Genomic Exploration of the Hemiascomycetous Yeasts: 10. *Kluyveromyces thermotolerans*

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Abstract A genomic exploration of *Kluyveromyces thermotolerans* was performed by random sequence tag (RST) analysis. We sequenced 2653 RSTs corresponding to inserts sequenced from both ends. We performed a systematic comparison with a complete set of proteins from *Saccharomyces cerevisiae*, other completely sequenced genomes and SwissProt. We identified six mitochondrial genes and 1358–1496 nuclear genes by comparison with *S. cerevisiae*. In addition, 25 genes were identified by comparison with other organisms. This corresponds to about 24% of the estimated gene content of this organism. A lower level of conservation is observed with orthologues to genes of *S. cerevisiae* previously classified as orphans. Gene order was found to be conserved between *S. cerevisiae* and *K. thermotolerans* in 56.5% of studied cases. © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

In this paper we describe results based on 2653 random sequence tags (RSTs) from the genome of *Kluyveromyces thermotolerans*. The type strain CBS 6340 of *K. thermotolerans*, previously named *Zygosaccharomyces thermotolerans* by Filippov, was isolated from plum jam and reclassified in the genus *Kluyveromyces* by Yarrow [1]. According to rDNA sequences, *K. thermotolerans* is a close relative of *Kluyveromyces waltii* and *Saccharomyces kluyveri* and is more distant from the *Kluyveromyces lactis* group [2–4]. Strain CBS 6340 is diploid as judged from its ability to sporulate (S. Casarégola, personal communication). It has seven chromosomes (haploid number) and a genome size of 10.6 Mb as estimated by pulsed field gel electrophoresis [5]. However, apart from these figures, *K. thermotolerans* has never been extensively studied. So far, only one nuclear gene, OLE1, has been partially sequenced [6]. The mitochondrial genome is about 23 kb in length [7] and the group I intron of the mitochondrial 21S rRNA gene was sequenced because it shows similarity to the one of *Saccharomyces cerevisiae* [8,9].

To explore the genome of this yeast we have compared the RSTs with *S. cerevisiae* and other organisms as described in [10]. We identified 1358–1496 new protein coding genes, 58 tRNA genes, and six mitochondrial genes by comparison with *S. cerevisiae*. In addition 25 genes have no orthologue in *S. cerevisiae*. The gene order conservation has been explored and 56.5% of conserved synteny is observed.

2. Materials and methods

Library construction was essentially as described in the *Pichia angusta* article [11]. Briefly, a random genomic library of strain CBS 6340 of *K. thermotolerans* was made by nebulization of total DNA (DNA Nebulizer, GATC GmbH, Germany). DNA fragments of 3–5 kb were purified from agarose gel and ligated to the plasmid vector. Actual size distribution of inserts was estimated on a random sample of 96 clones whose plasmid DNAs were purified, digested, and electrophoresed as described [11]. No empty vector was found and 60.9% of the clones contained inserts of 3–5 kb (average size of all inserts is 3.2 kb, S.D. 1.4). Subsequently, 1692 white bacterial colonies were cultivated in eighteen 96 well microtiter plates. Microplates were triplicated and stored at −80°C. Sequences were determined by the Génoscope [12].

3. Results and discussion

3.1. Random sequence tags of K. thermotolerans

In order to compare the *S. cerevisiae* and *K. thermotolerans* genomes, we have sequenced and analyzed RSTs of a *K. thermotolerans* random genomic library. The programs Phred and Phrap with standard parameters were used to construct contigs. The majority of contigs contain two or three RSTs (354 contigs) and up to a maximum of five (five contigs) except for a contig of 324 sequences which corresponds to the rDNA repeat (see below). Note that 107 short inserts were entirely sequenced from both ends and were considered in the contig assembly as one sequence. Using contig data we estimated a haploid genome size of about 11.8 Mb [10]. RSTs were also compared with *S. cerevisiae* rDNA, tRNA genes, Ty elements and 2μ plasmid sequences. No RSTs contained Ty-like elements or 2μ plasmid-like sequences.

