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Genomic Exploration of the Hemiascomycetous Yeasts: 11. *Kluyveromyces lactis*

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Abstract Random sequencing of the *Kluyveromyces lactis* genome allowed the identification of 2235–2601 open reading frames (ORFs) homologous to *S. cerevisiae* ORFs, 51 ORFs which were homologous to genes from other species, 64 tRNAs, the complete rDNA repeat, and a few Ty1- and Ty2-like sequences. In addition, the complete sequence of plasmid pKD1 and a large coverage of the mitochondrial genome were obtained. The global distribution into general functional categories found in *Saccharomyces cerevisiae* and as defined by MIPS is well conserved in *K. lactis*. However, detailed examination of certain subcategories revealed a small excess of genes involved in amino acid metabolism in *K. lactis*. The sequences are deposited at EMBL under the accession numbers AL424881–AL430960.

Key words: Random sequencing; Gene function; Synteny; Mitochondrial DNA

1. Introduction

Kluyveromyces lactis, closely related to *Saccharomyces cerevisiae* and initially called *Saccharomyces lactis*, has been studied since the early 1960s [1,2]. Recently, the elaboration of a replicative vector system [3], based on a new 2μ-like plasmid, pKD1 [4,5], has contributed to the development of molecular genetics of this yeast ([6], for a review).

Interest in *K. lactis* arose from its distinctive physiological properties compared to *S. cerevisiae*. *K. lactis* assimilates a wider variety of carbon sources, especially lactose [7]. It is a 'petite-negative' [8,9] and a 'Crabtree-negative' yeast [10]. The absence of glucose repression of respiration illustrates a major difference in the regulation of glucose metabolism as compared to *S. cerevisiae*. Some *K. lactis* strains contain cytoplasmic linear DNA plasmids conferring the killer phenotype. The state of the art in these various fields has been reviewed by Stark et al. [11], Wésolowski-Louvel et al. [6] and Chen and Clark-Walker [12]. Accumulating data concerning glucose metabolism and its regulation in *K. lactis* indicate that the redundancy of glycolytic genes that exists in *S. cerevisiae* is not found in *K. lactis* [13–17].

Besides fundamental research, *K. lactis* is used for industrial applications. For example, *K. lactis* has been utilized for many years as a source of β-galactosidase. Recently, *K. lactis* has been successfully used for high-level production of secreted heterologous proteins such as calf prochymosin, human serum albumin and human interleukin-1β [18–20].

Electrophoretic karyotyping distinguishes six chromosomes ranging in size from about 1 to 3 Mb [6] and the total genome is estimated to be 10–12 Mb. Five of the six centromeres have been characterized [21]. A genetic map of the *K. lactis* genome, describing 75 loci, has been published in 1995 [22] but an updated survey of databases reveals that 150 chromosomal genes of *K. lactis* have been cloned and entirely sequenced. The 'killer' and pKD1 plasmids have also been completely sequenced and partial sequences of the mitochondrial genome and of the nuclear ribosomal RNA genes are available. The sequence collection of *K. lactis* has been recently enriched by the addition of 588 random sequence tag (RST) sequences [23] identifying 292 novel genes.

2. Materials and methods

2.1. Strains and libraries

Strain CLIB 210, originally called 2359/152 (*MATa metA1-1*, K^+R^+), is an auxotrophic derivative of strain CBS 2359 [NRRLY-1140] which has been proposed as the reference strain of the *K. lactis* research community. The genomic library was prepared by nebulization of the genomic DNA and ligation into plasmid pCDNA2.1 as described in [24].

2.2. Sequence analysis

All sequences were treated as described in [25], and the computerized table was manually annotated with a specific interface in an Excel (Microsoft) environment with hypertext links. This helped to visually discriminate the different BLAST results.

All macros (in Visual Basic Application for Excel on Windows 98) are available on request at toffano@igmors.u-psud.fr.

