting kinetic parameters for LkAlt1 and KlAlt1 are presented in Table 3. It follows that the orthologous proteins LkAlt1 and KlAlt1 are alanine transaminases with similar kcat, and kin, in both cases the affinity for α-ketoglutarate was higher than the one for alanine (Table 3). Results from our laboratory indicate that S. cerevisiae ScAlt1 is also an alanine transaminase (Peñalosa-Ruiz et al., 2012) with close kinetic parameters to the ones observed in L. kluyveri LkAlt1 and K. lactis KlAlt1 (unpublished results).

![Figure 6](https://example.com/figure6.png)

**FIGURE 6**  | LkAlt1 and KlAlt1 are alanine transaminases with the characteristic ping pong mechanism. Initial velocities were measured at different alanine and α-ketoglutarate concentrations. Double reciprocal plots of (A) LkAlt1 and (B) KlAlt1. Global fit of (C) LkAlt1 and (D) KlAlt1 to the ping pong equation. The corresponding kinetic parameters are shown in Table 3.

| TABLE 3 | Kinetic parameters of L. kluyveri LkAlt1 and K. lactis KlAlt1. |
|----------|---------------------------|----------------|----------------|----------------|
| Enzyme   | kcat (s⁻¹) | kₐ (mM) | kₐ (mM) | R²          |
| LkAlt1   | 355         | 4.88    | 0.22    | 0.98         |
| KlAlt1   | 203         | 17.25   | 0.92    | 0.98         |

Data were fitted to the ping pong equation. kₐ represents the kₐ for alanine and kₐ for α-ketoglutarate.
Divergent Expression Profile between LkALT1 and KIALT1 Is Independent of Chromatin Organization

In *S. cerevisiae*, ScALT1 and ScALT2 are differentially expressed. Previous analysis revealed that the expression profile of ScALT1 resembles that of a catabolic gene, whose expression is induced in the presence of alanine. Opposed expression pattern is observed for ScALT2, which is repressed whenever alanine is present (Peñalosa-Ruiz et al., 2012). To investigate whether the expression pattern of LkALT1 and KIALT1 was similar to that of ScALT1 or ScALT2, Northern blot analysis were carried out in samples obtained from wild type strain cultures in different growth phases on either glucose-ammonium sulfate or glucose-alanine. LkALT1 was repressed in a medium with ammonium sulfate as sole nitrogen source and induced when alanine was the nitrogen source (Figure 7A), mirroring ScALT1 expression profile (Peñalosa-Ruiz et al., 2012). KIALT1 showed constitutive expression, was equally expressed in both glucose-ammonium sulfate and glucose-alanine conditions (Figure 7C). Enzymatic activity followed the expression profile observed for the two orthologous genes LkALT1 and KIALT1 (Table 2).

To analyze LkALT1 and KIALT1 promoter organization, nucleosome scanning assay (NuSA) was preformed. Samples obtained from exponential phase growth cultures (OD600 nm 0.3) on ammonium sulfate or alanine as sole nitrogen sources were analyzed to determine nucleosome positioning across the promoters of both genes. After yeast DNA-nucleosome interaction was fixated with formaldehyde, cells were spheroplasted and incubated with MNase. The resulting DNA fragments obtained after MNase digestion were analyzed by quantitative PCR (qPCR). For normalization *L. kluyveri* LkVCX1 locus (SAKL0F09878g), or *K. lactis* KlvCX1 locus (KLLA-ORF439) (Infante et al., 2011) were used. Nucleosome positioning was equivalent for LkALT1 and KIALT1 promoters when cells were grown on ammonium sulfate or alanine (Figures 7B,D), indicating that the different expression profiles observed for the LkALT1 promoter on ammonium sulfate or alanine was not related to nucleosome organization (Figure 7A).

