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Expression of the ADP/ATP carrier encoding genes in aerobic yeasts; phenotype of an ADP/ATP carrier deletion mutant of *Schizosaccharomyces pombe*

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Abstract

The expression of a key mitochondrial membrane component, the ADP/ATP carrier, was investigated in two aerobic yeast species, *Kluyveromyces lactis* and *Schizosaccharomyces pombe*. Although the two species differ very much in their respiratory capacity, the expression of the carrier in both yeast species was decreased under partially anaerobic conditions and was induced by nonfermentable carbon sources. The single ADP/ATP carrier encoding gene was deleted in *S. pombe*. The null mutant exhibits impaired growth properties, especially when cultivated at reduced oxygen tension, and is unable to grow on a nonfermentable carbon source. Our results suggest that the inability of *K. lactis* and *S. pombe* to grow under anaerobic conditions can be related in part to the absence of a functional ADP/ATP carrier due to repression of the corresponding gene expression.

Keywords: ADP/ATP carrier; Gene expression; Null mutant; (*Kluyveromyces lactis*); (*Schizosaccharomyces pombe*)

1. Introduction

The ADP/ATP carrier (Aacp or Ancp) of the inner mitochondrial membrane has entered into the focus of recent investigations because it affords a suitable model for elucidation of membrane transport phenomena [1,2] and because it could be intimately involved in cell growth control [3,4] and in programmed cell death [5]. In vivo, under aerobic conditions, the Aacp exchanges cytoplasmic ADP for intramitochondrially synthesized ATP, thus controlling the rate of oxidative phosphorylation together with the phosphate carrier and the ATP synthase. In contrast, under anaerobic conditions or in respiration-deficient mutants of yeast, the oxidative phosphorylation is not functional and the carrier works in the opposite direction, providing the glycolytically made ATP to mitochondria. In the mean time, due to the electrogenic character of the ADP/ATP exchange, it can create a membrane potential across the inner mitochondrial membrane. Previous work employing ADP/ATP translocator mutants of *Saccharomyces cerevisiae* demonstrated that the intrami-

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tochondrial ATP and/or mitochondrial membrane potential are required for growth [3,4,6].

In *S. cerevisiae*, the ADP/ATP carrier is encoded by three distinct genes, *AAC1*, *AAC2*, and *AAC3*, which are all functional [7]. It has been shown that they are all regulated by oxygen which in the case of *AAC2* and *AAC3* exerts its effect through heme [8,9]. Transcription of *AAC1* is also affected by oxygen, but in a heme-independent manner [10]. Under low oxygen tension, expression of *AAC2* is strongly repressed, whereas transcription of *AAC3* is derepressed several times [8–10]. It was previously demonstrated that deletion of *AAC2* and *AAC3* produced mutant cells unable to grow under anaerobiosis [7].

Under normal growth conditions, *AAC2* is the main expressed isoform and negative mutations in this gene yield cells unable to form *petite* (ρ^-) mutants [3,7], thus resembling petite-negative yeast strains, such as *Kluyveromyces lactis* and *Schizosaccharomyces pombe* [11]. These two species, although possessing high fermentative capacity, are unable to grow under strict anaerobic conditions [12]. With respect to the ADP/ATP carrier, *K. lactis* and *S. pombe* differ from *S. cerevisiae*, as they most probably have only one carrier encoding gene within their genomes [13,14]. The effect of oxygen on *AAC* gene expression in *S. cerevisiae*, as well as growth phenotype of *aac* mutants [6–10], suggests that insufficient ATP/ADP carrier function might become critical for the ability of aerobic yeast species to grow under reduced oxygen tension. To test this possibility, we have investigated the effect of oxygen on the expression of the ADP/ATP carrier encoding genes in the two most commonly used aerobic yeasts, *S. pombe* and *K. lactis*. In addition, we constructed an *anc1*Δ *S. pombe* mutant, and we will describe some of its properties.

2. Materials and methods

2.1. Yeast strains and growth conditions

K. lactis strain JBD100 (*trp1*, *lac4-1*, *ura3-100*) was provided by H.Y. Steensma (Kluyver Laboratory, University of Technology, Delft). The mutant *Klhap2* strain MW270-7B/16 (*MATa*, *uraA*, *leu2*, *metA1*, *hap2::URA3*) and the corresponding parent wild-type, MW270-7B, were provided by M. Bolotin-Fukuhara (Institut de Génétique et Microbiologie, Paris). *S. pombe* wild type strain was *h*$^-$ *972*. Disruption of the *ANC1* gene was made in MR1 (*h*$^-$, *ura4-D18*, *leu1-32*) (from Michel Rochet, Institut National Agronomique, Thivernal Grignon, France).

