APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1996, p. 41–46 Vol. 62, No. 1
0099-2240/96/$04.00+0
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Evaluation of Molecular Typing Techniques To Assign Genetic Diversity among *Saccharomyces cerevisiae* Strains

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Received 25 September 1995/Accepted 17 October 1995

Discrimination of strains within the species *Saccharomyces cerevisiae* was demonstrated by the use of four different techniques to type 15 strains isolated from spoiled wine and beer. Random amplified polymorphic DNA with specific oligonucleotides and PCR fingerprinting with the microsatellite oligonucleotide primers $(GAC)_5$ and $(GTG)_5$ enabled discrimination between the strains tested. Additionally, restriction enzyme analysis, with *Taq*I and *Mse*I, of PCR-amplified fragments from the complete internal transcribed spacer and nontranscribed spacer, both present in the rRNA-encoding gene cluster, proved to be suitable for generating intraspecies-specific patterns. Random amplified polymorphic DNA with primers 24 and OPA-11 and PCR fingerprinting with primer $(GTG)_5$ appeared to generate the highest degree of diversity. However, the results indicated that there was no single PCR-mediated typing technique enabling discrimination on the strain level. Discrimination of each individual strain was nevertheless possible by combining the results obtained with all typing techniques.

Yeasts are essential in the production of fermented foods such as bread, beer, wine, or cider. Unfortunately, yeasts are also involved in spoilage of food products, which is often caused by either failure in the fermentation management or postprocess contamination. Rapid and simple microbiological monitoring systems enabling the investigation of sources and routes of contamination in the food fermentation process and in the food production chain and the determination of the taxonomic position of microbial isolates may improve the microbiological quality of foods as was discussed by Hofstra et al. (9). Especially over the last decade with the rapid development of molecular biology, new techniques for the identification and typing of microorganisms have emerged. Previously, Baleiras Couto et al. (1) have shown that compared with tests based on physiological characteristics, the random amplified polymorphic DNA (RAPD) assay was a superior technique for differentiating between the species *Saccharomyces cerevisiae*, *Zygosaccharomyces bailii*, and *Zygosaccharomyces rouxii*. Recently, the applicability of the RAPD method as a tool for taxonomic purposes was demonstrated by correlating the RAPD data with restriction enzyme analysis of PCR-amplified small-subunit (ss) DNA coding for rRNA (rDNA) (2) and showed that both typing approaches provided information on the species level.

Since the food industry is highly interested in the route and source of contamination, there is a growing demand for typing techniques which enable differentiation at the strain level. A typing technique in which restriction fragment length polymorphism in the rDNA repeat of various isolates of *Candida albicans* was generated showed that this technique enabled discrimination on the intraspecies level (13). Chromosomal length polymorphism has also been shown in baker's (4, 20) and brewer's yeast strains (17, 18, 22). In addition, Blondin and Vezinhet (3), Petering et al. (19), and Vezinhet et al. (23) have shown that both electrophoretic karyotyping and mitochondrial DNA polymorphism are useful in differentiating wine yeast strains. Nevertheless, these approaches cannot be recommended for application in the food industry because they are complex and laborious techniques when compared with PCR-mediated typing techniques. Previous work (1, 2) indicated that in RAPD, the level of differentiation, either interspecies or intraspecies, depended highly on the primers used. Hamelin and coworkers (8) and Nicholson and Rezanoor (16) have used the RAPD assay to differentiate different fungal races of *Gremmeniella abietina* and to identify individual isolates of *Pseudocercosporella herpotrichoides*, respectively. Alternatively, DNA and PCR fingerprinting with the oligonucleotides $(GTG)_5$, $(GACA)_4$, and M13 core sequence seemed also to be suitable for discrimination on intra- and interspecies levels, respectively (12). As suggested by these authors, additional primer sequences and pair-wise combinations of primers may help in obtaining strain-specific patterns when PCR fingerprinting is used (12).

Spacer regions between the ss rDNA and the large-subunit rDNA (ls rDNA), i.e., the internal transcribed spacer (ITS) (25), and the spacer between the ribosomal gene clusters, i.e., the nontranscribed spacer (NTS) (15, 24), are highly variable between and within a species. This could be explained by the fact that both regions were conserved to a lesser degree than were the rRNA-encoding regions, since they are under fewer evolutionary constraints (14). The nucleotide sequences of rRNA genes and spacer regions have been used to analyze phylogenetic relationships among prokaryotes and eukaryotes (10, 25). Since nucleotide sequencing is rather laborious and time-consuming, it is an inappropriate tool for the food industry. Alternatively, the use of restriction enzymes to generate restriction patterns from the amplified rDNA regions is a more beneficial approach. Molina et al. (14) have amplified part of the NTS region composed of the 3′ external transcribed spacer and the intergenic spacer, which after digestion with *Msp*I allowed discrimination of strains between and within the species *S. cerevisiae*, *Saccharomyces pastorianus*, and *Saccharomyces carlsbergensis.*

