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Recent allopolyploid origin of *Zygosaccharomyces rouxii* strain ATCC 42981

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Abstract

Zygosaccharomyces rouxii **strain ATCC 42981 has been reported to have two copies of several genes including** ***HOG1*** **and** ***SOD2*****, whereas the type strain of** ***Z. rouxii*** **(CBS 732) has only one. To investigate the structure of the ATCC 42981 genome we sequenced random fragments from this genome and compared the data to the type strain. We found that ATCC 42981 contains two versions of the ribosomal RNA array, one of which is identical in the ITS1–ITS2 and 26S D1/D2 regions to** ***Z. rouxii*** **CBS 732, while the other is almost identical to a species provisionally named** ***Z. pseudorouxii.*** **We found that most genomic regions from** ***Z. rouxii*** **CBS 732 map in a one-to-two fashion to pairs of regions in ATCC 42981, with one of the ATCC 42981 regions having 97–100% DNA sequence identity to CBS 732 and the other having about 80–90% identity. Complete sequencing of regions containing 30 pairs of genes from ATCC 42981 and their orthologues in CBS 732 showed no evidence of the gene deletions or pseudogene formation that might be expected if ATCC 42981 had undergone whole-genome duplication several million years ago and was in the early stages of gene loss. Instead, we conclude that ATCC 42981 is a** ***Z. rouxii–Z. pseudorouxii*** **interspecies hybrid that was formed so recently that its genome has not had time to decay. Copyright © 2008 John Wiley & Sons, Ltd.**

Keywords: ***Zygosaccharomyces*****; allopolyploidy; hybridization; evolution**

Introduction

Zygosaccharomyces rouxii is a yeast species that is usually haploid and heterothallic (Wickerham and Burton, 1960; Mori and Windisch, 1982; Kurtzman and Fell, 1998). It is often referred to as osmophilic, but is more properly described as xerotolerant (Farkas, 2001) because it is able to grow at very low water activities. *Z. rouxii* is also acid-tolerant and can grow in media as acidic as pH 1.8 (Membre *et al*., 1999). *Z. rouxii* is used industrially in the production of soy sauce and miso paste (Onishi, 1963) and is also of economic importance as a causative agent of food spoilage, often being the first species to spoil items with high sugar concentrations, such as fruit juices (Deak and Beuchat, 1996; Spencer and Spencer, 1997; Brul *et al*., 2003). One mechanism that allows *Z. rouxii* to survive at high osmolarity is the production and accumulation of glycerol as a compatible solute, which protects the cell against lysis. Another key mechanism is the efflux of Na^+ from cells in high concentrations of salt. Several *Z. rouxii* genes involved in these processes have been characterized. Those involved in glycerol production and accumulation include *ZrHOG1* (Iwaki *et al*., 1999), *ZrFPS1* (Tang *et al*., 2005) and *ZrGPD1* (Iwaki *et al*., 2001). Those involved in Na^+ efflux from the cell include *ZrENA1* (Watanabe *et al*., 1999) and *ZrSOD2* (orthologue of *S. cerevisiae NHA1*) (Watanabe *et al*., 1995).

Some early studies that cloned genes from *Z. rouxii* used strain ATCC 42 981, which was originally isolated as a miso production strain (named S-96) from Niigata prefecture, Japan (Kiuchi *et al*., 1978). These studies reported that ATCC 42 981

contains two copies of several genes that are single-copy in *S. cerevisiae* and involved in osmotolerance, including *HOG1* (Iwaki *et al*., 1999), *SOD2* (Iwaki *et al*., 1998), *GPD1* and *GCY1* (Iwaki *et al*., 2001). The increased copy number of these genes in *Z. rouxii* was originally attributed to the osmotolerance of the species. In 2000, the Génolevures project included the type strain of *Z. rouxii* (CBS 732) among the species it surveyed by random plasmid end-sequencing (de Montigny *et al*., 2000). Rather surprisingly, the Génolevures data did not confirm any of the gene duplications previously reported in *Z. rouxii*. Sychrova and colleagues resolved the confusion by showing that *HOG1* and *SOD2* are both duplicated in ATCC 42 981 but not in CBS 732 (Kinclova *et al*., 2001). Moreover, one of the two copies of each gene in ATCC 42 981 was very similar to the CBS 732 gene, while the other was divergent in sequence (Kinclova *et al*., 2001).

