Hypothesis

Reoxidation of the NADPH produced by the pentose phosphate pathway is necessary for the utilization of glucose by *Kluyveromyces lactis rag2* mutants

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Abstract ***Kluyveromyces lactis*** **mutants defective in the glycolytic enzyme phosphoglucose isomerase are able to grow in glucose media and to produce ethanol, but they depend on a functional respiratory chain and do not grow in glucose-antimycin media. We postulate that this is due to the necessity of reoxidizing, in the mitochondria, the NADPH produced by the pentose phosphate pathway, which may be highly active in these mutants in order to bypass the blockade in the phosphoglucose isomerase step. This oxidation would be mediated by a cytoplasmic-side mitochondrial NAD(P)H dehydrogenase that would pass the electrons to ubiquinone. Data supporting this hypothesis are provided.**

Key words: rag 2; Pentose phosphate pathway; Mitochondrial respiratory chain; Ethanol production

1. Introduction

The Rag (resistance to antimycin on glucose) phenotype is an important tool for studying the glucose metabolism genes of the yeast *Kluyveromyces lactis*. Some natural strains of *K. lactis* were found to be able to grow in high glucose media in the presence of the respiratory inhibitor antimycin A, whereas others were not. Thus Rag^+ and Rag^- strains, respectively, were differentiated. This phenotype was initially found to be controlled by two unlinked nuclear genes: *RAG1* and *RAG2*. Either of their recessive alleles, *rag1* and *rag2*, led to the Rag^- phenotype [1]. *RAG1* encodes the low-affinity glucose transporter, and *RAG2* encodes the glycolytic enzyme phosphoglucose isomerase [2-5]. The Rag^- phenotype has been used to obtain mutations in genes related to glucose metabolism in *K. lactis*. At present, 12 additional complementation groups have been defined and are designated *rag3–rag14* [6,7]; the *RAG4* and *RAG8* gene products might interact specifically with the promoter region of the *RAG1* gene, *RAG5* encodes the unique hexokinase of *K. lactis* [8], *RAG14* is allelic to *KlPFK2*, *RAG3* is the homologue to *PDC2* from *Saccharomyces cerevisiae* [7] and *RAG6* is allelic to *KlPDCA* [9].

The inability of the *rag2* strains to grow in glucose when mitochondrial respiration is blocked by antimycin suggested the existence of a defect in the fermentative pathway of these strains. In fact, it was reported that the *K. lactis rag2* mutant could not produce ethanol from glucose [3], unlike the *rag1* mutant, which produces ethanol although at a lower rate than the Rag^+ strain [4]. We question the unexplained inability of *K. lactis rag2* mutants to produce ethanol. Recently, the production of ethanol by a double mutant *rag1rag2* strain in lactose was described [10]. We propose a hypothesis that explains the Rag^- phenotype without denying the fermentative capacity of the *rag2* mutant.

2. Hypothesis

If glycolysis is blocked at the level of phosphoglucose isomerase, glucose-6-phosphate enters the pentose phosphate pathway to yield 6-P-fructose and 3-P-glyceraldehyde, which are transformed to pyruvate via glycolysis. The NADH produced in glycolysis is then reoxidized to NAD by the mitochondrial respiratory chain or is utilized via ethanol fermentation.

Therefore, in a *K. lactis rag2* mutant strain, if the Krebs cycle is not active (Fig. 1A), the balance of glucose utilization (via the pentose phosphate pathway, glycolysis, and ethanol fermentation) would be

$$3\ \text{glucose} + 6\ NADP^+ + 5\ ADP + 5\ P_i \rightarrow$$
$$\rightarrow 5\ \text{ethanol} + 8\ CO_2 + 6\ NADPH + 6\ H^+ + 2\ H_2O + 5\ ATP$$

The balance of ethanol production via glycolysis directly, without the participation of the pentose phosphate pathway, would be

$$3\ \text{glucose} + 6\ ADP + 6\ P_i \rightarrow 6\ \text{ethanol} + 6\ CO_2 + 6\ ATP + 6\ H_2O$$

It is significant that fermentative glucose utilization based on the pentose phosphate pathway implies the net production of reduced NADP, while if the pentose phosphate pathway is not employed, there is no net production of reduced coenzymes.

