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Rapid Identification and Enumeration of *Saccharomyces cerevisiae* Cells in Wine by Real-Time PCR

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Despite the beneficial role of *Saccharomyces cerevisiae* in the food industry for food and beverage production, it is able to cause spoilage in wines. We have developed a real-time PCR method to directly detect and quantify this yeast species in wine samples to provide winemakers with a rapid and sensitive method to detect and prevent wine spoilage. Specific primers were designed for *S. cerevisiae* using the sequence information obtained from a cloned random amplified polymorphic DNA band that differentiated *S. cerevisiae* from its sibling species *Saccharomyces bayanus*, *Saccharomyces pastorianus*, and *Saccharomyces paradoxus*. The specificity of the primers was demonstrated for typical wine spoilage yeast species. The method was useful for estimating the level of *S. cerevisiae* directly in sweet wines and red wines without preenrichment when yeast is present in concentrations as low as 3.8 and 5 CFU per ml. This detection limit is in the same order as that obtained from glucose-peptone-yeast growth medium (GPY). Moreover, it was possible to quantify *S. cerevisiae* in artificially contaminated samples accurately. Limits for accurate quantification in wine were established, from 3.8×10^5 to 3.8 CFU/ml in sweet wine and from 5×10^6 to 50 CFU/ml in red wine.

Wine can become a growth substrate for a range of undesirable yeast species, both during and after fermentation. Uncontrolled yeast growth at either of these two stages can alter the chemical composition of wine, detracting from its sensory properties of appearance, aroma, and flavor. If these faults are severe, the wine is rejected by consumers. Thus, wine spoilage constitutes an important concern to wine producers.

It is well known that *Saccharomyces cerevisiae* plays a beneficial role in wine fermentation, in which it is the predominant species. Nevertheless, *S. cerevisiae* is able to spoil wine after fermentation if the yeast is not properly eliminated or if the bottle is contaminated by yeast cells present in the wine bottling line or in the cork. *S. cerevisiae* is an important spoilage yeast because it resists high ethanol concentrations. It has been found mainly in sweet wines, where fermentable sugar can support growth, and also in semidry bottled wines.

It is important to detect spoilage yeasts quickly to enable wineries to intervene rapidly and effectively. Methods based on PCR appear to be the best alternative. In the case of *S. cerevisiae*, several methods have been shown suitable for the detection of this species. This is the case of the restriction analysis of the rRNA region spanning the 5.8S gene and the two internal transcribed spacers (ITSs) (5.8S-ITS region). The amplification patterns of *S. cerevisiae* when its DNA is digested with the endonuclease CfoI, HaeIII, or HinfI identify this species accurately. These data are summarized in references 10 and 13 and are available online (http://www.yeast-id.com). Masneuf et al. (18) developed a similar system based on PCR amplification and subsequent restriction analysis using the nuclear gene MET2. These authors show that the endonucleases EcoRI, MaeIII, PstI, and NcoI are suitable for *S. cerevisiae* identification. Another technique used to detect yeast species is based on sequences of the D1/D2 domains of the 26S gene and subsequent comparison with those available in databases. This technique is now well accepted, given that it is possible to sequence PCR products directly and input automatic sequencers.

Unfortunately, all of these techniques, although rapid, require subsequent steps that slow down the results, such as DNA restriction and subsequent agarose gel electrophoresis for fragment separation or analysis of sequenced fragments. Also, they are not useful for determining the number of cells present in the sample, an important factor to take into account in the control of yeast spoilage. Neither the minimum acceptable number of yeasts in wine nor the spoilage potential of *S. cerevisiae* is clear yet. Studies focusing on *Zygosaccharomyces bailii*, considered the main cause of spoilage in bottled wines, show that only a few viable cells in a bottle may be sufficient to cause spoilage (5). This means that methods to detect spoilage yeasts in wine must be very sensitive, as yeast may be present in low quantities. Wineries routinely use membrane filtration and incubation of the filter to detect the minimum number of cells and the most probable number for quantification. Both techniques are time-consuming.

