Saccharomyces cerevisiae Bat1 and Bat2 Aminotransferases Have Functionally Diverged from the Ancestral-Like Kluyveromyces lactis Orthologous Enzyme

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Abstract

Background: Gene duplication is a key evolutionary mechanism providing material for the generation of genes with new or modified functions. The fate of duplicated gene copies has been amply discussed and several models have been put forward to account for duplicate conservation. The specialization model considers that duplication of a bifunctional ancestral gene could result in the preservation of both copies through subfunctionalization, resulting in the distribution of the two ancestral functions between the gene duplicates. Here we investigate whether the presumed bifunctional character displayed by the single branched chain amino acid aminotransferase present in K. lactis has been distributed in the two paralogous genes present in S. cerevisiae, and whether this conservation has impacted S. cerevisiae metabolism.

Principal Findings: Our results show that the KIBat1 orthologous BCAT is a bifunctional enzyme, which participates in the biosynthesis and catabolism of branched chain aminoacids (BCAAs). This dual role has been distributed in S. cerevisiae Bat1 and Bat2 paralogous proteins, supporting the specialization model posed to explain the evolution of gene duplications. BAT1 is highly expressed under biosynthetic conditions, while BAT2 expression is highest under catabolic conditions. Bat1 and Bat2 differential relocalization has favored their physiological function, since biosynthetic precursors are generated in the mitochondria (Bat1), while catabolic substrates are accumulated in the cytosol (Bat2). Under respiratory conditions, in the presence of ammonium and BCAAs the bat1Δ bat2Δ double mutant shows impaired growth, indicating that Bat1 and Bat2 could play redundant roles. In K. lactis wild type growth is independent of BCAA degradation, since a Klbat1Δ mutant grows under this condition.

Conclusions: Our study shows that BAT1 and BAT2 differential expression and subcellular relocalization has resulted in the distribution of the biosynthetic and catabolic roles of the ancestral BCAT in two isozymes improving BCAAs metabolism and constituting an adaptation to facultative metabolism.

Introduction

It is accepted that Saccharomyces cerevisiae genome arose from complete duplication of eight ancestral chromosomes; functionally normal ploidy was recovered due to the massive loss of 90% of duplicated genes. Analysis of the complete yeast genome sequence identified several interchromosomal duplicated regions [1,2] which constitute the molecular evidence of an ancient duplication of the entire yeast genome [3]. Gene duplication and the subsequent divergence of paralogous pairs play an important role in the evolution of novel gene functions. Several models have been proposed to account for the emergence, maintenance and evolution of gene copies. It has been shown that diversification of paralogous genes whose products are strictly involved in amino acid biosynthesis has led to functional diversification such that retention of both copies is needed to fulfill the function carried out by the original gene [4–6], thus supporting the duplication-degeneration-complementation model proposed by Force et al. [7]. The specialization or escape from adaptive conflict posed by Hughes [8] considers that if the original gene was performing two functions, that could not be independently improved, after duplication each gene copy could be driven by positive selection to improve one of the two functions. Aminotransferases constitute an interesting model to study diversification of paralogous genes carrying out two functions, both of which are needed to warrant metabolic provision, and which cannot be differentially improved, since aminotransferases constitute biosynthetic and catabolic pathways whose opposed action relies on a single catalytic site.
Furthermore, metabolite provision through the action of aminotransferases, is necessary when yeast is grown in either fermentable or non-fermentable carbon sources and thus, functional diversification of aminotransferase-encoding paralogous genes could play a fundamental role in the adaptation to facultative metabolism.

In the yeast *Saccharomyces cerevisiae*, the last step in the biosynthesis and the first step in the catabolism of branched chain amino acids (BCAAs), is achieved through the action of the branched chain aminotransferases (BCATs) encoded by the paralogous pair *BAT1* and *BAT2*, which form part of a duplicated chromosomal block generated from the Whole Genome Duplication (WGD) event [2,3] (http://www.gen.tcd.ie/~khwolfe/yeast/nova/index.html). An additional inspection using the Yeast Gene Order Browser (http://wolfe.gen.tcd.ie/sgob/) also suggests that *BAT1*/*BAT2* could be in a duplicated block. This evidence points to the origin of the *BAT1-BAT2* duplicated gene pair as part of the WGD duplication event rather than to an isolated gene duplication phenomenon. These enzymes catalyze the transfer of amino groups between the amino acids valine, leucine and isoleucine and their corresponding α-ketoacids, the biosynthetic precursors of fusel alcohols, which influence the aroma and flavor of yeast derived fermentation products such as beer and bread [9,10], and which have been recently found to regulate translation initiation by inhibiting eIF2B [11].

The lineage which gave rise to *Kluyveromyces lactis* (*K. lactis*) diverged before the WGD event, therefore, *K. lactis* genome does not harbor the duplication blocks present in *S. cerevisiae* [3]. In *K. lactis* the gene *KlBAT1* constitutes, the unique orthologue of the *S. cerevisiae* *BAT1* and *BAT2* paralogous gene pair encoding a branched chain aminotransferase (*KlBat1*). We have undertaken the study of the functional role played by *KlBat1*, *Bat1* and *Bat2*, in order to understand whether the role played by the ancestral-type enzyme has been conserved in *Bat1* and *Bat2* resulting in redundant function or whether it has been distributed between these two enzymes resulting in diversification.

*KlBat1* encoded protein is constituted by 407 amino acid residues and as well as *Bat1* it bears an amino terminal signal peptide which could direct its mitochondrial localization. It shares 82% amino acid identity with *Bat1* and 79% with *Bat2*. *BAT1* encodes a 393 amino acid residues mitochondrial protein, while the cytosolic *Bat2* is composed of 376 residues; these two enzymes show 81% identity. Previous results from other laboratories have shown that on glucose-containing media, *BAT1* single deletion impaired neither cell growth nor fusel alcohol production; however, drastic effects in fusel alcohol production were observed in a *bat2A* deletion mutant. Deletion of both genes resulted in branched chain amino acid auxotrophy, severe growth retardation and diminished fusel alcohol production [12]. The fact that the enzymes involved in the biosynthesis of the BCAAs are mitochondrially located has led to the notion that in *S. cerevisiae*, the biosynthetic process is mainly carried out in the mitochondria. However, the fact that *Bat1* and *Bat2* are located in both compartments indicates that the last step in BCAAs biosynthesis can be carried out in either the mitochondria or the cytoplasm. Furthermore, for the leucine biosynthetic pathway, Leu1 and Leu2 have been found in cytosol [13,14] indicating that the conversion of α-ketoisovalerate to α-isocaproate the immediate precursor of leucine is carried out in the cytoplasm and further transported to the mitochondria so that the last step in leucine biosynthesis can be carried out in either the mitochondria or the cytoplasm, through the action of either *Bat1* or *Bat2*. No analysis has been undertaken to determine the compartment in which BCAAs catabolism is carried out and the physiological role of differential *Bat1* and *Bat2* localization has not been analyzed.