Thus, we propose that *rag2* mutants are capable of fermentation, although they depend on an active mitochondrial respiratory chain to grow on glucose (as they do not grow on glucose-antimycin). This dependence may be explained by the necessity of reoxidizing the cytoplasmic NADPH produced during glucose utilization by the pentose phosphate pathway. If this reoxidation does not occur, the depletion of NADP would cause a block in the pentose phosphate pathway, the production of 6P-fructose and 3P-glyceraldehyde would cease, and ethanol would not be produced (Fig. 1B).

3. Data supporting this hypothesis

3.1. *K. lactis rag2 mutants produce ethanol from glucose*

As we found no theoretical impediment for glucose transformation to ethanol in the *rag2* mutants, we followed the kinetics of growth, glucose consumption and ethanol production of the *rag2* mutant strain PM5-2D [6] in glucose media. As shown in Fig. 2A, this strain produces appreciable amounts of ethanol in a glucose medium (more than 5 g/l of ethanol from 20 g/l of glucose). The A_{600} values reached are similar to those reported for the Rag^+ and *rag1* mutant strains, although they were achieved at a slower rate. The amount of ethanol accumulated in the culture medium was similar to that reported for the *rag1* mutant strain, although it

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had different kinetics of production, and was lower than that reported for the Rag^+ strain [4]. In the stationary phase, once the glucose is exhausted, PM5-2D oxidatively consumes the previously produced ethanol, as the concentration of ethanol in the culture medium reaches a maximum and then falls sharply. Previous investigators [3], who stated that *K. lactis rag2* mutants did not produce ethanol from glucose, apparently assayed the ethanol concentration only in stationary phase cultures, which explains why they did not find ethanol in the culture medium. Besides, the growth of *K. lactis rag2* mutants on fructose-antimycin [3] suggests the use of the fermentative pathway. Fructose enters directly to glycolysis without the participation of the pentose phosphate pathway.

3.2. *The pentose phosphate pathway is highly active in K. lactis*

K. lactis mutants for phosphoglycerate-kinase and hexokinase did not grow on glucose as the sole carbon source [8,11]. However, the growth of *rag2* mutant strains in glucose suggested that the pentose phosphate pathway should be active in *K. lactis* [3], since this pathway allows glucose-6-P to be transformed to fructose-6-P, bypassing the glycolytic step. This is well established by experimental data. The double null-mutant in the genes encoding the subunits of the *K. lactis* phosphofructokinase was still able to grow in glucose, but when the gene encoding transaldolase was also mutated, the triple mutant lost the ability to grow in glucose. Moreover, specific transaldolase activities are about five times higher in *K. lactis* than in *S. cerevisiae* [11]. Phosphoglucose isomerase (*pgi*) deletion mutants of *S. cerevisiae* cannot grow on glucose as the sole carbon source, suggesting that the pentose phosphate pathway in *S. cerevisiae* does not contribute substantially to glucose metabolism [12]. We consider it likely that the difference between the relative importance of the pentose phosphate pathway in *K. lactis* and *S. cerevisiae* is a consequence of the