Further recent studies have shown that various strains from yeast culture collections, all originally identified as '*Z. rouxii*', comprise at least two different genomic types: some that have genomes similar to CBS 732, with only one copy of each gene, and others that are like ATCC 42 981, with two copies of many genes, only one of which is highly similar to the CBS 732 sequence (James *et al*., 2005; Solieri *et al*., 2006, 2007). These results suggested that some strains of '*Z. rouxii*' were natural allopolyploid hybrids between one parent similar to *Z. rouxii* CBS 732 and another, more divergent, parent from elsewhere within the genus *Zygosaccharomyces*. This second parent was given the tentative name *Z. pseudorouxii*, but it should be noted that this species has not been formally described and only one isolate of it is known (NCYC 3042; James *et al*., 2005). Although *Z. pseudorouxii* is not a recognized species, for the purpose of this study we will refer to NCYC 3042 as a *Z. pseudorouxii* strain.

We hypothesized that the duplicated genes in *Z. rouxii* ATCC 42 981 might be the products of a hybridization event that occurred a long time ago. If so, ATCC 42 981 could represent a new allopolyploid species and its genome would provide a way to study the outcome of a second whole-genome duplication (WGD) event in a yeast lineage (*Zygosaccharomyces*) that is completely independent of the well-documented WGD that occurred in an ancestor of *Saccharomyces* (Wolfe and Shields, 1997; Kurtzman, 2003). To test this hypothesis, and more generally to understand the genomic relationship among *Z. rouxii* strains, we sequenced randomly chosen fragments of the ATCC 42 981 genome and compared them to CBS 732.

Methods

Z. rouxii strain ATCC 42 981 was purchased from the American Type Culture Collection. A clone library of random fragments of genomic DNA (*Sau*3AI partial digestion, average insert size approximately 8 kb) was constructed by AGOWA (**www.agowa.de**) and both ends of about 5000 plasmids were sequenced. After vector trimming and low-quality sequence removal, a total of 10 752 high-quality reads remained. ATCC 42 981 contigs were then assembled using PHRAP (**www.phrap.org**). We compared these data to the sequences of some sections of the *Z. rouxii* CBS 732 genome, containing the orthologues of all genes from *S. cerevisiae* chromosome III that we determined in another project (J.L.G. and K.H.W., in preparation). To test for evidence of genome degradation in ATCC 42 981 we completely sequenced plasmids from some regions where we had clone coverage from both of the subgenomes of ATCC 42 981 as well as from CBS 732, to obtain 15–20 kb of sequence around each of three loci (*SWE1, POP2* and *TIM23*) in each subgenome (Accession Nos AM989974-AM989979). For the ribosomal DNA analysis we first used PHRAP to assemble the complete sequence of the repeating unit of the rDNA array in CBS 732 (Accession No. AM943655) from random sequence data for that strain (de Montigny *et al*., 2000; J.L.G. and K.H.W., unpublished), and then used our ATCC 42 981 shotgun data to manually assemble contigs corresponding to the sequences of the two types of ITS–D1/D2 regions in ATCC 42 981 (Accession Nos AM943656 and AM943657).

Results and Discussion

Two types of ribosomal DNA array in ATCC 42 981

We obtained >10 000 random sequencing reads from a genomic plasmid library of ATCC 42 981.

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The contigs assembled from these reads revealed the presence of two different types of rDNA array in this strain. We were unable to assemble consensus sequences for the complete (~9.9 kb) repeating rDNA unit of each type, because the rDNAs appear to be almost identical over large stretches of the 18S and 26S rRNA genes, but we were able to assemble two types of consensus for a 1.7 kb segment that includes both the ITS1-5.8S-ITS2 region and the D1/D2 region at the 5′ end of the 26S rRNA gene.

