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Interactions between carbon and nitrogen sources depend on *RIM15* and determine fermentative or respiratory growth in *Saccharomyces cerevisiae*

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Abstract

Nutritional homeostasis is fundamental for alcoholic fermentation in *Saccharomyces cerevisiae*. Carbon and nitrogen have been related to this metabolic process; nevertheless, little is known about their interactions with the media and the energetic metabolism. Rim15p kinase is a point of convergence among different nutrient-activated signaling pathways; this makes it a target to investigate the relationship between nutritional status and energetic metabolism. To improve the current knowledge of nutrient interactions and their association with *RIM15*, we validated the doubling time as an indicator of growth phenotype, confirming that this kinetic parameter can be related to the cellular bioenergetic status. This endorses the usefulness of a threshold in doubling time values as an indicator of fermentative (≤ 6.5 h) and respiratory growth (≥ 13.2 h). Using the doubling time as response variable, we find that (i) two second-order interactions between type and concentration of carbon and nitrogen sources significantly affected the growth phenotype of *S. cerevisiae*; (ii) these metabolic interactions changed when *RIM15* was deleted, suggesting a dependence on this gene; (iii) high concentration of ammonium (5% *w*/*v*) is toxic for *S. cerevisiae* cells; (iv) proline prompted fermentative growth phenotype regardless presence or absence of *RIM15*; (v) *RIM15* deletion reverted ammonium toxicity when cells were grown in glucose (10% *w*/*v*); and (vi) *RIM15* deletion improves fermentative metabolism probably by a partial inhibition of the respiration capacity. This study reveals the existence of synergic and diverse roles of carbon and nitrogen sources that are affected by *RIM15*, influencing the fermentative and respiratory growth of *S. cerevisiae*.

Keywords *Saccharomyces cerevisiae* · Nutritional homeostasis · Carbon and nitrogen interactions · Respiro-fermentative metabolism · Rim15p

Introduction

S. cerevisiae uses alcoholic fermentation as a pathway to generate energy, and this occurs under specific culture conditions, such as high availability of fermentable carbohydrates (De Deken 1966) or anoxia (Dashko et al. 2014). Although the effect of carbohydrates on fermentative metabolism has been studied for years, little is known about the effect of other nutrients and their interaction on cell maintenance and growth during alcoholic fermentation. Carbon and nitrogen are important energy sources for *S. cerevisiae* cells and regulate a signaling network known as fermentable-growth-medium-induced (FGM) pathway (Thevelein 1994). The FGM activation is modulated by protein kinase A (PKA) that is not dependent on cAMP and

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requires glucose or other rapidly fermentable sugar, plus essential nutrients for growth (Crauwels et al. 1997). Moreover, in the presence of oxygen, carbon sources concentration play a major role in fermentative metabolism induction in *S. cerevisiae* cells (Crabtree 1929; Piškur et al. 2006; Hagman et al. 2014). On the other hand, although nitrogen sources have not been directly implicated in alcoholic fermentation, their availability and concentration have been associated with ethanol biosynthesis (Jiranek et al. 1995). For example, nitrogen starvation is one of the leading causes of sluggish and stuck fermentations (Varela et al. 2004; Marsit et al. 2015). Also, a relationship between certain nitrogen sources (Albers et al. 1996; Berthels et al. 2004; Kemsawasd et al. 2015) and their concentration (Mendes-Ferreira et al. 2004; Tesnière et al. 2013) with ethanol production has been established. Nitrogen has also been related to sugar consumption improvement in media containing a mixture of glucose and fructose (Berthels et al. 2004; Martínez-Moreno et al. 2012). Importantly, carbon and nitrogen signaling pathways are interconnected through Rim15p (Zaman et al. 2008; Conrad et al. 2014). This kinase is needed for entry into the quiescent state and regulates the activation of post-diauxic shift (PDS) and stress response element (STRE) genes (Swinnen et al. 2006). Deletion of *RIM15* gene has been associated with inhibition of UDP-glucose synthesis and improvement of the fermentation rate in *S. cerevisiae* (Watanabe et al. 2015), linking this gene with the carbon metabolism. Nonetheless, little is known about its relationship with nitrogen metabolism. Herein, we propose that metabolic interactions between carbon and nitrogen sources depend on *RIM15* gene, and these influenced the preference between a fermentative or respiratory metabolism in *S. cerevisiae*.

Thereby, the aim of this study is to provide a detailed description of the interaction of different carbon and nitrogen sources in the energetic metabolism of *S. cerevisiae*. We propose the use of a simple growth kinetic model, using a threshold of doubling time (D_t) values of 6 h for fermentation, and 13.2 h for respiration, to discriminate between fermentative and respiratory phenotypes of *S. cerevisiae*. We demonstrate that interactions among nitrogen and carbon sources and their concentrations changed when the *RIM15* gene was deleted, suggesting a dependence on this gene. Also, our data suggest an antagonistic participation of the *RIM15* gene during detoxification of toxic ammonium concentrations.

Materials and methods

Yeast strain and media

The strains used in this study were the BY4742 (Matα; *his3Δ*; *leu2Δ0*; *lys2Δ0*; *ura3Δ0*) of *S. cerevisiae* (wt) and its mutant in the gene *RIM15* (*rim15Δ*) (BY4742; MATα; ura3Δ0; leu2Δ0; his3Δ1; lys2Δ0; YFL033c::kanMX4), both acquired from EUROSCARF (Frankfurt, Germany). *S. cerevisiae* W303 was kindly donated by Dr. Luis Alberto Madrigal Perez. They were maintained in yeast extract-peptone-dextrose (YPD) medium (1% yeast extract, 2% casein peptone, and 2% glucose (Sigma-Aldrich, St. Louis, MO, USA)). Experiments were performed using synthetic complete (SC) medium, consisting of 0.18% yeast nitrogen base without amino acids (Sigma-Aldrich), 0.2% KH_2PO_4 (JT Baker, Center Valley, PA, USA), 1% drop-out mix without uracil (Sigma-Aldrich), and 400 μg/mL of uracil (Sigma-Aldrich). The SC medium was supplemented with glucose (Sigma-Aldrich), sucrose (JT Baker) or galactose (Merck, Darmstadt, Germany) as the carbon source and proline (Sigma-Aldrich), glutamate (Sigma-Aldrich), or ammonium sulfate (JT Baker) as the nitrogen source. For controls of respiratory phenotypes, we used yeast extract-peptone-ethanol (YPE) medium (1% yeast extract, 2% casein peptone, and 5% ethanol (Sigma-Aldrich)), yeast extract-peptone-glycerol (YPG) medium (1% yeast extract, 2% casein peptone, and 5% glycerol (JT Baker)), or SC supplemented with 5% ethanol or 5% glycerol. Percentages are *w*/*v*, except for ethanol and glycerol (*v*/*v*).

Experimental design

To identify interactions between carbon and nitrogen sources and their concentrations that could lead to a fermentative phenotype, we used a 3^4 full factorial randomized design. The following factors were evaluated: type of carbon source (CS) (levels: glucose, sucrose, and galactose), type of nitrogen source (NS) (levels: proline, glutamate, and ammonium sulfate), concentration of carbon in media ([C]) (levels: 0.01, 2, and 10%), and the concentration of nitrogen in media ([N]) (levels: 0.01, 0.5, and 5%). A total number of 81 nutritional conditions (Table S1) were assayed five times with two technical replicates. The experimental design was analyzed using the software JMP v.10 (SAS, Marlow, UK). The same experimental design was performed using the *rim15Δ* strain to verify whether interactions between carbon and nitrogen depend on the presence of this gene.

Growth curves and doubling time (D_t) calculation

Cultures were grown on honeycomb plates (growth curves, Piscataway, NJ, USA) with 145 μL of medium per well. Each well was inoculated with 5 μL of an overnight *S. cerevisiae* BY4742 culture, grown in YPD medium at 30 °C in an orbital shaker (MaxQ 6000, Thermo Scientific, Waltham, MA, USA) at 250 rpm. Samples were incubated at 30 °C for 48 h using a BioScreen (model C MBR, growth curves) programmed with continuous shaking at medium speed and readings at 600 nm, every 30 min. Then the D_t ($\ln 2/\mu$) was calculated fitting the exponential phase of the growth

curves with the exponential growth curve (Eq. 1). Data were analyzed using the GraphPad Prism v5 software (La Jolla, CA, USA).

$$X = X_0 e^{\mu t} \quad (1)$$

where X is cells concentration, X_0 is cells concentration at zero time, μ is the specific growth rate, and t is the time (h).

