A NADP-glutamate dehydrogenase mutant of the petit-negative yeast *Kluyveromyces lactis* uses the glutamine synthetase-glutamate synthase pathway for glutamate biosynthesis

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The activities of the enzymes involved in ammonium assimilation and glutamate biosynthesis were determined in wild-type and NADP-glutamate dehydrogenase (GDH) null mutant strains of *Kluyveromyces lactis*. The specific NADP-GDH activity from *K. lactis* was fivefold lower than that found in *Saccharomyces cerevisiae*. The glutamine synthetase (GS) and glutamate synthase (GOGAT) activities were similar to those reported in *S. cerevisiae*. The NADP-GDH null mutant was obtained by transforming the *uraA* strain MDU1 with a linearized integrative yeast vector harbouring a 390 bp fragment of the NADP-GDH structural gene. This mutant grew as well as the parent strain on ammonium, but showed GS and GOGAT activities higher that those found in the wild-type strain, implying that the GS-GOGAT pathway could play a leading role in glutamate biosynthesis in *K. lactis*. Southern blotting analysis of *K. lactis* chromosomes separated by contour-clamped homogeneous electric field electrophoresis, indicated that the NADP-GDH structural gene is localized on chromosome VI.

**Keywords:** nitrogen metabolism, glutamate biosynthesis

INTRODUCTION

Two pathways for ammonium assimilation and glutamate biosynthesis have been found in a variety of organisms. In one pathway, NADP-glutamate dehydrogenase (NADP-GDH; EC 1.4.1.4) catalyses the reductive amination of 2-oxoglutarate to form glutamate (Holzer & Schneider, 1957). The existence of an alternative pathway for the net biosynthesis of glutamate, was demonstrated by Tempest et al. (1970). In this pathway, glutamate is aminated to form glutamine by glutamine synthetase (GS; EC 6.3.1.2) and the amide group is then transferred reductively to 2-oxoglutarate by glutamate synthase (GOGAT; EC 1.4.1.13), resulting in the net conversion of ammonium and 2-oxoglutarate to glutamate. The GS-GOGAT pathway has been found in several microorganisms (Senior, 1975; Hummelt & Mora, 1980; Bravo & Mora, 1988; Marqués et al., 1992) and in higher plants (Miflin et al., 1980).

The presence of two pathways for glutamate biosynthesis has opened a discussion as to the need for two routes for the biosynthesis of the same end-product. In plants, the GS-GOGAT pathway constitutes the main route of ammonium assimilation (Miflin et al., 1980). In *Klebsiella aerogenes*, the GS-GOGAT pathway functions when the ammonium concentration is low, while the GDH pathway is more active when the cells are cultivated under ammonium excess (Tempest et al., 1970; Meers et al., 1970). In *Neurospora crassa*, it has been shown that the main function of GOGAT is to recycle some organic nitrogen from glutamine to glutamate, and that both pathways are involved in glutamate synthesis either on excess or limiting ammonium (Lomnitz et al., 1987).

In *Escherichia coli*, GDH and GOGAT activities are both present, but their precise functions are not known (Senior, 1975). It had been proposed that in this bacterium, the lack of GOGAT resulted in the incapacity to use a variety of nitrogen sources (Ntr− phenotype). However, it has now been established that the induction of Ntr enzymes is independent of GOGAT activity and mutants lacking GOGAT activity which are able to induce histidase have been isolated (Castaño et al., 1992). Recently, Helling (1994) proposed that in *E. coli*, glutamate biosynthesis
could proceed through NADP-GDH when the cell is energy-limited, while the GS-GOGAT pathway would function when the cell is not energy-limited.

The role of the GDH and GS-GOGAT pathways has been studied in some yeasts. Activities of the two ammonium-assimilation pathways in continuous cultures of *Candida albicans* and the isolation of *Schizosaccharomyces pombe* glutamate auxotrophs has indicated that the GS-GOGAT pathway is the major pathway for ammonium assimilation in these yeasts (Holmes et al., 1989; Barel & MacDonald, 1993). *Saccharomyces cerevisiae* mutants devoid of the biosynthetic NADP-GDH or of GOGAT have also been obtained (Folch et al., 1989; Miller & Magasanik, 1990; Cogoni et al., 1995). Those strains impaired in NADP-GDH show a twofold slower doubling time as compared to the wild-type strain when these are grown on ammonium as sole nitrogen source (Folch et al., 1989). The lack of GOGAT activity shows no phenotype when this yeast is grown on either high or low ammonium (Folch et al., 1989). Thus, in *S. cerevisiae* NADP-GDH has been assigned a role in glutamate biosynthesis, while the participation of GOGAT has remained unclear, and this activity would seem dispensable.