The parental *S. cerevisiae* strain and its cognate mutants containing deletions in *AAC* genes were previously described [7,15].

The *S. cerevisiae* and *K. lactis* cells were grown aerobically at 30°C in 1% yeast extract, 2% bactopeptone (YP) supplemented with the indicated carbon source. For cultivation of *S. pombe*, the bactopeptone was omitted and the yeast extract was lowered to 0.5% [16]. The anaerobic cultivation was performed in anaerobic jars using Anaerocult A system (Merck) as previously described [7,9]. The growth media for anaerobic growth were supplemented with 12 μg/ml ergosterol and 0.2% Tween 80. Semianaerobic conditions were achieved by static cultivation in small flasks tightly closed and filled up to the top with growth medium.

2.2. Disruption of SpANC1 gene in S. pombe

791 bp of the *SpANC1* open reading frame (969 bp) were deleted and replaced with the *S. cerevisiae LEU2* gene. In a first round of experiments, *ANC1* noncoding regions were amplified using PCR starting from plasmid pRS306-5.1 [14]. The following sets of primers were used: KS (5′-TCGAGGTCGACGGTATC-3′) (Stratagene) and ^{123}5′-GCGGTTTTgGATCCAGCAGCAG-3′^{110} to amplify the 5′ noncoding region and SK (5′-CGCTCTAGAACTAGTGGATC-3′) (Stratagene) and ^{901}5′-GTTGCTGGAtCcGGTGTCCTTTCC-3′^{924} to amplify the 3′ noncoding region. These primers allowed to introduce a *Bam*HI site (underlined) at one end of each fragment. The 5′ fragment (2.1 kb) generated by digestion with *Bam*HI and *Eco*RI was cloned into the corresponding restriction sites of phagemid pRS306, then the 3′ fragment (2.0 kb) was cloned into the relevant sites after digestion with *Bam*HI and *Xba*I. The recombinant plasmid was linearized with *Bam*HI to introduce a *Bgl*II fragment (2.8 kb) derived from plasmid CV9 [17] and containing the *S. cerevisiae LEU2* gene, which is able to complement a *LEU1*

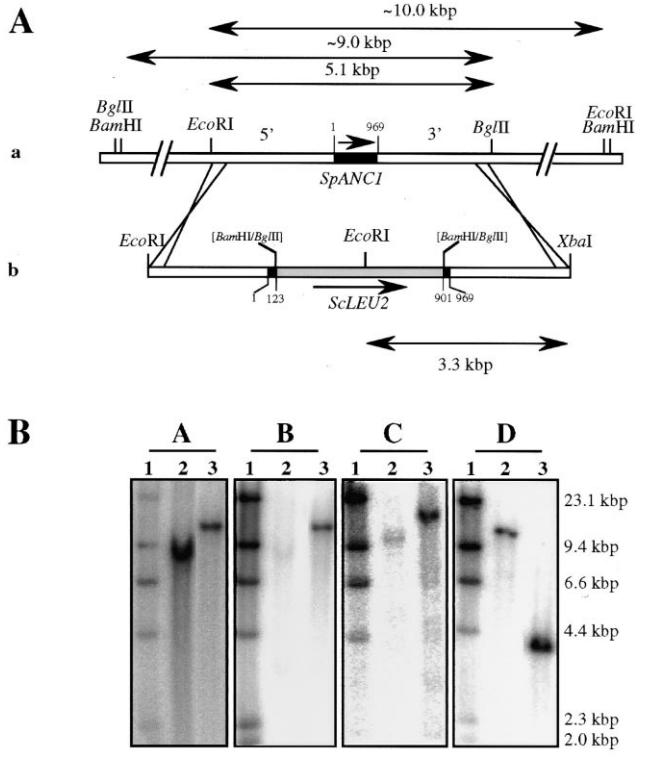

Fig. 1. Southern blot analysis of the *ANC1* gene disruption in *S. pombe*. (A) Schematic representation of the homologous recombination at the *ANC1* locus. (a) Chromosomal *ANC1* locus. (b) *Eco*RI-*Xba*I DNA fragment bearing the *LEU2*-disrupted *ANC1* locus. (B) Genomic DNA was prepared either from the parental strain (lanes 2) or from the *Spanc1* mutant cells (lanes 3). After digestion with *Bam*HI (A and B) or *Bgl*II (C) or *Eco*RI (D) genomic DNAs were probed either with a *Bgl*II fragment containing the *S. cerevisiae LEU2* gene (B) or a *Bam*HI-*Xba*I fragment corresponding to the 3′ noncoding region of *SpANC1*. Lanes 1: *Lambda* phage DNA digested with *Hin*dIII and probed with itself.