We refer to the two types of rDNA in ATCC 42 981 as 'T-type' and 'P-type' because they clearly originate from donors similar to the *Z. rouxii* type strain and to *Z. pseudorouxii*, respectively. The T-type and P-type sequences are substantially different from each other in the ITS region (Figure 1), and there are 15 substitutions

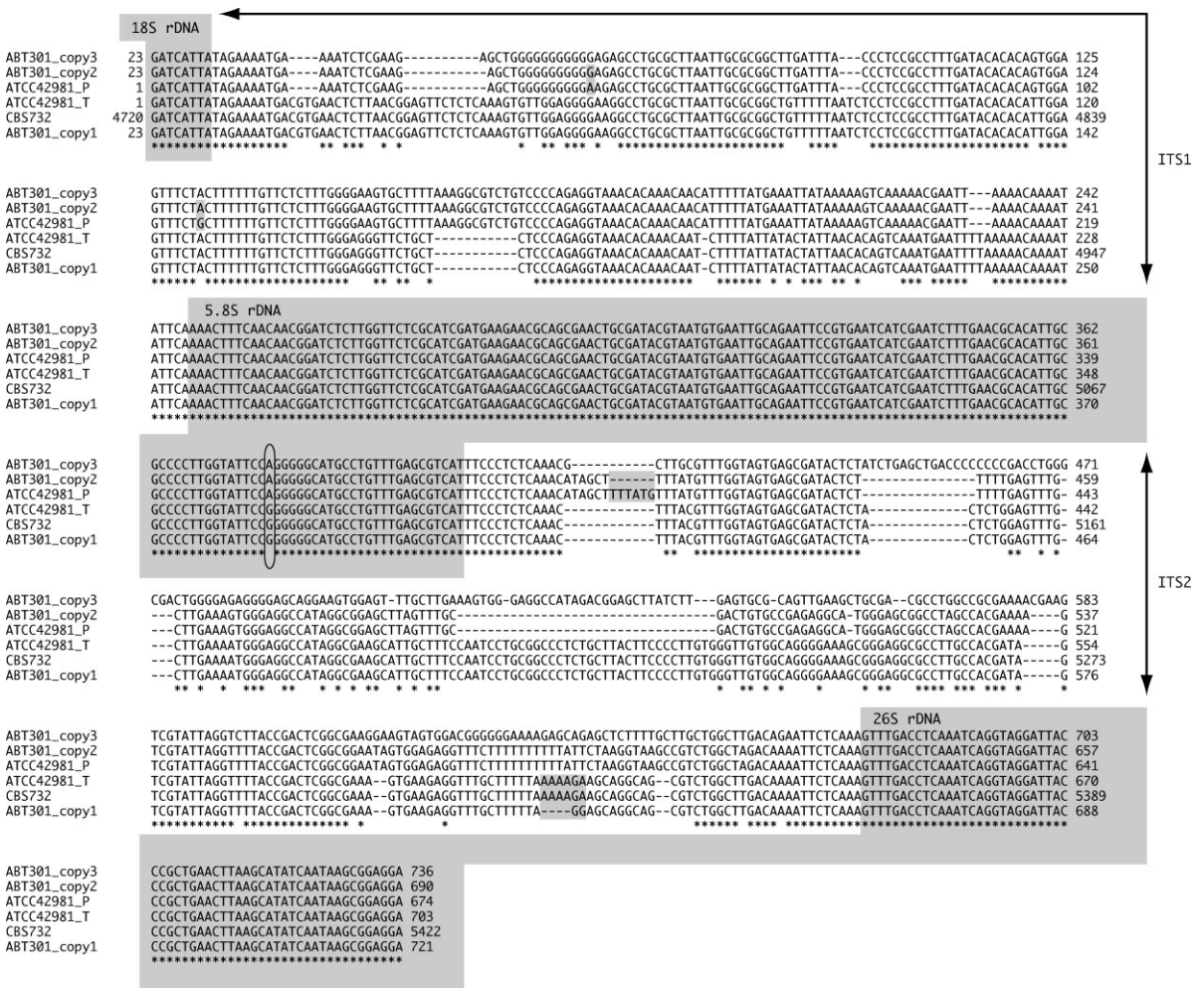

Figure 1. ClustalW alignment of the ITS1–ITS2 regions of rDNA from *Z. rouxii* strains ATCC 42 981 and CBS 732 (this study) and ABT 301 (data from Solieri *et al.*, 2007). The rRNA genes are shaded. Also highlighted are the three sites where the ATCC 42 981 P subgenome differs from ABT 301 copy 2, and the single site where ABT 301 copy 1 differs from CBS 732 and the ATCC 42 981 T subgenome. The site in the 5.8S gene where the P and T subgenomes differ is ringed. The numbering scheme for each sequence is that of the corresponding nucleotide database entry (in order from top to bottom: AM279696, AM279464, AM943657, AM943656, AM943655 and AM279465). We sequenced the complete rDNA array repeating unit (9940 bp) from CBS 732, and assembled 1.7 kb regions from the T and P subgenomes of ATCC 42 981 that include the D1/D2 region of 26S rDNA (not shown) as well as the ITS regions shown here. The 1.7 kb sequence from the ATCC 42 981 T subgenome is identical to the sequence from CBS 732, except for one point in the 26S rRNA gene where ATCC 42 981 has G_{10} and CBS 732 has G_8 (positions 6111–6118 of Accession No. AM943655)

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between their D1/D2 regions (not shown). The T-type sequence of ATCC 42 981 is identical to CBS 732 in the ITS region, and indeed over the whole 1.7 kb region except for a 2 bp difference in a homopolymer in the 26S rRNA gene (outside the D1/D2 region). It is also very similar to ITS copy 1 from strain ABT 301 (Solieri *et al*., 2007), differing by only one deletion (Figure 1). The 1.7 kb P-type sequence of ATCC 42 981 is identical in the D1/D2 region to the sequence of *Z. pseudorouxii* NCYC 3042 (Accession No. AJ555406). The ITS region of NCYC 3042 has not been sequenced, but the ATCC 42 981 P-type sequence shows only two differences from ITS copy 2 of ABT 301.

The rDNA structure of ATCC 42 981 thus contains both *Z. rouxii*- and *Z. pseudorouxii*-derived sequences. The rDNA arrangement in ATCC 42 981 is similar to that in ABT 301 (Solieri *et al*., 2007) but there are significant differences. ATCC 42 981 has two ITS and two D1/D2 sequences, as expected for a simple allopolyploid, but ABT 301 also has a third type of ITS (Figure 1) and has only a single (*Z. pseudorouxii*) type of D1/D2 sequence (Solieri *et al*., 2006, 2007).

Two subgenomes in ATCC 42 981

Many of the contigs we assembled from the ATCC 42 981 random sequencing data are similar but not identical to other contigs from the same strain. We compared these pairs of contigs to some completely sequenced sections of the *Z. rouxii* CBS 732 genome (J.L.G. and K.H.W., unpublished data) and find that in each case one ATCC 42 981 contig exhibits 97–100% nucleotide sequence identity to CBS 732, while the second contig generally has 80–90% identity (Figure 2B). A similar pattern is seen when we map pairs of end-reads from individual plasmids from the ATCC 42 981 library onto the CBS 732 data (Figure 2C). These results confirm that ATCC 42 981 is an allopolyploid containing a T subgenome derived from *Z. rouxii* and a P subgenome derived from *Z. pseudorouxii*. As far as we can tell from our limited data, this pattern of one-to-two mapping between CBS 732 and ATCC 42 981 is present across the whole genome; we did not find any evidence of large deletions from either of the ATCC 42 981 subgenomes.