2.3. Contig assembly

The assembly of RSTs into contigs was first performed on the SCF traces (on which no trimming was done) and its distribution revealed

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several large contigs (many reads reaching several kb) which turned out to correspond to the rDNA, the mitochondrial genome, or the pKD1 plasmid (see below). Surprisingly, we found two additional large contigs of chromosomal DNA (18 reads each, 5068 and 5259 bp respectively) which turned out to contain chromosomal genes as if those genes either were repeated or were localized in a chromosomal area preferentially selected during DNA breaking or cloning. Most of the reads, however, belonged to small contigs (two or three reads) or remained singletons.

3. Results and discussion

3.1. rDNA

One large contig of 10 924 bp (72 reads) was found which represented the complete rDNA unit (approximately 9 kb) of *K. lactis* (Fig. 1). The sequence alignments between the rDNA of *K. lactis* and the rDNA of *S. cerevisiae* revealed a highly conserved size, sequence (94–100% identity) and organization of rRNA genes between the two species.

3.2. Transposons and plasmids

When *K. lactis* sequences were first compared with the sequences of the various *S. cerevisiae* Ty elements no significant matches were obtained. However, comparison with *S. cerevisiae* Ty-encoded proteins revealed five contigs which showed similarity to Ty1 or Ty2. In two cases, the similarity was high (50% identity/67% similarity) while it was poor for three others (20%/40%). The number of detected transposons is therefore rather small. Considering that we analyzed at least one third of the *K. lactis* genome, the total number of transposons is estimated to be about 15, a number much smaller than what is usually found in *S. cerevisiae* strains. It is possible that either the *K. lactis* genome contains a smaller number of transposable elements or these transposons are present but have considerably diverged.

One 4692 bp contig with 109 reads could be identified by a search in the GPROTEOME database [25]. This contig contained the three genes of the circular plasmid pKD1 [5]. Analysis of the RSTs found in the contig (data not shown) indicated that it was covering the whole pKD1 sequence and both the A and B forms [5].

3.3. Mitochondrial DNA

Mitochondrial DNA could be reconstituted partially in three different steps. In a first screen, three contigs were identified by similarity with some pieces of *S. cerevisiae* mitochondrial DNA: 21S rRNA, *COX1*, and *ATP9* contigs. In a second screen, comparison with the GPROTEOME database of the remaining RSTs identified *K. lactis* mitochondrial open reading frames (ORFs) in six contigs: *COX1* (cytochrome oxidase subunit 1), *COX2* (subunit 2), *COX3* (subunit 3), *ATP9* (subunit 9 of the mitochondrial ATPase) and *VAR1* (ribosomal protein). Finally, the alignment of sequenced *K. lactis* DNA against mitochondrial tRNA genes of *S. cerevisiae* allowed us to detect five additional mitochondrial contigs in which 21 tRNA genes could be mapped.

The mitochondrial contigs thus represent 27 kb out of the 39 kb of the *K. lactis* mitochondrial DNA as established by restriction analysis [6]. Several of the mitochondrial genes of *K. lactis* have already been sequenced: *COX1*, *COX2*, *CYTB* [6], *COX3*, *ATP9* [26], and several tRNA genes [6]. However, the nucleotide sequences of the 21S rRNA gene and *VAR1* gene found in the present work were unknown before.

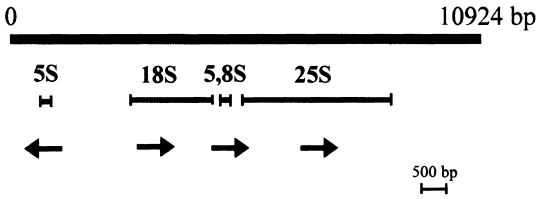

Fig. 1. Map of the rDNA contig. rRNA genes are positioned on the rDNA contig map represented by a line at the top. Arrows at the bottom indicate the orientation of transcription.

The *ATP9* and 21S rRNA gene sequences revealed the presence of intron sequences of 394 and 410 bp, respectively. The presence of these introns has been previously reported [6,26].

