Intergeneric hybrids between *Saccharomycopsis fibuligera* and *Yarrowia lipolytica*

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Saccharomycopsis fibuligera can utilize starch but not tributyrin as the sole carbon source, whereas *Yarrowia lipolytica* can utilize tributyrin but not starch. *S. fibuligera* mutant strain 193 *met* was crossed to *Y. lipolytica* A *his1* to produce hybrids. The intergeneric hybrids were able to utilize both starch and tributyrin as sole carbon source. They were mitotically unstable and gave segregants during prolonged vegetative culture. The majority of these segregants had the phenotype of the *S. fibuligera* parent, with a very low number of genetic recombinants. A more stable hybrid was produced by protoplast fusion between a mutant, *ade met*, obtained from UV treatment of a Met[−] mitotic segregant of the hybrid 14i and *Y. lipolytica* B *lys5 leu2 ade1 xpr2*. Sixteen of the 40 mitotic segregants of this hybrid were recombinants. Furthermore when a haploid mitotic segregant of *S. fibuligera* 193 *met, met glu*, was crossed to *Y. lipolytica* B *leu2 ade1 xpr2* more stable hybrids were also obtained. Eleven of the 35 mitotic segregants of the hybrid SA11 were recombinants. Such high frequencies of recombinant sectors from these hybrids should provide a system to establish genetic mapping analysis for the novel *Saccharomycopsis*–*Yarrowia* recombinant strains.

Introduction

The yeasts *Saccharomycopsis fibuligera* and *Yarrowia lipolytica* differ from one another in a number of respects (Table 1; Gaillardin *et al.*, 1973; Ogrydziak *et al.*, 1982; Kreger-van Rij, 1984). Their taxonomy has been described by a number of authors (Lodder, 1970; Barnett *et al.*, 1983; Kreger-van Rij, 1984). Gene cloning and transformation systems have been established in *Y. lipolytica* (Davidow *et al.*, 1985, 1987; Gaillardin *et al.*, 1985). In the case of *S. fibuligera*, genes for α -amylase, glucoamylase, acid protease and β -glucosidases have been cloned in *Saccharomyces cerevisiae* (Yamashita *et al.*, 1985a, b, 1986; Itoh *et al.*, 1987; Machida *et al.*, 1988).

Intergeneric hybrids involving either *S. fibuligera* or *Y. lipolytica* with other yeasts have been produced. Provost *et al.* (1978) found intergeneric hybrids produced by protoplast fusion between *Candida tropicalis* and *S. fibuligera* to be mitotically unstable, giving rise to the two parental strains in prolonged vegetative culture. Groves & Oliver (1984) produced intergeneric hybrids between *Y. lipolytica* and *Kluyveromyces lactis* by protoplast fusion. Although the hybrids had a morphology intermediate between the two parental strains they were also unstable and gave strains which had the phenotype of *Y. lipolytica*.

In the present study, intergeneric hybrids between *S. fibuligera* and *Y. lipolytica* were produced by mass mating and by protoplast fusion. Analysis of genetic segregation among the mitotic sectors of these hybrids was performed to determine the frequency of occurrence of recombinant types.

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Abbreviation: OFAGE, orthogonal field alternation gel electrophoresis.

Table 1. Differences between *Saccharomycopsis fibuligera* and *Yarrowia lipolytica*

	<i>Y. lipolytica</i>	<i>S. fibuligera</i>
Vegetative cells	Pseudohyphae; true hyphae with central pore	Pseudohyphae; true hyphae with septa having plasmodesmata
Fermentation of sugars	Sugars not fermented; shows oxidative metabolism	Slowly ferments sugars; fermentative
Mating system	Heterothallic	Homothallic
Genetic systems	Established	No information
Enzymes produced	Lipase	Glucoamylase α -Amylase

Methods

Strains. *S. fibuligera* strain 8014 was from the Malaysian Agricultural Research and Development Institute, Serdang, Malaysia. *S. fibuligera* 193 met was obtained from strain 8014 by UV mutagenesis. *Y. lipolytica* strains A his1, B leu2 ade1 xpr2 (reduced ability to produce alkaline protease) and B lys leu2 ade1 xpr2 were from the collection of H. Heslot. *Saccharomyces cerevisiae* SH964 was from the stock of Y. Oshima. A and B are the opposite mating-type alleles of *Y. lipolytica*. The strain met glu (glutamic acid requirement) is a haploid mitotic sector of *S. fibuligera* 193 met.