Validation of doubling time (D_t) as a preliminary parameter to determine growth phenotype of *S. cerevisiae*

To validate the usefulness of D_t for presumptive identification of the respiratory and fermentative growth, we determined the bioenergetic status of the cells. Six different concentrations of glucose were used (*w*/*v*): 0.01 (respiratory condition), 0.5, 1, 2, 5, and 10% (fermentative condition). The bioenergetic status was established by quantification of the basal mitochondrial respiration, the maximal respiration rate, and estimation of spare respiratory capacity (SRC). In addition, the glycolytic flux was measured, and these data were related to the growth phenotype. Finally, a threshold for D_t values was set up to discriminate between the respiratory and fermentative growth.

Determination of in situ mitochondrial respiration

The mitochondrial respiration was evaluated by oxygen consumption, measured by polarography at 28 °C using a Clark detector (model 5300, YSI, Yellow Springs, OH, USA) as described by Madrigal-Perez et al. (2016). Briefly, *S. cerevisiae* cultures were harvested by centrifugation at 5000 ×*g*, and 125 mg of cells was used for each consumption assay. Pellets were placed into 5 mL of MES-TEA buffer (10-mM 2-(*N*-morpholino) ethanesulfonic acid (Sigma-Aldrich), adjusted to pH 6.0 with triethanolamine (TEA) (Sigma-Aldrich)), in a closed chamber with constant stirring. Then, basal respiration was determined using 10-mM glucose as a substrate. Maximal rate of mitochondrial respiratory chain function was evaluated with the addition of the mitochondrial uncoupler CCCP (10 μM). The oxygen consumption due to non-mitochondrial sources was determined using antimycin A (Sigma-Aldrich). Three independent experiments with two technical replicates were carried out. The oxygen consumption was expressed as nat O/[(min) (mg of cells)]. SRC was calculated by subtracting the value of basal respiration from the maximum respiratory capacity (Nicholls and Ferguson 2013).

Extracellular acidification rate

To determine the glycolytic flux at basal state, we used the extracellular acidification rate (ECAR) as an indirect measurement and determined as reported by Madrigal-Perez et al. 2015. Briefly, yeast cells were grown in YPD medium with 0.01 or 10% glucose at mid-log phase (0.6 OD_{600}) at 30 °C with constant shaking at 250 rpm; cell pellets were collected by centrifugation for 5 min at 5000 rpm. Cells (2.16×10^5) were placed into 25 mL of deionized water, and pH changes were measured using the Titrando equipment (Mod. 902, Metrohm, Herisau, Switzerland). After 120 s, 1.4 mL of 1-M glucose was added, and pH was measured for 3 min. ECAR was calculated from the slope of a plot of pH (milliunits) vs. time (s) of the second minute after glucose addition, using the GraphPad v.5 software. Five independent experiments with two technical replicates were performed.

Ethanol, glycerol, and acetate quantification

Yeast cultures were grown at 30 °C with constant shaking at 250 rpm in SC medium supplemented with 0.01% sucrose + 5% proline, 2% sucrose + 5% ammonium, or 10% glucose + 5% ammonium for 16 h. Cultures supernatant were collected by centrifugation at 10,000 rpm for 10 min at 4 °C and filtered using nylon membranes (Millipore, Ireland) with a pore size of 0.20 μm.

Glycerol and ethanol quantification was performed by high-performance liquid chromatography (HPLC) (Mod. 1220 Infinity, Agilent Technologies, California, USA), fitted with refractive index detector, and the MetaCarb H Plus 300 × 7.8-mm column (Agilent Technologies). The mobile phase was 0.05-M sulfuric acid, at a flow rate of 0.35 mL/min, and running time of 35 min. The oven temperature was 75 °C and detector temperature of 55 °C. The sample injection volume was 20 μL.

Acetate quantification was performed by gas chromatography (GC) (Mod. 7890A Agilent Technologies) coupled to a mass spectrometry detector (model 5975C, Agilent Technologies). The incubation temperature of the samples was 60 °C for 30 min, and a headspace sampling protocol was used with splitless injection. The temperature ramp started at 40 °C and increased 5 °C/min up to 100 °C, then increased 30 °C/min until 280 °C, while the temperature of the injection port was maintained at 280 °C. A 60-m (250 × 0.25 μm) DV5 column (Agilent Technologies) was used. The flow rate was 1 mL/min, and the carrier gas was helium.

Carbon and nitrogen consumption

To quantify carbon consumption, the dinitrosalicylic acid (DNS) method for microplates was assayed (Wood et al. 2012). For sucrose determination, 20 μL of concentrated HCL (J.T. Baker) was added to 1 mL of sample and hydrolyzed at 90 °C for 5 min. Nitrogen consumption was determined by the ninhydrin assay using the microplate protocol (Abernathy et al. 2009).

Statistical analyses

The statistical significance of data from basal respiration, maximum respiratory capacity, SRC, and ECAR was evaluated by one-way ANOVA, followed by Tukey's test. D_t values were compared using one-way ANOVA, followed by a Dunnett's test to assess statistical significance; cultures grown in 10% glucose were the positive control for the fermentative phenotype. Results are presented as mean values ± standard deviation unless otherwise indicated. Pearson's correlation was used to evaluate the significant correlation between two variables. Statistical analyses were performed using the GraphPad Prism v.5 software.

Results

Doubling time validation as a preliminary parameter to determine growth phenotype of *S. cerevisiae*

To demonstrate the usefulness of D_t as a valid parameter to discriminate between the respiratory and fermentative phenotype preliminarily, we assessed the effect of glucose concentration in the mitochondrial respiration and glycolytic flux of *S. cerevisiae*. These data helped us to link the bioenergetic status of the cells with their growth phenotype.

Inhibition of mitochondrial respiration is the main characteristic of the Crabtree effect (Ibsen 1961). Therefore, to validate this phenotype, we measured mitochondrial respiration of *S. cerevisiae* cells grown in YPD medium supplemented with six levels of glucose (*w*/*v*): low (0.01%, respiratory), intermediate (0.5, 1, 2, and 5%), and high (10%, fermentative). As expected, we found a drastic inhibition of mitochondrial respiration at basal state and maximal respiratory capacity in *S. cerevisiae* cultures grown in 10% glucose in comparison to yeast cultures grown in 0.01% glucose, and the mitochondrial respiration inhibition was intensified as glucose concentration increased (Fig. 1a–b). SRC indicates the ATP yield that can be achieved using mitochondrial respiration as the primary pathway for energy production in response to a sudden energy demand (Brand and Nicholls 2011; Desler et al. 2012). Our data showed that SRC decreased in cells grown with high glucose concentration (Fig. 1c). According to these data, low glucose cultures respond to a sudden energy demand producing ATP through the oxidative phosphorylation pathway mainly, whereas high glucose cultures used the substrate-level phosphorylation as main energy producing pathway. Furthermore, the ATP yield in *S. cerevisiae* from oxidative phosphorylation is near 18 ATP molecules per glucose, which is higher than the ATP yield from substrate-level phosphorylation of 2 ATP molecules per glucose (Pfeiffer and Morley 2014). Therefore, fermentative cells compensate the low ATP yield increasing the glycolytic flux (Daran-Lapujade et al. 2007). Consequently, we measured glycolytic flux with ECAR (Fig. 1d). As expected, glycolytic flux increased as glucose concentration raised, showing the higher values at 10% glucose, corresponding to a fermentative growth phenotype. Thus, these data indicate that 10% glucose inhibits oxygen consumption and increases the glycolytic flux in *S. cerevisiae*, confirming the Crabtree effect under this condition. On the contrary, at 0.01% glucose, a fully respiratory phenotype is evident in *S. cerevisiae*. Interestingly, cultures supplemented with 0.5% glucose showed high mitochondrial respiration (Fig. 1a–b) and also high glycolytic flux (Fig. 1d), with an intermediate SCR value (Fig. 1c) indicating a mixed respiro-fermentative metabolism in this growth condition.

