Biochimica et Biophysica Acta 1850 (2015) 2222–2227

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

^{13}C NMR based profiling unveils different α-ketoglutarate pools involved into glutamate and lysine synthesis in the milk yeast *Kluyveromyces lactis*

D. Gorietti [a], E. Zanni [b], C. Palleschi [b], M. Delfini [a], D. Uccelletti [b], M. Saliola [b], C. Puccetti [a], A.P. Sobolev [c], L. Mannina [c,d], A. Miccheli [a,*]

[a] Dipartimento di Chimica, Sapienza Università di Roma, Piazzale Aldo Moro, 5, 00185 Rome, Italy
[b] Dipartimento di Biologia e Biotecnologie C. Darwin, Sapienza Università di Roma, Piazzale A. Moro, 5, 00185 Rome, Italy
[c] Istituto di Metodologie Chimiche (IMC) CNR, Via Salaria Km 29.300, Monterotondo 00015, Rome, Italy
[d] Dipartimento di Chimica e Tecnologie del Farmaco, Sapienza Università di Roma, Piazzale Aldo Moro, 5, I-00185 Rome, Italy

ARTICLE INFO

Article history:
Received 21 April 2015
Received in revised form 1 July 2015
Accepted 22 July 2015
Available online 30 July 2015

Keywords:
Yeasts
^{13}C NMR
Isotopomer analysis
Casein kinase
Lysine synthesis
Metabolic profiling

ABSTRACT

Background: The construction of efficient cell factories for the production of metabolites requires the rational improvement/engineering of the metabolism of microorganisms. The subject of this paper is directed towards the quantitative understanding of the respiratory/fermentative *Kluyveromyces lactis* yeast metabolism and its *rag8* casein kinase mutant, taken as a model for all *rag* gene mutations.
Methods: ^{13}C NMR spectroscopy and [1,2-$^{13}C_2$]glucose were used as metabolic stable-isotope tracer to define the metabolic profiling of a *K. lactis* yeast and its derivative mutants.
Results: Rag8 showed a decrease of all ^{13}C glutamate fractional enrichments, except for [4-^{13}C]glutamate that was higher than wild type ones. A decrease of TCA cycle flux in *rag8* mutants and a contribution of a [4-^{13}C]ketoglutarate pool not originating from mitochondria were suggested.
^{13}C lysine enrichments confirmed the presence of two compartmentalized α-ketoglutarate (α-KG) pools participating to glutamate and lysine synthesis.
Moreover, an increased transaldolase, as compared to transketolase activity, was observed in the *rag8* mutant by ^{13}C NMR isotopomer analysis of alanine.
Conclusions: ^{13}C NMR-based isotopomer analysis showed the existence of different α-KG metabolic pools for glutamate and lysine biosynthesis. In the *rag8* mutant, ^{13}C labeled pentose phosphate intermediates participated in the synthesis of this compartmentalized α-KG pool.
General significance: A compartmentalization of the α-KG pools involved in lysine biosynthesis has been revealed for the first time in *K. lactis*. Given its great impact in metabolic engineering field, its existence should be validated/compared with other yeasts and/or fungal species.

1. Introduction

Kluyveromyces lactis is both scientifically and biotechnologically one of the most important non-*Saccharomyces* yeasts commonly used for genetic studies and industrial applications, being one of the few yeasts capable of growing on lactose as a sole carbon source [1].

K. lactis has a number of advantages over other yeast expression systems, including easy genetic manipulation, availability of integrative and episomal expression vectors and a fully sequenced genome [2–4]. In addition, enzymes from *K. lactis* have GRAS (Generally Regarded As Safe) FDA status, permitting their use in various food and feed applications.

Moreover, the presence in *K. lactis* of a large number of respiratory and fermentative mutants with the desired engineering features makes this yeast a very interesting organism for biotechnological and industrial applications [5].

The improvement of *K. lactis* strains for the production of primary and secondary metabolites requires a quantitative analysis of their metabolism [6], to allow the development of network models for engineered cell factories.