defect in *S. pombe*. The final plasmid contained the *LEU2* gene bordered by the 5′ and the 3′ *ANC1* noncoding sequences on a 6.9-kb fragment, which was isolated by *Eco*RI and *Xba*I digests then used to transform MR1 strain. The transformants were selected for their ability to grow on a complete minimal medium deprived of leucine. Deletion of the *ANC1* gene was confirmed by Southern blot hybridization (Fig. 1).

2.3. Miscellaneous methods

Isolation of plasmid DNA, preparation of probes for hybridization, isolation of total RNA, blotting and hybridization were carried out as described in [18]. The probes for hybridization were: a 1.2-kb *Ban*II/*Dra*I fragment of *S. cerevisiae AAC2* gene, a 0.9-kb *Hin*dIII fragment of *S. pombe SpANC1* gene, and a 0.6-kb *Bam*HI fragment of *S. cerevisiae ACT1* gene.

3. Results

3.1. The KlAAC transcript

K. lactis is an aerobic, respiratory, petite-negative yeast species in which the mitochondrial ADP/ATP carrier is most probably encoded by a single nuclear gene, *KlAAC*. It is 84.4% identical, at the DNA level, with *AAC2*, the major carrier encoding gene in *S. cerevisiae* [13]. Northern blot hybridization analyses of total RNA from *K. lactis* show that transcription

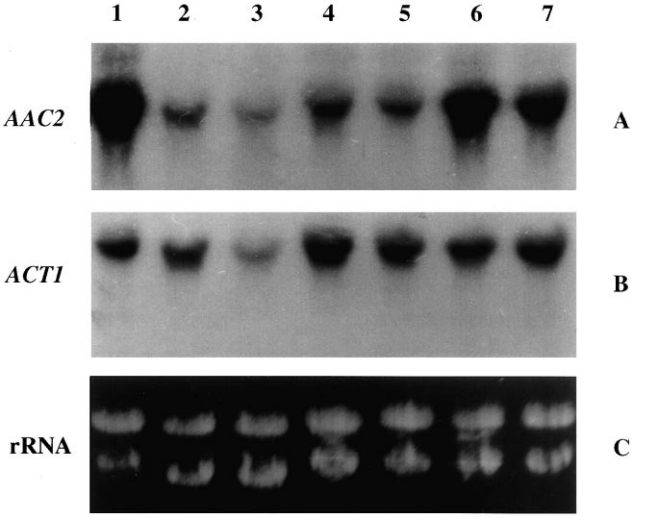

Fig. 2. Effect of various growth conditions of *K. lactis* cells on transcription of the ADP/ATP carrier encoding gene (*KlAAC*). Cells were grown in YPD medium under aerobic (lane 2) or semianaerobic (lane 3) conditions, as described in Section 2. Total RNA (20 μg in each lane) was isolated either from cells continuously grown in 2% glucose (lanes 2 and 3), or from cells grown overnight in 2% glucose and then shifted for 4 h to 2% glucose with (lane 5) or without 12 μg/ml hemin (lane 4). Cells were shifted for 4 h to YP medium supplemented with either 2% lactate (lane 6), or 3% glycerol+1% ethanol (lane 7). *S. cerevisiae AAC2* transcript is shown in lane 1. The probes used were (A) 1.2-kb *Ban*II-*Dra*I fragment of *S. cerevisiae AAC2* and (B) 0.6-kb *Bam*HI fragment of *ACT1* from *S. cerevisiae*. Ribosomal RNAs stained by ethidium bromide (C) are shown to give an estimate of the amounts loaded on the gels.