Results presented in this paper support the specialization model posed by Hughes [8], showing that i) *K. lactis* *KlBAT1* codifies a presumed mitochondrial localized BCAT, which participates in both, the biosynthesis and catabolism of BCAAs, which is unable to complement *S. cerevisiae bat2A* mutants, and that ii) in *S. cerevisiae* biosynthetic and catabolic roles have been distributed in two paralogous genes. *Bat1* is preferentially involved in BCAAs biosynthesis, while *Bat2* function is determinant for BCAAs catabolism, indicating functional diversification. The specialization has been afforded through differential subcellular localization of the encoded products and divergent gene expression patterns, which is reflected in enzyme activity under various physiological conditions.

**Results**

The ancestor-like branched chain aminotransferase *KlBat1* is a bifunctional biosynthetic and catabolic enzyme

A *Klbat1* mutant incubated on glucose and ammonium, displayed valine, isoleucine and leucine (VIL) auxotrophy (Table 1). Wild type growth was only attained when the three BCAAs were simultaneously added to the growth medium. The *Klbat1* mutant did not grow when branched chain amino acids were supplemented as sole nitrogen sources (Table 1), showing that this enzyme is also involved in BCAAs catabolism. These results indicate that no redundant pathways are involved in VIL biosynthesis and catabolism. As expected, *KlBat1* transformants carrying the *KIBAT1* gene on a centromeric plasmid displayed wild type phenotype when grown on either ammonium-glucose or VIL-glucose (Table 1), indicating that *KlBat1* is a bifunctional enzyme, which participates in VIL biosynthesis and catabolism.

In *S. cerevisiae*, biosynthetic and catabolic roles of the branched chain aminotransferases have been differentially distributed in the *BAT1* and *BAT2*-encoded isozymes

Single and double *bat1A* and *bat2A* mutants were constructed. As Table 2 and Figure 1A, CEN show, a double *bat1A bat2A* mutant displayed VIL auxotrophy when incubated on glucose and

### Table 1. *Klbat1A* mutants are impaired in VIL biosynthesis and catabolism.

| Strain (mutant) | Glucose | Relative growth (%) |
|----------------|---------|---------------------|
|                | NH₄⁺    | NH₄⁺ VIL           | VIL        |
| *KlWT*         | 100     | 100                 | 100        |
| *CLAS4 (Klbat1A)* | 0     | 96                  | 0          |
| *KlWT (pKD1)*  | 100     | 100                 | 100        |
| *CLAS4 (pKD1)* | 0       | N. D.²              | 0          |
| *CLAS4 (pKD1 KIBAT1)* | 100 | N. D.              | 100        |

*Values are shown relative to growth rate of the wild type strain (0.12 h⁻¹ and 0.13 h⁻¹ on NH₄⁺ and amino acids, respectively); and represent the means from three independent experiments (variation was always <10%).

*²Amino acids were supplemented at a concentration of 150 mg/l, 100 mg/l or 30 mg/l of valine (V), leucine (L) or isoleucine (I) respectively.

*¹N. D. not determined.

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ammonium; wild type growth was attained when this strain was grown in the presence of the three BCAAs (Table 2). BAT1 and BAT2 were independently cloned on centromeric plasmids and used to transform the bat1A bat2A mutant. Transformants carrying BAT1 recovered VIL prototrophy (Figure 1A, CEN BAT1), while those carrying BAT2 showed a braditrophy phenotype (Figure 1A, CEN BAT2), indicating that Bat1 had a more efficient biosynthetic role than that exerted by Bat2. When cultured on ammonium-glucose, the single bat1A mutant showed a significantly decreased growth rate (69%), as compared to the wild type strain however, it attained wild type growth rates by the sole addition of valine to the growth medium (94%) (Table 2), or when complemented with a centromeric plasmid harboring BAT1 (Figure 1A, CEN vs. CEN BAT1). BAT2 did not complement bat1A growth deficiency (Figure 1A, CEN BAT2). These results indicate that Bat1 activity is indispensable to fulfill valine requirement and that Bat2 is unable to fully replace Bat1, suggesting functional diversification. Accordingly, the single bat2A mutant grew as well as the wild type on ammonium-glucose, with or without amino acids (Table 2), confirming that Bat1 completely fulfilled biosynthetic needs. It has been established that Bat2 is cytosolic, while Bat1 is mitochondrially located [9] it could be considered that the valine pool generated through Bat2 might not be efficiently transported to the mitochondrion. In order to confirm in vivo enzyme localization, Bat1-yECitrine and Bat2-yECitrine tagged strains were constructed as described in Materials and Methods and subcellular localization was analyzed by confocal microscopy. As Figure 2 shows, Bat1 was found to be localized in mitochondria, while Bat2 was cytosolic, confirming previous observations [9]. It could thus be proposed that as mentioned above, the valine pool synthesized through Bat2 is not efficiently transported to the mitochondria or that valine synthesis through Bat2 is scarce leading to the observed valine braditrophy of a bat1A mutant.

