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Intergeneric Hybrids of *Saccharomyces cerevisiae* and *Zygosaccharomyces fermentati* Obtained by Protoplast Fusion

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To obtain strains that are able to efficiently produce ethanol from different carbohydrates, mainly cellulose hydrolysates, several species of the genus *Candida* and a *Zygosaccharomyces fermentati* strain were examined for their ability to utilize cellobiose and produce ethanol, as well as for their thermotolerance and the possibility of genetic manipulation. *Candida obtusa* and *Zygosaccharomyces fermentati* tolerated the maximal temperature for growth, possessed the highest cellobiase activity, and offered the possibility of genetic manipulation, although neither of them proved to be a good producer of ethanol. Intergeneric hybrids of *Saccharomyces cerevisiae* and *Z. fermentati* were obtained after protoplast fusion. They were selected as prototrophic strains, after isolation of auxotrophic mutants from *Z. fermentati* and fusion with an *S. cerevisiae* strain which was also auxotrophic. The hybrids, which appeared at a frequency of 2×10^{-7}, presented characteristics of both parents, such as resistance to certain drugs and the ability to grow with either cellobiose or lactic acid as the sole carbon source; they were very stable, even under nonselective conditions. These hybrids may have important industrial applications as good fermenting strains.

Ethanol is an ideal fuel and starting point for countless chemical transformations. Optimal conversion of carbohydrates to ethanol requires cells that are tolerant to high concentrations of both and are able to efficiently produce ethanol at relatively high temperatures (41). The most important substrate to be used for ethanol production is cellulose, since it is the most abundant and the easiest to obtain. Hydrolysis of cellulose with fungal cellulase complexes such as those obtained from *Trichoderma reesei* results in the liberation of glucose and cellobiose (26). The inability of industrial glucose-fermenting yeasts to ferment cellobiose results in the incomplete conversion of the cellulose hydrolysate to ethanol. This has given rise to an interest in yeasts that are capable of fermenting cellobiose (26).

Yeasts in general utilize glucose preferentially in mixtures of glucose and cellobiose (19). The former substrate prevents the uptake of the latter by catabolite regulation of the uptake system, resulting in the sequential utilization of the two substrates (5). The repression of cellobiose utilization as a result of the presence of glucose is a major factor limiting the industrial use of those strains that are able to utilize cellobiose. This drawback may be overcome by the selection of mutants that are able to utilize glucose and cellobiose simultaneously (4).

Furthermore, those yeasts which are able to utilize cellobiose, such as strains of the genera *Candida*, *Zygosaccharomyces*, *Pichia*, *Kluyveromyces*, *Brethanomyces*, and others (29), are not as efficient for ethanol production as *Saccharomyces* strains (10). For this reason it is desirable to obtain hybrids that are able to combine the best characterisitics of both types of yeast species, namely ethanol production and a capacity for cellobiose utilization. One approach toward achieving this hybrid formation is protoplast fusion. This process, rarely observed in a spontaneous way, can be easily achieved by using polyethylene glycol and Ca^{2+} (3, 35, 42, 43). Intraspecific protoplast fusion involving strains with the same mating types has been obtained with frequencies of about 1×10^{-3} to 5×10^{-3} in *Saccharomyces cerevisiae* (18), *Rhodosporidium toruloides* (39), *Candida utilis* (15), and *Candida albicans* (37). In filamentous fungi, protoplast fusion has been successfully used to produce heterokaryons between strains which are sexually incompatible (45) and has proved particularly valuable for basic genetic studies in *Candida* spp. (15), an organism in which the conventional forms of manipulation are difficult (8, 22, 46).

Although the main interest in the use of fusion techniques is the production of interspecific and intergeneric hybrids, the success of the hybrid formation mainly depends on the taxonomic proximity of the strains under study. Results of the earliest studies suggested that hybrid formation could be achieved only if closely related parental species were used (28). However, results of more recent experiments suggest that this might not be the case (33), since interspecific somatic hybrids between *Penicillium roquefortii* and *P. chrysogenum* (2), *Aspergillus nidulans* and *A. rugulosus* (24, 25), *Saccharomyces diastaticus* and *S. rouxii* (40), and between other species have been described. Furthermore, there are reports on intergeneric protoplast fusion between *Candida tropicalis* and *Saccharomycopsis fibuligera* (33) and *Saccharomycopsis diastaticus* and *Schizosaccharomyces pombe* (44). In these cases the fusion frequency is very low, about 10^{-6} to 10^{-7} (44). Many of the hybrids are unstable and readily dissociate into their parental strains (15); in others, parental nuclei fuse and either give stable hybrid progeny (15), or else the hybrid nuclei undergo random loss of chromosomes to stabilize at various levels of aneuploidy prior to segregation (33, 37).

Since there are some yeast strains that are able to efficiently produce ethanol and some others that are able to utilize cellobiose, the main aim of this study was to obtain an organism by intergeneric protoplast fusion which combines these characteristics currently associated with different yeast strains.