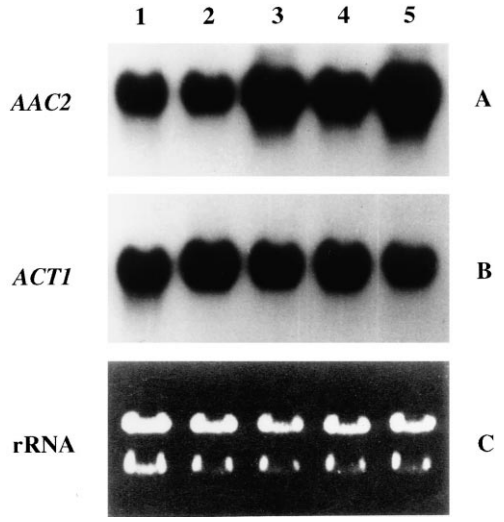

Fig. 3. Expression of *KlAAC* in the wild type strain 7B and in the *Klhap2* mutant strain 7B/16. The wild type (lanes 2 and 3) and mutant cells (lanes 4 and 5) were grown overnight in YP medium supplemented with 2% glucose and then shifted for 4 h to YP supplemented with either 2% glucose (lanes 2 and 4), or 3% glycerol+1% ethanol (lanes 3 and 5). Probes and other conditions are those described in Fig. 2.

of the *KlAAC* gene produces a transcript similar in size to that of *AAC2* transcript in *S. cerevisiae*, and that the level of *KlAAC* mRNA varies in cells depending on growth conditions. In contrast to the results reported earlier [13], derepressed conditions (Fig. 2, lanes 6 and 7) induce expression of the *KlAAC* gene, as compared to the repressed conditions in the presence of glucose. The derepression ratio is smaller than that observed with *AAC2* gene in *S. cerevisiae* [8]. This is in agreement with the results obtained by other authors [19,20], indicating that the respiratory genes that have a high repression ratio in *S. cerevisiae* show only a two-fold or smaller derepression ratio in *K. lactis*. On the other hand, semianaerobic conditions significantly reduces the level of *KlAAC* specific mRNA (Fig. 2, lane 2) as compared to aerobic conditions. It should be noted here, that under semianaerobic conditions the *K. lactis* were still able to grow on glucose, although with a reduced rate (not shown).

The effect of oxygen on expression of *KlAAC* in *K. lactis* could be mediated through heme and thus through heme-dependent transcription factors, as it is the case for *AAC2* gene in *S. cerevisiae* [8]. This question was addressed by adding hemin to the growth media. It induced expression of nuclear encoded mitochondrial proteins in both *K. lactis* [20] and *S. cerevisiae* [9]. Therefore, the effect of heme on *KlAAC* gene expression was also tested. Our results show that addition of hemin (Fig. 2, lane 5) to *K. lactis* does not significantly change the level of *KlAAC* transcripts, with respect to nonsupplemented cells. It should be noted here that for equivalent amounts of isolated total RNA, reduced levels of

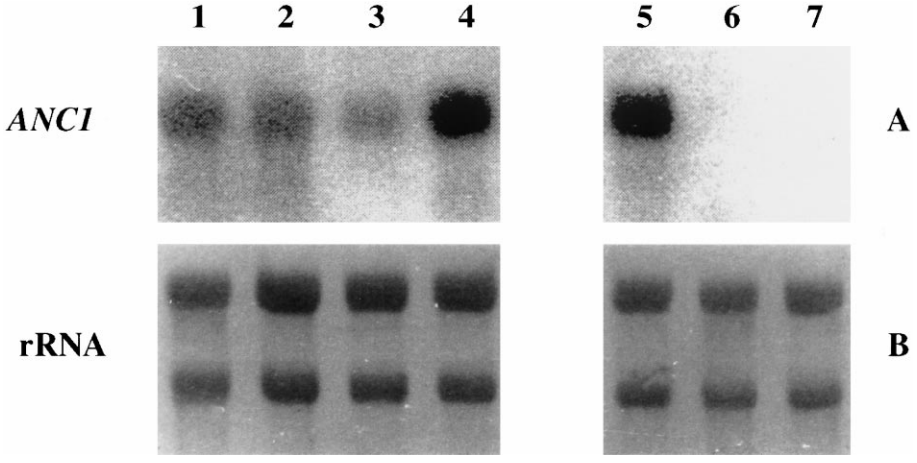

Fig. 4. Effect of carbon sources and semianaerobic conditions on the transcription of *SpANC1* in *S. pombe*. The wild type strain MR1 (lanes 1–5) and the *Spanc1* deletion mutant MR12 (lanes 6 and 7) were grown in YE medium supplemented with different carbon sources under either aerobic (lanes 1, 2, 4–6) or semianaerobic conditions (lanes 3 and 7) as described in Section 2. Total RNA was isolated from cells continuously grown in 3% glucose (lanes 1, 3 and 6), and from cells grown overnight in 3% glucose and then shifted to 8% glucose (lane 2), 3% glycerol+1% ethanol (lane 4), or 3% raffinose (lane 5). Ribosomal RNAs were estimated after methylene blue staining.