The establishment of fermentation as the major pathway to generate energy in *S. cerevisiae* cells may produce profound changes in the growth rate. To evaluate this effect, batch growth kinetics of *S. cerevisiae* were conducted. Cultures grown in 10% glucose displayed a faster growth than using 0.01% glucose (Fig. 2a), indicating faster growth when fermentation is the primary pathway for energy production than cells using respiration as the only path for energy output. Interestingly, the growth curve of cultures supplemented with 0.5% glucose showed a robust diauxic shift, with a smaller exponential phase than that of cultures supplemented with 10% glucose, probably due to glucose exhaustion in the medium. The rapid glucose depletion probably triggered the respiro-fermentative metabolism, which is consistent with the results from the bioenergetic status (Fig. 1). Additional growth kinetic experiments were performed to corroborate the respiratory growth phenotype using non-fermentable carbon sources that are exclusively metabolized by respiration in *S. cerevisiae*. The growth of *S. cerevisiae* in media supplemented with ethanol and glycerol showed similar behavior as when 0.01% glucose was used (Fig. 2b), in agreement with the respiratory growth performance previously reported (Yoboue et al. 2014). Thus, these data validated that the growth kinetics parameter D_t is useful to determine whether *S. cerevisiae* sustains growth by respiration or by fermentation in a preliminary way. The threshold values of D_t were set up to indicate respiratory ($\geq$ 13.2 h) or fermentative phenotypes ($\leq$ 6.5 h) (Fig. 2c), it is important to mention that this classification is presumptive and more experiments are required to corroborate the energy metabolism of the yeast.

Interactions among carbon, nitrogen, and their concentration affect *S. cerevisiae* phenotype

Although carbon and nitrogen have been identified as nutrients that influence fermentation, little is known about their interactions. Hence, we performed a full factorial experimental design expecting to find interactions between the carbon and nitrogen sources. Analysis of variance of the 3^4 factorial design showed that the empirical model fits the experimental data ($p < 0.001$) (Table S2). Ten significant effects were

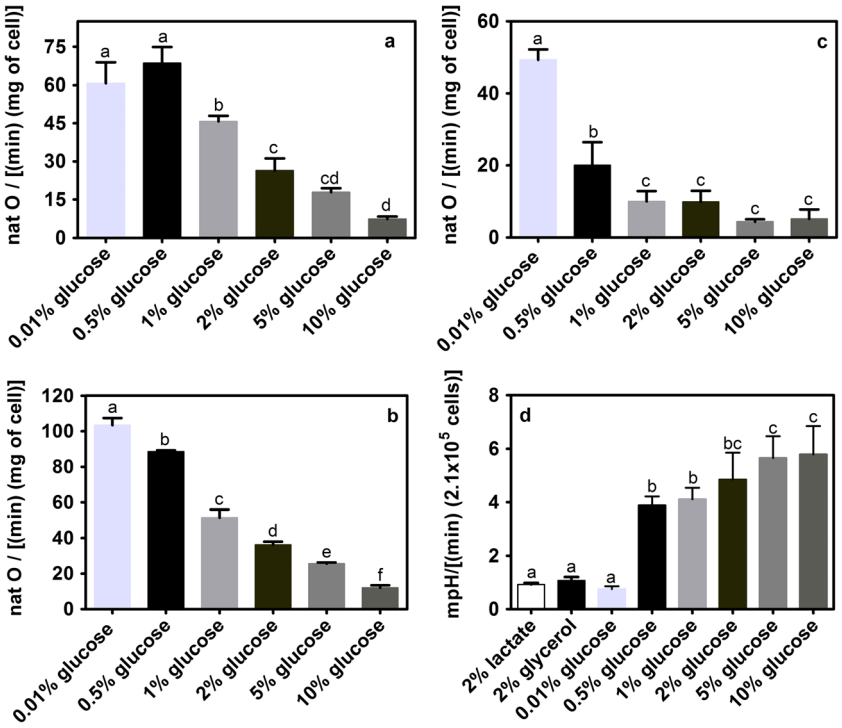

Fig. 1 Effect of glucose concentration on the mitochondrial respiration rate and glycolytic flux in *S. cerevisiae*. Cells for mitochondrial respiration in situ were cultured in YPD medium supplemented with 0.01 and 10% glucose at 30 °C at 200 rpm. **a** Basal respiration, **b** maximal respiratory capacity, **c** spare respiratory capacity (SRC), and **d** extracellular acidification rate. The results are presented as mean ± SD. The statistical significance of the measurements was determined using a one-way ANOVA followed by a Tukey's test (*$p < .01$)

observed ($p < 0.037$), three main factors, five effects with double factor interactions, and two effects with triple factor interactions. The higher-order significant interactions were further analyzed: [N]*CS*NS ($p < 0.007$) and [C]*[N]*NS ($p < 0.001$) (Table S3). They were chosen because valuable information would be lost if only the principal effects were analyzed, and thus we would be neglecting that the principal factors effects depend on each other. Additionally, according to the heredity principle, when an interaction is significant, at least one of its parental factors is significant. Therefore, their influence on the response variable is taken into account when the interaction is considered (Wu and Hamada 2009). For practical reasons, treatments where growth was inhibited were excluded from further analysis. Two interactions that affect *S. cerevisiae* growth were identified; the first one was among the nitrogen concentration, the types of carbon, and nitrogen

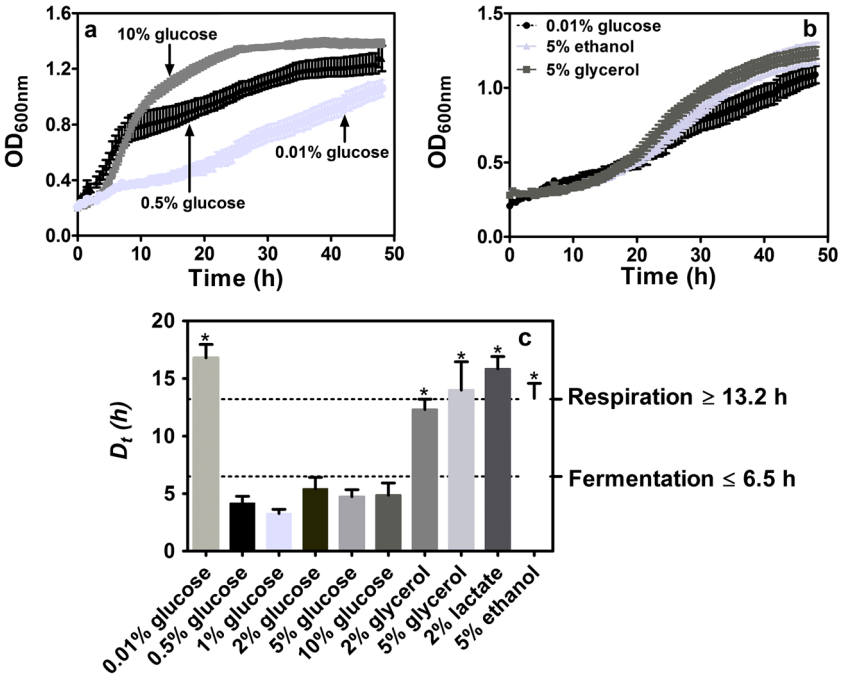

Fig. 2 Effect of carbon source and its concentration on *S. cerevisiae* growth phenotype and doubling time values. **a** Growth of *S. cerevisiae* cultures with different glucose concentrations; fermentative (10% glucose), respire-fermentative (0.5%), and respiratory (0.01% glucose). **b** Growth of *S. cerevisiae* in non-fermentable carbon sources 5% ethanol and 5% glycerol and with 0.01% glucose. Data are presented as mean ± SD and **c** D_t values of *S. cerevisiae* cultures under respiratory or fermentative growth. Data are presented as mean ± SD. Statistical analyses were performed using a one-way ANOVA followed by a Dunnett's test (*$p < 0.01$ vs. 10% glucose)

source ([N]*CS*NS) factors. The second interaction that significantly ($p < 0.000$) affects *S. cerevisiae* growth was among carbon concentration, nitrogen concentration, and type of nitrogen source ([C]*[N]*NS) factors (Fig. S1). These results indicate that nitrogen source and its concentration affect *S. cerevisiae* growth in a carbon source-dependent manner, and the effect of nitrogen and carbon concentrations upon *S. cerevisiae* growth phenotype is reliant on the nitrogen source used. We performed the same experimental design with the *S. cerevisiae* W303 strain, another representative laboratory strain, and the significant interactions pattern changed between BY4742 and W303 strains. However, the second-order interaction [C]*[N]*NS was significant for both strains, suggesting that there are common metabolic regulators in both strains (Table S4).