MATERIALS AND METHODS

Organisms. Yeast strains used in this study are listed in Table 1. *S. cerevisiae* ACA174 was isolated by J. A. Casas

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TABLE 1. Yeast strains used in this study

Strain	Species	Genotype	Source[a]
ACA174	*S. cerevisiae*	*MATα/a*	J. A. Casas
IFI256	*S. cerevisiae*	ND[b]	V. Arroyo
MMY1	*S. cerevisiae*	*MATα ura3-Δ52 cyh*^{r}	SERI
X30/3C	*S. cerevisiae*	*MATα ade2-1 his4-Δ15*	I. L. Calderón
S288C	*S. cerevisiae*	*MATα mal gal2 SUC2 CUP1*	J. Conde
K5-5A	*S. cerevisiae*	*MATα his4-Δ15 ade2-1 can1 kar1-1*	J. Conde
K5-13B	*S. cerevisiae*	*MATa his4-Δ15 can1 kar1-1*	J. Conde
CACA 3422	*C. cacaoi*	ND	N. van Uden
ATCC 38533	*C. lusitaniae*	ND	ATCC
ATCC 34449	*C. lusitaniae*	ND	ATCC
IFO 1664	*C. brassicae*	ND	G. H. Ermet
CBS 5814	*C. melibiosica*	ND	CBS
CBS 2162	*C. freyschussii*	ND	CBS
CBS 1944	*C. obtusa*	ND	CBS
CBS 4506	*Z. fermentati*	ND	CBS
APP1	*Z. fermentati*	*leu*	This study
APP2	*Z. fermentati*	*ura*	This study
H1 (CBS 4506/MMY1)	*Z. fermentati/S. cerevisiae*	ND	This study
H2 (CBS 4506/MMY1)	*Z. fermentati/S. cerevisiae*	ND	This study
H3 (CBS 4506/MMY1)	*Z. fermentati/S. cerevisiae*	ND	This study

[a] Sources are as follows: J. A. Casas and V. Arroyo, see text; SERI, Solar Energy Research Institute, Golden, Colo.; I. L. Calderón, Universidad de Sevilla, Seville, Spain; J. Conde, La Cruz del Campo, S. A., Seville, Spain; N. van Uden, Instituto Gulbenkiam, Oeiras, Portugal; ATCC, American Type Culture Collection, Rockville, Md.; G. H. Ermet, Biomass Research Center, Fayetteville, Ark.; CBS, Centraal Bureau voor Schimmelcultures, Delf, The Netherlands.
[b] ND, Not determined.

(Departamento de Microbiología, Facultad de Biología, University of Seville) from an Andalusian winery; *S. cerevisiae* IFI256, generously provided by Victor Arroyo (Instituto de Fermentaciones Industriales, Madrid), was isolated from wine. These two strains were used as a control of good fermenting yeast strains (10). The *Candida* and *Zygosaccharomyces fermentati* strains used have been described as being able to utilize cellobiose and grow at high temperatures, about 45°C (29). *Candida brassicae* IFO1664 has been described as a good ethanol-assimilating strain (1).

Chemicals. Chemicals were obtained from the following sources: helicase (Suc d'*Helix pomatia*) from L' Industrie Biologique FranÇaise, Clichy, France; *N*-glycosyl-polifungin (polifungin) from N. J. Pieniazek, Department of Genetics, University of Warsaw, Poland; glucose-oxidase, peroxidase, *o*-dianisidine, 2-mercaptoethanol, polyethylene glycol, cellobiose, cycloheximide, and deoxycholate from Sigma Chemical Co., St. Louis, Mo.; and finally *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (nitrosoguanidine) from the Aldrich Chemical Co. Ltd., Dorset, Great Britain.

Media. Growth media used in this study were YP medium (1% yeast extract [Difco Laboratories, Detroit, Mich.], 2% peptone) supplemented with the following carbon sources: 2% D-glucose (YPD); 2% D-glucose and different concentrations of ethanol (YPDE); 50% sucrose (YPS); 20% D-glucose (YPD20).

Minimal media (0.17% Difco yeast nitrogen base without amino acids and with 0.5% ammonium sulfate) was also prepared with the following carbon sources: 2% D-glucose (SD); 2% cellobiose (SC); 1% D-glucose and 1% cellobiose (SDC); 2% lactic acid (SL). Diploids were sporulated on SPO medium (0.1% Difco yeast extract, 1% potassium acetate, 0.05% D-glucose) following methods described by Sherman at al. (38) or by using media described by Pérez Bolaños and Herrera (34) for sporulation of *Candida* strains. To eliminate further growth when selecting auxotrophs by counterselection with polifungin, YCB medium (0.67% Difco yeast carbon base, 2% D-glucose) (38) was used. For protoplast fusion and regeneration, media were osmostabilized with 1 M sorbitol. Medium employed for protoplast regeneration was SOS (20) containing 1% yeast extract, 2% peptone, 2% glucose, 0.11% calcium chloride, and 18.2% sorbitol. Media were solidified by the addition of 2% agar. To supplement auxotrophic requirements, the appropriate amino acids or bases were added to the minimal medium (38). Cycloheximide was used at concentrations varying from 0.01 to 40 mg/liter and copper was used at a range of concentrations between 50 and 250 mg of copper sulfate per liter.

Culture conditions. Preliminary assays of fermentative capacity were made by inoculating 250-μl portions of stationary phase cultures into 10-ml tubes containing 4 ml of YPD supplemented with 10, 15, or 18% (vol/vol) ethanol (YPDE) or 50% sucrose (YPS) and incubated at 22 or 37°C. An inverted tube (Durham) was used to collect the CO_2 liberated through fermentation. A semiquantitative estimate of fermentative capacity was used, based on the amount of CO_2 liberated (from 0, zero capacity, to 3, maximal capacity).

Sucrose and glucose consumption and ethanol production. Sucrose was quantified by the anthrone (13) and phenol (14) methods. Glucose consumption was measured on a Yellow Springs Instrument model 25 glucose analyzer (Yellow Springs Instrument Co., Yellow Springs, Ohio). Ethanol concentrations were calculated from the remaining sucrose or glucose concentrations or were measured directly with alcohol dehydrogenase (23).

Genetic methods. The genetic methods used in this study were those described in the Cold Spring Harbor Laboratory yeast course manual (38), unless otherwise stated.