Denaturing gradient gel electrophoresis and real-time detection technology provide significant advances compared to PCR-based methods. They make quantification and specific detection possible simultaneously. Although denaturing gradient gel electrophoresis is becoming popular in food microbiology, it is neither as sensitive nor as fast as real-time techniques, and thus, it is less useful in spoilage preservation programs. Numerous real-time PCR systems have been developed to detect and enumerate bacteria, especially pathogens. Due to the advantages afforded by the technique, it was later extended to yeasts in the clinical setting (1, 22, 27, 29) and in food technology (2, 3). Currently, the detection of wine spoil-

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TABLE 1. Strains tested in this study and results of conventional and real-time PCR with primers SC1d and SC1r

Species	Reference strain(s)[a]	Result by:	
		Conventional PCR[c]	RTi-PCR[d]
Saccharomyces bayanus	CECT 1640, 1941^T, 1969, 1693, 10174; IF1369, 371	−	−
Saccharomyces cerevisiae	CECT 1462, 1475, 1477, 1483, 1485, 1881, 1882, 1883, 1894, 1895, 1942^{NT}, 1985, 10120, 10131, 10322, 10393, 10557, 10995; CR,[b] PV[b]	+	+
Saccharomyces paradoxus	CECT 1939^{NT}, 10175, 10176, 10308, 11143, 11152, 11158, 11422	−	−
Saccharomyces pastorianus	CECT 1940^{NT}, 1970, 11037, 11185	−	−
Candida rugosa	CECT 11889^T	−	−
Candida vini	CECT 11905^T	−	−
Candida zeylanoides	CECT 11907^T	−	−
Dekkera bruxellensis	IGC 4801	−	−
Issatchenkia orientalis	CECT 10027	−	−
Pichia membranifaciens	CECT 1115^T	−	−
Saccharomycodes ludwigii	CECT 10450^T	−	−
Schizosaccharomyces pombe	CECT 11197	−	−
Zygosaccharomyces bailii	CECT 11043	−	−

[a] Culture collections are abbreviated as follows: CECT, Spanish Type Culture Collection, Valencia, Spain; IFI, Instituto de Fermentaciones Industriales, Madrid, Spain; IGC, Portuguese Yeast Culture Collection, Portugal.
[b] *S. cerevisiae* commercial baker's strains.
[c] −, absence of PCR product; +, presence of PCR product (301 bp).
[d] −, absence of melting curve; +, presence of melting curve (T_m, 78.5°C); RTi-PCR, real-time PCR.

age yeast using this method is limited to the species *Dekkera bruxellensis* (21). In the present work, we have developed a real-time PCR to detect and enumerate *S. cerevisiae* directly in wine samples using the nonspecific fluorescent dye SYBR green.

MATERIALS AND METHODS

Strains and growth conditions. A total of 39 yeast strains belonging to the complex *Saccharomyces* sensu stricto were analyzed: 20 *S. cerevisiae* strains, 7 *Saccharomyces bayanus* strains, 8 *Saccharomyces paradoxus* strains, and 4 *Saccharomyces pastorianus* strains. One strain of each of the typical wine spoilage species (*Candida rugosa*, *Candida vini*, *Candida zeylanoides*, *D. bruxellensis*, *Issatchenkia orientalis*, *Pichia membranifaciens*, *Saccharomycodes ludwigii*, *Schizosaccharomyces pombe*, and *Z. bailii*) was also included. The origins and sources of these strains are listed in Table 1.

Yeast cells were grown in GPY medium (4% [wt/vol] glucose [Panreac], 0.5% [wt/vol] peptone [Oxoid], and 0.5% [wt/vol] yeast extract [Pronadisa]) with shaking at 28°C overnight. In the case of *D. bruxellensis*, 0.5% $CaCO_3$ (Probus) was added to the GPY medium.