Furthermore, we have taken advantage of the usefulness of D_t values to predict respiratory and fermentative growth of *S. cerevisiae* presumptively. Thereby, from the total of 81 cultures of *S. cerevisiae*, 45.7% displayed a fermentative phenotype, 29.6% respiro-fermentative behavior, 19.7% full respiratory growth, and 5% growth inhibition (Fig. 3a). Using proline as nitrogen source, 15 cultures of *S. cerevisiae* from a total of 27 were classified as fermentative phenotype, seven as respiro-fermentative, and five as respiratory phenotypes, making this amino acid the best for fermentative growth induction. The nitrogen source that showed an intermediary performance was glutamate, with a total of 13 cell cultures displaying fermentative phenotype, 11 as respiro-fermentative, and three as respiratory phenotypes (Fig. 3b). NH_4^+ prompted the poorest yield of cultures showing fermentative behavior with only nine cultures showing fermentative, six respiro-fermentative, and eight respiratory growths (Fig. 3b). Interestingly, four cultures displayed growth inhibition; three of them were supplemented with the highest level of NH_4^+ (5%) and the lowest level of carbon (0.01%); the remaining culture was supplemented with NH_4^+ (5%) and galactose (10%). Additionally, cultures with the highest concentrations of NH_4^+ (5%) and of carbon (10%) showed decreased growth rate (Fig. 3c), suggesting that NH_4^+ toxicity depends on the type and concentration of the carbon source. These results demonstrate that high levels of NH_4^+ are toxic to *S. cerevisiae* and suggest hindering of energetic metabolism.

On the other hand, glucose and sucrose produced the same trend in growth phenotypes: 15 cultures with fermentative behavior, six with respiro-fermentative growth, only five with full respiratory phenotype, and one with growth inhibition. These data indicate that glucose and sucrose metabolism in *S. cerevisiae* BY4742 respond to changes in nitrogen sources and their concentrations in a similar way. Moreover, galactose was the carbon source exhibiting the lowest fermentative behavior induction; this molecule displays only eight cultures of *S. cerevisiae* with fermentative phenotype, 15 with respiro-fermentative growth, two with full respiratory phenotype, and two with growth inhibition (Fig. 3e). These data agree with preferential respiro-fermentative metabolism for galactose catabolism (Fendt and Sauer 2010) and suggest that *S. cerevisiae* ferments galactose under more specific nutrient conditions (i.e., 2% galactose + 5% glutamate) as compared to glucose and sucrose. Unlike glucose and sucrose, that at a high concentration generally prompted a fermentative metabolism; galactose did not necessarily induce a fermentative metabolism in most of the conditions tested. Overall, these results indicate that although the nitrogen source influences fermentative growth, the mechanism implicated in this effect does not disturb the carbon preference of *S. cerevisiae*.

Deletion of *RIM15* changes significant interactions among carbon, nitrogen and their concentrations affecting the growth phenotype of *S. cerevisiae*

To confirm the hypothesis that Rim15p regulates the interactions among carbon, nitrogen, and their concentrations, we performed the same experimental design previously described, but using the *rim15Δ* strain. Analysis of variance of the 3^4 factorial design showed that the empirical model fits the experimental data ($p < 0.0001$) (Table S5). Eleven significant effects were observed ($p < 0.027$), four main factors, three effects with double factor interactions, three effects with triple factor interactions, and one effect of fourfold factor interaction (Table S6). Thus, deletion of *RIM15* changes the interactions between carbon and nitrogen sources affecting the growth phenotype of *S. cerevisiae,* suggesting a pivotal role of this gene in the energetic metabolism of this yeast. The four-factor interaction [C]*[N]*CS*NS was further analyzed. Again, the conditions that triggered inhibition or growth deceleration were omitted from the analysis (Fig. S2). This four-factor interaction suggests that deletion of *RIM15* affects growth behavior depending on: carbon and nitrogen concentration and carbon and nitrogen source used, suggesting that Rim15p participates in both carbon and nitrogen metabolism.

According to D_t value threshold previously defined (fermentative ≤ 6.5 h and respiratory ≥ 13.2 h) for the total of 81 cultures from the *rim15Δ* strain, 50.62% showed fermentative phenotype, 33.33% displayed respiro-fermentative behavior, 12.34% respiratory growth, and 3.31% growth inhibition (Fig. 4a). These results corroborate that deletion of *RIM15* enhances the fermentative metabolism. Moreover, the best nitrogen source was proline; 15 cultures using this nitrogen source displayed a fermentative phenotype, ten respiro-fermentative behaviors, and two respiratory growths. Glutamate was the nitrogen source that had an intermediary performance, 14 cell cultures presented fermentative behavior, ten respiro-fermentative growth, and only three cultures showed entirely respiratory phenotypes. NH_4^+ was the nitrogen source with the most deficient performance, 11 conditions using this molecule as substrate showed fermentative growth,

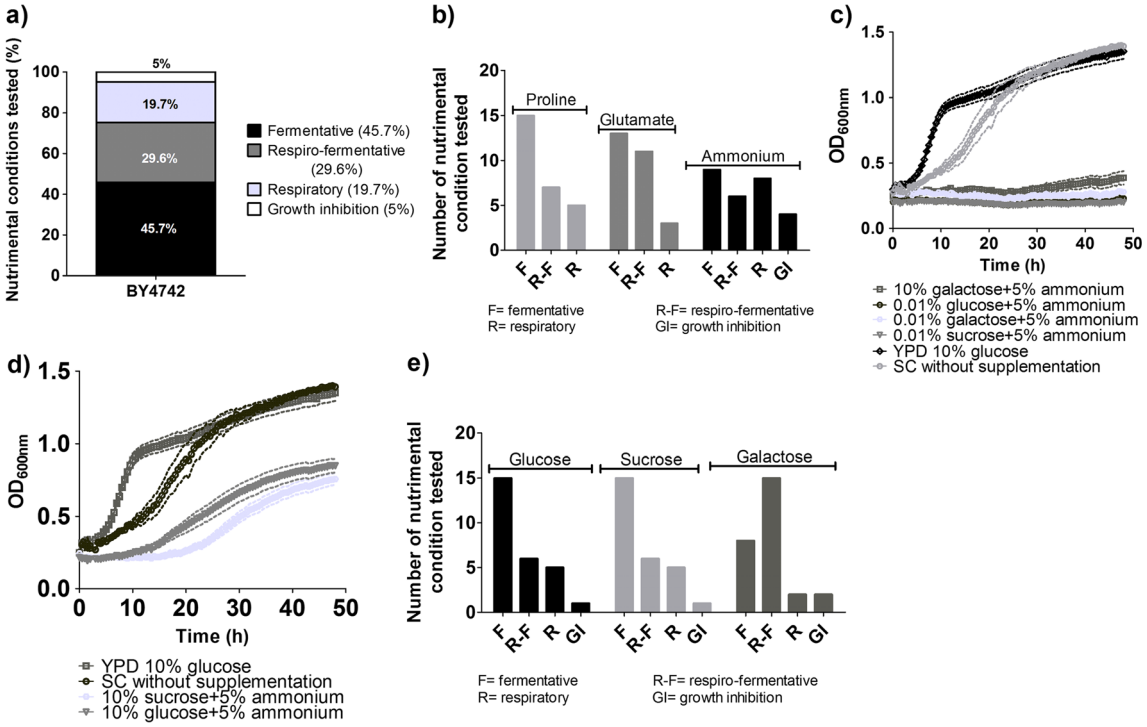

Fig. 3 Effect of the interaction among the factors: CS, NS, and [N] on the growth phenotype of *S. cerevisiae*. *S. cerevisiae* was grown under 81 different nutritional conditions at 30 °C. **a** Growth phenotypes percentage obtained with the wt strain, **b** distribution of phenotypes obtained with the carbon sources, **c** growth curves of *S. cerevisiae* where 5% NH_4^+ prompted growth inhibition, **d** growth curves of *S. cerevisiae* where 5% NH_4^+ induced growth deceleration, and **e** distribution of phenotypes obtained with the nitrogen sources