A number of eukaryotic metabolic models are available for microorganisms but, until now, only one model has been designed for the *K. lactis* yeast [7]. This model tries to reconstruct the metabolic network of this organism through the analysis of four compartments (extracellular milieu, cytoplasm, mitochondrion and endoplasmic reticulum) [8]. However, the reconstruction of the metabolic network of eukaryotic

* Corresponding author at: Dept. Chemistry, Sapienza-University of Rome, P.le A. Moro, 5, 00185 Rome, Italy.
E-mail addresses: daniela.gorietti@virgilio.it (D. Gorietti), elena.zanni@uniroma1.it (E. Zanni), claudio.palleschi@uniroma1.it (C. Palleschi), maurizio.delfini@uniroma1.it (M. Delfini), daniela.uccelletti@uniroma1.it (D. Uccelletti), michele.saliola@uniroma1.it (M. Saliola), caterinapuccetti@gmail.com (C. Puccetti), anatoli.sobolev@cnr.it (A.P. Sobolev), luisa.mannina@uniroma1.it (L. Mannina), alfredo.miccheli@uniroma1.it (A. Miccheli).

http://dx.doi.org/10.1016/j.bbagen.2015.07.008
0304-4165/

organisms is still a complex procedure and the use of different approaches/mutant strains will help to challenge this complexity from many different angles.

The use of *RAG8*, an essential *K. lactis* gene coding for the casein kinase I isoform (CKI) involved in a wide range of cellular processes coordinating morphogenesis and glycolytic flux with glucose/oxygen sensing [9] could be a useful tool to design an extended model for this milk yeast.

In particular, it has been demonstrated that Rag8 participates in the regulation of glucose metabolism regulating *RAG1* expression, coding for a low affinity glucose transporter, by two independent signaling pathways, the Rag4 glucose sensor and the Rgt1–Sck1 route, where Rgt1 is the repressor of Sck1 (**S**uppressor of **c**asein **k**inase I) [10,11]. Since the Rag4/Rag8 control of Rgt1 depends on glucose availability, it has been shown that Sck1 is unstable in strains harboring the rag8-1 allele [12]. This gene has a nonsense mutation coding for a Rag8-1 kinase protein devoid of its C-terminal domain required for its attachment to the plasma membrane (Marc Lemaire, personal communications).

In our previous paper, we showed that Rag8 has roles in vesicle transport, internalization of uracil permease, fatty acid/phospholipid fluidity and permeability [13], similar to the corresponding *Saccharomyces cerevisiae* ortholog activities. However, being the altered metabolic behavior of rag8 common to other *rag* mutants, the determination of the metabolic glucose fluxes in *rag8* constitutes a very interesting fermentative-deficient model for all *rag* mutants (at least 20 complementation groups) equivalent but alternative to the *S. cerevisiae* respiratory *petite* mutants.

To investigate the metabolic changes taking place in the *rag8* mutant, a metabolomic approach was performed by NMR spectroscopy. The metabolic network of the wild type and *rag8* mutant was built in our previous work by multivariate statistical model [13].

However, to define the metabolic behavior of this organism, and its response to the genetic mutation, cell metabolic network will be described by quantitative analysis of metabolic fluxes [14]. ^{13}C metabolic flux analysis is currently the most sophisticated and reliable method for determining intracellular fluxes and has become a widely used tool in system bio(techno)logy [15]. During the last decade, measurement of metabolic fluxes via ^{13}C-labeling has developed quickly across a diverse set of applications, including rational manipulation of cellular metabolism for product biosynthesis, gene function validation and drug-target search for a variety of diseases [14]. ^{13}C-labeling experiment is an ideally suited technique to determine flux ratios [16].

The differential ^{13}C-labeled isotopomer profiles can serve as a fingerprint of the metabolic network activity and could reflect both qualitative and quantitative differences in the metabolic pathways that lead to the synthesis of each metabolite [17].

In the present study, ^{13}C NMR based isotopomer analysis, with [1,2-$^{13}C_2$]glucose as metabolic stable-isotope tracer, was used to characterize the glycolysis, the re-cycling of pentose phosphate intermediates in glycolysis, TCA cycle and amino acid metabolism in *K. lactis* and in *rag8* mutant, taken as a model for all *rag* mutants.