Media. Complex YEPD medium consisted of 1% (w/v) peptone, 2% (w/v) glucose and 1% (w/v) yeast extract. Difco Yeast Nitrogen Base (without amino acids) (YNB) was used as minimal medium. Supplementation of this medium when required (for classification media) was as follows: 1.5% (w/v) starch, 2% (w/v) glucose, 1.5% (w/v) sodium citrate, 1% (v/v) tributyrin, 100 mg l⁻¹ each of methionine, histidine and leucine, 200 mg sodium glutamate l⁻¹ and 50 mg adenine l⁻¹. YPSS medium contained 4 g yeast extract, 15 g soluble starch, 1 g K₂HPO₄ and 0.5 g MgSO₄ · 7H₂O per litre. YMC medium contained 3 g malt extract, 5 g peptone, 3 g yeast extract and 0.5 g sodium citrate per litre. When necessary, media were solidified with 2% (w/v) agar.

Mass mating. *S. fibuligera* 193 met was crossed with *Y. lipolytica* A his1 using the method of Gaillardin *et al.* (1973). Putative hybrids were observed to grow at the meeting points between streaks of cells of the two parent strains on YMC. The hybrids were isolated on YNB supplemented with glucose (YNBG) after two subsequent subcultures on this medium. These were hybrids. The strain met glu was crossed with *Y. lipolytica* B leu2 ade1 xpr2 and the hybrids SA6 and SA11 were obtained by subculturing on YNBG.

Protoplast fusion. Strains *S. fibuligera* 193 met and *Y. lipolytica* B leu2 ade1 xpr2 were grown in 40 ml YE medium at 30 °C for 24 h with shaking. Then 10 ml portions of these cultures were added to 90 ml YE medium (5 g yeast extract, 30 g glucose and 15 g agar per litre) and grown to exponential phase. The cultures were harvested and washed in sterile distilled water. Cells were then resuspended in 10 ml PTP-sorbitol buffer, consisting of 1 M-sorbitol, 100 mM-Tris, 5 mM-EDTA and 20 mM-dithiothreitol. Cells of *Y. lipolytica* B leu2 ade1 xpr2 were treated for 5 min, those of *S. fibuligera* 193 met for 15 min. The tubes were kept in ice and the cells were spun down and washed in 8 ml MAP-sorbitol, consisting of 10 mM-2-amino-2-methyl-1,3-propanediol, 0.1 M-morpholinoethylene sulphate and 1 M-sorbitol, containing 60 mg of Novozym, and incubated at 30 °C for 30 min. The protoplasts were harvested and washed in MAP-sorbitol containing 5% (w/v) Ficoll. After centrifugation, the protoplasts were resuspended in 2 ml of an osmotic stabilizing buffer (OSB) consisting of 10 mM-Tris/HCl, 10 mM-

HEPES, 10 mM-CaCl₂ and 1 M-sorbitol. Protoplasts from the two strains were mixed in equal volumes, centrifuged, and the pellet was resuspended in 4 ml 25% (v/v) polyethylene glycol in OSB and incubated at 30 °C for 30 min. Finally an equal volume of OSB was added to the mixture, followed by centrifugation; the pellet was washed once in 2 ml OSB and resuspended in 2 ml OSB. Aliquots from this preparation, and diluted portions thereof, were dispensed into 3.5 ml molten YNBG agar containing 1 M-sorbitol (YNBGS) and overlaid onto YNBGS agar plates. The plates were incubated for 7–10 d at 30 °C, when prototrophic fusant hybrids could be observed on the media.