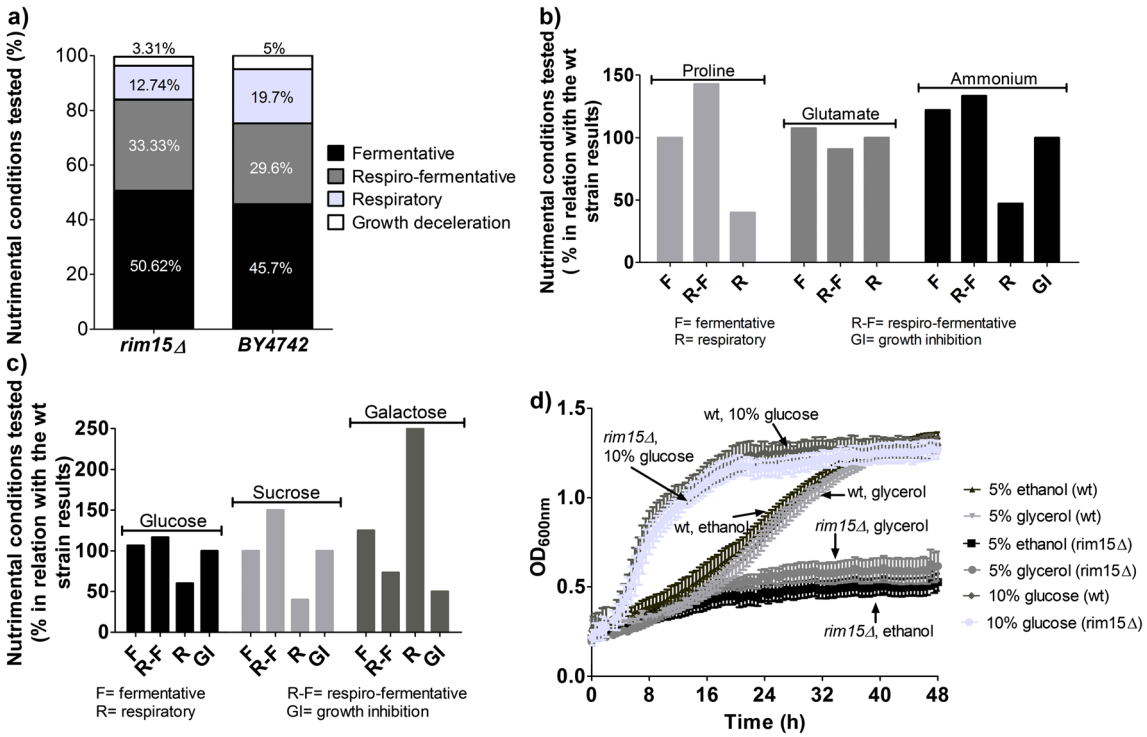

Fig. 4 Effect of the interaction among the factors: CS, NS, [N], and [C] on the growth phenotype of *S. cerevisiae*. *S. cerevisiae* was grown under 81 different nutritional conditions at 30 °C. **a** Growth phenotypes percentage obtained with the wt and the *rim15Δ* strains, **b** distribution of phenotypes of the *rim15Δ* obtained with the carbon sources in relation with the obtained with the wt strain, **c** distribution of phenotypes of the *rim15Δ* obtained with the nitrogen sources in relation with the obtained with the wt strain, and **d** growth curve comparison between the wt strain and the *rim15*Δ strain; the mutant strain showed growth problems under fully respiratory conditions; data are presented as mean ± SE

eight respiro-fermentative phenotypes, five full respiratory behavior, and three cultures showed growth inhibition (Fig. 4b). These results suggest that proline enhances fermentative metabolism in a mechanism that does not involve *RIM15*.

On the other hand, when the *rim15Δ* strain was used, the best carbon source was glucose, which induced a fermentative behavior in 16 cultures, seven cultures showed respiro-fermentative behavior, three displayed full respiratory phenotypes, and one exhibited growth inhibition. Sucrose had an intermediary performance prompting a fermentative metabolism in 15 cell cultures, nine with respiro-fermentative behavior, two with full respiratory phenotype, and one with growth inhibition. The carbon source inducing the lowest number of fermentative cultures was galactose, causing ten cell cultures with fermentative growth, 11 with respiro-fermentative behavior, five with respiratory phenotype, and one with growth inhibition (Fig. 4c). These results suggest that deletion of *RIM15* enhances fermentation and a respiro-fermentative metabolism independently of the carbon source used. Interestingly, *rim15Δ* strain displayed growth inhibition under full respiratory conditions in SC medium (5% ethanol and 5% glycerol) (Fig. 4d), suggesting a respiratory deficiency that could explain the enhancement of fermentative metabolism, although more evidence is needed to corroborate this hypothesis.

Additionally, deletion of *RIM15* prompted significant changes on D_t under 26 nutrimental conditions, while only eight conditions showed changes in growth phenotype. 15 cultures showed a fermentative phenotype, eight respiro-fermentative phenotypes, and only three full respiratory phenotypes (Fig. 5). Interestingly, in nutrimental conditions with the highest NH_4^+ concentration (5%) where the wild type strain showed growth deceleration, deletion of *RIM15* stimulated the reversion of this phenotype when 2 and 10% of glucose or sucrose were used. This behavior was further evident when media was supplemented with glucose (10%) and NH_4^+ (5%) (Fig. 6), suggesting an antagonistic participation of *RIM15* in NH_4^+ detoxification.

Deletion of *RIM15* redirection energy metabolism toward fermentation in *S. cerevisiae*

To prove the usefulness of D_t as a means of preliminary discrimination between cultures presenting a respiratory or fermentative metabolism, we chose three nutrimental conditions that prompted a different phenotype between the wt and mutant strains for further analysis. The selected conditions were as follows: 0.01% sucrose + 5% proline, 10% glucose + 5% ammonium, and 2% sucrose + 5% ammonium. Oxygen consumption and metabolite accumulation (ethanol, acetate, and glycerol) were determined to demonstrate that growth phenotype is related to the energetic metabolism. Carbon and nitrogen consumption was quantified to verify if changes in D_t were associated with energy metabolism. As anticipated, basal respiration was higher in the nutrimental conditions that led to D_t values ≥ 13.2 h than conditions prompting to D_t values corresponding to fermentative growth (≤ 6.5 h) (Fig. 7a). These variables showed a significant Pearson's correlation coefficient $r = 0.934$ ($p = 0.001$) (Fig. 7b), indicating that changes in D_t values are related to changes in the basal respiration, and both variables tend to increase or decrease accordingly. Also, the maximal respiratory capacity showed that mitochondrial respiration was more inhibited in *rim15Δ* than in the wt strain (Fig. 7c), corresponding to the fermentative and respiro-fermentative phenotypes induced by these nutritional conditions in *S. cerevisiae*. Moreover, SRC is also in agreement with culture phenotypes indicating higher ATP production capacity by oxidative phosphorylation in response to a sudden energy requirement, when a respiratory or respiro-fermentative growth phenotype was observed than in the fermentative ones (Fig. 7d). Also, we observed that in the BY4742 strain, when 5% ammonium was used as nitrogen source, a respiratory metabolism was predominantly present even when the carbon concentration (2% sucrose or 10% glucose) was sufficient to induce the Crabtree effect. These results suggest that 5% of ammonium caused a metabolic shift from fermentation to respiration when sucrose or glucose is used as carbon source in high concentrations. In general, *rim15Δ* strain showed less oxygen consumption than the wt strain; therefore, these results are in agreement with our hypothesis that *RIM15* deletion could inhibit the respiratory pathway driving the energetic metabolism toward fermentation.