Mutagenesis and counterselection with polifungin. *Candida* and *Z. fermentati* cells were mutagenized with nitrosoguanidine by the method described by Calderón and Cerdá-Olmedo (12). Auxotroph enrichment was attained by counterselection with polifungin as described by Polaina and Conde (36) for *S. cerevisiae*.

Protoplast formation. To obtain protoplasts, cells were grown in 200-ml flasks containing 100 ml of YPD without

TABLE 2. Ability of the different yeast strains to grow and ferment in the presence of ethanol and sucrose

Strain	Generation time (h) at the following temp in the indicated media:							Ethanol concn (% [vol/vol]) at which F = 0 at[a]:	
	22°C				37°C				
	YPD	YPDE (10%)	YPDE (15%)	YPS (50%)	YPD	YPDE (10%)	YPS (50%)	22°C	37°C
S. cerevisiae S288C	4.7	20.9	—[b]	38.0	2.8	—	27.0	10	10
S. cerevisiae ACA174	3.5	9.2	15.8	24.5	1.9	12.5	38.0	18	15
S. cerevisiae IFI256	4.2	7.0	25.0	28.0	2.1	18.0	25.0	15	15
C. lusitaniae									
ATCC 38533	10.0	—	—	29.0	6.5		23.0	10	<10
ATCC 34449	8.5	—	—	29.0	4.8	—	18.0	10	<10
C. obtusa	10.5	—	—	36.0	6.0	—	12.0	10	<10
C. freyschussii	13.0	—	—	40.0	10.0	—	16.0	10	<10
C. melibiosica	9.8	24.0	—	36.0	8.0	—	18.0	10	<10
C. brassicae	10.0	22.0	—	18.0	7.0	—	12.0	10	<10
C. cacaoi	11.0	—	—	39.0	6.8	—	15.0	10	<10
Z. fermentati	3.9	—	—	26.0	1.8	—	18.0	10	<10

[a] Maximal ethanol concentration at which fermentation (F) was detectable after 120 h in YPD with ethanol (YPDE) or sucrose (YPS). No strains grew in 18% ethanol at 22 or 37°C or in 15% ethanol at 37°C; all strains fermented in 50% sucrose at 22 and 37°C. Results are the average of three experiments.

[b] —, Nongrowth.

aeration and at 30°C until they reached an optical density at 660 nm (OD_{660}) of 0.5 (about 10^7 cells per ml, according to cell counts under the microscope). The cells were then centrifuged at 5,000 × *g* for 10 min, washed, and suspended in 10 ml of 1 M sorbitol to which 50 μl of 2-mercaptoethanol per ml had been added. In a previous experiment, different osmotic stabilizers were tested, with magnesium sulfate, manitol, and sorbitol proving to be the better ones and sorbitol the best of all. Cells were then incubated for 15 min at 30°C, washed, and newly suspended in 10 ml of 1 M sorbitol to which 25 μl of helicase per ml was added. The mixture was incubated at 30°C until a proportion of about 60% protoplasts was obtained. This was determined by measuring the difference in OD_{660} between a sample with the mixture of cells and protoplasts in 1 M sorbitol and a fraction in distilled water plus 10% sodium dodecyl sulfate. This percentage of protoplasts was obtained after incubation periods that varied between 25 and 40 min.

Protoplast fusion. Protoplasts were centrifuged and washed with 10 ml of 1 M sorbitol and suspended together with those with which fusion was to take place in a solution of 2 ml of 30% polyethylene glycol (molecular weight, 4,000) in 0.01 M $CaCl_2$. The mixture was incubated for 30 min at 30°C. Protoplasts were then centrifuged and washed with 10 ml of 1 M sorbitol and suspended in 0.5 ml of SOS medium for 60 min. The mixture was then added to soft agar, and 5 ml was spread onto each petri dish containing selective medium (either SD or SC, according to the strains to be fused). After 3 to 7 days of incubation, the colonies grown on the petri dishes were subjected to different growth tests and characterized.

Protein determination. Proteins were extracted as described by Benítez et al. (11) and then measured by the Folin reaction (30).

Cellobiase determination. Cells were grown in 100-ml flasks containing 20 ml of SD, SC, or SDC, with aeration and at 30°C, until exponential phase (OD_{660}, about 0.5). The cultures were then collected by filtration on Millipore filters, washed twice with 50 mM $MgCl_2$, and frozen at −20°C. For the enzymatic assays, cells were suspended in 2.7 ml of 50 mM phosphate buffer (pH 6.5). In a previous experiment, cellobiase activity was measured in a range of pHs between 4 and 9 by using different buffers. Activity was maximal at a pH of between 6 and 7, according to the strain. Further experiments therefore were carried out at pH 6.5, since this seemed to be the optimal pH for this enzyme. A toluene-ethanol mixture (0.3 ml) was added to each tube. The tubes were then thoroughly shaken for 5 min. From these tubes, 0.2-ml samples were taken and incubated with 1.1 ml of 2% cellobiose in 40 mM phosphate buffer (pH 6.5) for 0, 10, 20, 40, and 60 min at 37°C. The reaction was terminated by boiling the tubes in a water bath for 5 min. The glucose liberated after cellobiose hydrolysis was measured by the glucose-oxidase reaction (16). The specific activity was expressed as micrograms of liberated substrate (glucose) per hour and per microgram of protein.

In some experiments the enzymatic assays were performed on crude cell homogenates that were prepared as follows. The yeast cells were harvested by centrifugation and washed in 50 mM $MgCl_2$ at 4°C. The yeast pellet was then suspended in 5 ml of the same solution, and glass beads of 0.45 mm in diameter were added to the suspension. The cell suspension was then blended in a Braun homogenizer for a total of 90 s at 15-s intervals to break the cells. The resultant homogenate was centrifuged at 10,000 × *g* for 10 min to remove cellular debris and unbroken cells. This supernatant was used to assay for protein and enzyme activities.