S. cerevisiae has been studied extensively as to its importance in the production of various foods and beverages. However, this species is also able to cause spoilage; therefore, the food industry is interested in defining methods that can discriminate

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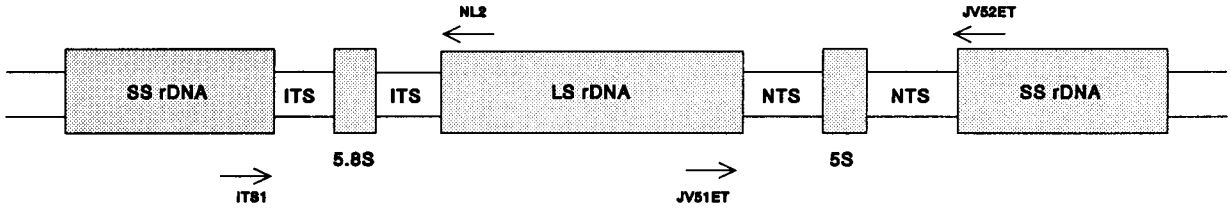

FIG. 1. Schematic presentation of the rDNA repeat unit and location of the ITS1 and NL2 primers used to amplify the complete ITS region and the JV51ET and JV52ET primers used to amplify the complete NTS region. The arrows indicate the directions of transcription.

between isolates of *S. cerevisiae*. In earlier work, we have characterized by RAPD with species-specific primers a collection of yeast strains isolated from spoiled products. A group of yeast isolates cluster together with well-typed strains of the species *S. cerevisiae* (2). The present work compares molecular techniques to characterize the genetic relatedness of these strains belonging to the *S. cerevisiae* complex. RAPD and PCR fingerprinting primers are tested for intraspecies-specific patterns. In addition, restriction enzyme analysis of PCR-amplified fragments from the complete ITS and NTS regions will be used in intraspecies differentiation. The discriminatory power at the intraspecies level of these PCR-based typing techniques is evaluated with 15 individual spoilage strains of *S. cerevisiae* isolated from wine and beer.

MATERIALS AND METHODS

Strains. Yeast strains were isolated from either spoiled wine or beer. Five strains were isolated from wine, namely, W5, W7, W8, W11, and W13, and 10 were isolated from beer, namely, B15, B22, B23, B32, B33, B34, B35, B36, B38, and B45. These strains were classified with the API Kit ATB 32 IC as described in the instructions of the manufacturer (Biomerieux SA, Marcy l'Etoile, France). The type strain of *S. cerevisiae* IGC 4455 was obtained from the Portuguese Culture Collection. The *S. cerevisiae* strains were cultured on yeast extract-glucose broth. Growth conditions and DNA isolation from the yeast strains have been described previously (1).

RAPD assay. The 10-mer primer 24 (5′-GCG TGA CTT G) and primer 28 (5′-AGG AGG AGG A) were synthesized on a DNA synthesizer (model 381A) from Applied Biosystems. Primer OPA-11 (5′-CAA TCG CCG T) was purchased from Operon Technologies Inc., Alameda, Calif. Conditions for the RAPD assay and gel electrophoresis were as published elsewhere (1).

PCR fingerprinting assay. The oligonucleotides $(GTG)_5$ and $(GAC)_5$ were synthesized on a DNA synthesizer (model 381A) from Applied Biosystems and used to generate PCR fingerprints. The composition of the 50-μl PCR mixture was as follows: 20 mM Tris-HCl (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$, 0.001% (wt/vol) gelatin, 200 μM each dATP, dCTP, dGTP, and dTTP, 0.2 μM primer, 0.25 ng of yeast genomic DNA, and 1 U of *Taq* polymerase. The PCR program was as follows: 5 min at 94°C, followed by 40 cycles of 15 s at 94°C, 45 s at 45 or 55°C for primer $(GAC)_5$ or $(GTG)_5$, respectively, and 1 min 30 s at 72°C. Finally, the mixture was heated at 72°C for 4 min and subsequently cooled to 4°C. PCR-amplified DNA was separated on a 1.2% agarose gel by electrophoresis as described previously (1).

Amplification of rDNA regions. The ITS was amplified with the primers ITS1 (5′-TCC GTA GGT GAA CCT GCG G) and NL2 (5′-CTC TCT TTT CAA AGT GCT TTT CAT CT) (25). The positions and directions of the primers used are indicated in Fig. 1. PCR conditions were identical to those described previously for the amplification of the ss rDNA region (2). The thermocycler program was as follows: 5 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C. Finally, the reaction mixture was heated to 72°C for 2 min and subsequently cooled to 4°C.