3.2. rDNA

A long contig of 324 RSTs, corresponding to rDNA, was assembled. The deduced consensus sequence of *K. thermotolerans* rDNA repeat unit is about 10 kb (similar to that of *S. cerevisiae*). The gene organization in this repeat unit is like that in *S. cerevisiae*. Comparison of the 18S rDNA sequence from our consensus with the previously identified 18S sequence (EMBL accession number X89526.1) shows 11 differ-

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ences, eight of which are due to ambiguous nucleotides in the public sequence. In the sequenced strain of *S. cerevisiae*, rDNA repeats represent 9.5% of the genome (140 repeats). By comparison, 13.1% of all RSTs of *K. thermotolerans* correspond to rDNA indicating the presence about 200 repeats of rDNA units in this yeast. Clonal size variation of the rDNA cluster (50–200 copies) has been reported in *S. cerevisiae* [13,14]. Although the figure for *K. thermotolerans* is high (especially with regard to other yeast species of this program), it is still compatible with the higher limit of *S. cerevisiae*.

3.3. Mitochondrial DNA

All RSTs were compared with the mitochondrial DNA sequences of *S. cerevisiae* and a few other yeast and fungal species [10]. The mitochondrial DNA of *K. thermotolerans* was previously characterized by restriction mapping [7]. With only 23 kb it is much smaller than the 86 kb *S. cerevisiae* mitochondrial genome [15]. We have found only 28 RSTs corresponding to mitochondrial DNA, 22 of them form seven small contigs. The mitochondrial RSTs represent 58.2% of entire mitochondrial genome. We found sequences exhibiting similarity with six *S. cerevisiae* mitochondrial genes (Table 1). The putative Var1 protein, involved in the protein translation machinery, is less conserved than the putative Cox3 and Cob proteins of the energy transfer complexes. Despite the small mitochondrial genome size, we also identified a new putative mitochondrial group I intron open reading frame (ORF) whose closest homologue is I-SceII [16]. Until now, only one putative intronic meganuclease from *K. thermotolerans* had been identified by sequence similarity with I-SceI [8,9]. Finally, one RST contains a short segment which, upon translation, shows 92% amino acid identity with the C-terminal part of a hypothetical ORF 11 of *S. cerevisiae*. In *S. cerevisiae*, this ORF is located 13 kb downstream of *COX3*, while in *K. thermotolerans* the equivalent sequence is only 170 bp downstream of *COX3* without conservation of the N-terminus. The hypothetical ORF 11 of *S. cerevisiae* may not be a gene in *K. thermotolerans*.

3.4. tRNA genes and the genetic code

By comparison with all *S. cerevisiae* tRNA gene families, we have identified 58 tRNA genes in *K. thermotolerans*. Genetic code and relative synonymous codon usage (RSCU) [17] were determined from the alignments of the *K. thermotolerans* sequences with the corresponding *S. cerevisiae* sequences [10]. The genetic code of *K. thermotolerans* is universal but the RSCU differs from that of *S. cerevisiae*. Consistent with the higher G+C content of the *K. thermotolerans* ORFs (47.2%) compared to *S. cerevisiae* (39.6%), the third position of codons is frequently biased in favor of G or C (data not shown).

3.5. Protein coding content of K. thermotolerans and functional classification

A total of 2277 RSTs (remaining after elimination of rDNA and mitochondrial DNA) were systematically translated in the six frames and compared with *S. cerevisiae*, SwissProt and other sequenced genomes as described in [10].

3.5.1. Comparison with S. cerevisiae. We identified 1358–1496 orthologues of our RSTs among *S. cerevisiae* genes. In a few cases, we were not able to determine the orthologous gene due to matches with several indistinguishable or closely related ORFs. In this category, we obtained 131 RSTs matching with 253 different genes from *S. cerevisiae* gene families [18].