Lack of genome degeneration

To investigate whether any degeneration of the ATCC 42 981 genome had occurred after the allopolyploidization, we identified and completely sequenced a number of plasmids from the ATCC 42 981 library (in total, 105 kb) that allowed us to make three-way comparisons between single regions in CBS 732 and pairs of regions in ATCC 42 981. Such trios of *Z. rouxii* sequences were collected from three different genomic regions, containing a total of 30 genes. These regions were chosen simply because they appeared to have a high gene density and clone coverage from both of the ATCC 42 981 subgenomes. In each region we saw conservation of protein-coding DNA and divergence of intergenic DNA (Figure 3). These regions did not show any evidence of gene structure degeneration, such as gene deletions or pseudogene formation; for all 30 loci strain ATCC 42 981 contains two copies of the gene that were both intact and presumably functional, and one of which was almost identical to the sequence from strain CBS 732. The average level of synonymous nucleotide substitution (estimated using the program yn00; Yang, 2007) between the ATCC 42 981 P and T subgenomes is 0.58 substitutions/site, which is similar to that seen among different *Saccharomyces sensu stricto* species, indicating a comparable divergence time of perhaps a few tens of millions of years (Kellis *et al*., 2003).

Apart from a few short in-frame length differences, we found essentially no changes in gene structures between the ATCC 42 981 T subgenome and CBS 732 in the 30 genes we examined. We did not, for example, find any frameshifts close to the 3′ end of genes, even though this is a frequent phenomenon among *Saccharomyces* species (Giacomelli *et al*., 2007). This lack of difference was surprising in view of the previous examples of gene structure divergence that were highlighted by Kinclova *et al*. (2001) as being of possible functional significance. In particular, in the *SOD2* homologues, there is an in-frame deletion of 45 basepairs near the 3′ end of the gene in the ATCC 42 981 T subgenome (*Z-SOD2*) relative to its homologues in CBS 732 (*ZrSOD2-22*) and the ATCC 42 981 P subgenome (*Z-SOD22*) (Kinclova *et al*., 2001). Our shotgun data confirms this deletion, which is not present in the corresponding T subgenome of strain ABT 301 (Solieri *et al*., 2007). We do not,

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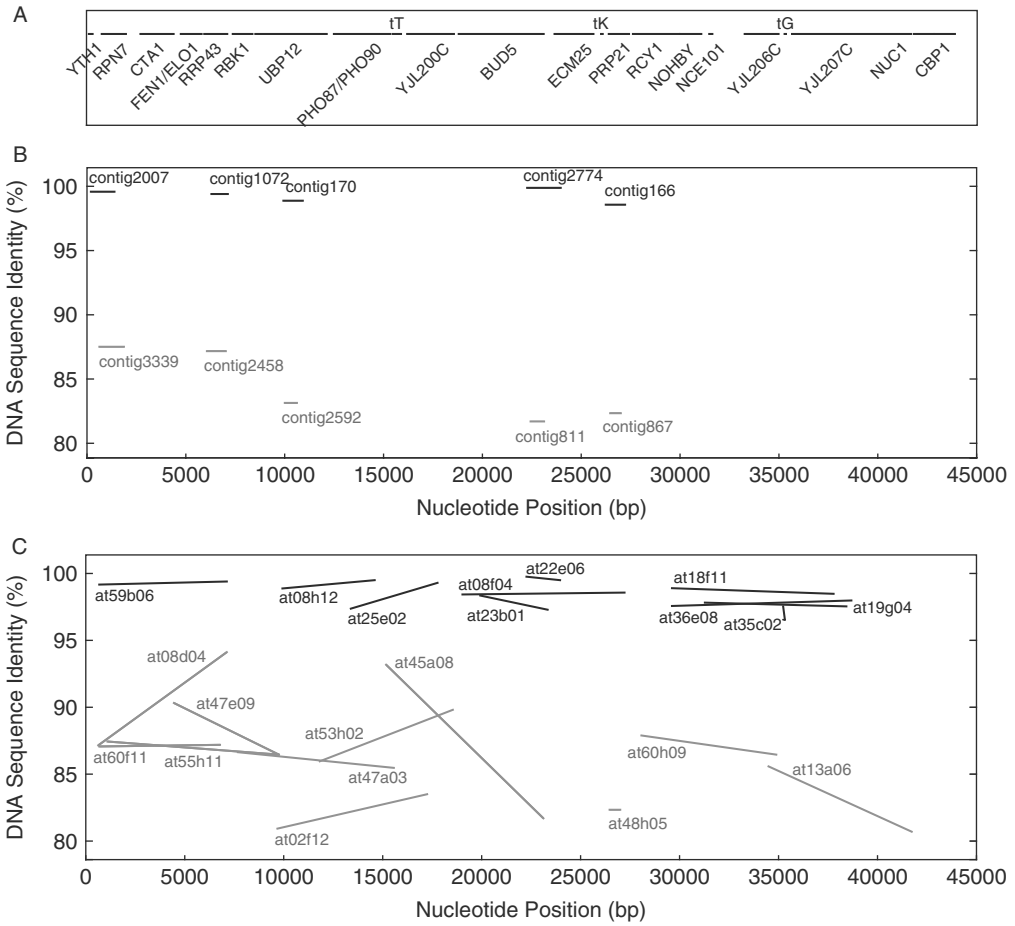