The definition of the primer JV52ET for the amplification of the NTS region was based on the comparison of the known ss rDNA sequences of several yeast species as described by Baleiras Couto et al. (2). Primer JV51ET was defined on the basis of the work of Molina et al. (14) and by comparison of the conserved regions in the ls rRNA of the yeast species *C. albicans*, *S. cerevisiae*, and *Schizosaccharomyces pombe* by alignment with the PC/GENE program (version 6.8; Amos Bairoch, University of Geneva, Geneva, Switzerland). The sequences of the primers used for the amplification of the NTS were, for JV51ET, 5′-TGA ACG CCT CTA AGY CAG AAT C and, for JV52ET, 5′-TTA TAC TTA GAC ATG CAT GGC. The positions and directions of the primers used are indicated in Fig. 1. Final concentrations in the PCR mixture were as described previously for the ss rDNA region (2). The PCR program was as follows: 5 min at 94°C, followed by 30 cycles of 30 s at 94°C, 1 min at 57°C, and 3 min at 72°C, after which the mixture was heated at 72°C for 2 min and subsequently held at 4°C.

Restriction enzyme digests of PCR-amplified fragments. Digestion with restriction enzymes took place in a 20-μl volume with 10 μl of processed PCR amplification mixture. The restriction enzymes *Mse*I, *Scrf*I, *Taq*I, *Msp*I, and *Ava*II, purchased from Boehringer GmbH (Mannheim, Germany) or Biolabs (Beverly, Mass.) were applied under the conditions recommended by the manufacturer. Digestion products were analyzed on a 2% agarose gel using 0.5× Tris-borate-EDTA buffer (21). Gels were stained with 0.4 μg of ethidium bromide per ml and photographed upon transillumination by UV light (254 nm).

RESULTS

RAPD assay. All 15 isolates were classified as *S. cerevisiae* with the API system (Biomerieux). To collect information on the intraspecies level, three different RAPD primers were used. Primers 24, 28, and OPA-11 gave rise to reproducible and intraspecies-specific patterns (Fig. 2). A high degree of heterogeneity was obtained with primer 24 (Fig. 2a). Although patterns shared several bands, e.g., a band of around 1,444 bp and another of 946 bp, the presence or absence of many other intense bands allowed differentiation of six groups within the 16 strains tested. In Table 1, the results are summarized by assigning a different letter to a pattern when it is different from the others in at least one intense band. With primer 28, all patterns appear to have in common a very intense band with a size of 1,800 bp (Fig. 2b). Apart from this band, the patterns vary among the strains because of differences in fragment number and size. Considering only the major bands in Fig. 2b, patterns can be grouped in the following way: the first group includes strains W5, W7, W8, W11, W13, B35, B45, and IGC 4455, the second group includes strains B22, B23, B32, B33, B34, B36, and B38, and the third group contains strain B15 (Table 1). RAPD analysis with primer OPA-11 also showed different pattern types within the species *S. cerevisiae* (Fig. 2c). These types are also summarized in Table 1. Types C, D, and E differ from type A especially as a result of the presence of bands with smaller molecular sizes. Type F, derived from strain IGC 4455, differs from type A in two bands of larger molecular size, i.e., around 3,350 bp. The observed heterogeneity among strains of the species *S. cerevisiae* assigned by the RAPD assay depends highly on the primer used.

PCR fingerprinting assay. PCR fingerprinting with the micro-satellite oligonucleotide primer $(GAC)_5$ resulted in two types of patterns (Fig. 3), allowing separation of the *S. cerevisiae* strains into two groups. Both types have many bands in common, suggesting a close relationship between the strains. PCR fingerprints of the strains with primer $(GTG)_5$ are shown in Fig. 4. The patterns obtained with this primer are also quite similar, although six groups of patterns were recognized on the basis of the presence or absence of major bands. These groups are type A, which includes strains W5 and W7, type B, which includes strains W8, W13, B35, and B45, type C, which includes strain W11, type D, which includes strains B22, B23, B32, B33, B34, and B36, type E, which includes strain B38, and type F,