Two bacteria (*Oenococcus oeni* CECT 217^T and *Acetobacter aceti* CECT 298^T) were used to study the contaminating effect of other microorganisms on the quantification of *S. cerevisiae*. *O. oeni* was grown in MLO medium (1% [wt/vol] tryptone [Pronadisa], 0.5% [wt/vol] yeast extract [Pronadisa], 1% [wt/vol] glucose [Panreac], 0.5% [wt/vol] fructose [Fluka], 0.02% [wt/vol] $SO_4Mg \cdot 7H_2O$ [Panreac], 0.005% [wt/vol] $MnSO_4H_2O$ [Merck], 0.35% [wt/vol] diammonium citrate [Sigma], 0.05% [wt/vol] L-cysteine hydrochloride [Merck], 0.1% [vol/vol] Tween 80 [Fluka], 10% [vol/vol] filtered tomato juice). The pH value was adjusted to 4.8, and the strain was grown at 30°C under microaerophilic conditions. *A. aceti* was grown at 30°C in yeast glucose agar for *Acetobacter* (10% [wt/vol] glucose [Panreac], 1% [wt/vol] yeast extract [Pronadisa], 0.2% [wt/vol] $CaCO_3$ [Probus], 1.5% [wt/vol] agar [Pronadisa]).

DNA extraction. Two methods were used to obtain genomic DNA. For the assays of standard PCR and specificity, DNA from *Saccharomyces* strains was extracted according to the method of Querol et al. (23). For non-*Saccharomyces* strains, enzymatic breakdown was performed with a mixture of 30 μl of 1-mg/ml Zymolyase 20T (Seikagaku, Tokyo, Japan) and 30 μl of 1-mg/ml Novozyme 234 (Interspex Products, Foster City, CA). For the real-time PCR experiments, DNA was obtained using the PrepMan kit (PE Applied Biosystems). For this purpose, 1 ml (or 4 to 8 ml when samples contained low cell numbers) of culture growth was used to collect more cells. Samples were centrifuged and pellet washed twice with sterile water. Previous to extraction, cells were subjected to physical breakdown as follows. Pellets were resuspended in PrepMan buffer and disrupted in a mini-bead beater (Biospec Products, Bartlesville, Okla.) by adding 0.3 g of 0.5-mm zirconia/silica beads. The setting chosen for cell disruption was in runs of three repetitions lasting 30 s at high speed. Samples were cooled on ice for 1 min between runs. The DNA was then extracted according to the PrepMan kit manufacturer's protocol (PE Applied Biosystems) followed by a precipitation step to minimize inhibitions. DNA was resuspended in 50 μl of sterile water, and its concentration was quantified using a DNA BioPhotometer (Eppendorf AG, Hamburg, Germany) and then confirmed by agarose gel visualization.

Cloning and sequencing. A random amplified polymorphic DNA (RAPD) fragment of 1,800 bp specific to *S. cerevisiae*, indicated in Fig. 1, was obtained by following the amplification conditions described by Fernández-Espinar et al. (11) using the primer named OPA-07 (GAAACGGGTG) (Operon Technologies) and the *S. cerevisiae* strain CECT 1885. The band was cut from a 1.4% agarose gel, and the DNA was purified using the Gene Clean system (BIO 101). DNA was blunt ended with a combination of the Klenow fragment of the polymerase I and T4 DNA polymerase, cloned in pBluescript vector, digested with EcoRV endonuclease, and dephosphorylated with alkaline phosphatase (Boehringer

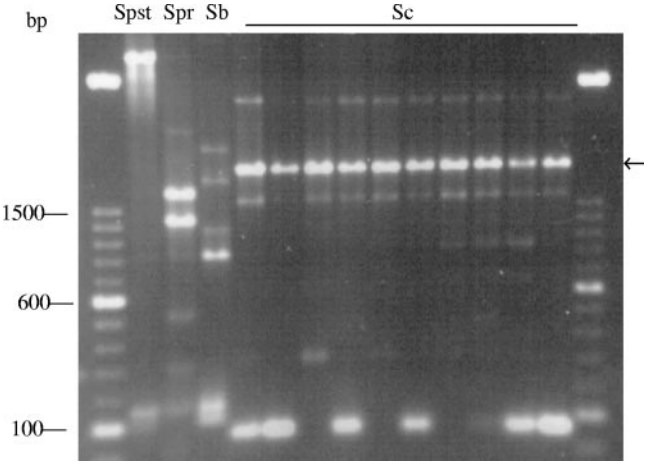

FIG. 1. RAPD patterns obtained with random primer OPA-07. Spst, *S. pastorianus* 1940 ^{NT}; Spr, *S. paradoxus* 1939^{NT}; Sb, *S. bayanus* 1941^T; SC, *S. cerevisiae* (1942^{NT}, 1475, 1477, 1483, 10393, 1485, 1881, 1883, 1894, 1895). The arrow indicates a RAPD fragment of 1,800 bp specific to *S. cerevisiae*. A 100-bp DNA ladder marker (Gibco BRL, Gaithersburg, MD) served as the size standard.