Genet redundancy is a major trait of the *S. cerevisiae* genome and about one third of yeast ORFs are members of structural families with two to 24 elements [19]. Genet redundancy is thought to permit accumulation of mutations and thus create new functions due to relaxation of selective constraints in one copy of the duplicated gene [20]. We used a classification of *S. cerevisiae* genes in structural families [19] to determine specific functional characteristics of *K. thermotolerans* [18,21]. We found 636–701 ORFs of *K. thermotolerans* matching with genes from structural families of *S. cerevisiae*. In general, the distribution of matches shows few differences between the two yeasts [18]. Nevertheless, four large families are without representatives in *K. thermotolerans* and it is striking that they are located in the subtelomeric regions in *S. cerevisiae* (P24.1f23.1, P9.4f8.1 and P26.1f13.1 [19]) or correspond to the DUP family. The DUP family (10 members of unknown function) was studied by Feuermann et al. [22] and corresponds to two clusters of tandem repeats on the right arm of *S. cerevisiae* chromosome I (six genes) and the left arm of chromosome VI (two members). The remaining two members of the DUP family are located as single copies on chromosomes III and VIII. We suggest that those tandem repeats appeared after the divergence of the *Kluyveromyces* and *Saccharomyces* genera. Largely, subtelomeric families are of unknown function and underrepresentation of these families may be correlated to general underrepresentation of subtelomeric genes [18]. Since *S. cerevisiae* has been used by humans for about 7000 years [23], selection may have amplified some interesting genes such as the *FLO* genes involved in flocculation during fermentation. The subtelomeric regions may be a preferred location of amplification due to the plasticity of these regions.

We have analyzed a potential specific functional bias be-

Table 1
Mitochondrial genes of *K. thermotolerans* found by similarity search with *S. cerevisiae*

RST	Position of alignment (aa)	Identity (%)	*S. cerevisiae* homologue	Functional annotation
AY0AA001A04DP1	86–316	62	*I-SceII*	group I intron ORF
AY0AA003C08DP1	109–269	84	*COX3*	cytochrome *c* oxidase subunit 3
AY0AA009F10TP1	1–206	78	*COX3*	cytochrome *c* oxidase subunit 3
AY0AA003C08TP1	109–134	92	*ORF11*	putative ORF (C-terminus only)
AY0AA004H12TP1	180–385	75	*COB*	apocytochrome *b*
AY0AA008G02TP1	1–207	80	*COB*	apocytochrome *b*
AY0AA008H02DP1	complete	93	*ATP9*	H^+-transporting ATPase, F_0 subunit 9
AY0AA014G11DP1	133–245	43	*VAR1*	mitochondrial ribosomal protein
XAY0AA002G03TP1	321–398	67	*VAR1*	mitochondrial ribosomal protein

The positions of the alignments refer to the *S. cerevisiae* proteins (amino acid numbers). The percentage of identity between *K. thermotolerans* and *S. cerevisiae* corresponds to BlastX aligned protein segments.

Table 2
K. thermotolerans putative ORFs identified by comparison to SwissProt and whole-sequenced genomes

Organism	Accession number	Gene name	Functional comment
Bacteria			
Thermotoga maritima	(2)	TM1867	L-lactate dehydrogenase
Pseudomonas aeruginosa	P51691	atsA	arylsulfatase
Burkholderia cepacia	P16932	dgdA	2,2-dialkylglycine decarboxylase
Escherichia coli	V00296	lacZ	β-galactosidase
Bacillus subtilis	(3)	YwqG	unknown protein
Bacillus stearothermophilus	P37113	AMAB	*N*-carbamyl-L-amino acid amidohydrolase
Synechocystis sp.	(4)	sll1305	unknown protein
Haemophilus influenzae	Q57051	HI0588	unknown protein
Archaea			
Aeropyrum pernix K1	(5)	APE1596	unknown protein
Pyrococcus horikoshii OT3	O59413	PH1704	protease I
Ascomycota			
Aspergillus nidulans	P48777	UAPC	purine permease
Schizosaccharomyces pombe	(1)	MLO2	unknown protein
	Q10449	SPAC12B10.16C	unknown protein
	Q09674	SPAC5H10.01	unknown protein
	(1)	SPBC660.12C	unknown protein
	P78771		unknown protein
	(1)	SPCC550.07	Acetamidase
	P32747	URA3	dihydroorotate dehydrogenase
	Q10082	SPAC11D3.03C	unknown protein
Candida albicans	P87218	SOU2	sorbitol utilization protein
	P87219	SOU1	L-sorbose dehydrogenase
Trigonopsis variabilis	Q99042	DAO1	D-amino acid oxidase
Other eukaryotes			
Rattus norvegicus	P05982	NMOR1	NAD(P)H dehydrogenase (quinone)
Caenorhabditis elegans	(6)	AAB92032.1	unknown protein
	(6)	T07D3.9	unknown protein
	(7)	Y119D3_446.d	unknown protein