To analyze the role of Bat1 and Bat2 on VIL catabolism, bat1A bat2A double mutant and single mutants were grown on glucose in the presence of the three BCAAs as sole nitrogen source. Under these conditions, the wild type strain and the bat1A mutant showed higher growth rates than those observed in the double and bat2A mutants indicating a catabolic role for Bat2 (Table 2 and Figure 1B, CEN). On VIL-glucose bat2A mutant was only able to achieve 70% of the growth rate displayed by the wild type strain, suggesting that Bat2-dependent VIL catabolism was required for wild type growth, and that Bat1 was unable to compensate lack of Bat2 (Table 2; Figure 1B, CEN). Accordingly, single bat2A and double mutants recovered wild type growth when transformed with a centromeric plasmid harboring BAT2, complementation failed with plasmids carrying BAT1 (Figure 1B, CEN vs. CEN BAT1 and CEN BAT2). Since on glucose-ammonium-VIL the double and single bat2A mutants showed growth rates which were equivalent to those displayed by the wild type strain, it can be concluded that in glucose VIL catabolism fulfills nitrogen requirements.

These results indicate that Bat2 has a prominent role in VIL catabolism, while Bat1 catabolic role is only evidenced in a bat2A genetic background. The fact that as Figure 2 shows, Bat1 mitochondrial localization is conserved in the presence of VIL as sole nitrogen source indicates that Bat1 catabolic character is exerted in this compartment. It could be proposed that under these conditions VIL accumulation in the mitochondria, would enhance Bat1 catabolic character.

Above presented results indicate that in a wild type strain Bat1 displays a biosynthetic character while Bat2 has a prominent catabolic role.

**KIBAT1 does not complement bat2A mutant strains**

To analyze whether the KIBat1 enzyme was able to replace Bat1 or Bat2 in *S. cerevisiae*, a monocopy plasmid harboring the KIBAT1 gene was independently transformed in both single mutants bat1A and bat2A and in the double mutant bat1A bat2A. Constructions were prepared in order to promote KIBAT1 expression from either its own promoter or by the heterologous BAT1 or BAT2 promoters. When grown on ammonium-glucose the bat1A mutant harboring KIBAT1 on a monocopy plasmid attained wild type growth regardless of the promoter used to drive its expression (Figure 1A). In the case of the bat1A bat2A double mutant, the presence of KIBAT1 only restored 72% of wild type growth (Figure 1A); indicating that KIBat1 could only partially substitute simultaneous lack of Bat1 and Bat2. When growing on VIL-glucose neither the bat2A nor the double mutant attained wild type growth when KIBAT1 expression was driven from the BAT1, BAT2 or KIBAT1 promoters (Figure 1B), although higher growth rates were attained with P_{BAT2}-KIBAT1 or P_{KIBAT1}-KIBAT1, suggesting that a promoter-dependent effect could enhance KIBAT1 capacity to complement lack of Bat2. It could be possible that either the KIBat1 heterologous enzyme has peculiar kinetic properties that do not allow full bat2A complementation, or that the differential subcellular localization of KIBat1 and Bat2, could hamper bat2A complementation, since as mentioned earlier, Bat2 is a cytosolic enzyme and although localization of KIBat1 has not been experimentally determined, an *in silico* analysis using Mitoprot and SignalP databases suggests that KIBat1 is located in the mitochondria.

| Table 2. In *S. cerevisiae* bat1A mutant is impaired in VIL biosynthesis, while a bat2A mutant is mainly impaired in VIL catabolism. |

| Strain | Glucose | NH4+ | NH4+ V | NH4+ I | NH4+ L | NH4+ VIL | V | I | L | VIL |
|--------|---------|------|--------|--------|--------|----------|---|---|---|-----|
| CLA1-2 (WT) | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| CLA31 (bat1A) | 69 | 94 | 65 | 65 | 100 | 97 | 88 | 78 | 100 |
| CLA32 (bat2A) | 100 | 95 | 93 | 100 | 100 | 61 | 52 | 78 | 70 |
| CLA33 (bat1A bat2A) | 0 | 0 | 0 | 0 | 91 | 0 | 0 | 0 | 0 |

*Values are shown relative to growth rate of the wild type strain (0.20 h⁻¹ and 0.11 h⁻¹ on NH₄⁺ and amino acids, respectively); and represent the means from three independent experiments (variation was always ±10%).

*Amino acids were supplemented at a concentration of 150 mg/l, 100 mg/l or 30 mg/l of valine (V), leucine (L) or isoleucine (I) respectively.

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**KIBAT1 has a biosynthetic-like expression profile**

Total RNA was prepared from *K. lactis* wild type strain grown on glucose as carbon source with various nitrogen sources. It was found that *KIBAT1* expression profile was that expected for a biosynthetic enzyme. Steady state mRNA levels were similar in total RNA samples obtained from cultures grown on either repressive (glutamine) or non-repressive nitrogen sources (GABA), indicating that the quality of the nitrogen source had no effect on *KIBAT1* expression (Figure 3A). However, expression was repressed in total RNA samples obtained from cultures grown in the presence of VIL as sole nitrogen source, or when combined with additional nitrogen sources such as ammonium or GABA, as compared to that found in the absence of VIL (Figure 3A). Worth of mention is the fact that VIL repression was not observed in

![Figure 1](#)

**Figure 1. Growth phenotype of single and double mutants complemented with plasmids harboring BAT1, BAT2 or KIBAT1.** Wild type, *bat1*D, *bat2*D and *bat1*D *bat2*D strains were grown on ammonium-glucose (A) or VIL-glucose (B). Values are shown relative to growth rate of the wild type strain (0.20 h⁻¹ and 0.13 h⁻¹ on ammonium-glucose and VIL glucose respectively) and represent the mean of three independent experiments ± S. D. Cells were complemented with a centromeric plasmid (CEN) harboring BAT1 (CEN BAT1), BAT2 (CEN BAT2) or the *K. lactis* orthologous gene KIBAT1 whose expression was driven by its own promoter (CEN P₂KIBAT1-KIBAT1) or by BAT1 (CEN P₂BATUS1) or BAT2 (CEN P₂BATUS2-KIBAT1) promoters. doi:10.1371/journal.pone.0016099.g001

![Figure 2](#)

**Figure 2. Bat1 is mitochondrially located, while Bat2 is cytoplasmic.** Fluorescence images showing the subcellular localization of the paralogous proteins Bat1 and Bat2. Samples were taken from exponentially grown cultures of tagged mutants grown on glucose-ammonium (A, B) or on glucose-VIL (C, D). Mitochondrial localization of Bat1, the signal of the Bat1-yECitrine fusion co-localizes with mitotracker signal (A, C). Cytoplasmic localization of the Bat2-yECitrine fusion (B, D).