RESULTS

Characterization of the different strains on the basis of their ability to grow and ferment in the presence of ethanol and sucrose. *Saccharomyces* strains have been described as being able to grow and ferment in 15% (vol/vol) ethanol and to ferment in 18% ethanol (10). These strains are unable to ferment or assimilate cellobiose. *Candida* and *Z. fermentati* strains can utilize cellobiose, but it is not known whether their fermenting capacity is good enough for it to be worthwhile to use some of them as substitutes for *Saccharomyces* strains for ethanol production from cellulosic residues. *Z. fermentati* and *Candida* strains therefore were tested for their ability to grow and ferment in ethanol and sucrose at 22 and 37°C. As controls, *S. cerevisiae* ACA174 and IFI256 as industrial yeast strains and S288C as a laboratory strain were also checked. The results (Table 2) show that none of the *Candida* or *Z. fermentati* strains were able to grow or ferment at ethanol concentrations at which *S. cerevisiae* is capable of doing so (15% ethanol at 22°C or 10% ethanol at 37°C).

When ethanol production from sucrose or glucose was

TABLE 3. Generation time (τ) of the different strains grown in YPD, SD, and SC and cellobiase-specific activity of these strains (measured in cell extracts) grown in SC[a]

Strain	τ (h) in:			Sp act (μg of glucose liberated/h per μg of protein)
	YPD	SD	SC	
S. cerevisiae				
ACA174	1.5	2.5	—[b]	~0
IFI256	1.5	2.0	—	~0
X30/3C[c]	1.7	4.1	—	~0
MMY1[c]	2.0	4.0	—	~0
C. cacaoi	2.5	5.0	5.5	0.45
C. lusitaniae				
ATCC 38533	2.7	3.0	3.8	1.79
ATCC 34449	2.2	3.5	3.9	1.58
C. obtusa	2.0	3.5	3.8	1.34
C. brassicae	2.2	3.5	—	~0
C. freyschussii	2.3	3.7	5.0	0.68
C. melibiosica	2.5	4.0	6.5	0.14
Z. fermentati	1.7	2.7	4.0	0.82

[a] Growth was at 30°C; results are the average of four to five experiments.
[b] —, Nongrowth.
[c] Since these strains are auxtrophs, their minimal media were supplemented with the appropriate amino acids and bases.

measured, whereas *S. cerevisiae* strains could produce up to 15% ethanol at 22°C and 12.5% at 37°C, as described previously (10), the best *Candida* and *Z. fermentati* strains produced no more than 7.5% ethanol at 37°C. Furthermore, this concentration varied between 3 and 6% at this temperature in the other strains (data not shown). At 22°C they were all so extremely slow that it was not possible to compare results with *Saccharomyces* strains. The maximal ethanol concentration went down to 5% (vol/vol) at 42°C in the best strain, *C. brassicae* (data not shown).

Growth of the different strains in YPD, SD, and SC. To ascertain the ability of *Candida* and *Z. fermentati* strains to utilize cellobiose, the cells were grown in SD, SC, and YPD at 30°C with aeration, and their generation time (τ) was calculated by measuring the absorbance at 660 nm (OD_{660}). *S. cerevisiae* ACA174 and IFI256 were also used as controls of industrial yeasts and X30/3C and MMY1 were used as controls of laboratory haploid strains. The results (Table 3) show that neither of the *S. cerevisiae* strains nor *C. brassicae* was able to utilize cellobiose. However, the rest of the strains were able to grow in SC, although at a slower rate than in SD, and after a lag time of 6 to 8 h.

Characterization of the different strains on the basis of their ability to grow at high temperatures. Since one of the main characteristics that is desirable for the hydrolysis and fermentation of cellulolytic residues is the capacity of the fermenting strains to tolerate high temperatures, this feature was tested in *S. cerevisiae*, *Z. fermentati*, and *Candida* strains. Cells were grown in YPD with aeration at 30°C for 2 days. Fractions of 10 μl of this culture were inoculated in a series of 20-ml tubes with 10 ml of YPD medium each; the tubes were then incubated at 22, 26, 30, 37, 40, 45, and 48°C in different water baths. The OD_{660} of the different cultures was measured periodically.

The results that were obtained indicate that at temperatures up to 40°C all the strains were able to grow exponentially, reaching the maximal OD measurable (1.5 to 2.0). However, at 45°C only *Candida lusitaniae* ATCC 38533 and ATCC 34449, *Candida cacaoi*, and *Z. fermentati* were able to grow exponentially. *Candida* strains stopped growing at an OD of about 0.7; *Z. fermentati* was the only strain that was able to grow at 48°C, thus being the best for thermotolerance selection.

Determination of cellobiase activity in the different strains. Cellobiase activity was measured as described above in all the strains capable of utilizing cellobiose to select those with the highest activity. Cells were grown in SD, SDC, and SC to see whether in all or any of the strains cellobiase activity was either constitutive (with activity in the three media), insensitive to catabolite repression (activity in SDC and SC), or inducible (activity only in SC). Cellobiase was measured in cell extracts and cells treated with toluene, with similar results. This activity was also measured in whole cells and in the supernatant after centrifugation of cells grown in SC. Activity was not detected in cells grown either in SD or in SDC, indicating that in all the strains the cellobiase activity was neither constitutive nor insensitive to catabolite repression. When cultivated in SC, there was no measurable activity in the supernatant of any of the strains. In whole cells, about 20% of the activity found in cell extracts was detected.