3.4. tRNA genes

After comparison with a database containing *S. cerevisiae* tRNA gene sequences and examination of contigs, a maximum of 64 different tRNA genes were identified (representing 27 different families out of the 52 families of *S. cerevisiae* genes). Taking into account the incomplete coverage of the *K. lactis* genome, this figure is slightly lower than expected if the number of tRNA genes were similar in the two species. As in *S. cerevisiae*, each tRNA gene exists in several highly conserved copies. If an intron was present in a *S. cerevisiae* tRNA gene, it was also detected in the corresponding *K. lactis* tRNA gene at the same position. Only the size of the intron was somewhat variable.

3.5. Centromeres

Five out of the six centromeres of *K. lactis* are known [21]. We compared their sequence to our data and identified only part of *KlCEN3* in one RST.

3.6. Identification of possible orthologues of the S. cerevisiae ORFs

ORFs were identified, translated, and compared to the *S. cerevisiae* database as described in [25]. Each of these comparisons was validated manually after examination of (i) the position of the protein sequence on the RST, (ii) the length of the protein as compared to the corresponding *S. cerevisiae* one, and (iii) the identity/similarity results. A total of 3383 clear-cut single *S. cerevisiae* ORF homologues were validated in 3129 RSTs, leading to the final identification of 1900 different ORFs. A second class of annotation corresponded to multiple matches. In this case, the *K. lactis* sequence was found to be homologous to a gene family rather than a single gene. A total of 1822 *S. cerevisiae* ORFs (in 749 RSTs) were annotated in this class. Among them, 324 were already found in the first class. This was due to the fact that the alignment concerned another region of the ORF, which allowed us to discriminate between the different members of the gene family. Therefore, the final classification for the second class was done on contigs rather than individual RSTs. Thus, 155 reads were found to correspond to singletons and 537 reads belonged to 277 different contigs, which finally yielded a maximum of 432 additional ORFS identified.

Two of the chromosomal *K. lactis* genes have been visually found to contain an intron. One was the orthologue of the *RPS7A*/*RPS7B* genes of *S. cerevisiae* which encode highly related ribosomal proteins, and contain an intron at the

same position in the two gene sequences. The corresponding *K. lactis* genes also contained an intervening sequence localized at the same position in the gene with the same splicing signals. Only the size of the intron differed (511 bp in *K. lactis* as compared to the 391 and 345 nucleotides of *RSP7A* and *RPS7B*, respectively). The other intron was found in the *K. lactis* orthologue of the *RPS17A*/*RPS17B* ribosomal protein genes.

Reciprocally, we examined orthologues of *S. cerevisiae* genes described as containing an intron [27]. Several of these ORFs started a few amino acids after the initiator Met. This suggests that the presence of a putative intron may have been missed on BLAST searches since very short homologous fragments would not have been detected. Clearly, *K. lactis*, like *S. cerevisiae*, has only a small number of genes with introns.

3.7. Comparison with other organisms

The reads which were not validated by comparison with the *S. cerevisiae* protein database were then compared to a much larger collection of proteins, GPROTEOME [25]. Interestingly, 56 *K. lactis* sequences with no *S. cerevisiae* homologues contained translation products similar to 44 distinct families of protein characterized in species other than *S. cerevisiae* (Table 1). We can arbitrarily distinguish three categories.

The first category included 10 genes of *K. lactis*, already cloned and sequenced, plus a β-glucosidase gene of the related species *K. marxianus*. Several of these genes have no functional equivalents in *S. cerevisiae*. Such is the case for the β-galactosidase (*LAC4*), lactose permease (*LAC12*) and the β-glucosidase genes which enable *K. lactis* to assimilate lactose and cellobiose (not used by *S. cerevisiae*). For some other genes of this category (*YAP1*, *ABF1*, *CBF1*) functional homologues do exist in *S. cerevisiae*, but the protein sequences have considerably diverged, except for interspersed conserved domains (probably absent in the present partial sequence of the gene). This situation was already known for several transcrip-

Table 1
Potential functions encoded by *K. lactis* RSTs having no validated homologues in the genome of *S. cerevisiae* (see 3.8)