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and BAT2 enzymatic activity is consistent with the expression profile of their encoding genes. Northern analysis was carried out. It was found that as well as KIBAT1, BAT1 was mainly expressed on ammonium-glucose exponential cultures (biosynthetic conditions), and repressed in the presence of VIL. BAT1 expression was not influenced by the quality of the nitrogen source, and VIL repression was observed on either repressive or non-repressive nitrogen sources (Figure 3B and 3C). Conversely, BAT2 showed a classic catabolic expression profile; responding to the quality of the nitrogen source; down-regulated in the presence of repressive nitrogen sources (glutamine) and derepressed in secondary non-repressive nitrogen sources such as GABA (Figure 3B and 3C). BAT2 expression was twelve-fold increased when total RNA was obtained from cultures in which VIL was provided as sole nitrogen source (catabolic conditions), as compared to that found when RNA was prepared from on ammonium-glucose cultures (Figure 4). BAT2 expression was also induced in a bat1A genetic background. Under derepressed conditions (GABA), the addition of the three branched chain amino acids, had a positive effect further inducing BAT2 expression (Figure 3B).

**KlBat1 enzymatic activity displays a biosynthetic character**

KlBat1 activity was determined in extracts obtained from cultures grown under biosynthetic and catabolic conditions (Figure 5A). Activity was similar in extracts obtained from ammonium-glucose with or without VIL (Figure 1A), indicating that the observed repression of KlBAT1 expression on VIL-ammonium did not result in decreased enzymatic activity. When ω-ketoisovalerate (ω-KIV) was used as substrate, activity was nearly two-fold higher to that found with ω-ketoisocaproate (ω-KIC), indicating differential kinetic properties for these substrates. Lowest activity was detected with ω-ketomethylvalerate (ω-KMV). In extracts obtained from VIL-glucose (catabolic conditions), KlBat1 activity was at least ten-fold lower than that observed on ammonium-glucose (biosynthetic conditions), confirming KlBAT1 expression profile (Figure 5A) and indicating that KlBat1 has a pronounced biosynthetic character.

**Bat1 and Bat2 enzymatic activity is consistent with BAT1 and BAT2 expression profile**

BCAT enzymatic activity was determined in extracts obtained from the wild type and the single bat1A and bat2A mutants. As Figure 5A shows, activity determined on ammonium-glucose (biosynthetic conditions) was higher in the bat2A (BAT1) mutant, as compared to that found in the bat1A (BAT2) mutant strain, although in the presence of VIL-ammonium Bat1 activity decreased, it was however higher than that observed for Bat2. Conversely, in the presence of VIL as sole nitrogen source (catabolic conditions) Bat2 activity was higher than that observed for Bat1. These results are in agreement with expression profile observed for either BAT1 or BAT2, which indicate that BAT1 expression is VIL repressed while that of BAT2 is induced in VIL as sole nitrogen source (Figure 4). These results support the proposition that BCAT biosynthetic and catabolic roles have been distributed between the two paralogous enzymes. Bat1 and Bat2 have diverged acquiring a biosynthetic or catabolic character, respectively. However, since only the double bat1A bat2A is a full VIL auxotroph unable to utilize VIL as sole nitrogen source, it can be concluded that the biosynthetic and catabolic roles of these enzymes is partially redundant. Highest activities were detected when ω-KIC (leucine) was provided as substrate, as compared to that for ω-KIV (valine) or ω-KMV (isoleucine), indicating differential kinetic properties for the various substrates that could reflect differential substrate affinities.

**BAT1 and BAT2 show differential expression profiles and enzymatic activity under respiratory conditions**

The collection of single and double bat1A and bat2A mutants was grown on ethanol as carbon source and ammonium as nitrogen source. Under this condition, growth of the double bat1A bat2A mutant was completely impaired, even in the presence of the three amino acids, indicating that VIL catabolism is compelling for growth in the presence of non-fermentable carbon sources, even on ammonium as nitrogen source (Table 3). Conversely, Klbat1A mutant was able to sustain growth in media supplemented with VIL-ammonium-ethanol, indicating that as opposed to that observed in *S. cerevisiae*, VIL catabolism is not necessary to achieve growth in the presence of ethanol as sole carbon source and ammonium as nitrogen source, underscoring the fact that *K. lactis* has a more efficient ethanol catabolism [15]. Accordingly, the *K. lactis* wild type strain achieved a higher growth rate than that attained by the *S. cerevisiae* wild type CLA1-2 strain when grown on ethanol as sole carbon source (0.21 vs. 0.12 h⁻¹). On ammonium-ethanol, the bat2A mutant and the wild type strain, showed equivalent growth rates, while bat1A showed a slightly decreased growth rate as compared to the wild type strain, which was alleviated in the presence of the three amino acids, thus confirming Bat1 biosynthetic role. As expected, single mutants and wild type strain showed equivalent growth rates in the presence of the three amino acids. When ethanol was provided as the sole carbon source and the branched amino acids as sole nitrogen source neither the *S. cerevisiae* wild type strain nor the single or double mutants grew, indicating that these amino acids are poorly catabolized and thus unable to allow growth under these conditions (Table 3). Conversely, on VIL-ethanol, *K. lactis* wild type and the Klbat1A mutant complemented with the centromeric plasmid harboring the KlBAT1 gene with its native promoter sequence, were able to sustain growth, confirming KlBat1 catabolic character (Table 3).

Northern analysis performed with extracts obtained from ammonium-ethanol grown cultures, showed that BAT1 and BAT2 displayed opposite expression profiles; BAT1 expression was five-fold repressed, while that of BAT2 was two-fold increased on ethanol as compared to those found on glucose. KlBAT1 showed a similar expression pattern to that of BAT1, since its expression was decreased on ethanol as compared to glucose (Figure 6A and 6B).