In Table 3 is expressed the results obtained with *Z. fermentati* and the *Candida* strains grown in SC. Four *S. cerevisiae* strains were included as controls; *C. brassicae* was also included to see whether its inability to grow in cellobiose is due to the lack of cellobiase activity or to some other factor. None of the strains of *S. cerevisiae* or *C. brassicae* possessed cellobiase activity under the experimental conditions, indicating that the lack of activity accounts for their incapacity to grow in SC. For the other strains, activity was only detected when the OD_{660} of the cultures was over 0.5. As shown in Table 3, the highest activity corresponds to *C. lusitaniae*, *Candida obtusa*, and *Z. fermentati*.

Genetic characterization of the strains. (i) Sporulation. To check sporulation capacity in the different strains, cells were grown to YPD for 2 days at 37°C and transferred (38) to the sporulation media described above. It was concluded that these strains either do not sporulate or do so scarcely.

(ii) Conjugation. Conjugation was attempted between each pair of the different strains used in this study and between these strains and the *S. cerevisiae* haploid strains MMY1 (α mating type) and K5-13B (**a** mating type). In this case the results were also negative. Either these strains do not conjugate or they are all of the same mating type and sufficiently distant taxonomically from *S. cerevisiae* as to be unable to respond to their mating hormones. Conjugation control of the α and **a** mating strains of *S. cerevisiae* yielded abundant zygotes.

(iii) Ploidy determination. The isolation of auxotrophic *Candida* or *Z. fermentati* strains was a requisite for further attempts at hybrid selection. Since the lower the ploidy of a strain the higher the auxotroph yield after mutagenesis, it was estimated to be convenient to establish the ploidy of the strains to be used in the fusion.

Survival to UV light has been reported to be proportional to ploidy for strains of *S. cerevisiae* (32). Consequently, UV radiation survival experiments were carried out with *Z. fermentati* and *Candida* strains, and the results were compared with those obtained with strains of *S. cerevisiae* of known ploidy (haploid and diploid).

C. obtusa and *Z. fermentati* displayed survival curves after UV irradiation similar to that of ACA174, the diploid control strain (data not shown). These two strains therefore could be diploids. No strain showed a level of ploidy lower than the diploid ACA174. *C. lusitaniae* ATCC 38533 and

TABLE 4. Cell survival, protoplast yield, and number of hybrids obtained in the fusions

Strains	No. of cells			
	Initial	Survival to helicase treatment[a]	Protoplasts[a]	Hybrids
S. cerevisiae X30/3C	1.2×10^9	9.6×10^7 (8.7)	6.7×10^7 (70.6)	—[b]
S. cerevisiae MMY1	1.0×10^9	9.5×10^7 (9.8)	6.2×10^7 (63.6)	—
Z. fermentati APP1	0.8×10^9	4.1×10^7 (8.3)	4.1×10^7 (60.1)	—
X30/3C and MMY1	—	—	—	105 (6.7×10^{-5})
X30/3C and APP1	—	—	—	0
MMY1 and APP1	—	—	—	3 (2.1×10^{-7})

[a] Percentages are given in parentheses.
[b] —, Nongrowth.

ATCC 34449 seemed to have a level of ploidy higher than diploid and were therefore provisionally discarded.

Mutagenesis of *C. obtusa* and *Z. fermentati* strains with nitrosoguanidine and counterselection with polifungin. As a previous experiment, fractions of *C. obtusa* and *Z. fermentati* either growing in SD (cells in active phase of growth) or maintained in YCB (in nongrowth phase) were incubated in the presence of concentrations of polifungin varying from 15 to 150 μg/ml. The results indicate that in *Z. fermentati* there are clear differences in mortality produced by the effect of the antibiotic, depending on whether or not the cells are growing. This difference was maximal at concentrations of polifungin of 50 μg/ml (80% survival for the nongrowing cells versus 2 to 3% for growing cells). In *C. obtusa* the best results were also obtained with 50 μg of polifungin per ml (30% survival for nongrowing cells as opposed to 10% for growing cells).

The selected strains, *C. obtusa* and *Z. fermentati*, were grown until exponential phase, centrifuged, washed, and mutagenized with nitrosoguanidine (12). Survival after the mutagenic treatment was 20% for *C. obtusa* and 27% for *Z. fermentati*. The cells were then incubated with YCB to exhaust their amino acid pool and incubated with polifungin (36). The survival percentage after polifungin treatment in the whole population was 4% for *C. obtusa* and 3% for *Z. fermentati*. After this treatment, the cells were washed, spread onto YPD petri dishes, and incubated at 30°C for 2 days. About 10^3 colonies from each strain were tested on SD medium. Four colonies from *Z. fermentati* which clearly showed a total incapacity to grow on SD were finally selected. After the strains were tested in SD supplemented with different amino acids and bases, one leucine-requiring (APP1) and one uracil-requiring (APP2) colony were selected for further experiments. None of the preselected colonies of *C. obtusa* proved to be clearly auxotrophic for any amino acids or bases.

Mutant characterization. The mutants isolated from *Z. fermentati* were characterized with regard to different physiological and genetic features. First, the spontaneous reversion frequency of the *leu* and *ura* mutations was determined. Both turned out to be less than 10^{-8}.

The generation time (τ) of APP1 and APP2 grown in different media (YPD, SD, SC, and YPDE supplemented with different concentrations of ethanol) and at different temperatures, was determined. Both mutants have τ values very similar to that of the parent *Z. fermentati* under any testing conditions. When checked for ethanol tolerance, the three strains stopped growing at ethanol concentrations of 8%. The generation times at lower concentrations were very similar for the three strains (data not shown). Cellobiase activity was also measured in the wild type and the mutants after the cells were grown in SC. This activity was slightly lower in the mutants than in the wild type (the specific activity was 0.56 for APP1 and 0.60 for APP2 mutants, whereas it was 0.81 for the wild type).