Organism	Accession number	Function, gene name
A: Known *K. lactis* ORFs		
Kluyveromyces marxianus	P07337 (5)	β-glucosidase
Kluyveromyces lactis	P00723	β-galactosidase (*LAC4*)
	P07921	lactose permease (*LAC12*)
	P49374	glucose permease, high affinity (*HGT1*)
	P52289	acid phosphatase (*PHO5*)
	P08540	potential acid phosphatase (*PHOX*)
	Q03215	phosphofructokinase α-subunit (*PFK1*)
	P56095	AP1-like transcription factor (*YAP1*)
	P26375	transcription factor (*ABF1*)
	P49379	centromere binding protein (*CBP1*)
	P87164	suppressor protein (*SEF1*)
B: ORFs with potential orthologues in *S. cerevisiae*		
Alcaligenes eutrophus	P14940	sorbitol dehydrogenase (YDL168w/YJR159w)
Mycobacterium tuberculosis	P71828	γ-glutamyltransferase (YLR299w)
Schizosaccharomyces pombe	Q10235	α-tubulin folding co-factor B (YNL148c)
	Q10193	pre-mRNA splicing (YNL016w)
	Q92341	putative major facilitator superfamily (YOL158c)
	O94562	aminotransferase (YOL140w)
Caenorhabditis elegans	O18237	GTP binding protein (YPL218w)
Drosophila melanogaster	P91622	pyruvate dehydrogenase kinase (YIL042c)
C: No detectable homology to any *S. cerevisiae* ORF		
Bacillus subtilis	P21340	repressor of sporulation
Haemophilus influenzae	Q57051	*N*-carbamyl-L-amino acid amidohydrolase
Klebsiella pneumoniae	P05192	β-lactamase
Pseudomonas aeruginosa	P51691 (3)	arylsulfatase
Aeropyrum pernix	Q9YAT7, Q9Y9E2	hypothetical proteins (two different ones)
Candida albicans	P87218 (2)	sorbitol utilization protein (Sou2p)
Schizosaccharomyces pombe	O43029	fructosyl amino acid oxidase (putative)
	O42887	arginase family
	Q9Y7N4(3)	D-amino acid oxidase (putative)
	O59832	cytoplasmic dipeptidase (putative)
	O59715	fatty acid desaturase (putative)
	L42550	involved in mitosis (Mlo2p)
	O59832, O42892	hypothetical proteins (four different ones)
	Q10341, Q10301	
	O94342	sugar transporter (putative)
	O94431	class V pyridoxal phosphate dependent amino transferase
Trigonopsis variabilis	Q99042	D-amino acid oxidase
Neurospora crassa	P38680	amino acid transporter
Aspergillus niger	Q12556	copper amine oxidase I
Brassica juncea	Q39287	endoplasmic fatty acid desaturase
Caenorhabditis elegans	O17337	hypothetical protein
Homo sapiens	Q16739	ceramide glucosyl transferase

The species names given are those for which the corresponding gene gave the best score. In the first category (A) the gene name is given in parentheses. In the second category (B) the ORF of *S. cerevisiae* that showed a lower score than the ORF of other organism is given in parentheses (see text). Accession number: SwissProt or TrEMBL database. Numbers in parentheses (column 2) indicate the number of distinct genes of *K. lactis* matching the same entry.

Table 2
List of presumed redundant genes in *K. lactis* which are single in *S. cerevisiae*

ORF	Copy number in *S. cerevisiae*	Copy number in *K. lactis*	Gene name	Presumed function
YBL022c	singleton	2	*PIM1*	mitochondrial protease
YDR278c	singleton	2		hypothetical
YEL046c	singleton	2	*GLY1*	threonine aldolase
YGL067w	singleton	2	*NPY1*	NADH pyrophosphatase
YGR037c	singleton	2	*ACB1*	acyl-CoA binding protein
YHL016c	singleton	2	*DUR3*	urea transport
YIL063c	singleton	2	*YRB2*	nuclear protein export
YIR042c	singleton	2		unknown
YJL127c	singleton	2	*SPT10*	chromatin accessory protein
YKL215c	singleton	3		amino acid hydrolase
YKL217w	singleton	2	*JEN1*	lactate permease
YMR091c	singleton	2	*NPL6*	nuclear protein localization factor
YNL024c	singleton	2		unknown

tional regulators [6] in which only the functional cores are well conserved.