In extracts prepared from ammonium-ethanol, Bat1 activity (bat1A) decreased when either one of the three ω-ketoacids were used as substrates, as compared to that found on glucose ammonium, while that of Bat2 (bat1A) was nearly two-fold increased as compared to that found on glucose (Figure 5A and 5B). These results suggest that under respiratory conditions, Bat1-dependent ω-ketoacid utilization would be diminished; avoiding increased carbon flux being channeled to VIL biosynthesis, while enhanced Bat2 activity would increase VIL utilization, favoring *S. cerevisiae* capacity to grow under respiratory conditions. On ammonium ethanol, Bat1 activity was equivalent to that found without VIL, and Bat2 activity was two or three-fold increased as compared to ammonium ethanol (Figure 5B), thus under VIL-ammonium-ethanol, Bat1 and Bat2 showed equivalent
Figure 3. *Saccharomyces cerevisiae* BAT1 and KIBAT1 expression is repressed by VIL. Northern analysis was carried out on total RNA obtained from *K. lactis* 155 (wild type) and CLA34 (Kibat1Δ) strains (A), and *S. cerevisiae* strain CLA1-2 (wild type B, C). Strains were grown on 2% glucose with either valine (V) (150 mg/l), leucine (L) (100 mg/l), isoleucine (I) (30 mg/l), γ-aminobutiric acid (GABA 7 mM), γ-aminobutiric acid+VIL (GABA VIL), VIL (valine+isoleucine+leucine), NH₄⁺ (40 mM NH₄ SO₄), NH₄ VIL (40 mM NH₄ SO₄+VIL), glutamine (GLN 7mM), glutamine+VIL (GLN VIL) as nitrogen sources. Filters were sequentially probed with the BAT1, BAT2, KIBAT1- specific PCR products described in experimental procedures and a BamH1-HindIII 1500 bp ACT1 DNA or an SCR 400bp PCR fragment as loading controls. Numbers indicate relative expression as compared to WT grown on ammonium-glucose. Four biological replicates were carried out, representative results are shown.

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enzymatic activities, indicating that both enzymes could equally contribute to VIL catabolism, in fact as Table 3 shows, growth rate of either \textit{bat1}D or \textit{bat2}D is similar, and growth is only impaired in the double mutant. It can be concluded that under respiratory conditions, Bat1 and Bat2 play partially biosynthetic redundant roles, and redundant catabolic roles.

As well as for Bat1, \textit{kIBat1} activity was three-fold decreased in extracts prepared from ammonium-ethanol grown cultures, as compared to those found on ammonium-glucose (Figure 5A and 5B) in agreement with the expression profile, and as well as for Bat1, addition of VIL to ammonium-ethanol growth medium did not affect activity, suggesting that under respiratory conditions, biosynthesis is decreased and catabolism is triggered, thus favoring an equilibrated consumption and synthesis of \(\alpha\)-ketoacids.

### Discussion

This study addresses the question of whether the biosynthetic and catabolic roles played by the ancestral-like \textit{KIBAT1} encoded aminotransferase present in \textit{K. lactis}, have been distributed in the paralogous \textit{BAT1} and \textit{BAT2} orthologous genes present in \textit{S. cerevisiae} and whether this subfunctionalization has improved branched chain amino acid metabolism constituting an adaptation to facultative metabolism.

\textit{BAT1} and \textit{BAT2} divergent expression profiles and differential subcellular localization contribute to Bat1 and Bat2 functional diversification

Presented results show that the \textit{kIBat1} orthologue codifies a bifunctional enzyme able to carry out BCAAs biosynthesis and catabolism and that this capacity has been distributed in the \textit{BAT1} and \textit{BAT2} \textit{S. cerevisiae} paralogous pair.

Under respiro-fermentative conditions \textit{BAT1} and \textit{BAT2} divergent expression has contributed to emphasize the biosynthetic function of Bat1 and the catabolic function of Bat2. The observation that \textit{BAT1} expression is four-fold higher than that of \textit{BAT2} when cells are grown on glucose ammonia, and that \textit{BAT2} expression is twelve-fold increased in the presence of a non-repressive nitrogen source and further enhanced when VIL is present as sole nitrogen source as compared to that found on ammonium, supports this proposition. Expression differences impact BCAT activity, in the presence of ammonium-glucose Bat1 activity is higher than that of Bat2 improving Bat1 biosynthetic capacity. Conversely, in the presence of glucose as carbon source and VIL as sole nitrogen source, Bat2 activity is enhanced, thus favoring its catabolic role. Bat1 has a limited catabolic character, which is most evident in the double \textit{bat1}D \textit{bat2}D mutant, which is completely unable to utilize VIL as nitrogen source. The fact that \textit{bat1}D is a valine braditroph indicates that Bat2 valine biosynthetic capacity is limited or that the cytosolic valine pool is unable to enter the mitochondria, and thus Bat1 constitutes a committed step to synthesize the mitochondrial pool. These observations underscore the role of differential localization in Bat2 and Bat1 divergence and put forward the possibility that restricted biosynthesis or transport of the cytosolic generated valine pool to the mitochondria could act as positive selection determining \textit{BAT1} retention and Bat1 mitochondrial localization.

\textit{BAT1} and \textit{BAT2} retention constitutes an adaptation to facultative metabolism

\textit{BAT1} expression is higher under fermento-respiratory conditions as compared to that detected under respiratory metabolism, while \textit{BAT2} expression is increased under respiratory conditions.

**Figure 4.** \textit{S. cerevisiae} \textit{BAT1} and \textit{BAT2} display divergent expression profile. Northern analysis was carried out on total RNA obtained from \textit{S. cerevisiae} strains CLA1-2 (wild type), CLA31 (\textit{bat1}A \textit{BAT2}) and CLA32 (\textit{BAT1} \textit{bat2}I). Strains were grown on 2% glucose with either 40mM NH\textsubscript{4} SO\textsubscript{2}, VIL (150 mg/l, 100 mg/l or 30 mg/l of valine (V), leucine (L) or isoleucine (I) respectively or NH\textsubscript{4} SO\textsubscript{2}+VIL as nitrogen sources. Filters were sequentially probed with a 1500 bp \textit{BAT1} fragment, a 1450 bp \textit{BAT2} and a \textit{BamH1-HindIII} 1500 bp \textit{ACT1} DNA fragment as loading control. Numbers indicate relative expression as compared to: Lane 1 the WT grown on glucose VIL, Lane 2 WT grown on glucose NH\textsubscript{4}. Four biological replicates were performed, and representative results are shown.