The table indicates the best homologue found to each of the *K. thermotolerans* RSTs having no homologue in *S. cerevisiae* with its accession number and gene name. The gene *URA3* of *S. pombe* corresponds to *URA1* in *S. cerevisiae*. Sequences not present in SwissProt are available as follows: (1) http://www.sanger.ac.uk/cgi-bin/yeastpub/GN_KW_search.p, (2) http://www.tigr.org/tdb/CMR/btm/htmls/GeneNameSearch.html, (3) http://bioweb.pasteur.fr/GenoList/SubtiList/, (4) http://www.kazusa.or.jp/cyano/kwd.html, (5) http://www.mild.nite.go.jp:11000/InterServ/APE/new/advanced.main, (6) http://www.sanger.ac.uk/Projects/C_elegans/wormpep/, (7) http://wormsrv1.sanger.ac.uk/cgi-bin/ace/simple/worm.

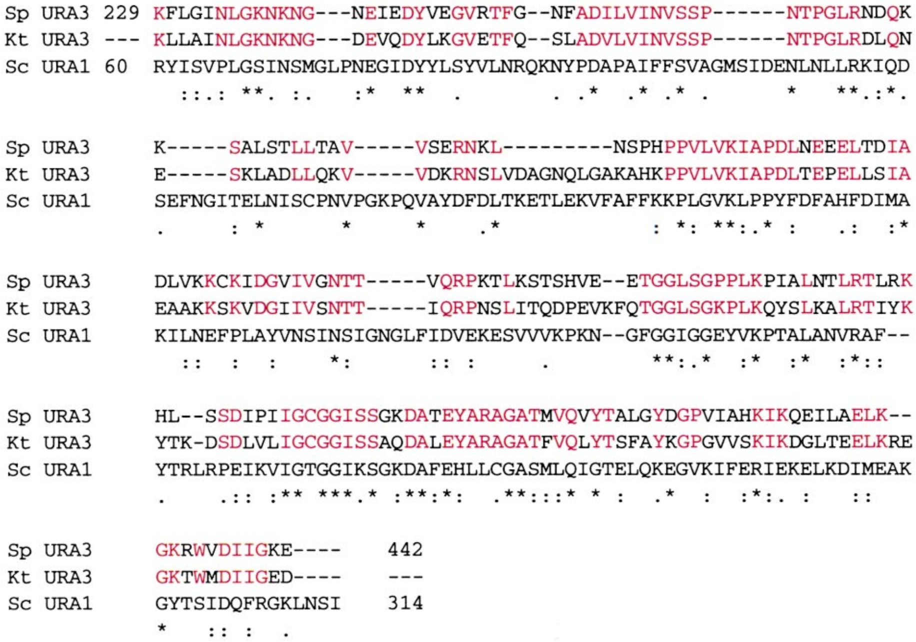
tween the two yeasts. To explore this field we propose the hypothesis that *K. thermotolerans* genes have the same function as their *S. cerevisiae* orthologous genes. A functional classification according to the orthologues in *S. cerevisiae* was performed using the PEDANT database [21,24]. A significant difference appears between *K. thermotolerans* and *S. cerevisiae* corresponding to functional class 05.01 of ribosomal proteins with only five genes found among RSTs compared to about 16 expected. Except for this category, these two yeasts seem to be very similar according to the functional classification of *S. cerevisiae* genes. Thus, despite the fact that the two species present physiological differences, such as the capacity to assimilate D-mannitol in *K. thermotolerans* [1], we were not able to identify significant variations in functional categories. The RST approach therefore is a powerful tool though not precise enough to identify minor variations which, on the other hand, could induce major physiological differences [21].