The second category includes eight ORFs which matched ORFs of *S. cerevisiae* in the first screen but were not validated because of low identity/similarity scores. Four of these potential orthologues (*YLR299w*, *YOL140w*, *YPL218w*, and *YIL042c*) also matched with high identity/similarity the translation products of other *K. lactis* RSTs. An example of this category is an RST sequence similar to an aminotransferase. It was closer to the *Schizosaccharomyces pombe* protein than to the *S. cerevisiae* one, Arg8p (encoded by *YOL140w*). Moreover, this sequence did not correspond to the *K. lactis ARG8* gene product which has been cloned and whose disruption led to an arginine auxotrophic phenotype [28]. Analysis of the *K. lactis* genes which belong to this second category showed that the difference between *K. lactis* and *S. cerevisiae* is not the presence/absence of orthologous genes but rather the presence/absence and the composition of gene families.

In the third category (Table 1C) we found 25 potentially new *K. lactis* genes with no known orthologues in *S. cerevisiae*. Most of them were found in the yeast *S. pombe* and in fungi, revealing the presence of specific ascomycetes genes [29] but some belong to various organisms from bacteria to human. At least 18 of these genes may encode proteins involved in metabolic pathways. Several of the new genes encode enzymes (amino acid oxidase, arginase or sorbitol utilization protein, etc.) which would allow *K. lactis* to assimilate carbon and nitrogen from sources that are not used by *S. cerevisiae*, like cadaverin, ethylamine, sorbitol, etc. [7]. In addition, several *K. lactis* gene products have an unexpected function. A putative β-lactamase showed an identity/similarity score of 51–72% over 135 amino acids when compared to the enzyme of *Klebsiella pneumoniae* which may suggest horizontal transfer.

3.8. Gene redundancy in K. lactis

The genes which are highly redundant in *S. cerevisiae* can occur as singletons in *K. lactis* as documented, for example, for genes involved in glycolysis and hexose transport. The reverse situation has also been found. When *K. lactis* RSTs from different contigs matched one *S. cerevisiae* ORF at the same positions within the sequence, the *K. lactis* ORFs were considered redundant. In other cases, the copy number could not be estimated with certainty. On these criteria, a minimum of 2235 ORFs and a maximum of 2601 ORFs were identified. Table 2 presents examples of singletons in *S. cerevisiae* which are duplicated (or triplicated) in *K. lactis*.

3.9. Repartition into functional categories

One can classify the *K. lactis* ORFs following the functional classification established for *S. cerevisiae* (MIPS Funcat catalogue, see also [30]). No striking differences were observed in the distribution, if one excludes the unclassified ORFs. The distribution reveals however some differences in some subcategories of 'metabolism'. We observed an increased number of genes for amino acid transporters (22 ORFs in *K. lactis* versus 11 in *S. cerevisiae*), for amino acid metabolism (119 versus 87), for nitrogen and sulfur metabolism (46 versus 32) and for lipid and fatty acid metabolism (110 versus 87). These differences between *K. lactis* and *S. cerevisiae* are probably significant since in other functional categories no such differences were seen.

Among the ORFs redundant in *K. lactis* and singular in *S. cerevisiae*, several are involved in metabolism (see Table 2). Such a variation in gene redundancy might be a way for a given organism to fine-tune its metabolism to its specific habitat.

3.10. Synteny

According to the criteria defined in [31], we observed 47.3% syntenic pairs among the RSTs, a value consistent with what we could expect from the fragmentary data collected from published sources. The biological significance of this finding for evolution is discussed in this issue [31].

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