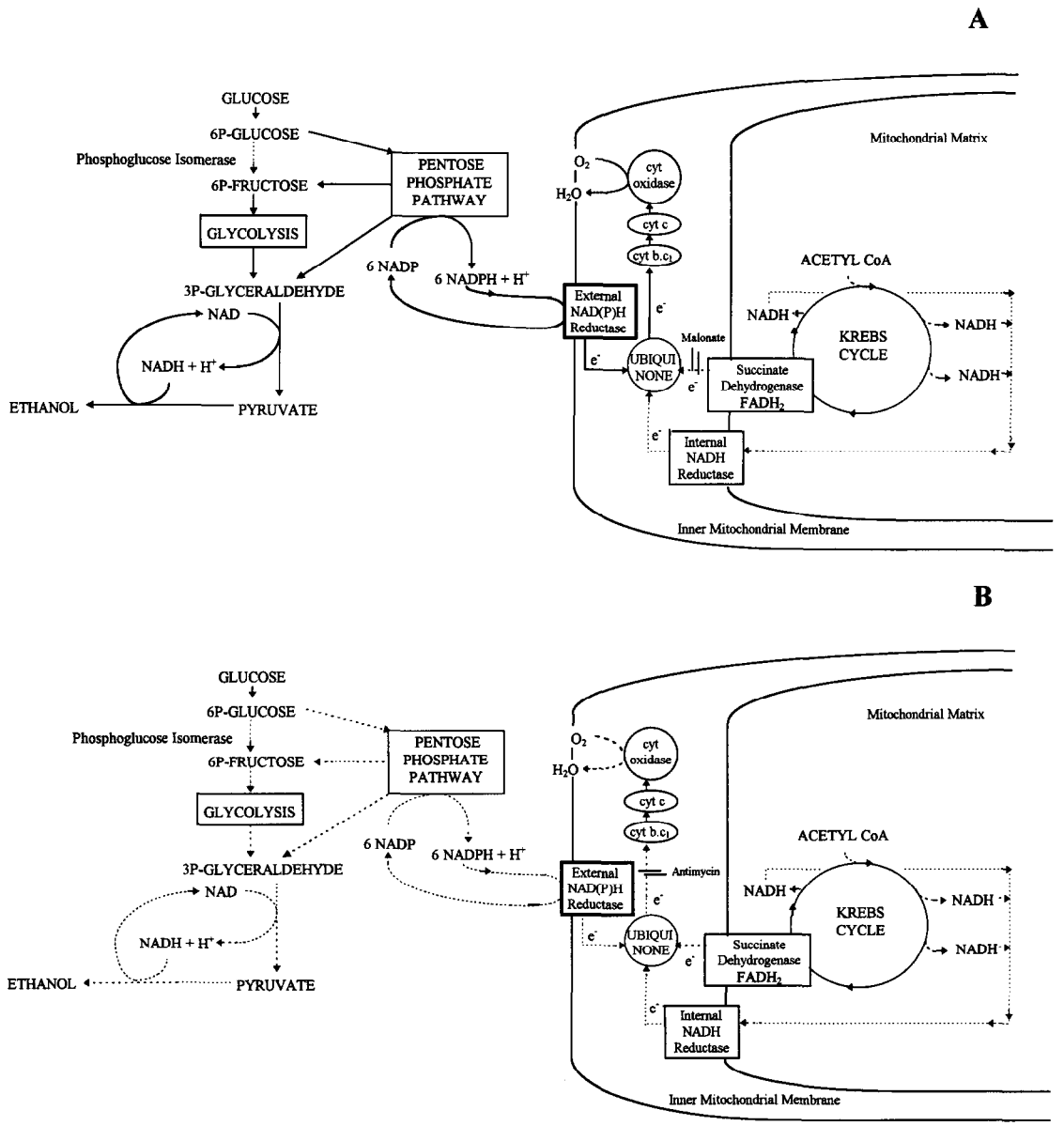

Fig. 1. Scheme illustrating the hypothesis that the *K. lactis* phosphoglucose isomerase mutants need mitochondrial oxidation of the NADPH produced by the pentose phosphate pathway to grow and produce ethanol from glucose. Non-functional reactions are indicated by dotted lines (A) when succinate dehydrogenase is blocked with malonate, and (B) when the mitochondrial respiratory chain is blocked with antimycin.