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Reduced B\textit{AT1} expression under respiratory conditions could contribute to decreased metabolite flow to amino acid biosynthesis favoring energy yielding pathways. Conversely, increased Bat1 activity under fermento-respiratory conditions, would not hinder energy provision, since under these conditions decreased intermediate flow through the tricarboxylic cycle does not hamper energy provision, constituting an adaptation to facultative metabolism.

Under fermentative or respiratory conditions, \textit{Kl} \textit{bat1} \textit{D} mutant is able to grow in the presence of the three BCAAs, indicating that catabolism is not required for growth, however the \textit{bat1} \textit{D} \textit{bat2} \textit{D} mutant is not.
Table 3. Growth phenotypes of single and double bat1Δ and bat2Δ mutants.

| Strain | Relative growth* (%) | Ethanol | NH₄⁺ | NH₄⁺ VIL | VIL |
|--------|----------------------|---------|-------|----------|-----|
| CLA1-2 (WT) | 100 | 100 | 0 |
| CLA31 (bat1Δ) | 87 | 93 | 0 |
| CLA32 (bat2Δ) | 100 | 95 | 0 |
| CLA33 (bat1Δ bat2Δ) | 0 | 0 | 0 |
| KWT | 100 | 100 | 71 |
| CLA34 (Klbat1Δ) | 0 | 64 | 0 |
| CLA34 (CEN KlBAT1) | 90 | 100 | 85 |

*Values are shown relative to growth rate of the wild type strain (0.20 h⁻¹ and 0.11 h⁻¹ on NH₄⁺ and amino acids, respectively); and represent the means from three independent experiments (variation was always ≤10%).

†Amino acids were supplemented at a concentration of 150 mg/l, 100 mg/l or 30 mg/l of valine (V), leucine (L) or isoleucine (I), respectively.

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Concluding remarks

Genetic redundancy is a major feature of virtually all species; duplication of functional genes constitutes a source of new or specialized functions of the encoded proteins. Duplicate genes that are retained either provide an increased dosage of the same product or go through a process of subfunctionalization, during which both copies of the gene lose a subset of their ancestral functions, while acquiring new properties [7,8,18].

BAT1 and BAT2 retention and acquisition of divergent expression profiles, warrants amino acid and α-ketoacid provision under fermento-respiratory and respiratory conditions. In addition, distribution of the biosynthetic and catabolic character of the BCAAT in two isozymes could contribute to the avoidance of futile cycles since the independent regulation of each gene determines the presence of the pertinent isozymes under either biosynthetic or catabolic physiological conditions. The divergent physiological role played by Bat1 and Bat2 is further enhanced through differential localization; each enzyme is located in the compartment in which the pertinent substrates are produced. The fact that KlBat1 is mainly biosynthetic and its catabolic role is only exerted when VIL is added to the medium excludes the operation of futile cycles.

It has been proposed that the specialization of the GDH1- and GDH3-encoded NADP-dependent glutamate dehydrogenases and the LJS2/LJS21-encoded homocitrate synthases could result in the formation of hetero-oligomeric structures showing biochemical properties distinct from those displayed by the homo-oligomers, and which could play an important role under certain environmental conditions [4,6]. Building up of Gdh1-Gdh3 or Lys20-Lys21 hetero-oligomeric isoforms is possible since both enzymes are located in the same subcellular compartment. For Bat1 and Bat2, constitution of hetero-oligomeric isoforms would be hindered by differential localization. Since in many cases oligomerization domains are conserved in paralogous proteins, differential subcellular localization would avoid hetero-oligomerization, preventing the formation of hybrid isozymes whose biological activity could be hindered. The fact that the bifunctional role played by the ancestral-like KlBat1 has been distributed in Bat1 and Bat2, which could be presumed to be oligomeric enzymes [19–21] suggests that for this case, formation of hetero-oligomeric forms could hinder their biological activity, underscoring the role of enzyme relocalization on the functional diversification of duplicate genes.

This study provides an example indicating that the improvement of the functions carried out by a bifunctional gene product can be achieved through gene duplication and further subfunctionalization as has been shown to be the case for the genetic switch...
controlling the yeast galactose utilization pathway. In *S. cerevisiae*, two paralogous genes encode the Gal3 co-inducer and the GAL1-encoded galactokinase, which in *K. lactis* are contained in a single bifunctional ancestral-like gene [22].

Finally and worth mentioning is the existence in *S. cerevisiae* genome of three pairs of duplicated genes respectively encoding piruvate, aspartate and aromatic aminotransferases (*ALT1-ALT2, AAT1-AAT2 and ARO8-ARO9*). *ALT1-ALT2* belong to the duplicated blocks acquired after the WGD event, while *AAT1-AAT2* and *ARO8-ARO9* correspond to independent duplication events. Thus, the described duplication and further diversification of *BAT1-BAT2* may be representative of a general mechanism through which *S. cerevisiae* has improved amino acid metabolism.

**Materials and Methods**

**Strains**

Table 4 describes the characteristics of the strains used in the present work. Independent *bat1A* and *bat2A* derivatives of the CLA1-2 (*ura3 leu2::LEU2*) [4] were obtained using the PCR-based gene replacement protocol described by Wach et al. [23], with *kanMX4* as a marker. Four deoxyoligonucleotides were designed respectively based on the *BAT1* (*M1 and M2*) or *BAT2* (*M3 and M4*) nucleotide sequences and that of the multiple cloning site present in the pFA6a vector [23] (oligonucleotides used for this study are described in Table S1). QIAGEN purified pFA6a DNA was used as template for PCR amplification in a Stratagene Robocycler 40 using standard amplification protocols. The obtained 1584-bp and 1586-bp PCR products respectively harboring *BAT1* or *BAT2* sequences were gel-purified and used to transform strain CLA1-2, generating strains CLA31 (*bat1A::kanMX4 BAT2 ura3 leu2::LEU2*) and CLA32 (*BAT1 bat2A::kanMX4 ura3 leu2::LEU2*).

A CLA1-2 *bat1A bat2A* derivative (CLA33) was isolated from a nourseothricin resistant derivative of the CLA1-2 *bat1A* single mutant obtained by transforming this strain with p4339 EcoRI digested plasmid, which bears a copy of clonNAT gene [23], that replaces the *kanMX4* module by homologous recombination, generating strain CLA31-a (*bat1A::natMX4 BAT2 ura3 leu2::LEU2*). A *bat2A* derivative was generated from CLA31-a, as described above.