Mannheim, Germany). *Escherichia coli* transformation and plasmid isolation were carried out using standard protocols (26).

The first 600 nucleotides of the 5′ end were sequenced in an ABI Prism 310 Genetic Analyzer using the dRhodamine Ready Reaction DyeDeoxy Terminator Cycle Sequencing kit (PE Applied Biosystems) and KS primer (5′-CGAGGT CGACGGTATCG-3′). A single strand was obtained, and ambiguities were removed by comparison with the *S. cerevisiae* genomic sequence using the basic local alignment search tool (WU-BLAST2) program at the EBI website (http://www.ebi.ac.uk/blast2/index.html).

Primer selection and optimization of primer concentrations. Using the Primer Express, version 2.0, software (PE Applied Biosystems), two primers named SC1d (5′-ACATATGAAGTATGTTTCTATATAACGGGTG-3′) and SC1r (5′-TGGTGCTGGTGCGGATCTA-3′), which generate a 301-bp product, were defined in the sequenced *S. cerevisiae* region. The primers were compared to the database by using BLAST to confirm specificity. To determine the optimal concentration of primers in the PCR, preliminary tests were performed by combining the primers in three final concentrations: 30, 60, and 100 mM.

Real-time PCR conditions. Amplification and detection were performed with the GeneAmp 5700 sequence detection system (PE Applied Biosystems). Duplicate samples were used. The PCR mixture contained 12.5 μl of SYBR green PCR master mix (PE Applied Biosystems), 2.5 μl of DNA at 10 ng/ml, and 60 mM concentrations of each primer in a 25-μl final volume. To minimize inhibition by substances present in the culture medium or in the wine, bovine serum albumin (BSA) was added to the PCR mixture in a final concentration of 0.05 μg/μl. The reaction conditions were 95°C for 10 min and 40 cycles of 95°C for 15 s, 60°C for 1 min, and 72°C for 1.5 min.

Standard curves and efficiency. All samples were automatically processed for melting curve analysis of amplified DNA using the software Dissociation Curve, version 1.0, provided with the GeneAmp 5700 system. The T_m (melting temperature) is specific to each amplicon. Standard curves were created by plotting the C_T (cycle threshold) values of the real-time PCR performed on dilution series of DNA (ABI Prism Sequence Detection software, version 1.3) against the log input cells/ml. From the slope of the standard curve, the amplification efficiency (E) was estimated by the formula $E = 10^{-1/\text{slope}} - 1$. Standard and amplification curves were generated by using Sigma Plot, version 8.0.

Standard PCR conditions. Standard PCRs containing 1 U of Biotools DNA polymerase were performed in the same thermocycler and with the same reaction conditions described above for real-time PCR. Amplicons were analyzed by conventional DNA electrophoresis on a 1.4% agarose gel.

Real-time PCR in artificially contaminated wine samples. Dessert sweet wine (Moscatel grape variety) was purchased in a local supermarket, and red wine (50% Bobal and 50% Monastrell grape varieties) was provided by a Spanish winery. Three aliquots (in duplicate) of 8 ml of the dessert wine were artificially contaminated with 6.8×10^6, 6.8×10^3, and 6.8×10^1 CFU of *S. cerevisiae* (CECT 1485), respectively, per ml of wine. In the case of red wine, the 8-ml samples were contaminated with 5×10^4, 5×10^2, and 5×10^1 CFU per ml of wine. Each 8-ml sample was subjected to DNA extraction with PrepMan as described above and used as a template for the real-time PCR protocol developed and melting curve analysis. The experiment was repeated two times.