2. Materials and methods

2.1. Strains, media and culture conditions

The strains used in this work were: PM6-7A (*MATa ade-T600 uraA1-1*) and PM6-7AVV30 (*MATa ade-T600 uraA1-1 rag8-1*) [9]. Cultures were grown under shaking conditions at 28 °C in YP (1% Difco yeast extract, 2% Difco Bacto-peptone) supplemented with glucose at the concentration of 0.5 g/L. Four samples were prepared from the same cellular pool, for each strain (wild type and *rag8* mutant). When the cells reached 0.7 OD_{600}, glucose was added to the media of two samples, for each strain, at 1.6 g/L (named control samples), whereas [1,2-$^{13}C_2$]-glucose was supplied to the other two samples (enriched samples). The cells were grown until late exponential phase to 3.0 OD_{600}, to ensure that both metabolic and isotopic steady-states were reached.

2.2. Cellular extraction procedure

Cell cultures grown as described above were washed three times with cold double distilled water (H_2O_{dd}) and suspended and broken with glass beads in 900 μL of cold methanol (−20 °C) to quench intracellular metabolism. To extract the metabolites the method reported in [18] was followed. Briefly, 1.1 mL of methanol, 2 mL of chloroform and 1 mL of H_2O_{dd} were added to keep the final volume in a 2:2:1 ratio, respectively; polar and organic phases were separated by centrifugation at 10,500 rpm at 4 °C for 30 min. The polar phase (top layer) was collected separately from the organic phase (bottom layer), dried under N_2 flux and stored at −80 °C until NMR analysis.

2.3. Sample preparation for ^{13}C NMR analysis

The freeze-dried polar samples were re-dissolved in 0.5 mL of D_2O phosphate buffer solution (pH = 7.4) and transferred to 5 mm NMR glass tubes for analysis. As ^{13}C quantitative reference a coaxial tube, containing 70 μL of pure acetonitrile (CH_3CN) was inserted into the samples.

2.4. NMR spectroscopy

The calculation of each carbon enrichment is achieved by the acquisition of 1D ^{13}C NMR spectra, at 298 K, using a Bruker Avance AQS600 spectrometer operating at the proton frequency of 600.13 MHz. Since the observation of ^{13}C NMR signals fine structure relative to the coupling pattern between adjacent ^{13}C, fundamental for isotopomer identification, crucially depends on the efficient removal of the large $^{1}J_{CH}$ scalar coupling interactions [16], an inverse gated decoupling pulse sequence was adopted. This avoids the effects of NOE that could compromise ^{13}C quantitative analysis. The decoupler was gated only during the acquisition of the ^{13}C FID, after a single 90° detection pulse, calibrated before the acquisition of each spectrum. The D1 relaxation delay was set to 7 s for all the spectra, acquiring 64 k data point in about 0.83 s. The spectral width was set to ~39,370 Hz (~260 ppm) and 8192 scans were collected for each spectrum to achieve an acceptable signal-to-noise ratio.

2.5. Data analysis

^{13}C NMR spectra (Figs. S1 and S2) were processed using the 1D-NMR Manager ver. 12.0 software (Advanced Chemistry Development, Inc., Toronto, Ontario, Canada).

The assignment of the peaks to specific metabolites was achieved by standard two-dimensional (2D) ^{1}H–^{1}H total correlation spectroscopy (TOCSY), ^{1}H–^{13}C heteronuclear single quantum correlation (HSQC), and Heteronuclear Multiple Bond Correlation (HMBC) and confirmed using an internal library of compounds and by comparison with literature data [19–23].

2.6. Fractional enrichment

The interpretation of ^{13}C NMR spectra in terms of flux through metabolic pathways requires the quantification of the ^{13}C incorporated in specific carbons. This is done by expressing ^{13}C incorporation as a fractional ^{13}C enrichment in carbon C_i (Y_{Ci}) [23]. The increased intensity of the resonances observed in the spectrum obtained after infusion of ^{13}C-labeled substrate, indicates that the ^{13}C label has been incorporated in the metabolites.

The difference between the intensity of the natural abundance signal (control samples) and the total intensity of the corresponding multiplet resonance in the spectrum obtained with ^{13}C-labeled