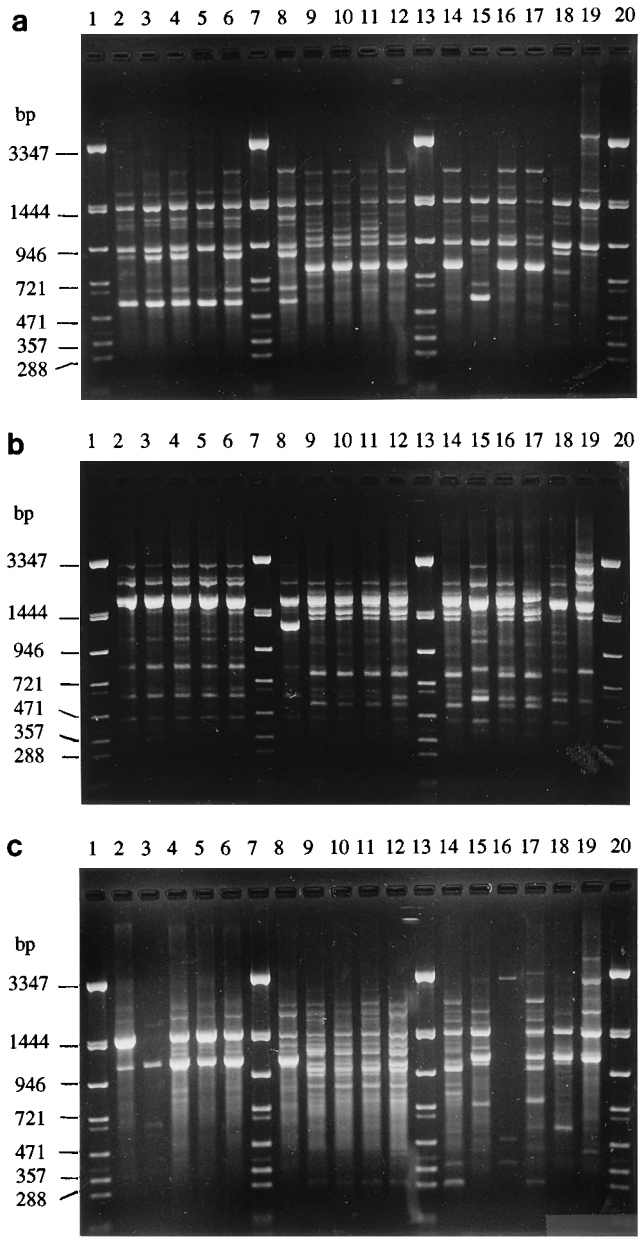

FIG. 2. RAPD patterns of *S. cerevisiae* strains generated with primers 24 (a), 28 (b), and OPA-11 (c). Lanes: 2, strain W5; 3, strain W7; 4, strain W8; 5, strain W11; 6, strain W13; 8, strain B15; 9, strain B22; 10, strain B23; 11, strain B32; 12, strain B33; 14, strain B34; 15, strain B35; 16, strain B36; 17, strain B38; 18, strain B45; 19, strain IGC 4455; 1, 7, and 20, PEMBL 8 marker.

which includes strain IGC 4455. Strain B15, despite giving rise to a very faint pattern, resembles type F. The results of both PCR fingerprinting assays, summarized in Table 1, showed that the $(GTG)_5$ primer allowed discrimination at the lowest taxonomic level.

Restriction enzyme analysis of the amplified ITS region. Figure 5 shows the patterns of *Taq*I digests, which differ from each other in the presence of an extra restriction fragment between the two larger-molecular-size bands, except for strain B38, in which a lower band of around 100 bp is missing. Therefore, strains are separated mainly into two groups, except for strain B38, which displays an aberrant pattern. The profiles

TABLE 1. Pattern types obtained from RAPD, PCR fingerprinting, and restriction enzyme analysis of fragments from the ITS and NTS amplified regions[a]

Strain	Pattern type obtained by: RAPD with primer:				PCR fingerprint with primer:		Restriction enzyme analysis of:			Overall pattern
							ITS with:		NTS with	
	15[b]	24	28	OPA-11	$(GAC)_5$	$(GTG)_5$	*Taq*I	*Mse*I	*Taq*I	
W5	A	A	A	A	A	A	A	A	A	A
W7	A	A	A	A[c]	A	A	B	B	A	B
W8	A	A	A	A	A	B	B	B	A	C
W11	A	B	A	A	A	C	B	B	A	D
W13	A	A	A	A	A	B	B	C	A	E
B15	A	A	B	A	A	F[c]	B	C	A	F
B22	B	C	C	B	B	D	A	D	B	G
B23	B	C	C	B	B	D	A	D	B	G
B32	B	C	C	B	B	D	A	D	B	G
B33	B	C	C	B	B	D	A	D	B	G
B34	B	D	C	B	B	D	A	D	B	H
B35	B	B	A	C	A	B	B	D	B	I
B36	ND[d]	D	C	ND	B	D	A	D	B	J
B38	B	D	C	D	B	E	C	C	C	K
B45	B	E	A	E	A	B	A	C	A	L
IGC 4455	ND	F	A	F	A	F	A	C	ND	M

[a] Identical pattern types are represented by an identical letter code. The data in the last column, overall pattern, were obtained from combining the results of each individual technique. A different letter was given to a new combination of pattern types.
[b] Data in this column are derived from a previous paper (1).
[c] Faint pattern.
[d] ND, not determined.

obtained after digestion with the restriction enzyme *Mse*I are shown in Fig. 6. New groups were obtained when this enzyme was used (Table 1). Similar profiles were generated with strains W7, W8, and W11 (type B). Another group (type C) contains strains W13, B15, B38, B45, and IGC 4455, and a third group (type D) includes strains B22, B23, B32, B33, B34, B35, and B36. Strain W5 gave rise to a unique profile (type A) with this approach. Restriction enzyme analysis of a PCR-amplified fragment from the complete ITS region with *Taq*I and *Mse*I

