Heterologous complementation of the Klaac null mutation of Kluyveromyces lactis by the Saccharomyces cerevisiae AAC3 gene encoding the ADP/ATP carrier

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Abstract

The KIAAC gene, encoding the ADP/ATP carrier, has been assumed to be a single gene in Kluyveromyces lactis, an aerobic, petite-negative yeast species. The Klaac null mutation, which causes a respiratory-deficient phenotype, was fully complemented by AAC2, the Saccharomyces cerevisiae major gene for the ADP/ATP carrier and also by AAC1, a gene that is poorly expressed in S. cerevisiae. In this study, we demonstrate that the Klaac null mutation is partially complemented by the ScAAC3 gene, encoding the hypoxic ADP/ATP carrier isoform, whose expression in S. cerevisiae is prevented by oxygen. Once introduced into K. lactis, the AAC3 gene was expressed both under aerobic and under partial anaerobic conditions but did not support the growth of K. lactis under strict anaerobic conditions.

Introduction

The ADP/ATP antiporter protein, located in the inner mitochondrial membrane, plays a key role in the energy metabolism of eukaryotic cells (Klingenberg, 1979). The genes encoding the carrier protein are localized in the nucleus and have been cloned from a variety of organisms; they are highly conserved in all eukaryotic organisms. In the yeast Schizosaccharomyces pombe, three genes, AAC1, AAC2, AAC3, whose expression is differently regulated by environmental factors, encode the ADP/ATP carrier (O’Malley et al., 1982; Lawson & Douglas, 1988; Kolarov et al., 1990; Sabová et al., 1993; Betina et al., 1995; Gavuriniková et al., 1996). AAC1 and AAC3 are not essential for growth on respiratory carbon sources and their disruption, either separately or together in the same cell, does not affect the mitochondrial ADP/ATP translocation (Drgon et al., 1992). AAC2 encodes the major translocator protein and is essential for growth on non-fermentable carbon sources, such as glycerol, ethanol and lactate. An aac2 yeast mutant, named op1 for oxidative phosphorylation defect, has been isolated as a spontaneous mutant (Kovácová et al., 1968). The central role played by the AAC2 gene in the cellular energy metabolism of S. cerevisiae can be deduced from the observation that, in an op1 genetic background, S. cerevisiae is unable to produce viable mitochondrial petite mutants, and thus resembles petite-negative yeasts (Bulder, 1964). A corresponding gene, KIAAC, has been identified and characterized in the petite-negative yeast Kluyveromyces lactis in our laboratory (Viola et al., 1995). Unlike in the case of S. cerevisiae, in K. lactis the ADP/ATP carrier is encoded by a single gene (Viola et al., 1995, 1999), similar to the situation seen in Schizosaccharomyces pombe (Couzin et al., 1996) and in the pathogenic yeast Candida parapsilosis (Nebohacová et al., 1999). The K. lactis gene complements the petite-negative phenotype exhibited by the S. cerevisiae op1 mutant, as well as its inability to grow on non-fermentable carbon sources (Viola et al., 1995). The KIAAC gene codes for a 305-amino-acid protein having a significant degree of similarity with the ADP/ATP antiporter of S. cerevisiae. A comparison of KIAAC with AAC1, AAC2 and AAC3 gene products shows an identity of 74.1%, 87.5% and 81.4%, respectively. Disruption of the single KIAAC gene is non-lethal in our haploid DPV1/27A K. lactis strain (Viola et al., 1995), but
is reported to be lethal in a different *K. lactis* strain (Clark-Walker & Chen, 2001). Moreover, deletion of SpANCl, the single gene encoding the ADP/ATP carrier in *Sch. pombe*, was also proved to be non-lethal (Couzin *et al.*, 1996). The *Klaac* null mutant in our strain is respiratory deficient and unable to grow on galactose, maltose and raffinose, consistent with the Kluyver-positive character of the species *K. lactis* (Entian & Barnett, 1983; Goffrini *et al.*, 1989, 2002). The phenotype of the *Klaac* null mutation was fully complemented, both in multicopy and monocopy plasmids, not only by AAC2, the major gene for the ADP/ATP carrier in *S. cerevisiae*, but also by the AAC1 gene, which is poorly expressed in this species. When AAC1 was introduced into *K. lactis* it was highly transcribed, which is consistent with the normal growth on glycerol that had been restored in the transformed mutant (Viola *et al.*, 1999).