Characterization of the putative hybrid strains. Putative intergeneric hybrids between *S. fibuligera* and *Y. lipolytica* were tested for their ability to grow on YNBG and to utilize starch, tributyrin or citrate as the sole carbon source.

Mitotic sectoring of putative hybrid strains. The aim of this method is to assess the frequency of obtaining mitotic sectors from the hybrid strains grown as single colonies on YPSS for 14 d at 30 °C. The mitotic sectors were characterized on classification media.

Preparations of gel inserts of yeast chromosomal DNA. The method used was that of De Jonge *et al.* (1986). Yeasts were grown to exponential phase in YEPD broth at 30 °C in 2-litre Erlenmeyer flasks. Approximately 10¹⁰ cells were harvested and washed in 20 ml 0.05 M-EDTA, pH 7.5, at room temperature. Following a second centrifugation the cell pellet was resuspended in 20 ml buffer containing 0.05 M-EDTA, 10 mM-Tris/HCl and 10 mM-dithiothreitol and incubated at 30 °C for 15 min with gentle shaking. The cells were centrifuged and the pellet resuspended in 20 ml CPE buffer (CPE: mix 100 ml 40 mM-citric acid, 120 mM-Na₂HPO₄, pH 6.0, with 4 ml 0.5 M-EDTA, pH 7.5) and resuspended in 3 ml CPES buffer (CPES: CPE containing 1.2 M-sorbitol and 5 mM-dithiothreitol) and left at 38 °C for 5 min. To prepare spheroplasts, 40 mg Novozym 234 (Novo Biolabs) was dissolved in 5 ml 1% (w/v) low-melting-point agarose, prepared in CPE buffer, cooled to 38 °C and immediately mixed with the cell suspension. The mixtures were pipetted into precooled insert plastic moulds and allowed to gel at 0 °C. The agarose blocks were removed from the moulds and kept overnight at 30 °C in 20 ml CPE buffer. The CPE buffer was drained off and the inserts were then treated with 0.02 g proteinase K in 20 ml NDS (NDS: 0.5 M-EDTA, pH 7.5, 10 mM-Tris/HCl and 1% (w/v) sodium *N*-lauroylsarcosinate) for 48 h at 50 °C in a water bath. The agarose blocks were then treated with RNAase A (500 µg ml⁻¹ in 10 mM-Tris/HCl and 15 mM-NaCl) by incubating the inserts for 2 h at 37 °C with gentle shaking in 30 ml electrophoresis buffer (TBE: 90 mM-Tris base, 90 mM-boric acid, 2.5 mM-EDTA, pH 8.2) containing 1.5 ml RNAase. RNAase was removed from the inserts by rinsing twice with TBE.

Pulsed-field gel electrophoresis. Electrophoresis (OFAGE) was carried out using an LKB/Pharmacia Pulsaphor apparatus with a hexagonal electrode array. The gel was run in TBE under the following conditions: at 47 V with a pulse regime of 3600 s for 96 h, then of 1800 s for 48 h; and at 92 V with a pulse regime of 550 s for 48 h. At the end of the run, the gels were stained with ethidium bromide (0.5 µg ml⁻¹) for 1 h and destained overnight in TBE.

Results and Discussion

Intergeneric hybrids between *S. fibuligera* 193 met and *Y. lipolytica* A his1

Prototrophic intergeneric hybrids were produced between *S. fibuligera* 193 met and *Y. lipolytica* A his1 by

Table 2. Classification of the hybrids obtained by mass mating and protoplast fusion

	Ability to grow on*:			
	YNB+ citrate	YNB+ tributylin	YNB+ starch	YNB+ glucose
Parent strains				
193 <i>met</i>	—	—	+	+ b
<i>A his1</i>	+	+	—	—
Hybrid strains (Met ⁺ His ⁺)				
7i	+	+	+	+
9i	+	+	+	+
14i	+	+	+	+
15i	+	+	+	+
Parent strains				
193 <i>met</i>	—	—	+	+ r
<i>B leu2 ade1 xpr2</i>	+s	+	—	—
Hybrid strains (Met ⁺ Leu ⁺ Ade ⁺)				
F ₄	—	+	+	+
F ₆	+s	+	+	+
F ₁₀	+s	+	+	+
F ₁₆	—	+	+	+

* + or —, ability or inability to grow; +s, slow growth; +b, basal growth; +r, reduced growth.