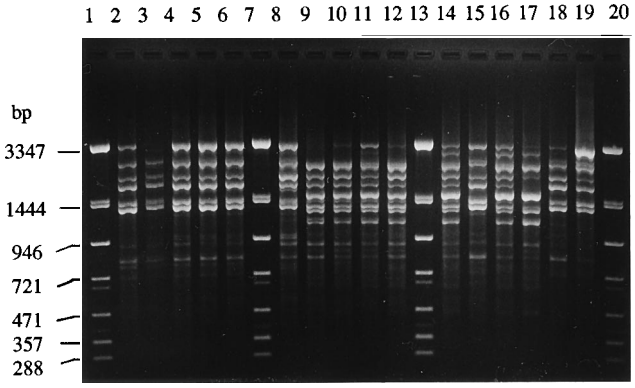

FIG. 3. PCR fingerprinting of *S. cerevisiae* strains primed with the minisatellite oligonucleotide primer $(GAC)_5$. Lanes: 2, strain W5; 3, strain W7; 4, strain W8; 5, strain W11; 6, strain W13; 8, strain B15; 9, strain B22; 10, strain B23; 11, strain B32; 12, strain B33; 14, strain B34; 15, strain B35; 16, strain B36; 17, strain B38; 18, strain B45; 19, strain IGC 4455; 1, 7, and 20, PEMBL 8 marker.

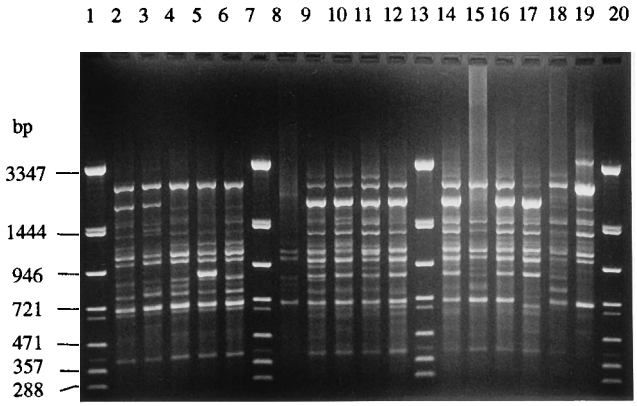

FIG. 4. PCR fingerprinting of *S. cerevisiae* strains primed with the minisatellite oligonucleotide primer $(GTG)_5$. Lanes: 2, W5; 3, strain W7; 4, strain W8; 5, strain W11; 6, strain W13; 8, strain B15; 9, strain B22; 10, strain B23; 11, strain B32; 12, strain B33; 14, strain B34; 15, strain B35; 16, strain B36; 17, strain B38; 18, strain B45; 19, strain IGC 4455; 1, 7, and 20, PEMBL 8 marker.

allowed the separation of *S. cerevisiae* strains into three and four groups, respectively.

Restriction enzyme analysis of the amplified NTS region. The primer pair JV51ET-JV52ET allowed the amplification of the complete NTS between the rDNA clusters of *S. cerevisiae*. The PCR products of all 15 isolates had identical molecular sizes of about 2,640 bp. The results of restriction enzyme analysis with *Taq*I are shown in Fig. 7. From this figure, three groups of patterns could be identified, which are indicated in Table 1. Restriction enzyme analysis of the PCR-amplified products with the restriction enzymes *Mse*I, *Msp*I, and *Ava*II resulted in an identical clustering of the strains as was shown with *Taq*I (results not shown). The restriction enzyme *Scrf*I could only discriminate between strain B38 and the rest of the strains (results not shown). Restriction enzyme analysis of the amplified NTS region allowed discrimination at the intraspecies level depending on the restriction enzyme chosen.

DISCUSSION

Nowadays, the food industry is eager to have new methods suited to rapidly identify possible routes of contamination and therefore to trace a spoilage outbreak. Thus, identification of individual strains is often required. In the present study, four PCR-based typing techniques were evaluated for intraspecies differentiation. Fifteen spoilage strains isolated from wine and beer, all belonging to the species *S. cerevisiae*, were typed to assess intraspecies diversity. The inclusion of the well-typed strain IGC 4455 in this study sustained the previous identification of the 15 isolates as *S. cerevisiae* by means of RAPD analysis with primer 13 and ss rDNA restriction analysis (2). RAPD analysis with primer 15 enabled the discrimination of two groups within the species *S. cerevisiae* (1). These groups are shown in Table 1. The RAPD primers tested in the present study, namely, 24, 28, and OPA-11, allowed the clustering of the 16 strains of *S. cerevisiae* into different groups. Apart from the results for strains B15, B35, and B45, similar groups were observed by use of RAPD with either primer 15 or 28 (Table 1). A higher degree of diversity was obtained with primers 24 and OPA-11, which gave rise to six different types, than with primers 15 and 28. RAPD with the primers described above enabled discrimination at the intraspecies level. From the data in Table 1, it can be concluded that the level of discrimination by means of RAPD depends highly on the primer used. Apparently, strain-specific patterns were not generated. This study also showed that, with few exceptions, RAPD analysis enabled separation between the strains of the species *S. cerevisiae* isolated from wine and beer. Both primers 15 and OPA-11 directed the beer isolate B15 to cluster with the wine isolates. Strains B15 and B35 cluster with the wine isolates as a result of RAPD with primer 24, while RAPD with primer 28 grouped strains B35 and B45 with the wine isolates. The different pattern types observed for the strains isolated from wine and most of the strains isolated from beer may suggest a close relationship between RAPD types and some physiologic characteristics of the strains. It can be speculated that the observed differences are related to ethanol tolerance. This needs to be tested in the future.