In the present study, we demonstrate that the phenotype caused by the disruption of *Klaac* can be complemented by the third AAC gene of *S. cerevisiae*, AAC3, which enabled the cells to utilize the fermentable carbon sources maltose, raffinose and galactose, and the respiratory carbon source lactate, and improved the growth under partial anaerobiosis, but did not confer upon *K. lactis* the ability to grow under strict anaerobic conditions.

### Materials and methods

#### Yeast strains and growth conditions

The parental *Kluyveromyces lactis* strain and its cognate mutant containing the deletion of the *KLAAC* gene have been described previously (Viola *et al.*, 1995, 1999).

The complete medium (YP) contained 1% Bacto-yeast extract and 2% Bacto-peptone. The minimal medium contained 0.7% yeast nitrogen base without amino acids (Difco, Detroit, MI), supplemented with appropriate amino acids and bases to a final concentration of 40 μg mL⁻¹. The carbon sources were added at 2% (w/v) except where indicated otherwise. Lactate was a commercial racemic product (Fluka, St Louis, MO). Media were solidified with Bacto agar at 2%. The *K. lactis* cells were grown aerobically with vigorous shaking at 28 °C in theYP medium supplemented with the indicated carbon source. The growth medium for anaerobic and semi-anaerobic growth was complete medium supplemented with 12 μg mL⁻¹ ergosterol and 0.2% Tween 80 and the appropriate carbon source at 2%.

The anaerobic cultivation was performed using the AnaeroGen COMPACT AN010C/AN020C (Oxoid, Basingstoke, UK), which is a simple system for the anaerobic incubation of up to four Petri dishes. The plates were seeded and incubated at 28 °C for 5 days. Under the strict anaerobic conditions, the control *K. lactis* strain is unable to grow.

Semi-anaerobic conditions were achieved by AnaeroGen AN 25 (Oxoid). When an AnaeroGen sachet is placed in a sealed jar, the atmospheric oxygen in the jar is rapidly absorbed, with the simultaneous generation of carbon dioxide. The AnaeroGen sachet reduces the oxygen level in the jar to below 1% within 30 min. Under semi-anaerobic conditions the control *K. lactis* strain was still able to grow on glucose.

#### Plasmids

The following vectors were used: pUK-S11, a *K. lactis* multicopy vector (Chen & Fukuhara, 1988); and KCp491, a *K. lactis* monocopy vector (Prior *et al.*, 1993). Plasmid pKA3 was constructed by insertion of the 1.8-kb *Nhe*I–*Bam*HI DNA fragment containing AAC3 and its flanking regions into the *Nhe*I–*Bam*HI sites of the KCp491 vector and into the *Xba*I–*Bam*HI sites of the pUK-S11 vector. This AAC3-containing fragment was obtained by PCR amplification of the genomic DNA of the W303-1B strain of *S. cerevisiae*.

The following primers were employed: forward: 5’-GCTAGCCGCTTCAACTGAGACCGCCC-3’ corresponding to position −572 to −550 of the AAC3 gene; this primer contained the *Nhe*I sequence; reverse: 5’-GGATCCTGATAGCGCAAGGTACTTAAAGG-3’ corresponding to position +1282 to +1257 of the AAC3 gene; this primer contained the *Bam*HI sequence.

#### Cytochrome spectra and respiration

Differential spectra between reduced and oxidized cells of a suspension of cells at 60 mg mL⁻¹ (wet weight) were recorded at room temperature, using a Cary 219 spectrophotometer (Varian, Palo Alto, CA). Oxygen uptake rate was measured at 30 °C using a Clark electrode in a reaction vessel of 3 mL of air-saturated respiration buffer (0.1 M phthalate-KOH, pH 5.0), 10 mM glucose, starting the reaction with the addition of 20 mg of wet weight of cells, according to Ferrero *et al.* (1981).

#### RNA preparation and Northern analysis

Total RNA was extracted with hot acidic phenol (Ausubel *et al.*, 1994), from cells grown in YNB supplemented with the indicated carbon source. Northern analysis was carried out as previously described (Sherman *et al.*, 1983). The AAC3 probe was a 700-bp fragment obtained by PCR amplification with pKA3 plasmid as a template. The primers used for the amplification were forward 5’-GGTTTATCTCATTGGGAGG-3’ and reverse 5’-CAAGATCATTTAAGG-3’.