Protoplast formation and fusion. Experiments were carried out to fuse *Z. fermentati* and *S. cerevisiae* protoplasts. The *Z. fermentati* parental strain used was the *leu* mutant APP1. It was fused in different experiments to *S. cerevisiae* MMY1 and X30/3C. Protoplasts were obtained as described above. The length of incubation time at which the viability of the protoplasts was maximal (indicated by the highest proportion of protoplasts able to regenerate) was first determined. It was observed that this occurred when the percentage of protoplasts was about 60 to 70% (the average of three experiments was 71% protoplasts obtained from strain X30/3C, 64% from strain MMY1, and 61% from strain APP1).

The protoplasts were then harvested and fused as described above. Protoplasts from *Z. fermentati* APP1 were mixed with protoplasts from either strain MMY1 or X30/3C. As a control to the fusion procedure, protoplasts of the two *S. cerevisiae* strains, MMY1 and X30/3C, were also mixed together. Since they are both of the same mating type (α), the expected frequency of spontaneous diploid formation by conjugation was about 10^{-7} (9). Protoplast mixtures were spread onto petri dishes with YPD as a control of viability or, as selective media, either SD for intraspecific fusion or SC for intergeneric fusion. Petri dishes were incubated at 30°C for 7 days. The results obtained in one of the experiments are expressed in Table 4.

In similar experiments carried out subsequently, 86 intraspecific and 2 interspecific hybrids were obtained, again between strains APP1 and MMY1, giving fusion frequencies of about 10^{-4} for intraspecific fusion and 2×10^{-7} for intergeneric fusion.

In the intergeneric fusion experiments, two kinds of colonies were observed in selective medium: one of standard size and the other of abnormally small size. The very small colonies were immediately discarded because they were either the result of residual growth or were unstable heterokaryons which had fused cytoplasm but whose nuclei had not fused. Since the concern of this study was the formation of stable hybrids, from all the colonies which appeared in selective medium after intergeneric fusion, only those which displayed vigorous growth in selective medium (a total of three) were chosen.

Characterization of the hybrids between *Z. fermentati* and *S. cerevisiae*. Those potential hybrids obtained after protoplast fusion of *Z. fermentati* and *S. cerevisiae* strains were subjected to different physiological and genetic tests for the purpose of characterization. Generation time of the two parental strains, APP1 and MMY1, and the three selected hybrids H1, H2, and H3 was measured by growing

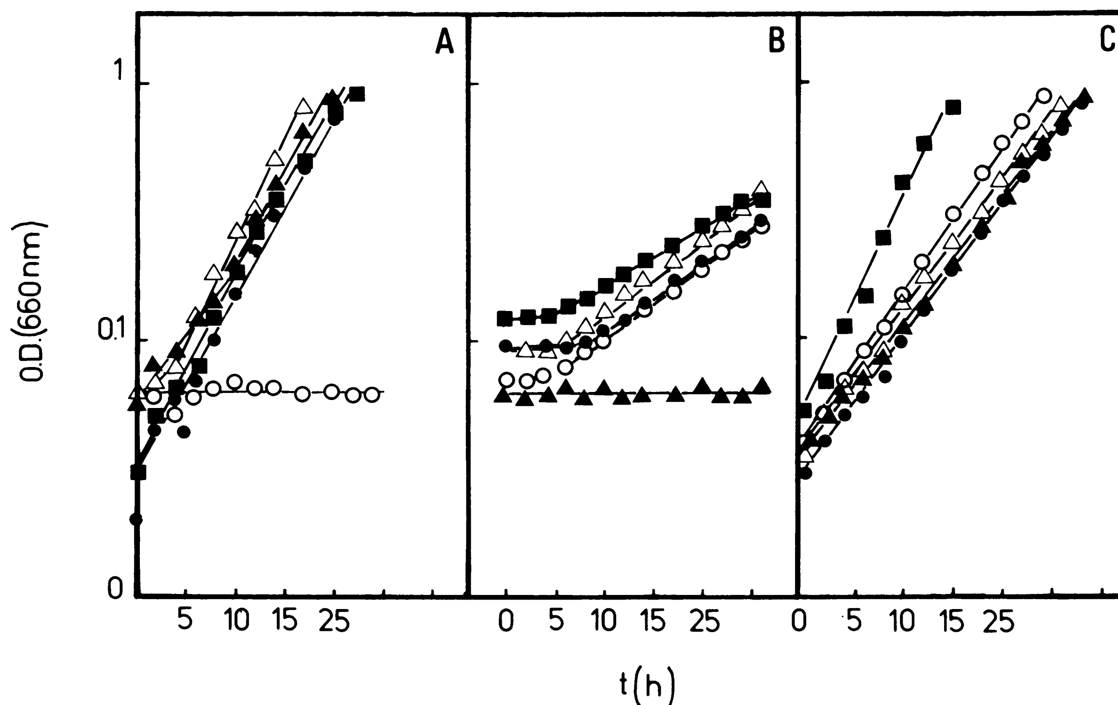

FIG. 1. Growth in SC (A), SL (B), and YPDE (6% ethanol) (C) of the following strains: *S. cerevisiae* MMY1 (○) and *Z. fermentati* APP1 (▲) and the intergeneric hybrids H1 (■), H2 (●), and H3 (△). Results are the average of three experiments.

the cells at 30°C in YPD, SD, SC (cellobiose as the sole carbon source can only be used by *Z. fermentati*) (29), SL (lactic acid as the sole carbon source can only be used by *S. cerevisiae*) (29), and YPDE (6% ethanol). Minimal media were supplemented with the requirements of the auxotrophic parents. Figure 1A, B, and C shows the results that were obtained. Whereas the parental strain MMY1 was completely unable to grow in SC, the three hybrids could grow as satisfactorily as their *Z. fermentati* parent (Fig. 1A). Similarly, whereas the parental strain APP1 cannot grow in SL, the three hybrids could grow and in a similar way to their *S. cerevisiae* parent (Fig. 1B). While growing in YPDE (6% ethanol) the three hybrids behaved like their *S. cerevisiae* parent (Fig. 1C).