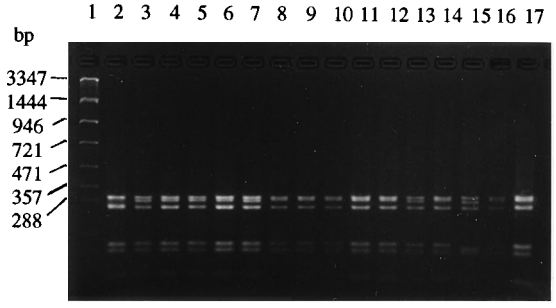

FIG. 5. Restriction digests of the PCR-amplified ITS region of *S. cerevisiae* with *Taq*I. Lanes: 2, strain W5; 3, strain W7; 4, strain W8; 5, strain W11; 6, strain W13; 7, strain B15; 8, strain B22; 9, strain B23; 10, strain B32; 11, strain B33; 12, strain B34; 13, strain B35; 14, strain B36; 15, strain B38; 16, strain B45; 1 and 17, PEMBL 8 marker.

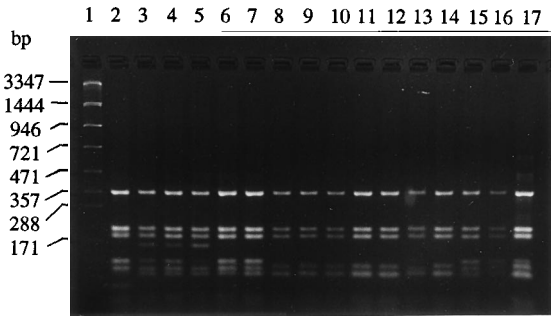

FIG. 6. Restriction digests of the PCR-amplified ITS region of *S. cerevisiae* with *Mse*I. Lanes: 2, strain W5; 3, strain W7; 4, strain W8; 5, strain W11; 6, strain W13; 7, strain B15; 8, strain B22; 9, strain B23; 10, strain B32; 11, strain B33; 12, strain B34; 13, strain B35; 14, strain B36; 15, strain B38; 16, strain B45; 1 and 17, PEMBL 8 marker.

PCR fingerprinting of the 16 strains with $(GTG)_5$ allowed separation of these strains into six different groups. In contrast to the observation of Lieckfeldt et al. (12), the present report shows that PCR fingerprinting with $(GTG)_5$ under more-stringent PCR conditions (annealing temperature of 55°C) allowed intraspecies discrimination. However, it is important to note that this study was executed with *S. cerevisiae* isolates different from those analyzed by Lieckfeldt et al. (12). The PCR fingerprinting primer $(GTG)_5$ enabled a higher level of discrimination than the primer $(GAC)_5$, by which strains were separated into only two different groups (Table 1). The groups observed upon PCR fingerprinting with primer $(GAC)_5$ correlate with those obtained with RAPD primer 15, with the exception of strains B35 and B45.

The ITS region of the *S. cerevisiae* strains showed a molec-

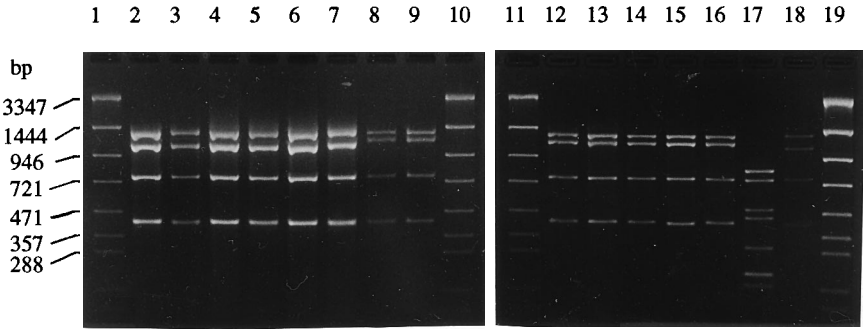

FIG. 7. Restriction digests of the PCR-amplified NTS region of *S. cerevisiae* with *Taq*I. Lanes: 2, strain W5; 3, strain W7; 4, strain W8; 5, strain W11; 6, strain W13; 7, strain B15; 8, strain B22; 9, strain B23; 12, strain B32; 13, strain B33; 14, strain B34; 15, strain B35; 16, strain B36; 17, strain B38; 18, strain B45; 1, 10, 11, and 19, PEMBL 8 marker.

ular size of about 1,200 bp. This size differed from that of the amplified ITS region of other yeast species tested, including *Z. bailii*, *Z. rouxii*, *Candida valida*, and *Candida lipolytica* (results not shown). It has been shown that the ITS regions have a significant level of length and sequence polymorphism across both bacterial and fungal species (5, 7, 11, 25). Digestion of the amplified ITS fragment from the *S. cerevisiae* strains with *Taq*I and *Mse*I resulted in three and four clusters of patterns, respectively. Therefore, sequence polymorphism within the species *S. cerevisiae* is demonstrated and allows discrimination on the subspecies level. The level of discrimination depends on the number and type of restriction enzymes used. Hence, the use of multiple enzymes may allow the discrimination of individual strains.