The amount of RNA loaded on the gel was estimated by hybridization with an actin gene probe (*ACTI*). It should be
noted here that, in agreement with results reported previously (Trezeguet et al., 1999), our data disqualify the use of the actin gene as a reference in particular conditions. In fact, for equivalent amounts of isolated total RNA, reduced levels of the control actin mRNA were observed under semi-anaerobiosis. Therefore, to estimate the quantity of total RNA loaded on the gels, we examined the amount of ribosomal RNAs after ethidium bromide staining.

All the probes were labelled with [α-32P]dCTP using the rediprime DNA labelling system (Amersham, Little Chalfont, UK).

Miscellaneous

Published procedures were used for transformation of K. lactis (Bianchi et al., 1987). Probes for Northern analysis were labelled with α-32P dCTP using the rediprime DNA labelling kit (Amersham).

Results

Complementation of the Klaac mutation by the Saccharomyces cerevisiae AAC3 gene

The Klaac null mutant is respiratory-deficient and unable to grow on galactose, maltose and raffinose. The AAC2 and the AAC1 genes, either in multicopy or in monocopy, fully complement the Klaac mutation (Viola et al., 1995, 1999). We asked the equivalent question of whether the Klaac mutation could be complemented by the Saccharomyces cerevisiae AAC3 gene.

As a host for this heterologous complementation, the Klaac mutant strain (disrupted by the URA3 gene, and made ura3) was used (DPV1/27-5A) (Viola et al., 1995). This ura3 host was transformed with the AAC3 gene either in multicopy or in monocopy. These recombinant plasmids were transformed into yeast strain DPV1/27-5A in which Klaac has been deleted and which is unable to grow either on non-fermentable or on fermentable carbon sources, with the exception of glucose. Transformants were plated on appropriate selective media (lacking uracil), and the resulting colonies were spotted onto media with different carbon sources to test for the ability of AAC3 to complement the Klaac phenotype. As a negative control, the empty plasmid was used. No complementation was observed with the control plasmid lacking ADP/ATP-carrier DNA. AAC3, either in multiple copy, or in single copy, fully complemented the growth defect of the Klaac null mutant on raffinose, galactose (Figs 1a and b) and maltose (data not shown), indicating that AAC3 can replace the inactivated Klaac.

Unlike the AAC1 and AAC2 genes of S. cerevisiae, which fully complement the negative phenotype of the Klaac null mutant, the AAC3 gene was unable fully to complement its growth defect. Although it enabled the mutant to grow on fermentable carbon sources, it only partially restored growth on non-fermentable carbon sources. In particular, transformants containing either the multicopy or the monocopy plasmid showed no complementation of the glycerol-negative phenotype (Fig. 1c), nor of the ethanol- nor of the pyruvate-negative phenotype (data not shown). The AAC3 gene did, however, enable the Klaac null mutant to grow on lactate (Fig. 1d). An analysis of the cytochrome spectra showed that the addition of AAC3 partially restored aa3 and b cytochromes and respiratory activity (Fig. 2 and Table 1), as expected by the complementation of the galactose- and raffinose-negative phenotype. Indeed, in K. lactis utilization of these carbon sources is prevented by the respiratory-deficient condition.

![Fig. 1. Growth phenotype of the Kluyveromyces lactis deletion mutant ΔKlaac with Saccharomyces cerevisiae AAC3 in monocopy and multicopy plasmid. Growth under aerobic conditions was scored after three days. Serial dilutions of (1) KJAAC parental strain; (2) Klaac null mutant; (3) Klaac null mutant transformed with S. cerevisiae AAC3 in monocopy; (4) Klaac null mutant transformed with S. cerevisiae AAC3 in multicopy were spotted on minimal medium plus fermentable carbon sources galactose (a) and raffinose (b) and on non-fermentable carbon sources glycerol (c) and lactate (d).](image-url)
**Kluyveromyces lactis under partial and strict anaerobic conditions**

*Kluyveromyces lactis*, although possessing high fermentative capacity, is unable to grow under strict anaerobic conditions. Under semi-anaerobic conditions *K. lactis* was able to grow on glucose but grew poorly on raffinose (Figs 3a and b, row 1). On the other hand, when the *Klaac* gene was disrupted almost no growth was observed (Figs 3a and b, row 2), indicating that the gene product of *KlAAC* is essential for growth under reduced oxygen concentration. AAC3, either in multicopy or in monocopy, was able to restore the growth under these conditions. It should be noted here that under partial anaerobiosis any of the *S. cerevisiae* AAC genes (*AAC1, AAC2* and *AAC3*) supported the growth on raffinose better than did *KlAAC* (Fig. 3, rows 3–5).