Statistical procedures. Confidence intervals for the study of reproducibility were calculated by Student's t test with a significance level of 5% (9).

Multifactor analysis of variance was performed using Statgraphics (version 5.1). The F test was used to evaluate the significance of the effect of contaminating microorganisms on *S. cerevisiae* cell number determination by quantitative PCR (QPCR). A P value of <0.05 was considered significant.

RESULTS

Primer design and specificity of PCR. *S. cerevisiae* is very closely related to the species *S. bayanus*, *S. pastorianus*, *S. paradoxus*, *Saccharomyces cariocanus*, *Saccharomyces mikatae*, and *Saccharomyces kudriavzevii*. These six species, together with *S. cerevisiae*, constitute the *Saccharomyces* sensu stricto complex. Among these species, only *S. cerevisiae*, *S. bayanus*, *S. pastorianus*, and *S. paradoxus* are associated with fermentation processes. However, *S. pastorianus* is only present in beer making and *S. paradoxus* has been isolated only once in wine (25). *S. cerevisiae* and *S. bayanus* are by far the most common species in wine fermentation, but in a previous work, we showed that all of the commercial *S. bayanus* strains characterized (12 strains) were in fact *S. cerevisiae* as result of incorrect classifications (12). This is why we have considered interesting design primers for the specific identification of strains belonging the species *S. cerevisiae*. The phylogenetic proximity among these four species makes their differentiation very difficult. In a previous study, we showed that these four species can be distinguished by RAPD-PCR analysis (11), and we decided to exploit this fact. In the aforementioned study, we showed that amplification with a decamer primer named OPA-07 (GAAACGGGTG) generated a 1,800-bp band specific to the species *S. cerevisiae*. This band was present in the 10 *S. cerevisiae* strains tested but not in the other three species (Fig. 1). Thus, it was cloned and subsequently sequenced using the DNA of one of these strains (CECT 1485). Using the Primer Express software and the sequence obtained, we selected two primers named SC1d (sense), which conserved the last 8 nucleotides (underlined) of OPA-07 at its 3′ end (5′-ACATATGAAGTATGTTTCTATATAACGGGTG-3′), and SC1r (antisense) located downstream within the sequenced RAPD band. These primers generated a PCR product of an appropriate size for quantitative PCR (301 bp). The specificity of both primers was checked against sequences available in current databases using the Blastn server. SC1d showed significant homology only to *S. cerevisiae*; in the case of SC1r, other significant entries were identified but never to yeast or bacteria known to have been isolated from wine.

To evaluate the effectiveness and specificity of this primer pair, conventional PCR was performed using purified DNA from different strains of *Saccharomyces* listed in Table 1. Additionally, nine strains were tested that belong to species associated with wine and soft drink spoilage (*C. rugosa*, *C. vini*, *C. zeylanoides*, *D. bruxellensis*, *I. orientalis*, *P. membranifaciens*, *S. ludwigii*, *S. pombe*, and *Z. bailii*). A DNA fragment of the expected size (301 bp) was obtained only in the case of *S. cerevisiae* strains. The final concentration of primers chosen for the experiments was 60 mM because it provided the lowest C_T and the best specificity.

Real-time PCR in pure cultures. (i) Detection. DNA (10 ng/μl) from the *S. cerevisiae* strain CECT 1485 was subjected to real-time PCR using the double-stranded DNA binding dye SYBR green. The melting curve obtained for the amplicon generated is shown in Fig. 2, with a T_m of 78.5°C. The specificity of the system was assessed using the same strain species as in conventional PCR. Again, amplification, represented by a melting curve with the T_m expected, was only detected for *S. cerevisiae* (Table 1), providing evidence of the specificity of the system developed. Nonspecific side products and primer dimmers showed lower T_ms.

(ii) Sensitivity. To determine the minimum number of cells per milliliter that the method could detect, DNA obtained from an *S. cerevisiae* culture growth with a concentration of 5.6×10^7 CFU/ml was serially diluted 10-fold. Each DNA dilution was subjected to real-time PCR to obtain amplification curves. The results were reported as threshold cycle numbers versus fluorescence intensities (Fig. 3). The amplification curves showed positive signals for concentrations in cell numbers between 5.6×10^6 CFU/ml and 5.6 CFU/ml.