the control actin mRNA were observed in *K. lactis* cells grown under semianaerobiosis (Fig. 2B). This disqualifies the use of actin gene as a reference in these particular conditions. Therefore, to estimate the quantity of total RNA loaded on the gels, we examined the amount of ribosomal RNAs after ethidium bromide staining (Fig. 2C).

Two sequences, RTCRYNNNNNACG and TGATTGGT, which are identical with the consensus sequences for recognition of respectively the ABF1 factor and the oligomeric complex HAP2/3/4/5 in *S. cerevisiae*, were identified in the upstream region of *KlAAC* gene [13]. Both factors play an important role in the regulation of genes encoding mitochondrial proteins in *S. cerevisiae*, including the *AAC2* gene [8,19,20]. Therefore, the effect of *Kl*HAP2 on *KlAAC* expression was tested. Fig. 3 demonstrates that deletion of *KlHAP2* gene neither changes significantly the expression of *KlAAC* nor influences induction of the gene expression by nonfermentable substrate. It should be noted here that efforts were made by ourselves to inactivate *KlAAC* but no viable cells could be obtained. The reason for this is not clearly understood.

3.2. The SpANC1 transcript

S. pombe is an aerobic, fermentative, petite-negative yeast species in which the mitochondrial ADP/ATP carrier is encoded by a single gene (*SpANC1*) [14]. Its expression under different growth conditions was investigated using as a probe a 0.9-kb (*Hin*dIII) ^{32}P-labeled DNA fragment encompassing two thirds of the *SpANC1* coding region. Northern blot analyses show that the *SpANC1* gene is transcribed to relatively low levels in cells grown in media containing either low or high concentration of glucose (Fig. 4, lanes 1 and 2). The level of *SpANC1* specific mRNA is further reduced by cultivation under semianaerobic conditions (Fig. 4, lane 3). As observed with *K. lactis*, expression of *SpANC1* gene was induced in *S. pombe* cells under derepressed conditions, though to a lower extent. This is documented by the high level of *SpANC1* specific mRNA in cells grown in media containing either glycerol, or raffinose as carbon sources (Fig. 4, lanes 4 and 5). The successful deletion of *SpANC1* (see below) is corroborated by the absence of specific hybridization when total RNA was isolated from the deletion mutant grown under either aerobic or semianaerobic conditions (Fig. 4, lanes 6 and 7). These results are consistent with previous works demonstrating the absence in *S. pombe* of another *ANC* gene induced under hypoxic conditions [14].