Table 3. Classification of spontaneous sectors from the hybrid strains obtained from the cross between *S. fibuligera* 193 *met* and *Y. lipolytica* 1529 *A his1*

Hybrid strain (Met ⁺ His ⁺)	No. of sectors with phenotype shown:					Total no. of sectors tested
	Met [—]	His [—]	Met [—] Glu [—]	Met [—] His [—] Glu [—]	Met ⁺ His ⁺	
7i	86	0	2	0	1	89
9i	233	0	2	2	1	238
14i	208	0	3	2	0	213
15i	232	0	8	0	0	240
						780

mass mating. These strains were able to utilize starch, tributyrin or citrate as the sole carbon source (Table 2). Colonies of the hybrid strains were grown on YPSS for 14 d at 30 °C, after which mitotic sectors were observed. Each colony gave 12–15 mitotic sectors. A total of 780 mitotic sectors were isolated and characterized on classification media (Table 3). Four were Met[—]His[—]Glu[—] and 15 Met[—]Glu[—]. This result indicated that most of the mitotic sectors had the phenotype of the parental strain, *S. fibuligera* 193 *met*, and only Met⁺His⁺ and Met[—]His[—]Glu[—] types were recombinants (unpublished data). When cells of the two Met[—]His[—]Glu[—] sectors from the hybrid 14i were streaked onto YE agar, colonies

Table 4. Classification of the sectors from the hybrid strains obtained by protoplast fusion between *S. fibuligera* 193 *met* and *Y. lipolytica* *B leu2 ade1 xpr2*

Hybrid strain	No. of sectors with phenotype shown*:				Total no. of sectors tested
	Met ⁺ Leu ⁺ Ade ⁺	Met [—]	Met [—] Glu [—]	Met [—] Glu [—] Ade [—] Leu [—]	
F ₄	0	76	3	1	80
F ₄	1	170	1	0	172
F ₁₀	0	72	2	1	75
F ₁₆	1	74	0	0	75
					402

* None of the Glu⁺ sectors showed requirements for leucine or adenine.

which grew were Met[—]Glu[—] and Met[—]His[—]Glu[—]. These strains were mitotically stable and are presumed to be haploids or stable aneuploids. This result may be taken to indicate that the mechanism of sectoring is mitotic non-disjunction.

The occurrence of sectors with a Met[—]Glu[—] phenotype was not expected. In order to ascertain the origin of these, a test for sectoring was carried out for *S. fibuligera* 193 *met*. Seven out of 240 mitotic sectors were Met[—]Glu[—] and 233 were Met[—], suggesting that *S. fibuligera* 193 *met* may, indeed, be a naturally occurring diploid heterozygous for *GLU/glu* (Abu Bakar, 1990).

Intergeneric hybrids between S. fibuligera 193 *met* and *Y. lipolytica* *B leu2 ade1 xpr2*

A corresponding set of intergeneric hybrids between *S. fibuligera* 193 *met* and *Y. lipolytica* *B leu2 ade1 xpr2* was produced by protoplast fusion. The characteristics of these hybrids are given in Table 2. When colonies of the hybrid strains were grown on YPSS for 14 d at 30 °C, 402 mitotic sectors were obtained and characterized (Table 4). Two recombinant sectors were obtained. The sectors were predominantly of the phenotype Met[—]. Met[—]Glu[—]Ade[—]Leu[—] recombinants are mitotically stable and are presumed to be haploids or stable aneuploids.