As the *AAC3* gene, in *S. cerevisiae*, encodes the hypoxic isoform of the mitochondrial ADP/ATP carrier, we hypothesised that *K. lactis* is an aerobic yeast because a gene equivalent to *AAC3* is absent from its genome. This was not the case, however, because under strict anaerobiosis no growth was observed either in the wild-type strain or in the *Klaac* null mutant transformed with *AAC3* either in multicopy or in monocopy (data not shown).

**Expression of AAC3**

The ability of *AAC3* to complement the *Klaac::ura3* mutation both under reduced oxygen pressure and in aerobic conditions prompted us to analyse the expression of *AAC3* in *K. lactis* by Northern analysis. Total RNA was extracted from the DPV1/27-5A/F *Klaac::ura3* mutant transformed with the KCp491 monocopy plasmid carrying the *AAC3* gene, grown in a variety of conditions, and hybridized with an *AAC3* probe. The expression of the *AAC3* gene was also detected in cultures grown in aerobicosis in the presence of glucose or raffinose (Fig. 4, lanes 1 and 2). Semi-anaerobic conditions caused an increase of the level of *AAC3*-specific mRNA (Fig. 4, lanes 3 and 4). Unlike in *S. cerevisiae*, the presence of oxygen did not prevent the expression of *AAC3*; however, the reduced oxygen concentration did increase the gene expression.

**Discussion**

We had demonstrated previously that the *Saccharomyces cerevisiae* genes *AAC1* and *AAC2*, which encode the

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Table 1. Effect of the *ScAAC3* gene on respiration of the *Klaac* null mutant

<table>
<thead>
<tr>
<th>Strains</th>
<th>Respiration (µL of oxygen consumed h⁻¹ mg⁻¹ of dry mass)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Kluyveromyces lactis</em> <em>KlAAC</em></td>
<td>50.0</td>
</tr>
<tr>
<td><em>Klaac</em> null mutant</td>
<td>8.9</td>
</tr>
<tr>
<td><em>Klaac</em> null mutant/<em>AAC3</em></td>
<td>21.0</td>
</tr>
</tbody>
</table>

Cells were grown on 2% glucose. All the values are the average of three independent experiments. In no case was the variation higher than 15%.

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Fig. 2. Cytochrome profile of *Klaac* null mutant (profile a), *KlAAC* parental strain (profile b) and *Klaac* null mutant transformed with *Saccharomyces cerevisiae* *AAC3* in monocopy plasmid (profile c). Cells were grown on minimal medium plus 2% glucose.

Fig. 3. Growth phenotype in semi-anaerobic conditions, on glucose (a) and raffinose (b), of the *KlAAC* parental strain (1), *Kluyveromyces lactis* deletion mutant Δ*Klaac* (2) and *K. lactis* deletion mutant Δ*Klaac* transformed by *ScAAC1* (3), *ScAAC2* (4) and *ScAAC3* (5) (all genes were in monocopy plasmids).

Fig. 4. Northern blot analysis of total RNA extracted from the DPV1/27-5A/F *Klaac::ura3* mutant transformed with the KCp491 monocopy plasmid carrying the *AAC3* gene, grown in aerobicosis in the presence of glucose (lanes 1 and 2) or raffinose (lanes 3 and 4).
nucleotide carrier, are able to complement the pleiotropic negative phenotype of the Klaac null mutant of K. lactis (Viola et al., 1995, 1999). In Table 2 all pairs of homologous and heterologous complementation involving Klaac, AAC1, AAC2 and AAC3 genes are reported.

Here we show that wild-type K. lactis, which is unable to grow under strict anaerobiosis, grows under partial anaerobiosis, and that the deletion of the Klaac gene considerably affects its growth rate in this condition. Taken together, these findings indicate that, for fermentative aerobes such as K. lactis, an intact mitochondrial ADP/ATP carrier is required for growth in the presence of reduced oxygen tension.