Resistance to antibiotics and drugs was also tested. It has previously been observed that *Z. fermentati* is able to grow in the presence of cycloheximide at concentrations at least as high as 40 μg/ml. *S. cerevisiae* MMY1 withstood up to 10 μg/ml, and the other strains used in this study withstood between 0.1 and 1 μg/ml (data not shown). When the hybrids were checked for cycloheximide resistance, it was observed that the three of them were as resistant to the antibiotic (having the capacity to grow in the presence of 40 μg of cycloheximide per ml) as their *Z. fermentati* parent.

With regard to copper resistance, it was also observed that *S. cerevisiae* MMY1 withstood up to 150 μg of copper sulfate per ml, whereas *Z. fermentati* could only grow at copper sulfate concentrations of about 50 to 60 μg/ml. The hybrids again were shown to be just as resistant as their more resistant parent, *S. cerevisiae*, being able to grow in the presence of copper sulfate concentrations of about 130 to 150 μg/ml.

The capability of the three hybrids to conjugate was also tested. Unfortunately, only strains of *S. cerevisiae* of either the α (K5-5A) or **a** mating type (K5-13B) were available. No zygotes were observed when either the parental APP1 or any of the three hybrids were mixed with either strain K5-5A or K5-13B. This indicates that the hybrids are unable to conjugate.

Similarly, when sporulation capacity was checked in SPO medium, whereas the control strain *S. cerevisiae* ACA174 formed four spore asci after a 4-day incubation period at

TABLE 5. Physiological and genetic characteristics of parental strains MMY1 (*S. cerevisiae*) and APP1 (*Z. fermentati*) and hybrids H1, H2, and H3[a]

Characteristics	Strains				
	Parents		Hybrids		
	APP1	MMY1	H1	H2	H3
Generation time (h) in:					
YPD	2.29	1.95	3.42	3.66	3.42
SD	3.71	3.85	3.77	3.98	3.91
SC	5.10	—[b]	5.18	5.91	5.93
SL	—[b]	8.94	7.08	9.30	9.19
YPDE (6% ethanol)	4.50	5.50	6.00	6.10	6.50
Sporulation	−	−	−	−	−
Conjugation with:					
*S. cerevisiae MAT***a**	−	+	−	−	−
*S. cerevisiae MAT*α	−	−	−	−	−
Resistances to:					
Cycloheximide (μg/ml)	>40	10	>40	>40	>40
Copper sulphate (μg/ml)	50	150	150	150	150

[a] Results are the average of four experiments.
[b] —, Nongrowth.

22°C, neither the parental APP1 nor any of the three hybrids was able to sporulate. These three strains therefore seem to be real hybrids, since they display characteristics of one parent or the other. In Table 5 is summarized the results of the different tests carried out to characterize the selected hybrids.

To establish whether the hybrids were stable, cells grown in SC were suspended in YPD and spread onto YPD solid medium. About 20 petri dishes with between 100 and 200 cells per dish were inoculated and incubated at 30°C for 5 days. The colonies grown were then replica-plated onto SC medium. All the replicated colonies were able to grow on minimal medium. They were homogenious in size and they did not form nongrowth sectors, indicating that the isolated hybrids were very stable. Nor was segregation found for other features, i.e., pink colonies resulting from the *ade2-1* mutation, when a single colony grown on SC was resuspended and the cells were spread on YPD, which also points to the stability of the hybrids.

DISCUSSION

The main aim of this study was the isolation of strains which have characteristics which favor ethanol production from carbohydrates, mainly cellulose hydrolysates. These characteristics are the ability to use cellobiose, produce ethanol at high speeds, and tolerate industrial culture conditions (high temperatures and high concentrations of substrate and ethanol, as well as heavy metals such as copper, etc.) (27). To this goal, *Saccharomyces* strains had previously been selected because of their high fermenting ability (10). In this study, *Candida* and *Z. fermentati* strains described as being able to utilize cellobiose (29) were also characterized to fuse the best hydrolytic strain with a *Saccharomyces* strain with high fermentative capacity.

All the strains studied were able to grow in SD medium, indicating that either they are not auxotrophic or that the strains, not being haploids, are heterozygous for the auxotrophy. All the *Candida* and *Z. fermentati* strains, except *C. brassicae*, grew in SC. The strains had lag periods between 6 and 8 h before they started to grow, suggesting that the enzyme cellobiase is inducible. This activity has previously been described as inducible in several yeast strains such as *Rhodotorula* (17) and *Kluyveromyces lactis* (7). In *Kluyveromyces fragilis*, however, this enzyme seems to be constitutive (21). Although the existence of nonconstitutive cellobiase means that cellobiose utilization can be repressed by glucose (5), preliminary results (data not shown) indicate that it is possible to obtain strains that are able to efficiently utilize cellobiose in the presence of glucose, by the isolation of 2-deoxy-D-glucose-resistant mutants, in a way similar to that described for galactose-constitutive mutants (4).

The experiments were carried out without any previous knowledge of the regulation and location of cellobiase enzymes in *Candida* and *Zygosaccharomyces* strains. If cellobiase had been excreted into the medium, it would have been more difficult to select the hybrids since the excreted enzyme would have hydrolyzed the cellobiose which was the sole carbon source. The results indicate that these strains do not excrete cellobiase and that the enzyme is located, at least partially, in the periplasmic space. These results agree with those described for *Rhodotorula* and *Kluyveromyces* strains and other yeasts in which most hydrolases, among them cellobiase, are not excreted; furthermore, they are partially located between the membrane and the cell wall (7).