Intergeneric hybrids between a mutant, ade met, of a Met[—] mitotic segregant of the hybrid 14i, and Y. lipolytica *B lys5 leu2 ade1 xpr2*

A Met[—] haploid mitotic segregant of the hybrid 14i was mutagenized by UV treatment to give a mutant, *ade met*. A hybrid, 20.1, was produced by protoplast fusion between *ade met* and *Y. lipolytica* *B lys5 leu2 ade1 xpr2*. This hybrid utilized starch, tributyrin or citrate as the

Table 5. Classification of spontaneous sectors from the hybrid 20.1

Type	Phenotype	No. of sectors
1	Ade ⁻ Met ⁻ Leu ⁻ Lys ⁺	4
2	Ade ⁻ Met ⁺ Leu ⁺ Lys ⁻	1
3	Ade ⁻ Met ⁺ Leu ⁺ Lys ⁺	4
4	Ade ⁻ Met ⁻ Leu ⁻ Lys ⁻	1
5	Ade ⁻ Met ⁻ Leu ⁺ Lys ⁻	1
6	Ade ⁻ Met ⁻ Leu ⁻ Lys ⁻	5
7	Ade ⁻ Met ⁻ Leu ⁺ Lys ⁺	24

sole carbon source. It was unable to grow on media not supplemented with adenine, suggesting that *ade* of the strain *ade met* is allelic to *ade1* of *Y. lipolytica*. When colonies of the hybrid were cultivated on YPSS for 14 d at 30 °C, mitotic sectors were obtained at a frequency of 0.7 per colony. Sixteen of the 40 sectors were recombinants (types 1–6; Table 5).

The five type 6 sectors were not able to grow on media supplemented with all the requirements of the parent strains but grew when glutamate was added. When further classified on appropriate media, two were Leu⁺Lys⁺, one Leu⁻Lys⁺, and two Leu⁻Lys⁻. These results suggest that the *ade met* parent of the hybrid may indeed be an aneuploid heterozygous for *GLU/glu*.

Intergeneric hybrid between a mitotic segregant of S. fibuligera 193 met and Y. lipolytica B leu2 ade1 xpr2

A haploid strain *met glu* which is a mitotic segregant of *S. fibuligera* 193 *met* was crossed to *Y. lipolytica* B *leu2 ade1 xpr2* to produce prototrophic hybrids SA6 and SA11. Both the hybrids utilized starch, tributyrin or citrate as the sole carbon source. When colonies of the hybrids were grown on YPSS for 14 d at 30 °C, mitotic sectors were obtained at a frequency of 0.8 to 1.44 per colony. Twenty-six mitotic sectors from SA11 were characterized as follows: 14 were Met⁻, two Met⁻Leu⁻, one Met⁻Glu⁻, and nine contained a mixed population of cells of Met⁻ and Met⁻Leu⁻ types. In this case therefore 11 out of 35 mitotic segregants of SA11 were recombinants. This result also suggests that Met⁻ segregants are likely to be the progenitors of Met⁻Leu⁻ types. Mitotic non-disjunction is probably the underlying mechanism for the change from Met⁻ to Met⁻Leu⁻. Met⁻Leu⁻ recombinants are mitotically stable and are presumed to be haploids or stable aneuploids. Furthermore when four of the 14 Met⁻ segregants from SA11 were grown on YPSS at 30 °C for 14 d, three of them gave Met⁻ mitotic sectors at different frequencies and were putative aneuploids whilst the other, which did not give sectors, was

Fig. 1. DNA bands of yeast strains on OFAGE. Lanes: 1, *Saccharomyces cerevisiae* SH964; 2 and 3, *Y. lipolytica* A *his1*; 4 and 5, *Y. lipolytica* B *leu2 ade1 xpr2*; 6 and 7, *S. fibuligera* 193 *met*; 8, mitotic segregants of the intergeneric hybrid 9i; 9, mitotic segregants of the intergeneric hybrid 14i.

presumably a haploid (G. H. Loh, unpublished data). The three strains that gave sectors differed from the fourth strain in having a reduced growth rate.