We report here catalytic activities of NADP-GDH, GS and GOGAT and the isolation of a NADP-GDH null mutant from *Kluyveromyces lactis*. The NADP-GDH-less mutant strain has no growth-defective phenotype and shows higher GOGAT and GS activities than the wild-type strain, indicating that in this yeast the GS-GOGAT pathway could play a major role in glutamate biosynthesis and ammonium assimilation.

**METHODS**

**Growth conditions.** Strains were routinely grown on minimal medium (MM) containing salts, trace elements, and vitamins following the formula of yeast nitrogen base (Difco). Glucose (2%, w/v) was used as the carbon source, and 38 mM (NH₄)₂SO₄ was used as the nitrogen source. Amino acids needed to satisfy auxotrophic requirements were added at 0.01% (w/v). Cells were incubated at 30°C with agitation. Growth was monitored by measuring optical density at 600 nm (Hewlett Packard model 8452A spectrophotometer).

**Determination of NADP-GDH, GS and GOGAT activities.** Soluble extracts for enzyme assays were prepared by grinding whole cells suspended in 0.1 M potassium phosphate, pH 7.5, 1 mM EDTA, with glass beads and a Vortex mixer (six cycles of 1 min). NADP-GDH, GS and GOGAT were assayed by the methods of Doherty (1970), Ferguson & Sims (1974) and Roon et al. (1974), respectively.

**Oligonucleotide design and PCR amplification of *K. lactis* genomic DNA.** Two pairs of degenerated deoxyoligonucleotides were designed based on the amino acid sequence of *S. cerevisiae* GDH (Nagasu & Hall, 1985; Moye et al., 1985) and *K. lactis* codon usage (Lloyd & Sharp, 1993).

The deoxyoligonucleotide F₁, TT(G + A) GA(A + G) GA(C + T) TC(T + C) AC(T + C) CT(T + C) TT(T + C) GA was designed from the sequence LEDSTLFE located at position 17–24 in the GDH sequence. The deoxyoligonucleotide R₁, TT(C + T) AA(G + A) GC(G + A) GC(G + A) TA(T + C) TG(A + G) GC(A + G) AC(G + A) TT was designed from the sequence NVAQYAAALK located at position 227–235. The deoxyligoligonucleotide F₂, TT(G + A) GA(A + G) CA(A + G) AT(C + T) GT(C + T) AA(C + T) GA(G + A) TA(C + T) TC was designed from the sequence LEQIVNEY located at position 277–285. The deoxyoligonucleotide R₂, CAT (A + G) AT(T + C) CT(T + C) TT(C + T) AA(C + T) TC(T + C) TG(G + A) TC was designed from the sequence DQELKRM located at position 400–407.

Total DNA from *K. lactis* strain WM37 (MAT a, his 5) was used as template for amplification by PCR. It was carried out in a Coy TempCycler II with the following program: one denaturation cycle for 10 min at 94°C, followed by 50 cycles of 30 s denaturation at 94°C, 45 s annealing at 45°C and 2 min extension at 72°C, with a final 10 min extension at 72°C. A 390 bp PCR product obtained from primers F₂–R₂ was gel-purified and ligated into the pcRII vector (Invitrogen). This subclone was sequenced by primer extension using the sequenase V.2 kit (USB).

**Chromosome separation.** Chromosomes from strain WM37 were separated by contour-clamped homogeneous electric field (CHEF) electrophoresis as described by Miranda et al. (1995).

**Southern blot analysis.** The agarose gels containing BglII restricted DNA from the wild-type and NADP-GDH null mutant and that containing separated chromosomes were exposed to short wavelength UV for 5 min to break the DNA, then denatured, neutralized and transferred to nylon membranes as described in Sambrook et al. (1989). DNA blots were probed with the 390 bp PCR fragment labelled with [α²³P]dCTP.