Figure 2. Bimodal distribution of DNA sequence identity between ATCC 42 981 and CBS 732. The horizontal scale in all three panels indicates nucleotide position in a completely sequenced 44 kb region of the CBS 732 genome. (A) Gene content of this region in CBS 732. Genes are named according to their *S. cerevisiae* orthologues. Names written above the horizontal line are tRNA genes. (B) Plot of sequence identity between CBS 732 and pairs of contigs assembled from the ATCC 42 981 shotgun sequencing data. We infer that the contigs labelled in black are derived from the T subgenome, and those labelled in grey are derived from the P subgenome. (C) Plasmid clones from the ATCC 42 981 library that map to this 44 kb region were identified by BLASTN searches of their end-sequence data. Their locations and levels of DNA sequence identity to CBS 732 are plotted, joined by a straight line for each plasmid. We infer that the plasmid clones labelled in black are derived from the T subgenome, and those labelled in grey are derived from the P subgenome

however, confirm the reported differences between the *HOG1* genes of CBS 732 and the ATCC 42 981 T subgenome. Instead, we found that there are frameshift errors near the 3′ ends in the sequences reported by both Iwaki *et al*. (1999) and Kinclova *et al*. (2001). When corrected, the resulting proteins have identical C-termini that are also more similar to the C-termini of Hog1 proteins in other species. We have submitted our sequences of the 3′ end of *HOG1* from the ATCC 42 981 T subgenome and CBS 732 to the databases (Accession Nos AM944569 and AM944570); we do not have data for the 5′ end of the gene or for the ATCC 42 981 P subgenome.

Implications for laboratory strains

The discovery of allopolyploidization in *Zygosaccharomyces* makes it necessary to revisit the origins of the strains used in *Z. rouxii* genetics and molecular biology. Wickerham and Burton (1960) isolated a heterothallic pair of mating strains, NRRL Y-2547 (mating type *MT***a**) and NRRL Y-2548 (mating type *MT*α). One of these, NRRL