Activity was only detected when the OD_{660} of the cultures was over 0.5. This may be due to the fact that cellobiose is contaminated with small amounts of glucose, since during the enzymatic assays the control of substrate without cells shows some activity. Although small, this amount of glucose could be sufficient to repress, at low cell density, the expression of the cellobiase activity. It is only when this glucose is completely depleted from the medium, at higher cell density, that enzyme activity is detected. The other possibility is that cellobiase-specific activity changes with growth, so that at the beginning of the exponential phase it is so low that is almost undetectable, as is the case with other enzymes (31).

The establishment of ploidy by means of the comparison of UV survival curves between different species is not entirely valid. However, the results reveal that, whereas the genomes of *Z. fermentati* and *C. obtusa* were close to that of the diploid strain *S. cerevisiae* ACA174, *C. lusitaniae* ATCC 38533 and ATCC 34449 had higher levels of ploidy, rendering difficult the isolation of auxotrophs. Because of their high cellobiose activity, these strains will be considered for experiments similar to those described in this report, although a previous lowering of their ploidy should be attempted.

One way of lowering the level of ploidy of the strains to facilitate auxotroph formation may be to sporulate them and select those spores with the most favorable physiological characteristics, i.e., high cellobiase activity but only half of the chromosome complement. This is only possible, under appropriate conditions for sporulation, if the strains are heterothallic. It is known that some of the industrial *S. cerevisiae* strains are homothallic, either diploid or aneuploid, and that their sporulation conditions (10) have been established. However, little is known about the possibilities of genetic manipulation of the strains *Candida* and *Z. fermentati*. Attempts were made to induce sporulation in these strains, but they were unsuccessful, in spite of the use of methods similar to those employed with other taxonomically close strains, such as *C. utilis* (34). One possible explanation for this failure could be that these strains are aneuploids and they do not sporulate because of the lack of certain specific chromosomes. This should imply that although they are aneuploids, some of them might be able to conjugate. However, attempts to conjugate these strains were also unsuccessful.

The survival of *C. obtusa* and *Z. fermentati* obtained after nitrosoguanidine treatment (27 and 20%, respectively) was similar to that already described for *S. cerevisiae* (12). However, survival to polifungin treatment (2 to 3%), as well as the frequency of auxotroph mutants that were obtained (less than 0.1%), was significantly different from those reported for *A. nidulans* (6) and, above all, *S. cerevisiae* (36). Since this antibiotic acts on the membrane lipids, mainly sterols (47), it might be that lipid composition in these yeasts is very different from that of *S. cerevisiae* or *A. nidulans*. This would explain the different efficiency of the polifungin.

The strains used for protoplast fusion were *Z. fermentati* APP1 and *S. cerevisiae* MMY1 and X30/3C. Although these last two strains are not particularly suitable for ethanol production, they each possess a deletion which has a reversion frequency to prototrophy of almost zero. Therefore, these two strains could help to establish whether the formation of hybrids between the genera *Zygosaccharomyces* and *Saccharomyces* is feasible, however low the hybrid formation frequency might be.

When hybrids were selected between *S. cerevisiae* and *Z. fermentati* after protoplast fusion, colonies were obtained

with a frequency of 2×10^{-7}. This frequency was similar to those described for other intergeneric fusions (25, 44). Similarly, the frequency found for the intraspecific control between X30/3C and MYY1 was about 10^{-4}, which is comparable to frequencies reported by Ferenczy and Maraz (18), 4×10^{-4}, and Sipiczki and Ferenczy (39), 5.6×10^{-5}, in *S. cerevisiae* and *Schizosaccharomyces pombe*, respectively. Since the reversion frequency of the *leu* mutation was below 10^{-8}, it was expected that most of the colonies that were obtained would be hybrids. The fact that the isolated colonies normally grow in minimal medium indicates that *S. cerevisiae* and *Z. fermentati* are able to complement their auxotrophs.

Growth tests indicate that the colonies selected are real hybrids since they are able to grow either with cellobiose or lactic acid as the sole carbon source (29). The hybrids obtained in all the experiments were those between strains APP1 and MMY1. The fact that no hybrids could be isolated from APP1 and X30/3C may be due to incompatibility between the genomes of these two strains. This suggestion is based on the fact that although stable colonies of regular size were not isolated, there were numerous abortive colonies, of reduced size, which could well be unstable heterokaryons that were unable to grow on minimal medium. These types of abortive colonies also have been reported by Delgado and Herrera (15) in protoplast fusion experiments with *C. utilis* and by Tamaki (44), who suggested that after the nuclear fusion, chromosomal reorganization frequently occurs, making the cell unable to survive, in spite of the fact that it possesses chromosomes from both parents.

The isolated hybrids were unable to conjugate with either α or **a** mating type *S. cerevisiae* strains. They were also unable to sporulate. This may be due to the fact that either the appropriate conditions for inducing sporulation were not found or the genetic features of *Z. fermentati* make the strain unable to sporulate. Similar results have been described even after intraspecific protoplast fusion (18), and a mechanism of chromosomal reorganization after protoplast fusion has been suggested as the reason accounting for the lack of sporulation capacity (42).

As for their physiological characteristics, the hybrids that were obtained behaved similarly to their parents with regard to their ability to grow in different media and to tolerate certain drugs such as cycloheximide and copper (Table 5). They also seemed to be very stable, since they did not segregate after they were grown on YPD for many generations. This result is very promising for future fusions of *Z. fermentati* and industrial strains of *S. cerevisiae*.

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