We found a significant correlation between D_t and the basal respiration under a broad number of nutritional conditions (Fig. S3). A significant correlation ($p = 0.038$) for 92.3% of the data was observed, and 18% of the variance is shared between the two variables. In the remaining 7.7% of the data, representing two nutrimental conditions tested, no significant correlation was found. A particular behavior was detected at both conditions, such as excessive oxygen consumption (487.3 ± 8.35 nat O/[(min) (mg of cells)]) corresponding to 0.01% glucose + 0.5% glutamate (Fig. S4) and low oxygen consumption with slow growth at 10% sucrose + 5% ammonium (Figs. S3 and S4). More experiments are required to obtain reasonable explanations about these phenotypes. Overall, these data confirm that D_t is a useful tool to discriminate between fermentative and respiratory metabolism in *S. cerevisiae*. However, it is important to highlight that we propose its use as a preliminary screening tool and that additional experiments are needed to confirm the primary energy production pathway.

Ethanol, glycerol, and acetate were quantified to verify if fermentation was the primary energy production pathway. As expected, when cultures showed a higher value of D_t (Fig. 5), fermentative metabolite accumulation was lower than in those

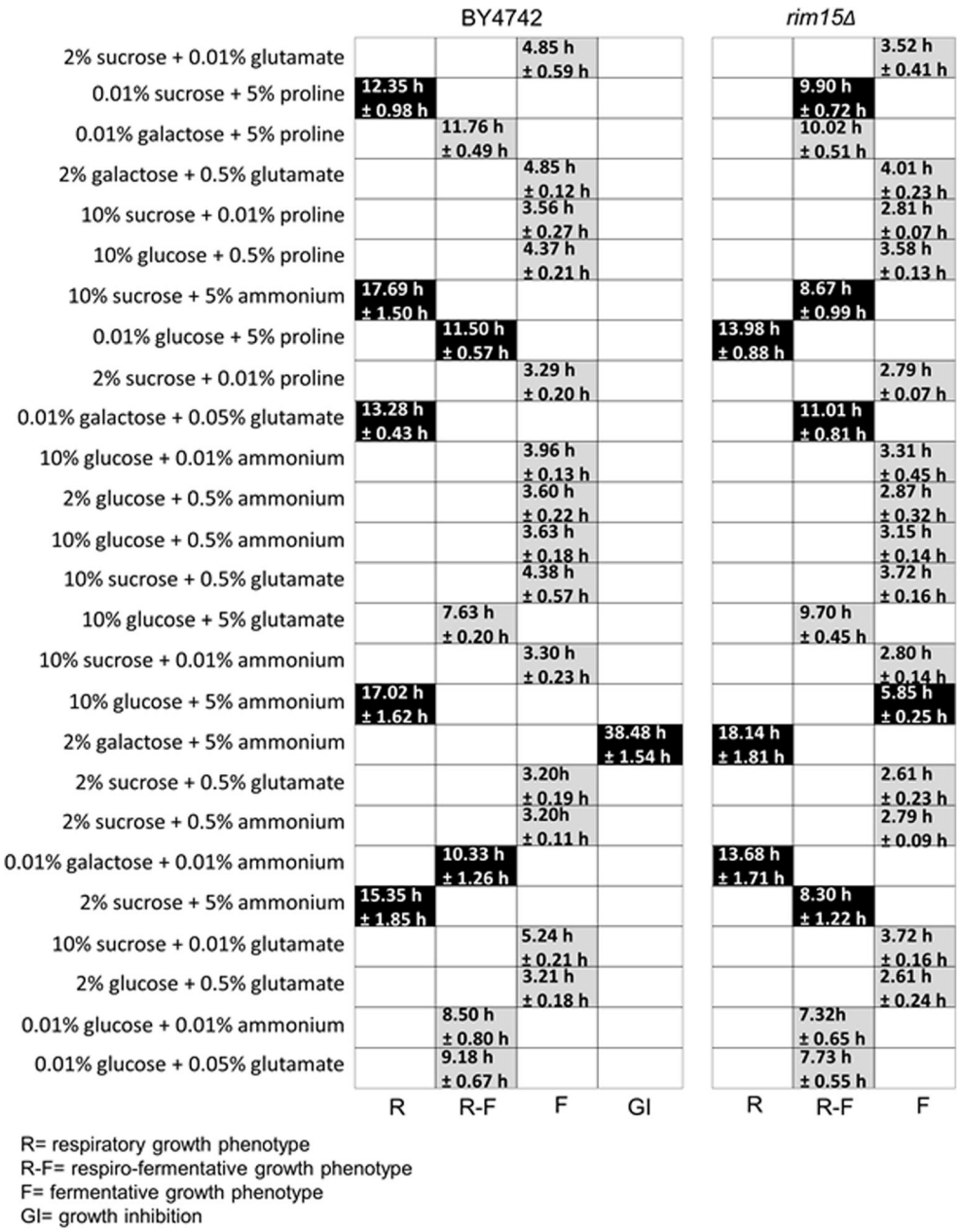

Fig. 5 Effect of deletion of *RIM15* on doubling time values and growth phenotype of *S. cerevisiae*. The wt and *rim15Δ* strains were grown at 30 °C on media supplemented with different carbon and nitrogen sources. Only nutritional conditions with significant differences between D_t values of the wt and mutant strain are shown. Black boxes represent a significant change in D_t values and growth phenotype when *RIM15* was deleted, while gray boxes represent only a significant change in D_t values in absence of *RIM15*. The statistical significance of the measurements was determined using the Student's *t* test with a two-tailed non-paired comparison (*$p < .0.01$)

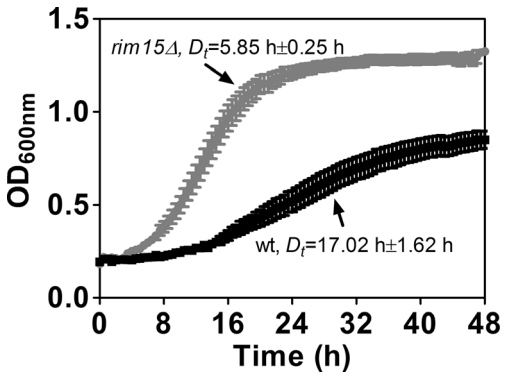

Fig. 6 Effect of deletion of *RIM15* at 5% ammonium and 10% glucose supplementation

cultures with smaller D_t values (Fig. 8). The strain *rim15Δ* showed higher accumulation of ethanol (Fig. 8a), glycerol (Fig. 8b), and acetate (Fig. 8c) than the wt strain when using 10% glucose or 2% sucrose. Interestingly, even at 0.01% of sucrose, higher accumulation of glycerol was observed for the mutant strain (respiro-fermentative behavior) (Fig. 8b), than for the wt strain (fully respiratory phenotype). Also, fermentative metabolites could not be detected in wt strain cultures, confirming that deletion of *RIM15* improves fermentative metabolism.