3.3. Deletion of SpANC1 gene and phenotype of the null mutant

The ADP/ATP carrier encoding gene was deleted

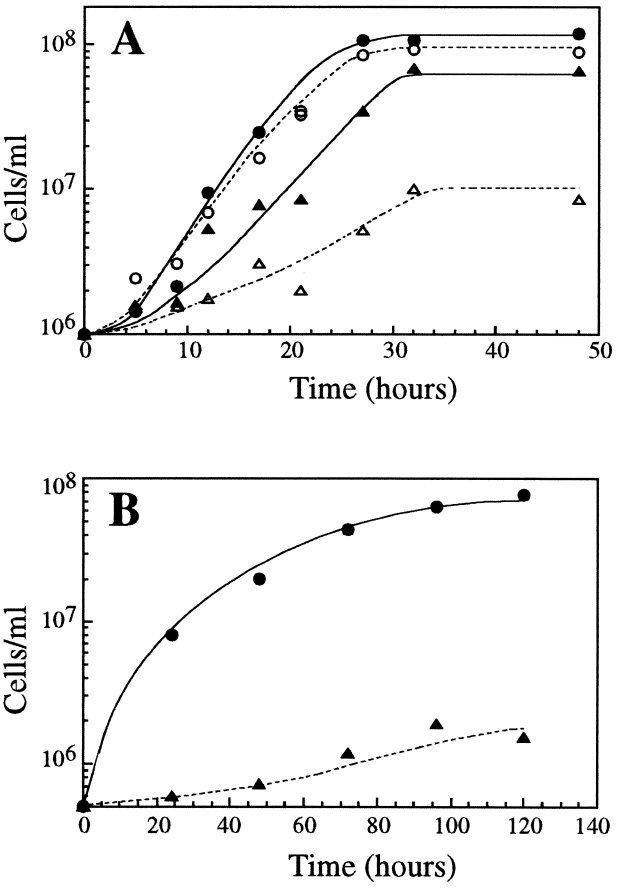

Fig. 5. Growth of *S. pombe* wild type and *Spanc1*Δ mutant under aerobic and semianaerobic conditions. The wild type *S. pombe* MR1 (●, ○) and *Spanc1*Δ mutant (▲, △) grown in YE medium supplemented with 3% glucose, were inoculated (A) to 10^6 cells/ml into rich medium supplemented with either 3% glucose (●, ▲) or 3% raffinose (○, △) and cultivated aerobically with vigorous shaking. (B) The wild type and the mutant were inoculated to 5×10^5 cells/ml into bottles filled up to the top with YE medium and incubated without shaking. Bottles were sequentially opened at the time indicated and the cells were counted.

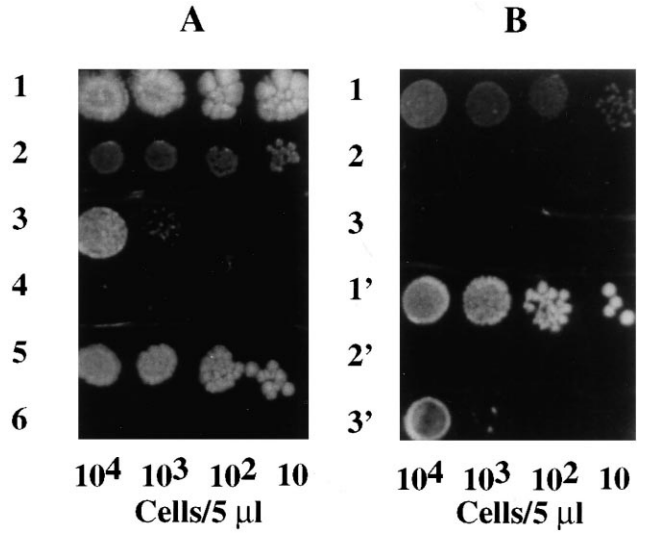

Fig. 6. Growth of *S. pombe* wild type and *Spanc1*Δ mutant on solid media supplemented with different carbon sources. (A) The wild type (1, 3 and 5) and mutant (2, 4 and 6) cells were first grown in YE with 3% glucose and 5 µl aliquots of serial dilutions were spotted on YE with 3% glucose (1 and 2), 3% glycerol+1% ethanol (3 and 4) or 2% raffinose (5 and 6). In (B) the wild type (1), the *Spanc1*Δ mutant (2) and the *S. cerevisiae* Δ*aac1-3* triple mutant (3) cells were spotted on YE with 3% glucose, incubated for five days under strict anaerobiosis (1–3) and then transferred to aerobiosis (1′–3′).

in *S. pombe*, as described in Section 2. Surprisingly, although *S. pombe* is a petite-negative yeast species, the *S. pombe* mutant cells, in which the *SpANC1* gene is deleted, are still viable though their growth properties are impaired.

In glucose-containing liquid medium and under aerobic conditions, the *Spanc1* mutant cells grew at a rate comparable to that of the corresponding wild-type strain. However, the former cells grew slower and present lower growth yield in raffinose-containing liquid medium (Fig. 5A). As expected, they could not grow on nonfermentable carbon sources (not shown). Wild type *S. pombe* cells could still grow under semianaerobic conditions, but deletion of the ADP/ATP carrier encoding gene considerably affected growth rate in glucose-containing medium (Fig. 5B). This deletion also prevented growth on glucose-containing medium supplemented with antimycin A. Cells were dead after a 24-h incubation and unable to recover after removing antimycin A from the culture medium (not shown).

Growth defects of the *Spanc1* null mutant were even more prominent on solid media (Fig. 6). Alike true petite yeasts, the mutant cells form small colonies on glucose plates and do not grow on solid medium containing either glycerol plus ethanol or raffinose (Fig. 6A). Furthermore, an interesting phenotype was observed when the cells were kept under tighter anaerobic conditions (anaerobic jars). Indeed, both the wild type *S. pombe* and the *S. cerevisiae* triple deletion mutant, JLY1-3 (Δ*aac1-3*), were able to recover after oxygen is supplied again while the *S. pombe* mutant cells were evidently dead (Fig. 6B).