In earlier work, Molina et al. (14) have shown that *Msp*I digests of only the left part of the NTS region (designated as the 3′ external transcribed spacer and the intergenic spacer) generated different banding patterns in each of the four strains of *S. cerevisiae* tested. These authors suggested that restriction enzyme analysis of the 3′ external transcribed spacer and intergenic spacer should be useful in the identification of strains in which the 5S rDNA is part of the rDNA repeated gene cluster. The fact that the 5S rDNA is sometimes absent from the rDNA repeat unit (6) limits this method from being used universally. Therefore, we have designed new primers and optimized the PCR conditions to amplify the complete NTS region. Amplification of the complete NTS region was also successful in other yeast species, such as *Candida colliculosa*, *Candida famata*, *Candida pelliculosa*, and *Rhodotorula mucilaginosa*. However, the amplification did not succeed with strains of the species *C. valida, Candida krusei*, *Z. bailii*, or *Z. rouxii* (results not shown). Thus, this approach is not generally applicable. Since the complete NTS region of *S. cerevisiae* strains was used for restriction enzyme analysis, a greater variability between the patterns was anticipated, taking into account the results of Molina et al. (14). Surprisingly, restriction enzyme digests of the PCR-amplified NTS region of the genome from the 15 strains tested did not show a high degree of variability. For the 15 strains tested in this study, only three different types of patterns were recognized upon digestion with *Taq*I. Molina et al. (14) obtained different banding patterns for each of the four strains of *S. cerevisiae* when the small part of the NTS region was digested with *Msp*I. Unfortunately, although five restriction enzymes were tested, the discrimination level observed by Molina et al. (14) could not be obtained.

On the basis of the overall results of the different typing approaches tested, some conclusions can be drawn. Genetic diversity within the species *S. cerevisiae* was determined by all techniques tested. Discrimination below the species level was obtained, and the 16 *S. cerevisiae* strains were clustered in a minimum of two and a maximum of six groups, depending on the technique used. From an evaluation of the level of resolution of the typing techniques tested by the number of types of patterns generated, RAPD with primers 24 and OPA-11 and PCR fingerprinting with primer $(GTG)_5$ appeared to give the highest resolution (Table 1). Restriction enzyme digests of the ITS region appeared also to be relatively discriminative. This region displayed a higher level of heterogeneity than the NTS region, therefore allowing more detailed information on the subspecies level. If the results of the different typing techniques are combined, 13 different types for 16 strains could be identified (Table 1). Only four strains, i.e., B22, B23, B33, and B34, showed a similar combined type. It is likely that these strains are actually replicates of the same isolate because they were collected from the same factory. Discrimination of each individual strain was not possible with a single typing technique. This shows the necessity of combining the typing results of more than one technique. Since the typing techniques presented are rapid and relatively easy to perform, multiple tests can be executed, enabling rapid identification at the strain level. This clearly demonstrates that these techniques are suitable for tracing routes of contamination. Since different levels of discrimination are obtained depending on the technique used, the decision of which technique(s) should be applied for typing strains depends on the level of discrimination required. In the future, additional efforts are necessary to develop a single PCR-based typing technique to discriminate at the strain level.

ACKNOWLEDGMENTS

This work was partially supported by grant AIR2-CT 93-0830 from the Agri-Industrial Research Programme of the Commission of the European Communities and by a research grant provided by Junta Nacional de Investigação Científica e Tecnológica of Portugal to M. M. Baleiras Couto.

REFERENCES

1. **Baleiras Couto, M. M., J. M. B. M. van der Vossen, H. Hofstra, and J. H. J. Huis in't Veld.** 1994. RAPD analysis: a rapid technique for differentiation of spoilage yeasts. Int. J. Food Microbiol. **24:**249–260.
2. **Baleiras Couto, M. M., J. T. W. E. Vogels, H. Hofstra, J. H. J. Huis in't Veld, and J. M. B. M. van der Vossen.** 1995. Random amplified polymorphic DNA and restriction enzyme analysis of PCR amplified rDNA in taxonomy: two identification techniques for food-borne yeasts. J. Appl. Bacteriol. **79:**525–535.
3. **Blondin, B., and F. Vezinhet.** 1988. Identification de souches de levures oenologiques par leurs caryotypes obtenus en electrophorese en champ pulse. Rev. Fr. Oenol. **28:**7–11.