To obtain a *Klbat1A* mutant, from the *Kluyveromyces lactis* (*K. lactis*) orthologous *BAT1/BAT2* gene (KLLA0A10307g; *KlBAT1* in this study) *KlBAT1* was replaced by homologous recombination using a module containing the *kanMX4* cassette flanked by 95 bp of 5′ UTR (−105 to −10) and 101 bp of 3′ UTR (+1228 to +1329) sequences of *KlBAT1* gene. The module was amplified from pFA6a plasmid by using deoxyoligonucleotides M5 and M6 (Table S1). The PCR product was purified by using the Wizard SV Gel and PCR Clean-Up System (PROMEGA) and used as template for a second PCR in order to extend the homologous recombination regions. Second PCR was amplified with oligonucleotides M7 and M8 (Table S1). Yeasts were transformed by the method described by Ito et al. [24]. Transformants were selected for either G418 resistance (200 mg/l; Life Technologies, Inc.), or nourseothricin resistance (100 mg/l; Werner BioAgents) or both, on yeast extract-peptone-dextrose (YPD)-rich medium.

**Growth conditions**

Strains were routinely grown on MM containing salts, trace elements, and vitamins following the formula of yeast nitrogen base (Difco). Glucose (2%, w/v) or ethanol (2%, w/v) was used as a carbon source, and 40 mM ammonium sulfate was used as a nitrogen source. Valine (150 mg/l), leucine (100 mg/l), isoleucine (30 mg/l), adenine (20 mg/l), histidine (20 mg/l) or uracile (20 mg/l) were added at the indicated final concentrations when required. 7 mM glutamine or GABA were supplemented when needed. Cells were incubated at 30°C with shaking (250 rpm).

**Construction of low copy number plasmids bearing *BAT1*, *BAT2* or *KlBAT1* genes**

All standard molecular biology techniques were followed as described by Sambrook et al. [25]. *BAT1* or *BAT2* were PCR-amplified together with their 5′ promoter sequence and cloned into the pRS416 (*CEN6 ARSH4 URA3*) low-copy-number plasmid [26,27]. For *BAT1*, a 2313 bp region between −1080 from the start codon and +293 from the stop codon was amplified with deoxyoligonucleotides M9 and M10 (Table S1) generating plasmids pRS416- *BAT1* and pRS426- *BAT1*. For *BAT2*, a 1681 bp region between −471 from the start codon and +79 from the stop codon was amplified with deoxyoligonucleotides M11 and M12 generating plasmids pRS416- *BAT2* and pRS426- *BAT2*. For *KlBAT1* a 1791 bp region between −500 from the start codon and +67 from the stop codon was amplified with deoxyoligonucleotides M13 and M14 (Table S1) The PCR fragment was then cloned into YeplKDS352 (pKD1 ori URA3) plasmid (kindly provided by Dr. Roberto Coria) or pRS416 (*CEN6 ARSH4 URA3*) plasmid generating YEpKD352-*KlBAT1* and pRS416-*KlBAT1* respectively. DNA sequencing was carried out, using the T3/T7 priming sites of pRS316 and pRS426, at the Unidad de Biología Molecular, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México (UNAM). Plasmids were subsequently transformed into CLA1-2 and isogenic *bat1A* and *bat2A* single mutants and *bat1A bat2A* double mutant or *K. lactis* wild type strain and *Klbat1A* mutant.

**Construction of *BAT1* and *BAT2* chimerical fusion plasmids**

Fusions containing either the *BAT1* promoter and the *KlBAT1* coding sequence or the *BAT2* promoter and the *KlBAT1* coding sequence were generated by overlapping PCR amplification. For this purpose, primers M9 and M15 were used to obtain a 1092 bp product corresponding to the *BAT1* promoter sequence and the first 29 bp of the *KlBAT1* coding sequence; this was overlapped with the 1303 bp product of primers M16 and M14, which included the complete *KlBAT1* coding sequence. Similarly, primers M11 and M17 were used to obtain a 483 bp product corresponding to the *BAT2* promoter sequence, together with the first 29 bp of the *KlBAT1* coding sequence, and overlapped with the 1303-bp product of primers M16 and M14, which included

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**Table 4. Strains used in this work.**

| Strain     | Relevant phenotype | Source |
|------------|--------------------|--------|
| CLA1-2     | MATa BAT1 BAT2 ura3 leu2::LEU2 | [4]    |
| CLA31      | MATa bat1A::kanMX4 BAT2 ura3 leu2::LEU2 | This study |
| CLA32      | MATa BAT1 bat2A::kanMX4 ura3 leu2::LEU2 | This study |
| CLA33      | MATa bat1A::NAT bat2A::kanMX4 ura3 leu2::LEU2 | This study |
| **Kluyveromyces lactis** 155 | MATa ade2 his3 ura3 | [37] |
| CLA34      | K. lactis 155 Klbat1A::kanMX4 | This study |

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the complete KlBAT1 coding sequence. The modules obtained were cloned into the pRS416 plasmid, thus generating pRS416 P_{BAT1-yEC} and pRS416 P_{BAT2-yEC} plasmids. These plasmids were subsequently transformed into the CLA1-2 and isogenic bat1A and bat2A single mutants and bat1A bat2A double mutant.

Construction of BAT1 and BAT2 β-galactosidase fusion plasmids

Transcriptional fusions of BAT1 or BAT2 promoters to the coding region of lacZ gene of Escherichia coli were generated by cloning the promoter regions into the YEp353 (2μ ori URA3) plasmid [28]. M9 and M18 deoxyoligonucleotides were used to amplify a 1122 bp sequence corresponding to the BAT1 promoter and M11 and M19 deoxyoligonucleotides were used to amplify a 510 bp region corresponding to the BAT2 promoter. Plasmids generated were YEp353 P_{BAT1} and YEp353 P_{BAT2}. These plasmids were transformed in CLA1-2 wild type strain.