Nitrogen and carbon consumptions were quantified to know if *RIM15* deletion improves fermentative metabolism by increasing nutrient uptake (Fig. 9). Carbon consumption improved when *RIM15* was deleted only when

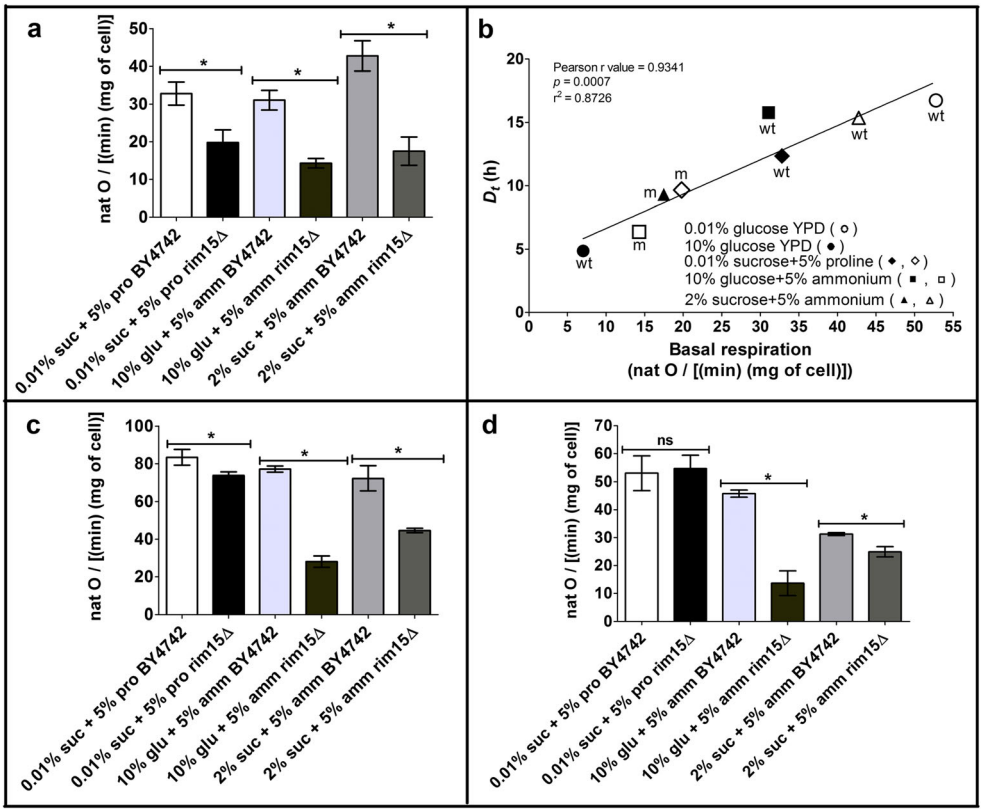

Fig. 7 Effect of *RIM15* deletion in the bioenergetic status of *S. cerevisiae* BY4742. **a** basal respiration, **b** Pearson's correlation between the basal respiration and the D_t, **c** maximal respiratory capacity, and **d** spare respiratory capacity (SRC). The results are presented as mean ± SD. The statistical significance of the measurements was determined using a Student's *t* test (wt vs. *rim15Δ*) (*$p < .01$)

the carbon source was sucrose (Fig. 9a), whereas nitrogen consumption only improved when proline was used as nitrogen source (Fig. 9b). These results indicate that deletion of *RIM15* could improve fermentative metabolism not only by inhibiting respiration but also by improving uptake of some nutrients. Nonetheless, more experiments are required to confirm this hypothesis.

Discussion

It is well known that nutritional status influences fermentation performance in *S. cerevisiae* (Rollero et al. 2014) and therefore its energetic metabolism. For example, during winemaking, nitrogen supplementation of musts is a common practice for enhancement of fermentation kinetics (Clement et al. 2013); and during beer fermentation, one of the crucial factors to achieve high fermentation performance is the nutritional status of the wort (Bokulich and Bamforth 2013). Two essential nutrients for yeast growth are carbon and nitrogen, but little is known about their interactions and influence on induction of fermentative growth. To gain insight of the carbon and nitrogen interactions, we performed growth experiments on strain BY4742 of *S. cerevisiae* under 81 culture conditions with several concentrations of different carbon and nitrogen sources using D_t values to discriminate between respiratory and fermentative phenotypes. Herein, we validated that basal respiration and maximal respiratory capacity are inhibited at high glucose concentration (10%), indicating that the SRC is high when cells are forced to use the respiratory metabolism, i.e., low levels of glucose (0.01%) or respirable carbon sources such as ethanol. Then, we confirmed that glycolysis is more active when *S. cerevisiae* is grown under fermentative (10% glucose) than under respiratory conditions (0.01% glucose), as reported by van den Brink et al. (2008) using a chemostat. Also, *S. cerevisiae* showed a particular growth phenotype under fermentation, which is different to the respiratory phenotype. These data evidenced the bioenergetic status of yeast cells and validate the usefulness of parameter D_t as an indicator of fermentative and respiratory metabolism. Previously, a relation between growth rate (this parameter is proportional to D_t) and degree of respiration was found by Fendt and Sauer (2010), showing that growth rate decreases while the level of respiration increases, corroborating the use of D_t as a good parameter to identify respiratory of fermentative metabolism of *S. cerevisiae*. Therefore, we used D_t as the response variable for the experimental design and proved that two interactions among carbon and nitrogen sources and both concentrations influenced the induction of the fermentative phenotype in *S. cerevisiae*. Furthermore, deletion of *RIM15* affects these interactions leading to changes in this pattern, resulting in a significant third-order interaction among

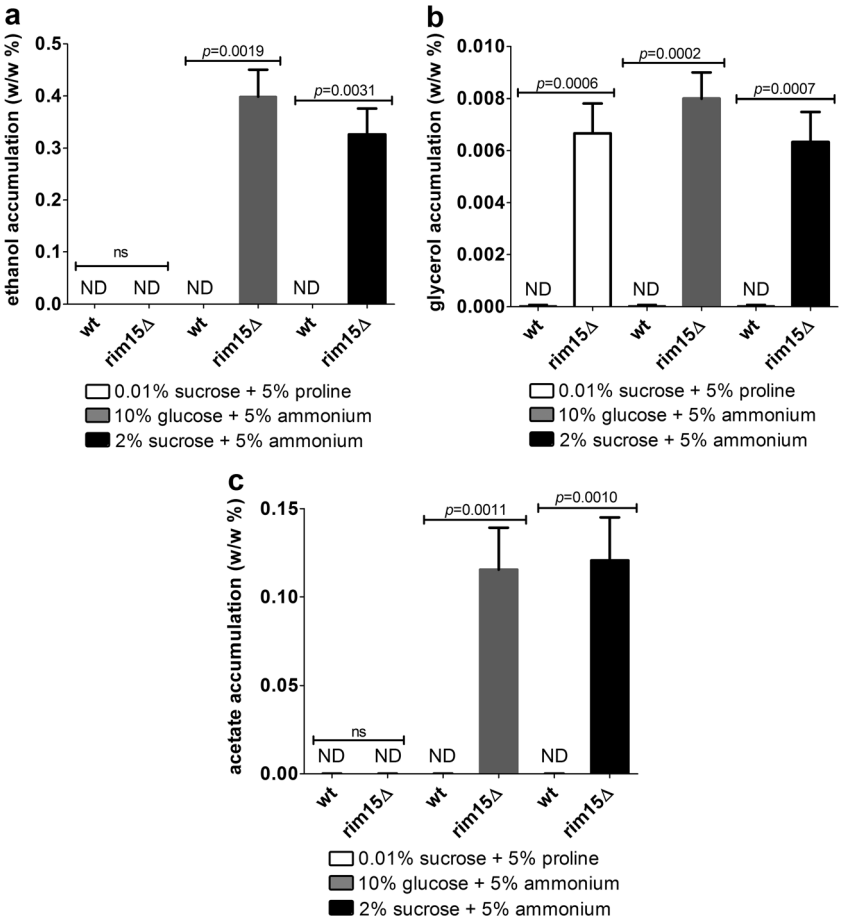

Fig. 8 Effect of *RIM15* deletion in the accumulation of fermentative metabolites. **a** Ethanol accumulation, **b** glycerol accumulation, and **c** acetate accumulation. The results are presented as mean ± SD. The statistical significance of the measurements was determined using a Student's *t* test (wt vs. *rim15Δ*) (*$p < .01$)

the four factors tested. Overall, these data suggest the participation of *RIM15* during energetic metabolism regulation and indicate the impact of interactions between carbon and nitrogen during the fermentative growth induction of *S. cerevisiae*.