4. **Casey, G. P., A. T. Pringle, and P. A. Erdman.** 1990. Evaluation of recent techniques used to identify individual strains of *Saccharomyces* yeasts. Am. Soc. Brew. Chem. **48:**100–106.
5. **Feibelman, T., P. Bayman, and W. G. Cibula.** 1994. Length variation in the internal transcribed spacer of ribosomal DNA in chanterelles. Mycol. Res. **98:**614–618.
6. **Gerbi, S. A.** 1985. Evolution of ribosomal DNA, p. 419–517. *In* R. MacIntyre (ed.), Molecular evolutionary genetics. Plenum Press, New York.
7. **Guillot, J., and E. Gueho.** 1995. The diversity of *Malassezia* yeasts confirmed by rRNA sequence and nuclear DNA comparisons. Antonie van Leeuwenhoek **67:**297–314.
8. **Hamelin, R. C., G. B. Ouellette, and L. Bernier.** 1993. Identification of *Gremmeniella abietina* races with random amplified polymorphic DNA markers. Appl. Environ. Microbiol. **59:**1752–1755.
9. **Hofstra, H., J. M. B. M. van der Vossen, and J. van der Plas.** 1994. Microbes in food processing technology. FEMS Microbiol. Rev. **15:**175–183.
10. **James, S. A., M. D. Collins, and I. N. Roberts.** 1994. Genetic relationships among species of the yeast genus Zygosaccharomyces as revealed by small-subunit rRNA gene sequences. Yeast **20:**871–881.
11. **Jensen, M. A., J. A. Webster, and N. Straus.** 1993. Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphisms. Appl. Environ. Microbiol. **59:**945–952.
12. **Lieckfeldt, E., W. Meyer, and T. Borner.** 1993. Rapid identification and differentiation of yeasts by DNA and PCR fingerprinting. J. Basic Microbiol. **33:**413–426.
13. **Magee, B. B., T. M. D'Souza, and P. T. Magee.** 1987. Strain and species identification by restriction fragment length polymorphisms in the ribosomal DNA repeat of *Candida* species. J. Bacteriol. **169:**1639–1643.
14. **Molina, F. I., S.-C. Jong, and J. L. Huffman.** 1993. PCR amplification of the 3′ external transcribed and intergenic spacer of the ribosomal DNA repeat unit in three species of *Saccharomyces*. FEMS Microbiol. Lett. **108:**259–264.
15. **Musters, W., R. J. Planta, H. van Heerikhuizen, and H. A. Raue.** 1990. Functional analysis of the transcribed spacer of *Saccharomyces cerevisiae* ribosomal DNA: it takes a precursor to form a ribosome, p. 435–442. *In* W. E. Hill, A. Dahlberg, R. A. Garret, P. B. Moore, D. Schlessinger, and J. R. Warner (ed.), The ribosome: structure, function, and evolution. American Society for Microbiology, Washington, D.C.
16. **Nicholson, P., and H. N. Rezanoor.** 1994. The use of random polymorphic DNA to identify pathotype and detect variation in *Pseudocercosporella*. Mycol. Res. **98:**13–21.
17. **Pedersen, M. B.** 1986. DNA sequence polymorphism in the genus *Saccharomyces*. IV. Homologous chromosomes III of *Saccharomyces bayanus*, *S. carlsbergensis* and *S. uvarum*. Carlsberg Res. Commun. **51:**185–202.
18. **Pedersen, M. B.** 1987. Practical use of electro-karyotypes for brewing yeast identification. Proc. Congr. Eur. Brew. Conv. **21:**489–496.
19. **Petering, J., P. Langridge, and P. Henshke.** 1988. Fingerprinting wine yeasts. The application of chromosome electrophoresis. Aust. N. Z. Wine Ind. J. **3:**48–52.
20. **Rank, G. H., G. P. Casey, W. Xiao, and A. T. Pringle.** 1991. Polymorphism within the nuclear and 2μm genomes of *Saccharomyces cerevisiae*. Curr. Genet. **20:**189–194.
21. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
22. **Sheehan, C. A., A. S. Weiss, I. A. Newsom, V. Flint, and D. C. O'Donnell.** 1991. Brewing yeast identification and chromosome analysis using high resolution CHEF gel electrophoresis. J. Inst. Brew. **97:**163–167.
23. **Vezinhet, F., B. Blondin, and J.-N. Hallet.** 1990. Chromosomal DNA patterns and mitochondrial DNA polymorphism as tools for identification of enological strains of *Saccharomyces cerevisiae*. Appl. Microbiol. Biotechnol. **32:**568–571.
24. **Welsh, J., and M. McClelland.** 1992. PCR-amplified length polymorphisms in tRNA intergenic spacers for categorizing staphylococci. Mol. Microbiol. **6:**1673–1680.
25. **White, T. J., T. Bruns, S. Lee, and J. Taylor.** 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, p. 315–322. *In* M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (ed.), PCR protocols: a guide to methods and applications. Academic Press, Inc., San Diego, Calif.