As expected for KIALT1, which displays constitutive expression, NuSA analysis showed similar nucleosome organization on both growth conditions (Figure 7D). Since the nucleosome positioning did not change in either one of the promoters tested it can be hypothesized that the most likely transcriptional factors involved in LkALT1 and KIALT1 transcriptional regulation should have their binding sites in the Nucleosome Free Region (NFR). Accordingly, an “*in silico*” analysis of the LkALT1 and KIALT1 promoter sequences was performed1, considering the consensus and conserved binding elements known for *S. cerevisiae*. In both promoters, *cis* elements for Gcn4 and Rtg3 in either free or nucleosome protected regions were found, additionally, in KIALT1 promoter region an Nrg1 sequence was found. This analysis suggests that LkALT1 could be regulated by the combined action of Gcn4 and Rtg3 orthologous transcriptional factors, while that of KIALT1 would also combine Nrg1. Further research on this matter will be required to determine whether the predicted regulators are involved in LkALT1 and KIALT1 transcriptional regulation.

DISCUSSION

Here, we present a combined phenotypic, biochemical and transcriptional regulation analysis of the *L. kluyveri* LkALT1 and *K. lactis* KIALT1 genes, which shows that both encode transaminases involved in alanine biosynthesis and catabolism. Interestingly, the *S. cerevisiae* isozyme encoded by ScALT1, retained alanine transaminase activity, while ScALT2 encoded enzyme did not conserve this activity (Peñalosa-Ruiz et al., 2012). In all three cases transaminase activity followed gene expression levels of their encoding genes. Accordingly, while ScALT1, LkALT1 and KIALT1, were capable of reciprocally complementing their role in alanine metabolism, ScALT2 was not. Moreover, it was discovered that LkALT1 and KIALT1 harbors an additional function besides that of alanine transaminase, which was neither retained by ScALT1 nor ScALT2.

Insights into the Evolution of Alanine Metabolism

Of the three yeasts, which were used as experimental models in this work, *K. lactis* was the first one to diverge from the lineage leading to *S. cerevisiae* (Merico et al., 2007; Huerta-Cepas et al., 2014; Marceau-Houben and Gabaldón, 2015), thus it can be considered that this yeast is an appropriate representative of KLE progenitor physiology. In *K. lactis* alanine metabolism is accomplished by more than one pathway; trough the Kialt1 alanine transaminase, and must likely by another yet unidentified alanine transaminase. Nevertheless, the existence of alternative pathways involved in alanine metabolism cannot be ruled out for example glutamine transaminase, which is known to exist in *S. cerevisiae* (Soberón and González, 1987), that could support alanine biosynthesis from pyruvate and glutamine. In *K. lactis*, the expression of KIALT1 and the activity displayed by the encoded protein is equivalent under biosynthetic or catabolic conditions. Also, the KIALT1 alanine transaminase activity detected in a *Klalt1*Δ mutant shows similar enzymatic activity in both conditions, suggesting that KIALT1 is similarly specialized in alanine biosynthesis and catabolism. Nevertheless, while a *Klalt1*Δ strain displays wild type growth rate under biosynthetic conditions, it shows decreased growth rate when grown on alanine as sole nitrogen source, indicating that the alternative pathways involved in alanine metabolism cannot fully compensate KIALT1 catabolic role.

As well as *K. lactis*, *L. kluyveri* can also be considered a KLE ancestral type yeast. In *L. kluyveri*, alanine biosynthesis and catabolism can be achieved by more than one pathway, since an *Lkalt1*Δ mutant is able to grow on ammonium or alanine as sole nitrogen sources. In *L. kluyveri* LkALT1 expression is increased when alanine is provided as sole nitrogen source.

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which results in higher alanine transaminase activity, constituting a specialization of LkAlt1 catabolic role. In addition, under this condition, the LkAlt1 independent alanine transaminase decreases its activity, enhancing the leading role of LkAlt1 in alanine catabolism. The expression pattern observed in LkALT1, favoring its catabolic role, was maintained by ScALT1, but the degree of specialization in alanine catabolism increased to the extent in which ScAlt1 is the only enzyme capable of catabolizing alanine, since the alternative pathway for alanine catabolism present in K. lactis and L. kluyveri was lost in S. cerevisiae. It is not surprising that the specialization of ScAlt1 and LkAlt1 towards alanine catabolism was achieved through a change in gene expression pattern, since the reaction catalyzed by transaminases, is close to equilibrium, and thus the direction of the reaction largely depends on substrates and products concentration, and given the enzymatic mechanism of the reaction, modification in the kinetic parameters of the enzyme would affect in the same proportion the biosynthetic and catabolic direction of the reaction (Nisonoff et al., 1953). It could be considered that the catabolic specialization present in these enzymes is compelling in order to improve their anaplerotic character which enables them to furnish pyruvate to the tricarboxylic acid cycle (Land, 2002). It can be considered that the anaplerotic role of alanine transaminase is less compelling for K. lactis than for S. cerevisiae and L. kluyveri, since K. lactis metabolism is constitutively respiratory (Breunig et al., 2000) and S. cerevisiae and L. kluyveri have a facultative metabolism (Moller et al., 2002; Merico et al., 2007). Hence, L. kluyveri and S. cerevisiae catabolic specialization arrived to the extreme in which S. cerevisiae lost the alternative pathways for alanine catabolism present in