Construction of BAT1 and BAT2 yECitrine tagged mutants

BAT1-yCE and BAT2-yCE were prepared as described by Longtine et al. [29]. Two pairs of oligonucleotides were designed, based on either the BAT1 (M20-M21) or BAT2 (M22-M23) coding sequence and that of pKT175 deoxyoligonucleotides were used to amplify four PCR fragments, plasmids generated were transformed into the BY4741 yeast strain (Table 4). yECitrine-fusion constructs on the carboxy-end of either BAT1 or BAT2 was carried out as previously described [29], and PCR confirmed.

Fluorescent microscopy

Bat1-yECitrine and Bat2-yECitrine tagged strains were used to assay these proteins subcellular localization through confocal microscopy. To confirm mitochondrial localization the strain Bat1-yECitrine was stained with Mito-Tracker Red CMXRos (Molecular Probes) according to manufacturers specifications. Co-localization between the Mito-Tracker and yECitrine was determined through sequential imaging. Confocal images were obtained using a Fluoview FV1000 laser confocal system (Olympus) attached/interfaced to an Olympus IX81 inverted light microscope with a 60× oil immersion objective (UPLSAPO 60× O N.A:1.35), zoom x20.0 and 3.5 μm of confocal aperture. The excitation and emission settings were as follows: yECitrine excitation at 488 nm; emission 520 nm BF 500 nm range 30 nm; Mito-Tracker excitation 543 nm; emission 598 nm, BF 555 nm range 100 nm. The images were collected in a sequential mode z-stack (5μm/slice) using a Kalman integration mode. The subsequent image processing was carried with Olympus Fluoview FV1000 (version 1.7) software.

Cell extract preparation

Cell extracts were prepared using a modified protocol from Rigaut et al. [30] and the NCRR Yeast Resource Center. Briefly, a 21 yeast culture was grown to exponential phase in YPD, cells were collected by centrifugation at 4°C; pellets were washed with bi-distilled cold water and then with cold NP-40 buffer (NαHPO4 15 mM, Na2HPO4·H2O 10 mM, NP-40 1%, NaCl 150 mM, EDTA 2 mM, NaF 80 mM, Na3VO4 0.1 mM, DTT 1 mM, BSA 0.1%, PMSF 1mM, pH 7.2). Cells are collected by centrifugation, suspended in 15–20 ml of NP-40 buffer and transferred to the 50 ml chamber of a bead beater. The same volume of glass beads was added to the suspended cells. Cells were then lysed in an ice bath with 7 cycles of 1 min on/1 min off, with a 3 min off period half-way through. Lysate was transferred to 50 ml Falcon tubes and clarified at 3,000 rpm for 10 min in a refrigerated centrifuge. The supernatant was collected and enzymatic activity was determined immediately.

Branch chain aminotransferase enzymatic assay and protein determination

A previously described assay [31], coupled branch chain aminotransferase activity to NAD(P)H oxidation catalyzed by NAD(P)H Glutamate Dehydrogenase (GDH). However, under our experimental conditions NADP-GDH was able to use the branched-chain α-ketoacids as substrates, thus uncoupling the NAD(P)H oxidation from the branched-amino acid transaminase activity. An alternative method to measure branched-amino acid transaminase activity using the multienzyme α-ketoglutarate dehydrogenase complex from porcine heart (α-KGDH), was developed. Bat1 and Bat2 enzymes used the branched-chain α-ketoacids (BCKA) and glutamic acid as substrates to produce branched chain amino acids (BCAA) and α-ketoglutarate (α-KG), using pyridoxal 5′-phosphate (PP) as cofactor. Then, the (α-KG) produced can be used as substrate, along with Coenzyme A (CoA) and NAD^+ by α-KGDH producing succinyl-CoA and CO₂ thus reducing the NAD^+ to NADH. NAD^+ reduction was monitored measuring Absorbance at 340 nm along the time. The final volume of the assay was 1 ml containing 50 mM MOPS pH 7.1, 1 mM DTT, 0.1 mM CaCl2, 0.47 MgCl2, 1 mM thiamine pyrophosphate (C8754, SIGMA), 0.25 mM CoA (C4780, SIGMA), 0.25 mM pyridoxal 5′-phosphate (PP2955, SIGMA), 0.25 U α-KGDH (K1502, SIGMA), 1 mM NAD^+ (N7004, SIGMA), 5 mM potassium phosphate buffer pH 7.0 and variable concentrations of BCKA and glutamic acid (1501, SIGMA). The reaction was started with the addition of crude cell extracts. All assays were carried out at 30°C in a Varian Cary 400 spectrophotometer with a 1 cm path length. BCKA used in this assay were: α-Ketoisocaproic acid sodium salt (α-KIC; K0629, SIGMA), DL-α-Keto-β-methylvaleric acid sodium salt (α-KMV; K7125, SIGMA) and Sodium methyl valerate (α-KIV; 151395, ICN).

β-galactosidase activity determination

Soluble extracts were prepared by resuspending whole cells in the corresponding extraction buffer [32], cells were lysed with glass beads. β-galactosidase (β-Gal) activities were determined as previously described [33,34]. Specific activity was expressed as nmol of o-nitrophenol produced per minute per milligram of protein. Protein was measured by the method of Lowry [35] using bovine serum albumin as a standard.

Northern blot analysis

Northern analysis was carried out as described previously [34]. Total yeast RNA was prepared as described by Struhl & Davis [36] from exponentially grown cells (OD_{600} 0.4–0.6) or stationary grown cells (3–5 days) in 100 ml cultures. BAT1, BAT2 and KIBAT1 probes were amplified using M24 and M10, M25 and M26, and M16 and M27 deoxyoligonucleotides. BAT1, BAT2 and KIBAT1. Probes include the whole coding region and promoter of each gene. Blots were scanned using the program ImageQuant 5.2 (Molecular Dynamics). Either a 473 bp KSCR1 fragment amplified on K. lactis genomic DNA preparation, using deoxyoligonucleotides M28 and M29 or a 1200 bp ACT1 fragment were used as loading controls.

Supporting Information

Table S1 Deoxyoligonucleotides used in this study. (DOC)
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Author Contributions

Conceived and designed the experiments: MC HQ AG. Performed the experiments: MC FH KL JG GL CA. Analyzed the data: MC HQ AG. Contributed reagents/materials/analysis tools: AG. Wrote the manuscript: AG MG.