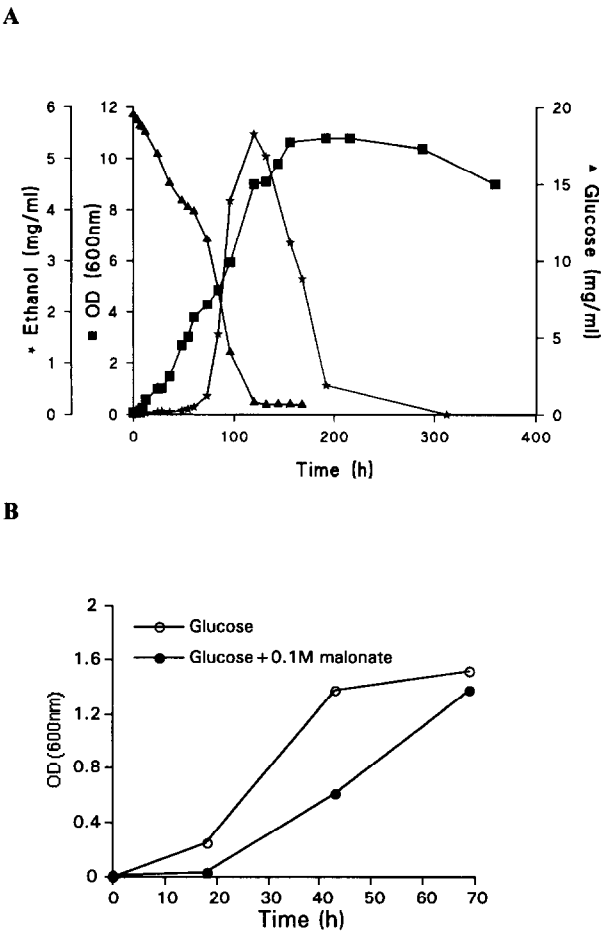

Fig. 2. A: Growth, glucose consumption, and ethanol production of the *K. lactis rag2* mutant PM5-2D [6] in YPD medium (1% yeast extract, 2% bactopeptone, 2% glucose). Growth was measured as OD at 600 nm, reducing sugars in culture supernatants (glucose) were assayed by the 3,5-dinitrosalicylic acid method [26], and ethanol concentrations in culture supernatants were determined by using a kit from Boehringer Mannheim (Germany), following the instructions of the supplier. Growth conditions: a 1% inoculum from an exponential phase preculture in the same medium into 200 ml culture medium/500 ml flask in an orbital shaker at 30°C and 200 rpm. B: Growth (OD at 600 nm) of the *K. lactis rag2* mutant PM5-2D [6] in liquid CM-3% glucose [24] media with and without 0.1 M sodium malonate. Conditions: a 0.4% inoculum from a 3 day preculture in the same medium was placed in 25 ml culture medium/100 ml flask in an orbital shaker at 30°C and 200 rpm.

differences in the mechanisms allowing reoxidation of NADPH in both yeasts.

3.3. *Utilization of the pentose phosphate pathway in K. lactis is related to an increase in the activity of the mitochondrial respiratory chain*

Direct (without shuttle mechanisms) oxidation of cytoplasmic NAD(P)H by yeast mitochondria has been reported [13], although its relative importance in metabolism seems to be species-dependent. In *C. utilis*, oxidation of NADPH via the respiratory chain, and linked to only site II and III phosphorylation, has been established, whereas in *S. cerevisiae*, it remains under discussion [14-16]. The absence or low efficacy of this direct NADPH reoxidation mechanism in *S. cerevisiae* is supported by the results of Boles et al. [17], who proposed that the growth defect of the *pgi* mutants in glucose is due to a rapid depletion of cytoplasmic NADP, which is needed as a cofactor in the pentose phosphate pathway. They proved that this defect could be alleviated by adding oxidizing agents, or over-expressing the NADH-dependent glutamate dehydrogenase thus increasing NADPH consumption [17].

Experimental data directly supporting the occurrence of mitochondrial NADPH oxidation in *K. lactis* are not available, but there is some evidence that when *K. lactis* utilizes glucose via the pentose phosphate pathway (as occurs in *rag2* mutants), the mitochondrial respiratory chain is more active. Thus, in batch, fully aerated, fermenter cultures of *K. lactis* NRRL-Y-1140 (CBS-2359, Rag^+) [6,18] and MW98-8c (*rag1rag2*) [19] in YP-0.5% glucose media, we observed that in order to reach the same A_{600}=0.75 value, MW98-8c utilized much more oxygen than Y-1140 (the dissolved oxygen decreased about 20% for the former and 1% for the latter) [10]. MW98-8c needs to use the pentose phosphate pathway to metabolize glucose, whereas Y-1140 does not, because in this strain glycolysis is not blocked at the phosphoglucose isomerase step.

In addition, we have observed that the levels of *KlCYC1* (cytochrome *c* gene) mRNA (quantified densitometrically using the SI system from Molecular Dynamics) for the *K. lactis* MW98-8c (*rag1rag2*) mutant growing aerobically in glucose are 8.5-fold higher than in fructose (Fig. 3). The same effect is observed for the *rag2* mutant PM5-2D although the ratio is lower probably due to higher intracellular levels of glucose causing repression. This difference has not been observed in the Rag^+ strain NRRL-Y-1140. In glucose, the *rag2* mutant needs to perform the pentose phosphate pathway to bypass the phosphoglucose isomerase reaction, and there is concomitant production of NADPH that has to be reoxidized. Fructose enters directly into glycolysis at the 6-P-fructose level, and the pentose phosphate pathway is not necessary for its utilization.

Therefore, the possibility exists for *K. lactis*, as described for other yeasts [13], of performing direct oxidation of cytoplasmic NADPH by an external mitochondrial dehydrogenase, which would pass the electrons to the mitochondrial respiratory chain. It is also possible that cytoplasmic substrate cycling occurs, thus allowing oxidation of NADPH with concomitant reduction of NAD, which would be reoxidized in the mitochondria. The effectiveness of this oxidation would be facilitated by the high activity of the respiratory chain found in these yeasts, which have unlimited respiration [10], high levels of expression of respiration related genes [20,21] and no pyruvate overflow when the first step of the fermentative pathway is interrupted [9].