3.5.2. Comparison with SwissProt and complete genome sequences. We found 25 genes not previously identified by comparison with *S. cerevisiae* (Table 2). Comparison with other hemiascomycete yeasts yielded 11 new genes and comparisons with bacteria, *Caenorhabditis elegans* and Archaea revealed eight, three and two genes, respectively. Finally, one gene was identified by similarity with a rat sequence, corresponding to a NAD(P)H dehydrogenase gene. A striking observation is that RST AY0AA013G08TP1 matches with the *URA3* gene of *Schizosaccharomyces pombe*. Gene *URA3* of *S. pombe* corresponds to the *S. cerevisiae URA1* gene encoding dihydroorotate dehydrogenase [25] but the primary sequences are quite divergent (Fig. 1). The *S. pombe* protein is localized in the mitochondria whereas its homologue from *S. cerevisiae* is found to be cytosolic. Thus, *K. thermotolerans* dihydroorotate dehydrogenase is a close relative of the *S. pombe* protein and may have a similar cellular localization.

Moreover, a homologue to β-galactosidase of *Escherichia coli* was found (this gene has no known homologue in *S. cerevisiae*). The yeast *S. cerevisiae* cannot assimilate L-sorbose in contrast to *K. thermotolerans* [1]. Genes involved in L-sorbose metabolism have also been found by comparison with the *Candida albicans SOU1* and *SOU2* genes [26].

3.6. Sequence divergence between various classes of genes

Previously, one third of *S. cerevisiae* ORFs had no identified homologue in general sequence databases and other whole-sequenced genomes other than the ascomycete phylum (orphans or ascomycete specific genes) [27]. We identified homologues of ascomycete specific genes in *K. thermotolerans* and we have calculated the identity of orthologous genes at the amino acid level. We observed that orthologues of other genes (conserved genes, median 59% identity) are less divergent than orthologues to ascomycete specific genes (median 45.6% identity). In addition, orphans represent 29% of *S. cerevisiae* genes and we found that only 20% of *K. thermotolerans* identified genes are orthologous to the previous orphan genes. Similar results have been obtained from the other spe-



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Sp URA3 229 KFLGINLGKNKNG---NEIEDYVEGVRTFG--NFADILVINVSSP------NTPGLRNDQK
Kt URA3 --- KLLAINLGKNKNG---DEVQDYLKGVETFQ--SLADVLVINVSSP------NTPGLRDLQN
Sc URA1 60  RYISVPLGSINSMGLPNEGIDYYLSYVLNRQKNYPDAPAIFFSVAGMSIDENLNLLRKIQD
              ::.: **. :.   :*  **  .       . .*  .* .* .      *   **.:*.

Sp URA3     K-----SALSTLLTAV-----VSERNKL---------NSPHPPVLVKIAPDLNEEELTDIA
Kt URA3     E-----SKLADLLQKV-----VDKRNSLVDAGNQLGAKAHKPPVLVKIAPDLTEPELLSIA
Sc URA1     SEFNGITELNISCPNVPGKPQVAYDFDLTKETLEKVFAFFKKPLGVKLPPYFDFAHFDIMA
            .     : *      *     *    .*            : *: **:.* :   .:  :*

Sp URA3     DLVKKCKIDGVIVGNTT-----VQRPKTLKSTSHVE--ETGGLSGPPLKPIALNTLRTLRK
Kt URA3     EAAKKSKVDGIIVSNTT-----IQRPNSLITQDPEVKFQTGGLSGKPLKQYSLKALRTIYK
Sc URA1     KILNEFPLAYVNSINSIGNGLFIDVEKESVVVKPKN--GFGGIGGEYVKPTALANVRAF--
              ::  :  :    *:      :: :     .        **:.*  :*  :*  :*::