The growth of K. lactis on the fermentative carbon source raffinose is inhibited by partial anaerobiosis, such as occurs in the presence of respiration inhibitors and in respiratory-deficient conditions. ScAAC3 and the other two genes, ScAAC1 and ScAAC2, promoted growth under these conditions. The ability of the S. cerevisiae genes and the inability of the K. lactis gene to support growth on raffinose under semi-anaerobic conditions is in agreement with previous observations that KIAAC gene expression is significantly reduced in semi-anaerobiosis (Trezeguet et al., 1999). In that condition, the level of ADP/ATP carrier is reduced, and consequently the translocation of ADP/ATP in the cell could be insufficient to sustain cell multiplication.

Moreover, results presented here demonstrate that the ScAAC3 gene was also able to complement the lactate-negative, but not the glycerol- and ethanol-negative, phenotype. On the other hand, the partial restoration of the cytochromes profile and respiration is in contrast to the absence of growth on the respiratory carbon sources glycerol and ethanol, but not on lactate. Our results are consistent with those seen in previous work with a K. lactis mutant of the gene KISDH1, which encodes the flavoprotein subunit of succinate dehydrogenase (SDH) complex and which is essential for the aerobic utilization of carbon sources (Saliola et al., 2004). This mutant, just as our transformed strain, although possessing a high respiration rate, was unable to grow on any respiratory carbon source with the exception of lactate. It was hypothesized that lactate utilization in K. lactis can be achieved through an alternative pathway bypassing SDH; in particular, lactate-derived pyruvate would be utilized through the alternative pyruvate decarboxylase pathway (Saliola et al., 2004).

Regarding the inability of the AAC3 gene to support growth on the respiratory carbon sources ethanol and glycerol, this can be explained by in vitro experiments, which indicate that the AAC3 isofrom is more effective than the other isoforms for the inverse exchange of cytosolic ATP for mitochondrial ADP, thus preventing efficient oxidative phosphorylation under the conditions used (Sokolikova et al., 2000). Our data in vivo support this Kolarov hypothesis. The inverse exchange has also been postulated for ANT2, a human isofrom preferentially expressed under

![Fig. 4. Effect of aerobic and semi-anaerobic conditions and carbon sources on the transcription of ScAAC3. Total RNA was extracted from the Klaac::ura3 mutant transformed with the AAC3 gene in monocopy plasmid, grown under aerobiosis in the presence of glucose and raffinose (lanes 1 and 2), and in semi-anaerobic conditions in the presence of glucose and raffinose (lanes 3 and 4) and hybridized with an AAC3 probe.](image)

<table>
<thead>
<tr>
<th>Strains</th>
<th>Carbon source</th>
<th>AAC1</th>
<th>AAC2</th>
<th>AAC3</th>
<th>KIAAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saccharomyces cerevisiae+O₂</td>
<td>Glycerol</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Kluuyveromyces lactis+O₂</td>
<td>Glycerol</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Kluuyveromyces lactis+O₂</td>
<td>Lactate</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Kluuyveromyces lactis+O₂</td>
<td>Galactose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Kluuyveromyces lactis+O₂</td>
<td>Raffinose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Kluuyveromyces lactis low O₂</td>
<td>Glucose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Kluuyveromyces lactis low O₂</td>
<td>Raffinose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Kluuyveromyces lactis low O₂</td>
<td>Glucose</td>
<td>nd</td>
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</tr>
</tbody>
</table>

The results refer to the gene in monocopy plasmid.

nd, not determined.
glycolytic metabolism (Giraud et al., 1998) and for the mouse ANT2 gene, which is constitutively expressed in tissues other than skeletal muscle (Levy et al., 2000).

It is interesting to note that, unlike in S. cerevisiae, where the ScAAC3 gene is expressed only under strict anaerobiosis, in K. lactis it is expressed in aerobicosis, and the increased gene expression seen in reduced oxygen conditions indicates that it maintains a certain regulation. In addition, the gene expression seen in reduced oxygen conditions indicates that K. lactis is capable of growth under anaerobic conditions, although it is still able to survive exposure to reduced oxygen tension. Here we have demonstrated that the inability of K. lactis to grow under anaerobic conditions is not as a result of the absence from its genome of a gene equivalent to the hypoxic isoform of S. cerevisiae, AAC3, because its addition does not confer on K. lactis the ability to grow under strict anaerobic conditions.

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