Amplification efficiency. The C_T values obtained for concentrations in cell numbers ranging from 5.8×10^6 CFU/ml to 5.8 CFU/ml showed good linearity (Fig. 4) for triplicate sam-

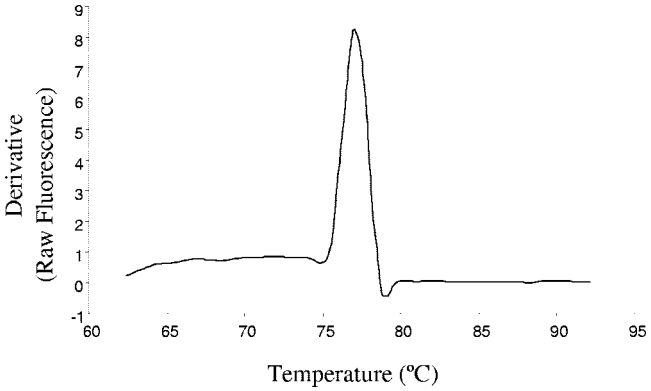

FIG. 2. Melting curve analysis of the amplified PCR product of *S. cerevisiae*.

ples. The amplification plot generated a slope of −3.53 with a correlation coefficient of 0.990878 and an efficiency of 0.92. Therefore, the approach provided data for quantification to be expressed in terms of CFU per PCR.

The assay was linear over 6 orders of magnitude. These results indicate that it is only possible to quantify samples of unknown concentration accurately within this range of concentrations.

In addition, the reproducibility of the method was tested in GPY growth medium. For this purpose, 8 standard curves were obtained from independent experiments. As a result, parallelism of the linear regression analysis was observed (data not shown) and the confidence interval values calculated by Student's *t* test for the intercept and slope of each standard curve were comparable (Table 2).

(iii) Quantification (correlation of real-time PCR and plate count technique). The quantification ability of the method was tested by correlating the cell number concentration estimated by plate count and real-time PCR. A cell suspension was serially diluted 10-fold in wine (Moscatel) and in GPY medium to create samples of known concentration. The *S. cerevisiae* population was determined in each sample by QPCR and correlated with that obtained by plating (Fig. 5). The correlation was excellent, as demonstrated by the regression analysis that produced high R^2 values: 0.983 in an assay performed in GPY medium and 0.999, 0.999, and 0.991 in three assays performed on three separate cultures in Moscatel wine.

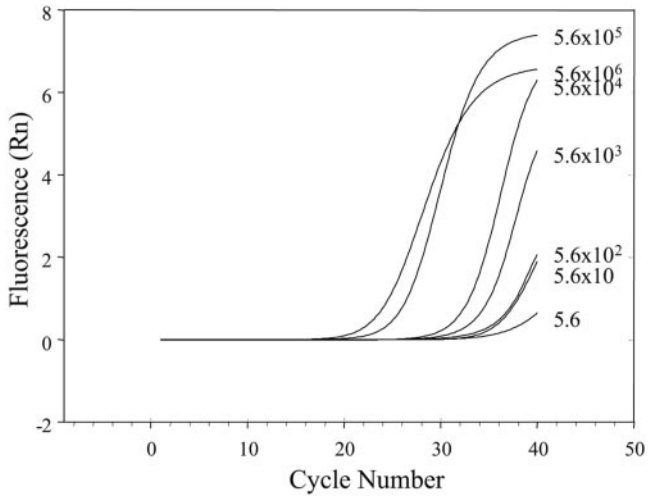

FIG. 3. Specific *S. cerevisiae* real-time SYBR green PCR (strain CECT 1485). The curve represents fluorescence changes over cycles of a 10-fold serial dilution series of cells from strain CECT 1485. Concentrations are expressed in CFU/ml.