**FIGURE 7** LkALT1 and KIAALT1 expression profile and NuSA assay. Northern blot analysis shown expression profile of LkAlt1 (A) and KIAALT1 (B), in both cases Lk18S or K118S ribosomal RNA was used as control in glucose-ammonium sulfate or glucose alanine. (C) Nucleosome scanning assay (NuSA) LkALT1 gene promoter in glucose-ammonium (black line) or glucose-alanine (gray line); and (D) Nucleosome Scanning Assay (NuSA) KIAALT1 gene promoter in glucose-ammonium (black line) or glucose-alanine (gray line); nucleosomes are shown in gray ovals and vertical lines shown DNA binding sites. Error bars represent the standard deviations from two independent experiments.
ScALT2 Evolutionary History

Although the closest taxonomic group of ScALT2 parental strain is the KLE clade, ScAlt2 is not an alanine transaminase, nor displays the alternative function encoded by LkALT1 and KIALT1. A structural analysis comparing ScAlt1 and ScAlt2 sequences demonstrated that ScAlt2 has conserved the binding site for alanine and for pyridoxal-5-phosphate which is the coenzyme which enables transaminase activity (García-Campusano, 2009). Together, these data indicate that in the KLE parental strain the ancestral form of ScALT2 codified for an alanine transaminase. Thus, it can be considered that when the hybrid was formed the KLE parental strain contributed with at least two alanine transaminases, one of them endowed by the ancestral form of ScALT2, and another one codified by a yet unidentified gene encoding the observed transaminase activity present in the Lkalt1Δ and Klalt1Δ mutants. In addition, the ZT parental strain provided a third alanine transaminase, which corresponded to the ScALT1 ancestral form, which probably was already specialized towards alanine catabolism through gene expression, since this tendency is already present in L. kluyveri LkALT1.

The specialization of ScALT1 towards alanine catabolism and its contribution to alanine biosynthesis, not only relaxed the selective pressure that would favor the maintenance of an ScALT1 and ScALT2 independent alternative catabolic pathways, provided by the KLE parental, but also flexibilized the need to conserve ScALT2 encoded alanine transaminase activity. Hence, the divergence between ScALT1 and ScALT2 can be explained by loss-of-function, sub-functionalization, and neo-functionalization models posed to explain the fate of duplicated genes. The scenario provided by the loss-of-function model, is the most unlikely, since ScALT2 promoter has retained the capacity to respond to alanine concentration and a complete soluble protein with no transaminase activity is synthesized (Peñalosa-Ruiz et al., 2012). If the divergence of ScALT1 and ScALT2 had occurred through sub-functionalization, one would expect that the transaminase activity present in LkAlt1 and in KIALT1 would have only been retained by ScAlt1 and lost by ScAlt2, as shown in this work; whereas, the alternative function provided by LkALT1 and KIALT1 would have been delegated to ScALT2 and lost from ScALT1, which is not consistent with the finding that ScAlt2 does not complement neither Lkalt1Δ nor Klalt1Δ mutants. Finally, under a neo-functionalization model, it would be expected that ScAlt2 function would be absent from either ScALT1 LkALT1 or KIALT1. The data herein presented support this later scenario; nevertheless, further studies are needed to understand the role of ScAlt2 and to distinguish between the above presented scenarios.

AUTHOR CONTRIBUTIONS

AG designed experiments, wrote the MS, obtained funding. XE-F performed and designed experiments, wrote the MS. CC-B performed experiments. MC performed and designed experiments, contributed to MS writing. JG performed experiments. DM performed experiments.

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Supplementary Material

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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