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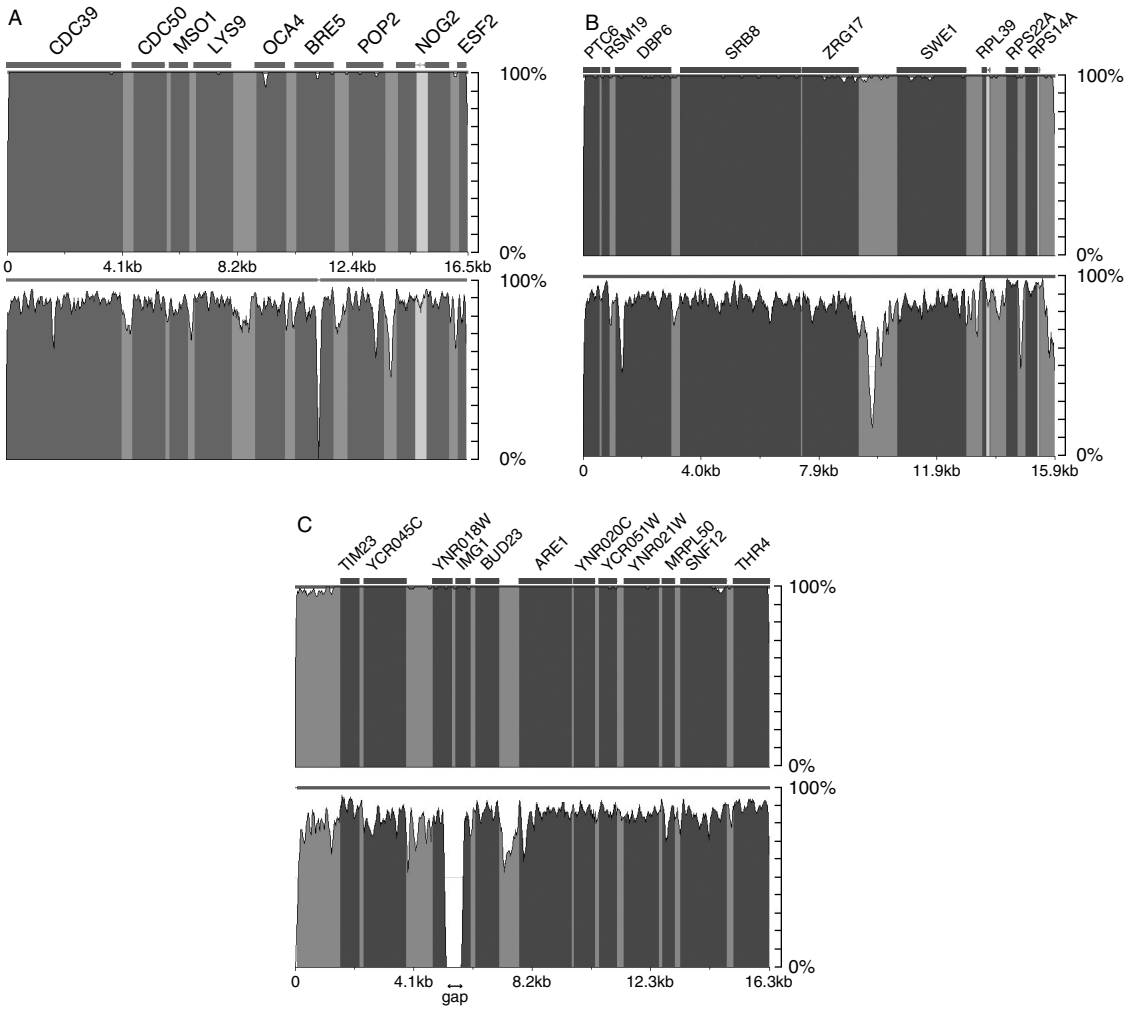

Figure 3. Plots of DNA sequence identity across three genomic regions. The upper graph in each panel shows a comparison between the ATCC 42 981 T subgenome and CBS 732, and the lower graph shows a comparison between the ATCC 42 981 P subgenome and CBS 732. Gene locations are indicated at the top. Each comparison spans approximately 16 kb. Lighter shading shows the locations of intergenic regions and introns (in *NOG2, RPL39* and *RPS14A*). The region of zero identity in (C) is an artifact caused by a small unsequenced gap in the P subgenome data. The drop-off in the *BRE5* gene in the lower part of (A) is due to an in-frame deletion of 108 bp from a repetitive region in the P subgenome relative to CBS 732. The plots were generated by zPicture (**http://zpicture.dcode.org/**)

Y-2547 (= NCYC 1682), is a *Z. rouxii–Z. pseudorouxii* hybrid (James *et al*., 2005) and unfortunately the status of the other, NRRL Y-2548, has not yet been investigated. The type strain, CBS 732 (= IFO 1130 and NCYC 568) is non-hybrid and mating type *MAT***a** (Ushio *et al*., 1988). Some commonly used laboratory strains are therefore hybrids (ATCC 42 981, NRRL Y-2547 and MA11-3, which is a derivative of NRRL Y-2547), and others such as strain ME3 are the products of laboratory crosses between hybrid and non-hybrid strains (Ushio *et al*., 1988). A further complication is that the convention that has been used to name mating types in *Zygosaccharomyces* turns out to be opposite to that used in *Saccharomyces*: the type strain CBS 732 behaves as mating type *MAT***a** (Ushio *et al*., 1988) but the DNA sequence of its mating type loci (both its *MAT* locus and its two *HM*-type cassettes) is homologous to the *MATα* idiomorph of *S. cerevisiae* (Butler *et al*., 2004; J.L.G. and K.H.W., unpublished). Thus, it is important to distinguish between

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behavioural (*MT*) and molecular (*MAT*) genotypes.