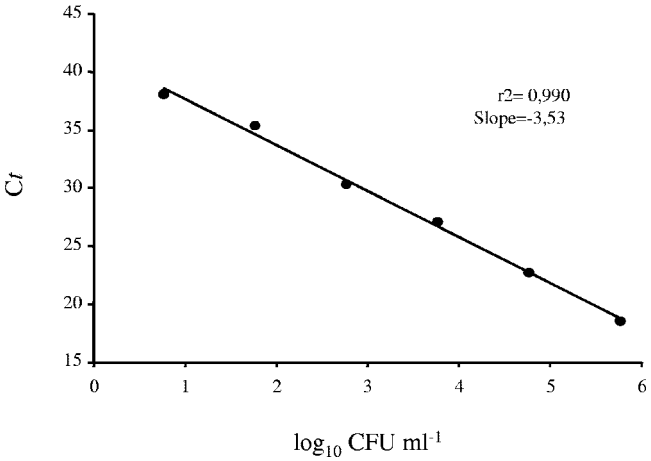

FIG. 4. Standard curve obtained from a GPY culture showing the correlation between cycle number (C_T) and initial CFU/ml ($\log_{10}$ CO). C_T values are averaegs of three replicates.

Real-time PCR with wine. (i) Artificially contaminated wine samples. The ability of real-time PCR to detect *S. cerevisiae* cells in wine was evaluated. Two different commercial wines were tested, sweet (Moscatel) and red (50% Bobal, 50% Monastrell). For the analyses, sweet wine was artificially contaminated at 6.8×10^6, 6.8×10^3, and 6.8×10 CFU/ml and the red wine was artificially contaminated at 5×10^4, 5×10^2, and

TABLE 2. Confidence intervals for intercept and slope of several standard curves obtained from GPY cultures, sweet wine, and red wine[a]

Matrix	Standard curve no.	Confidence interval for:	
		Intercept	Slope
GPY medium	1	41.64 ± 1.30	−3.96 ± 0.35
	2	40.20 ± 2.25	−3.37 ± 0.61
	3	43.13 ± 2.01	−3.69 ± 0.54
	4	40.18 ± 3.23	−3.61 ± 0.88
	5	42.67 ± 2.92	−3.99 ± 0.79
	6	43.13 ± 3.26	−3.64 ± 0.88
	7	42.60 ± 2.63	−3.82 ± 0.71
	8	42.11 ± 2.02	−2.77 ± 0.55
Sweet wine (Moscatel)	1	44.33 ± 2.19	−3.95 ± 0.50
	2	41.30 ± 2.42	−3.56 ± 0.50
	3	40.16 ± 2.54	−3.49 ± 0.53
	4	42.09 ± 2.13	−3.89 ± 0.45
Red wine (Bobal/Monastrell)	1	46.06 ± 2.47	−3.83 ± 0.54
	2	45.01 ± 1.17	−4.47 ± 0.29
	3	44.06 ± 1.71	−4.46 ± 0.41
	4	41.86 ± 3.10	−3.17 ± 0.68

[a] A *P* value of <0.05 was considered significant.

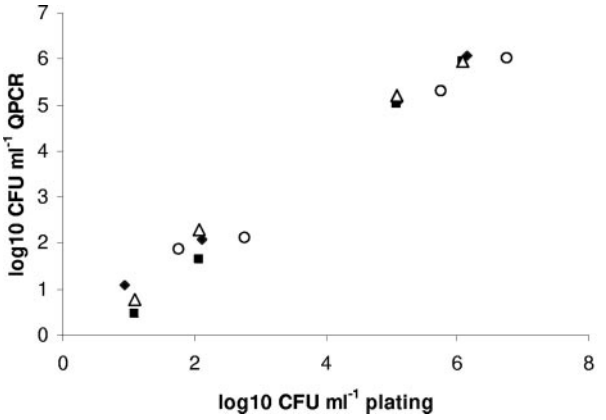

FIG. 5. Linear relationship between results of numbers of cells of *S. cerevisiae* obtained by QPCR and plating. The correlation was studied with GPY medium (assay 1, ○) and with three separate cultures of Moscatel wine (assay 2, ◆; assay 3, ■; assay 4, △). R^2 values are as follows: assay 1, 0.983; assay 2, 0.999; assay 3, 0.999; assay 4, 0.991.