In *S. cerevisiae*, carbon and nitrogen are necessary nutrients for cellular maintenance and participate in the regulation of metabolism (Conrad et al. 2014). Furthermore, these nutrients have been associated with regulation of the fermentative pathway in this yeast (Brice et al. 2014; Otterstedt et al. 2004). One of the primary signal transduction pathways activated by carbon is the cAMP-PKA, which is regulated by glucose, and stimulates fermentation and cellular proliferation (Conrad et al. 2014). Other nutrients, such as nitrogen, activate PKA but independent of cAMP (Alberghina et al. 2012). Moreover, one of the main nitrogen signaling routes, the target of rapamycin (TOR) pathway, can be regulated at Gln3p level because of glucose availability, through Snf1p (Bertram et al. 2002). Indicating that nutrient signaling pathways are interconnected and can be activated by more than one nutrient supports the hypothesis of carbon and nitrogen source interactions and their concentrations that are regulated by the Rim15p kinase. This protein has been proposed as an integrative protein of different nutrient signals (Conrad et al. 2014; Swinnen et al. 2006) and as a target protein for alcoholic fermentation investigations (Oomuro et al. 2016; Watanabe et al. 2015; Watanabe et al. 2017). Also, it has been observed that during fermentations using maltose or glucose as carbon sources, the nitrogen source complexity affects the biomass yield and fermentation performance (Cruz et al. 2002). Besides, Martínez-Moreno et al. (2012) reported the influence of nitrogen availability, type of nitrogen source, and sugar concentration on *S. cerevisiae* nitrogen requirements, when a mixture of glucose and fructose was fermented. Interestingly, our results showed that these three factors that influence the nitrogen requirements interact with each other, and their interaction affects the fermentative phenotype of *S. cerevisiae*. Furthermore, it is shown that a second three-factor interaction among the type of carbon and nitrogen sources and their concentration also affect the fermentative growth induction. To our knowledge, this is the first report where two second-order interactions among the carbon and nitrogen sources and their levels in the culture media influence alcoholic fermentation of *S. cerevisiae*. However, when *RIM15* was deleted, a four-factor interaction was significant, indicating the dependence of growth phenotype behavior (respirative, respiro-fermentative, or fermentative) on this interaction. Second-order interactions observed in the wt strain depend

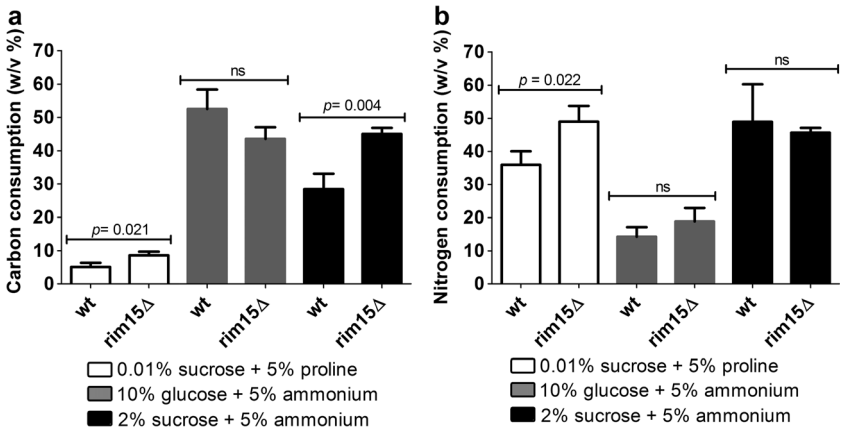

Fig. 9 Effect of *RIM15* deletion in carbon and nitrogen consumption. **a** Carbon consumption and **b** nitrogen consumption. The results are presented as mean ± SD. The statistical significance of the measurements was determined using a Student's *t* test (wt vs. *rim15Δ*) (*$p < .0.01$)

on the presence of *RIM15*, suggesting a role of this protein in the energetic metabolism. Also, more studies are needed to determine how cellular nutritional status affects the energetic metabolism of this yeast, considering that interactions between essential nutrients and micronutrients play a significant role in yeast metabolism.

Nitrogen source quality is classified for *S. cerevisiae* as preferred and non-preferred, based on how well the compounds support cells growth, and if they suppress the use of other nitrogen sources (Godard et al. 2007; Ljungdahl and Daignan-Fornier 2012). Based on this, proline is classified as an intermediate preferred nitrogen source, even when it is considered as non-metabolizable (Martínez-Moreno et al. 2012). Thus, we expected a little number of cultures with fermentative growth phenotype in media supplemented with this amino acid, opposite to glutamate and NH_4^+ that are considered preferred nitrogen sources. Surprisingly, 55.5% of cultures using proline showed a fermentative phenotype, which could be due to its protective effect against oxidative stress caused by ethanol production (Takagi et al. 2016). However, we hypothesize that the ability of proline to promote fermentative growth phenotype could be associated with the energetic metabolism via NADH production during its catabolism by the delta-1-pyrroline-5-carboxylate dehydrogenase (Brandriss and Magasanik 1979). Nonetheless, we have not rejected the possibility of a synergistic effect between proline and other compounds in the culture media. Interestingly, in the wt and *rim15Δ* strains, proline induced fermentative phenotype cultures, suggesting a mechanism that does not involve *RIM15*. Moreover, according to Cooper (1982) and Magasanik (2003), the primary source of cellular nitrogen is glutamate, and it was expected to obtain fermentative type cultures supplementing this nitrogen source. Nevertheless, only 48.1% of cultures presented a fermentative phenotype, in agreement with a report where excessing amounts of glutamate and glutamine showed toxic effects on *S. cerevisiae* (Tesnière et al. 2013). Wt and *rim15Δ* strains supplemented with glutamate showed similar performance during fermentative metabolism. However, increased number of mutant strain cultures exhibited a fermentative and respiro-fermentative behavior. Moreover, NH_4^+ is classified as a preferred nitrogen source (Ljungdahl and Daignan-Fornier 2012) and is mostly used in wine and beer industries, but high concentrations caused cellular toxicity (Hess et al. 2006; Tesnière et al. 2013), which is consistent with our results for the wt (only 33.3% cultures displayed a fermentative behavior). Furthermore, we observed that NH_4^+ toxicity is more evident at low carbon levels, or when galactose is used as carbon source. The mechanism of NH_4^+ toxicity and its dependence on carbon source remain to be investigated. Interestingly, in the *rim15Δ* strain, the influence of NH_4^+ toxicity on *S. cerevisiae* growth is less evident, especially when 10% glucose was used as carbon source. These results were unexpected, since deletion of *RIM15* has been related with a deleterious effect on stress response (Kessi-Pérez et al. 2016; Watanabe et al. 2017), as result of a defective activation of *MSN2* and *MSN4* by Rim15p. These genes codify for the transcriptional factors Msn2p/4p that modulate genes with STRE (stress responsive element) elements (Estruch 2000). Hence, it would be interesting to study the mechanism by which the deletion of *RIM15* decreases ammonium toxicity under certain nutritional conditions. Our results proved that under certain nutrimental conditions, deletion of *RIM15* redirects the energetic metabolism from respiration toward fermentation and that this could be due a partial respiratory inhibition. *GIS1* is a transcriptional factor that regulates genes important during the stationary phase when cells are prepared to use ethanol to obtain energy using the oxidative phosphorylation pathway (Galdieri et al. 2010). Therefore, a possible explanation for the redirection of energy metabolism in *rim15Δ* is that *GIS1* could not be activated by Rim15p, leading to lack of gene transcription with PDS elements (Pedruzzi et al. 2000) causing deregulation of the respiratory metabolism. Furthermore, deletion of *RIM15* improved sucrose consumption that can be explained by an increase in the transcription of *AGT1* (alpha-glucoside transporter 1) and

MAL2T (maltase permease) genes which code for two sucrose carriers for its transport to the cytoplasm where it is further metabolized by the cytoplasmic invertase (Stambuk et al. 2000; Batista et al. 2005; Marques et al. 2016). In this way, carbon flux could be increased leading to the redirection of metabolism from respiratory to mostly fermentative. However, more experiments are required to verify the mechanism through which *RIM15* deletion improves the fermentative metabolism.

In conclusion, two interactions among carbon, nitrogen, and their concentrations that depend on *RIM15* influenced the growth phenotype of *S. cerevisiae*, suggesting that this gene is necessary for carbon and nitrogen metabolism. Also, proline could be used as an enhancer for the fermentative metabolism in *S. cerevisiae* in wine and beer production, and we proved that deletion of *RIM15* improved fermentative metabolism under certain nutrimental conditions.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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