According to the results described in the following section, we think that this external mitochondrial dehydrogenase passes electrons to the ubiquinone pool.

3.4. *If the respiratory chain is blocked before ubiquinone, K. lactis rag2 mutants continue to grow on glucose*

Since *K. lactis rag2* mutants do not grow on glucose plus antimycin, which blocks the respiratory chain after ubiquinone, we blocked the respiratory chain at a preceding level and tested the ability of the mutants to grow in glucose under these conditions. In order to control the effectiveness of the inhibition, we verified the absence of growth in lactate, a non-fermentable carbon source.

Previous trials using rotenone as an inhibitor of the respira-

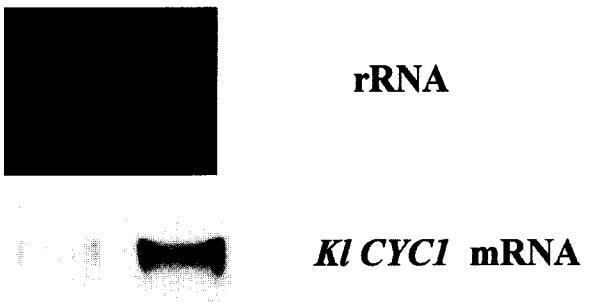

Fig. 3. Northern analysis of RNA isolated from the *K. lactis* strain MW98-8c (*rag1rag2*) [19] grown aerobically in YP medium (1% yeast extract, 2% bactopeptone) with 2% fructose or glucose. The upper part of the figure shows the intensity of the ethidium bromide stained ribosomal RNA bands, used for loading corrections. The lower part corresponds to the blot showing the mRNA levels of the cytochrome *c* gene (*KlCYC1*). Northern analysis was performed as previously described [27].

tory chain, which blocks the internal NADH-reductase [22,23], showed that not only the *rag2* mutant PM5-2D, but also the other *K. lactis* strains NRRL-Y-1140 (Rag^+), MW98-8c (*rag1rag2*), and PM4-4B/*RAG1::URA3* [4] were able to grow in solid CM-3% glucose and CM-2% lactate media [24] supplemented with rotenone at the assayed concentrations (0.3–300 μM). Therefore, rotenone was not useful for our purpose, but this result led us to think that in *K. lactis*, the NADH-ubiquinone-dehydrogenase involved in the oxidation of intramitochondrial NADH may be resistant to rotenone, as has been reported for *S. cerevisiae* and other yeasts [14,25].

We then assayed the effect of malonate on the growth of the *K. lactis rag2* mutant PM5-2D; this compound inhibits the succinate dehydrogenase complex [23]. Growth was tested in solid media supplemented with sodium malonate at concentrations ranging from 0.1 mM to 1 M. It was observed that after 3 days at 30°C, PM5-2D scarcely grew in CM-2% lactate medium with malonate concentrations of 0.1–0.5 M, but it grew abundantly in CM-3% glucose medium supplemented with the same concentration of malonate. Higher concentrations of the inhibitor (about 1 M) prevented growth even on glucose and at concentrations lower than 0.1 M, growth was abundant in both glucose and lactate media. In liquid media (Fig. 2B), the *K. lactis rag2* mutant retained the ability to grow on glucose plus 0.1 M sodium malonate, although at a slower initial rate. When the respiratory chain is blocked at the level of the succinate dehydrogenase complex, which passes electrons to ubiquinone, mitochondrial respiration is inhibited, but the external NAD(P)H dehydrogenase would allow reoxidation of the cytoplasmic NAD(P)H, thus explaining the capacity of *rag2* mutants to grow in the presence of this inhibitor (Fig. 1A).

4. Conclusion

K. lactis rag2 mutants produce ethanol (at lower levels than the Rag^+ strain) when grown in a complete glucose medium. This is because in *K. lactis*, the highly active pentose phosphate pathway allows the blocked phosphoglucose isomerase step to be bypassed. Fermentation in these mutants becomes dependent on a functional mitochondrial respiratory chain, since the NADPH produced by the pentose phosphate pathway has to be reoxidized, probably by an external mitochondrial dehydrogenase that would pass electrons to ubiquinone. The *rag2* mutants are not able to grow in glucose-antimycin media (the Rag^- phenotype) because NADP depletion would inhibit the pentose phosphate pathway, and both respiration and fermentation would be impaired.

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