Sp URA3     HL--SSDIPIIGCGGISSGKDATEYARAGATMVQVYTALGYDGPVIAHKIKQEILAELK--
Kt URA3     YTK-DSDLVLIGCGGISSAQDALEYARAGATFVQLYTSFAYKGPGVVSKIKDGLTEELKRE
Sc URA1     YTRLRPEIKVIGTGGIKSGKDAFEHLLCGASMLQIGTELQKEGVKIFERIEKELKDIMEAK
            .    .:: :** ***.* :**:*:  .**:::* * :  .*  :  :*:. :   ::

Sp URA3     GKRWVDIIGKE----    442
Kt URA3     GKTWMDIIGED----    ---
Sc URA1     GYTSIDQFRGKLNSI    314
            *   :: :  .
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Fig. 1. ClustalW alignment of *S. pombe URA3* (Sp URA3), *K. thermotolerans URA3* (Kt URA3) and *S. cerevisiae URA1* (Sc URA1). Identical amino acids between Sp *URA3* and Kt *URA3* are colored in red. The identity level between Kt *URA3* and Sp *URA3* is 56.5% and the common part between all the aligned sequences represents 17.2% of the amino acids. The numbers indicate amino acid coordinates in the different sequences.

cies of the present program [28]. In addition, we observed that genes designated 'conserved' are more likely to be involved in the general processes of cell metabolism and that orphan genes are more likely involved in specific structures or processes such as the cell wall or the pheromone response pathway [28]. A pool of less constrained genes is of obvious evolutionary interest for the emergence and natural selection of advantageous mutations in a particular environment. Such genes may clearly represent an important contributor to the speciation mechanism. Another striking observation is that RSCU values for ascomycete specific genes are less biased than universal genes [28]. Consistent with the fact that these genes evolve rapidly, a higher mutation rate may induce an equilibration of codon usage.

3.7. *Comparison of maps between S. cerevisiae and K. thermotolerans*

The 'two end' sequences of inserts allow us to describe gene order conservation (synteny conservation) between *S. cerevisiae* and *K. thermotolerans* for at least two genes identified in an insert. Among 682 couples of genes [29], we observed 56.5% conserved synteny between the two yeasts. This value is close to *S. kluyveri* (53.5%)[30], *K. lactis* (47.3%)[31], and *K. marxianus* var. *marxianus* (49.8%) [32]. We analyzed the 297 remaining pairs and we identified 173 pairs corresponding to an ancestral gene organization as described in [29]. Further, we have identified 44 trans-chromosomal series (see [29] for a global analysis).

3.8. *Chromosome organization: the role of reciprocal translocation*

In 1963, Mayr wrote: " There was a widespread belief among early cytogeneticists that chromosomal rearrangement was the essential step in speciation. Proposed as an alternative to geographic speciation, the chromosomal speciation is not valid" [33]. Numerous examples of species which have speciated without any significant change in chromosome morphology are documented. Nevertheless, chromosomal rearrangement is an actor in speciation [34]. Among yeast species, chromosome pattern variation is frequently observed [5,35]. Some authors suggested a possible role for reciprocal translocation in speciation of *Saccharomyces bayanus* versus *S. cerevisiae* [36] but this does not establish that this was the primary event of speciation. Reciprocal translocations are thought to be the main form of large-scale rearrangement in the yeast genomes [37]. A recent article shows that speciation in sensu stricto yeasts of the genus *Saccharomyces* is not related to reciprocal translocation [38]. To explore the importance of reciprocal translocation, we performed a systematic analysis of local gene orientation [29]. A reciprocal translocation must conserve relative gene orientation with respect to the centromere. In our strain, we obtained respectively 2.1% (8/385) and 5.2% (9/173) local gene inversion in syntenic pairs and in trans-chromosomal pairs. These values are very similar. The remaining 124 non-syntenic pairs show 46.6% local gene inversion compared to *S. cerevisiae*. This high value is not compatible with reciprocal translocation which conserves relative orientation. Therefore, the reciprocal translocation cannot be considered the major event of chromosome rearrangement due to the high proportion of inverted pairs [29].

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