**RESULTS AND DISCUSSION**

**Determination of the enzymes involved in glutamate biosynthesis.** Glutamate biosynthesis can be achieved through the action of the NADP-GDH or through the concerted action of GS and GOGAT. To determine whether these two pathways were present in *K. lactis* the activity of these enzymes was measured (Fig. 1). In extracts of the wild-type strain grown on ammonium as sole nitrogen source, GS activity increased slightly in the stationary growth phase, while GOGAT activity was highest during the exponential growth phase. The specific activities of these two enzymes were similar to those found in *S. cerevisiae* (González et al., 1985a; Folch et al., 1989). NADP-GDH showed the highest activity during exponential phase of *K. lactis*, although the specific activity was fivefold lower than that reported for *S. cerevisiae* (González et al., 1985b). It is worth noting that even though *K. lactis* showed a considerably lower NADP-GDH activity than *S. cerevisiae*, both strains grew on ammonium with the same doubling time (2 h). This result implies that in *K. lactis* the growth rate on ammonium is not limited by the capacity to assimilate ammonium through the NADP-GDH, but that the normal assimilation of ammonia in *K. lactis* could depend on the GS-GOGAT pathway. To resolve this question, *K. lactis* mutants devoid of NADP-GDH activity were isolated.

**Isolation and characterization of a NADP-GDH null mutant.** Based on the observation that there is a high degree of amino acid sequence similarity between various proteins from *S. cerevisiae* and *K. lactis* (Hendriks et al., 1992) and
the fact that codon usage is very similar in these two yeasts (Lloyd & Sharp, 1993), two pairs of deoxyoligonucleotides were designed as amplification primers to be used on total DNA from K. lactis strain WM37. According to the S. cerevisiae NADP-GDH sequence (Moye et al., 1985; Nagasu & Hall, 1985), primers F1/R1 (see Methods), were expected to amplify a 654 bp fragment; primers F2/R2 were expected to amplify a 390 bp fragment, while the combined primers F1-R2 were expected to amplify a 1170 bp fragment. Only the F2/R2 couple amplified the expected product. This 390 bp fragment was subcloned into the pCRII vector and sequenced in its entirety. The deduced amino acid sequence showed a high degree of similarity (75% similarity, 67% identity) with the corresponding region of the NADP-GDH protein from S. cerevisiae (Fig. 2).

To obtain a K. lactis NADP-GDH null mutant, we constructed plasmid pJR1 by cloning the 390 bp PCR fragment into YIp352 integrative vector, which harbours the URA3 yeast marker. The resulting plasmid, pJR1, was linearized by digesting at the single ClaI site located at position 214 of the PCR fragment. Strain MD2/1 (MATa lys4 arg4 ura4) was transformed to uracil prototrophy. Chromosomal DNA was isolated from 10 transformants and digested with HindIII. Southern analysis was carried out with the 390 bp PCR fragment as the probe. The pattern of transformants clearly indicated the insertion of the construct in the wild-type genomic sequence of NADP-GDH (Fig. 3). The null mutants obtained were completely devoid of NADP-GDH activity (Fig. 1a). The doubling time of the wild-type and of the NADP-GDH mutant strains when both were grown on ammonium as sole nitrogen source, was 120 min (data not shown). These results imply, that in K. lactis, NADP-GDH could be a non-essential enzyme under these growth conditions.
conditions. GOGAT and GS specific activities were 1.5- to 2-fold higher in the NADP-GDH mutant strain than those found in the wild-type strain (Fig. 1b, c). These results imply that the GS-GOGAT pathway could be either a compensatory mechanism for the lack of NADP-GDH or the primary route of ammonium assimilation and glutamate biosynthesis and that the NADP-GDH could have only a collateral significance. In C. albicans and S. pombe, the GS-GOGAT pathway plays an essential role in ammonium assimilation (Holmes et al., 1989; Barel & MacDonald, 1993). In S. pombe, mutants devoid of GOGAT activity grow slowly and increase fivefold their NADP-GDH levels implying that the GS-GOGAT pathway is the primary route of glutamate biosynthesis (Barel & MacDonald, 1993). It can be concluded that while the yeasts studied to date have both routes for glutamate biosynthesis, the primary route is not always the same.

Chromosomal localization of the NADP-GDH structural gene

Using the CHEF conditions described in Methods, six bands were separated that corresponded to chromosomes from strain WM37 (MATa, his3) (Fig. 4, a), that match with those reported from the strain CBS 2360 (Sor & Fukuhara, 1989). These chromosomes, numbered I to VI, were immobilized on a nylon membrane. The K. lactis GDH1 (NADP-GDH) gene was localized on chromosome VI using the PCR-amplified fragment as probe (Fig. 4, b). The K. lactis GDH1 gene encoding the NADP-GDH should be added to the growing list of genes mapped in this organism.

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