Conclusions

Our results show that the previous reports that ATCC 42 981 contains two copies of some genes can be extended to the whole genome. This strain is an allopolyploid formed by hybridization between strains of *Z. rouxii* and *Z. pseudorouxii.* The two parental genomes are quite divergent (averaging about 85% nucleotide sequence identity) but two lines of evidence indicate that the hybridization event that formed ATCC 42 981 happened relatively recently. First, the two subgenomes of ATCC 42 981 do not show any evidence of degeneration. From observations made in the *Saccharomyces* lineage, both on evolutionary (Scannell *et al*., 2007) and laboratory timescales (Antunovics *et al*., 2005), we expect that a polyploidization event will be followed quickly by the deletion of unnecessary extra copies of genes. Such gene loss has not happened in ATCC 42 981, most probably because there has not been enough time for it to happen. Second, the T subgenome is approximately 99% identical in DNA sequence to the CBS 732 genome. This low divergence implies that their last common ancestor existed recently, but the allopolyploidization must have occurred after this point because it happened specifically on the lineage leading to the T subgenome after it had split from the CBS 732 genome. It will be interesting to investigate whether the other apparent *Z. rouxii–Z. pseudorouxii* hybrids (James *et al*., 2005; Solieri *et al*., 2006) all share a single origin, or whether there have been multiple events of similar interspecies hybridizations, as seems to have occurred among *Saccharomyces sensu stricto* species (Groth *et al*., 1999; Gonzalez *et al*., 2006, 2008; Lopandic *et al*., 2007).

Pulsed-field gel analysis by Pribylova *et al*. (2007a) showed that ATCC 42 981 has eight chromosomal bands and CBS 732 has seven. They commented that the ATCC 42 981 genome is larger but did not estimate its size. Our results indicate that the genome of ATCC 42 981 contains approximately twice as much DNA as the genome of CBS 732. This difference seems more substantial than the difference observed in the karyotypes, and suggests that some of the chromosomal bands in the ATCC 42 981 karyotype could be doublets. One chromosome of CBS 732 (chromosome V) clearly lacks a same-size counterpart in ATCC 42 981 (Pribylova *et al*., 2007a), so the T subgenome of the hybrid must have undergone some structural rearrangement relative to the type strain, despite being almost identical in sequence.

Pribylova *et al*. (2007a; 2007b) also documented numerous morphological and physiological differences between *Z. rouxii* CBS 732 and strain ATCC 42 981. These differences could be the results of the duplicated nature of ATCC 42 981, although some of them may reflect the different origins of these two strains: ATCC 42 981 originates from industrial miso fermentations, whereas CBS 732 was isolated from concentrated grape must (Kurtzman and Fell, 1998). The ATCC 42 981 cells are larger than CBS 732 cells, which is expected for a hybrid cell with a larger genome. Physiologically, ATCC 42 981 is capable of growing in more extreme conditions than CBS 732, although it grows less efficiently in normal media (Pribylova *et al*., 2007a). The cell wall of ATCC 42 981 is more flexible and elastic, which may be linked to both the larger size of the cell and its ability to survive in harsher conditions than its parental species (Pribylova *et al*., 2007b). The data from comparisons between ATCC 42 981 and CBS 732 indicate that there is possibly a cost in growth efficiency in exchange for the ability to withstand harsh environments. If this is true, then polyploidization could be an evolutionary device to deal effectively with strong selection pressures during times of environmental instability by doubling all genes, including any that may increase the survival of the organism. In many respects the relationship of the *Z. rouxii* type strain CBS 732 to the industrial strain ATCC 42 981 resembles that of *S. cerevisiae* laboratory strains to the allopolyploid (*S. pastorianus*) strains used in the brewing industry, although the latter show evidence of a more complex history than simple allopolyploidization (Bond *et al*., 2004).

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