The observation that the intergeneric hybrids between *S. fibuligera* 193 *met* and strains of *Y. lipolytica* were more unstable than the other hybrids was not unexpected as these hybrids were constituted from *S. fibuligera* and *Y. lipolytica* strains which have different karyotypes (Fig. 1). *Y. lipolytica* strains have four bands (1–4) on OFAGE, *S. fibuligera* has five bands (1–5), and the hybrids between *S. fibuligera* 193 *met* and *Y. lipolytica* A *his1* have four bands. In earlier studies, De Jonge *et al.* (1986) reported that the OFAGE banding pattern for *Saccharomycopsis lipolytica* showed one band; Johnston *et al.* (1988) observed three bands for *S. fibuligera* in field inversion gel electrophoresis studies on chromosomal DNAs of yeast strains. Bands 1, 2 and 4 of *Y. lipolytica* seem to correspond to bands 3, 4 and 5 of *S. fibuligera*. The intergeneric hybrids 14i and 9i have four bands (not shown in Fig. 1) which appear to be similar to those of some of their mitotic segregants. Band 4 of the intergeneric hybrids seems to correspond to bands 4 and 5 of *Y. lipolytica* and *S. fibuligera*, respectively. Bands 1, 2 and 3 do not correspond to those of either of the parental strains. We take these results to suggest that intergeneric hybrids were indeed produced between *Y. lipolytica* and *S. fibuligera* in our studies.

The high frequency of occurrence of Met⁻ mitotic sectors in all the intergeneric hybrids seems to suggest a high fidelity of replication of the chromosomes of the *S. fibuligera* parent strain followed by the segregation of a complete set of these chromosomes in cell division in mitosis. The hybrid 20.1 gave 24 Ade⁻ Met⁻ sectors out of 40. In the case of SA11, 23 out of 35 mitotic segregants were Met⁻. Both these results indicate a significant decrease in frequency of Met⁻ segregants when compared with those described above.

Provost *et al.* (1978) reported that *Candida tropicalis*-*S. fibuligera* hybrids from protoplast fusion were unstable. In prolonged culture the hybrids gave the two parental strains. The authors suggested that the hybrids consist of the genome of one parental strain with a few chromosomes of the other. In another study, Groves & Oliver (1984) showed that intergeneric hybrids between *Y. lipolytica* and *Kluyveromyces lactis* had a colonial morphology intermediate between those of the two parental strains. They suggested that the cells of the hybrids gave cells that contained only the genome of *Y. lipolytica* in subsequent mitotic divisions. In both these studies the intergeneric hybrids had a strong tendency to give segregants of one or both parental types in mitotic divisions. An important difference between the results of these previous studies and our present work is that no genetic recombinants were obtained from the mitotic segregants of their intergeneric hybrids.

Our finding that the hybrids 20.1 and SA11 gave a relatively high frequency of genetic recombinants in mitotic sectoring may, indeed, provide a significant tool to facilitate development of genetic systems for *S. fibuligera* and for *Saccharomycopsis*-*Yarrowia* recombinants. This is especially important because there is no available information on the genetics of *S. fibuligera*; this line of study is at present being investigated in our laboratory.

The isolation of recombinant mitotic sectors such as *met glu leu2 ade1* and *met leu2* from the intergeneric hybrids *S. fibuligera* 193 *met/Y. lipolytica* B *leu2 ade1 xpr2* and *met glu/B leu2 ade1 xpr2*, respectively, is important. We believe that such strains may be used as recipients to establish a transformation system in *Saccharomycopsis*-*Yarrowia* recombinants using the YEp *LEU2* cloning vectors.

Some recombinants obtained may prove to be useful in industrial processes based on substrates such as tapioca and sago starch which are available in abundance in the countries of South-East Asia. *S. fibuligera* can utilize starch but not citrate. We obtained Met⁻ mitotic segregants from SA11 that are able to utilize starch and citrate. As far as we are aware this is the first report of the isolation of such *Saccharomycopsis*-*Yarrowia* recombinants.

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