4. Discussion

K. lactis and *S. pombe* are two petite-negative yeast species which differ very much in their respiratory capacity (Q_{O_2} = 134 and 10 µl/h/mg d.w., respectively [12]) but both are known to strongly ferment glucose (Q_{CO_2} = 185 and 404 µl/h/mg d.w., respectively [12]) and to require the presence of oxygen for growth. As demonstrated previously by Southern blot analyses [13,14] and confirmed in this work by Northern blot analyses, both *K. lactis* and *S. pombe* have a single ADP/ATP carrier encoding gene in their genome. Results presented above demonstrate that both *KlAAC* and *SpANC1* are inducible by growth on nonfermentable carbon sources, although not to a similar degree. On the other hand, under semianaerobic conditions their expressions are repressed in both species. The negative effect of semianaerobic conditions on the carrier encoding gene is important enough to allow us to extrapolate that under more strict anaerobic conditions, a complete absence of functional translocator, and consequently an arrest of cellular growth, can take place.

It was recently demonstrated that expression of the *K. lactis* genes related to mitochondrial functions can be induced by heme as it is the case in *S. cerevisiae* [21]. It was also shown that the 5-aminolevulinate synthase encoding gene in *K. lactis* (*KlHEM1*) is induced under reduced oxygen tension [22]. Both findings suggest that *K. lactis* cells own regulatory mechanisms similar to those of *S. cerevisiae* in order to respond at the gene level to heme as well as to anaerobiosis. Yet, they show that aerobic respiratory yeast possess within their genomes hypoxic genes which are induced under low oxygen tension. Our results demonstrate that the level of *KlAAC* specific

mRNA responds neither to heme addition nor to mutated *Klhap2* factor. They also show that *KlAAC* is negatively regulated by the absence of oxygen, suggesting that *KlAAC* regulation resembles rather that of *AAC1* than that of *AAC2* in *S. cerevisiae* [8,10]. It was previously shown that *AAC1* encodes a minor isoform of the ADP/ATP carrier and that its expression is almost completely switched off by anaerobiosis or in respiratory-deficient mutants [10]. This is achieved in an heme-independent manner in contrast to what is observed with *AAC2*. Accordingly, *S. cerevisiae* double deletion mutant (*Δaac2*, *Δaac3*), in spite of an intact *AAC1* gene, does not grow under anaerobic conditions and does not form viable petite (ρ^-) mutants [15]. *K. lactis* cells also present this phenotype.

The role of heme in *SpANC1* regulation was not investigated in this paper. We used another approach and, indeed, we succeeded in deleting the *SpANC1* gene and in preparing an oxidative phosphorylation-deficient mutant of a petite-negative yeast. To our knowledge, this is the second example of a null *S. pombe* mutant of a nuclear encoded mitochondrial component [23] and we anticipate that the *Spanc1* mutant will be a useful host to study the expression of heterologous ADP/ATP carrier proteins, including those encoded by the human genes.

The growth phenotype of the *S. pombe anc1* mutant resembles that of *S. cerevisiae aac1-3* triple deletion mutant [6,15]. The *Spanc1* mutant does not grow on nonfermentable carbon sources and a reduced growth on glucose-containing medium under aerobic conditions is also observed. Furthermore, it was completely inhibited in the presence of antimycin A, a respiration inhibitor, thus confirming that the essential pool of intra-mitochondrial ATP in the mutant is only maintained by respiration and not through a passive diffusion between the different cellular compartments. Interestingly, in contrast to the *S. cerevisiae* triple *Δaac1-3* mutant, the *Spanc1* mutant does not grow on raffinose and viability of the cells is markedly affected by anaerobic cultivation. This could be accounted for by a greater sensitivity of the null mutant, as compared to the parent, to the oxidative stress induced by supplying oxygen to the anaerobic culture. Last, partial anaerobiosis inhibits growth of the mutant cells to a larger extent than it inhibits growth of the parental cells, consistent with the petite negative phenotype of *S. pombe*.

Taken together, these findings demonstrate that for fermentative aerobes such as *S. pombe* an intact mitochondrial ADP/ATP carrier is required for growth more in the presence of reduced oxygen tension than under normal aerobic conditions.

Acknowledgements

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