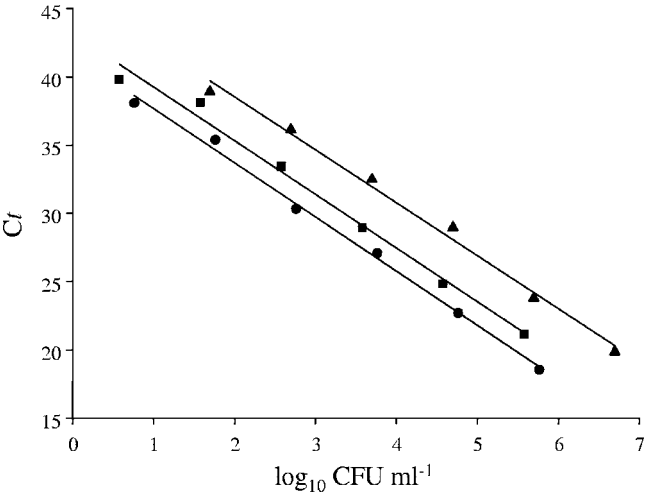

FIG. 6. Standard curves for *S. cerevisiae* obtained from a GPY culture (●), sweet wine (■), and red wine (▲).

5×10 CFU/ml. In both cases, real-time PCR showed successful amplification in all of the contaminated samples.

The detection limits were assessed. For this purpose, 5-ml volumes of sweet and red wine were inoculated to final concentrations of 3.8×10^6 and 5×10^6 CFU/ml, respectively, and used for DNA extraction. Tenfold serial dilutions of the DNA were performed in both cases, and each dilution was used to generate standard curves ranging from 3.8×10^6 to 3.8 CFU/ml in sweet wine and from 5×10^6 to 5 CFU/ml in red wine. Positive signals were obtained down to 3.8 CFU/ml in sweet wine and 5 CFU/ml in red wine. These values were compared with those obtained when starting from GPY cultures (5.6 CFU/ml), and we observed that samples obtained from wine do not influence the sensitivity of the assay.

The impact of contaminating DNA on *S. cerevisiae* cell number determination in wine by QPCR was also studied. This DNA could correspond to other yeasts and bacteria (lactic and acetic) that can be found at high levels in wines at later stages. The study was performed using *D. bruxellensis* (strain CECT 1451^T) as a representative yeast and *O. oeni* (strain CECT 217^T) and *A. aceti* (strain CECT 298^T) as representative lactic and acetic bacteria, respectively. Two concentrations of *S. cerevisiae* (10^2 and 10^6 CFU/ml) were combined in a wine (Moscatel) matrix with two concentrations (10^2 and 10^6 CFU/ml) of the contaminating microorganism, generating four samples in each case. The DNAs obtained were subjected to QPCR, the results were evaluated in terms of C_T, and a multifactor variance analysis (analysis of variance) was performed. No significant effects of the presence of contaminating yeasts or bacteria were observed on the number of *S. cerevisiae* cells, as indicated by the *P* values obtained (0.4565, 0.8294, and 0.0688). Moreover, we obtained the *P* values by analyzing the interaction between the numbers of *S. cerevisiae* cells and contaminating microorganism cells. These *P* values were higher than 0.3 in all three cases, indicating that the presence of other microorganisms does not have significant effects on quantification independent of *S. cerevisiae* concentration.

Another aspect studied was the influence of wine on the efficiency of the real-time PCR system, which was analyzed by comparing standard curves obtained from wine with that from the GPY cultures (Fig. 6). The curve obtained for sweet wine showed a good linearity response ($R^2 = 0.990$) for concentrations ranging from 3.8×10^5 CFU/ml and 3.8 CFU/ml, with an efficiency of 0.882. In the case of red wine, the curve obtained showed a good linearity response ($R^2 = 0.996$) for concentrations ranging from 5×10^6 CFU/ml and 50 CFU/ml, with an efficiency of 0.7939. When we compared both standard curves with one obtained for DNA from GPY cultures, we observed parallelism (Fig. 6). However, we also observed that, in the case of sweet and red wine, the C_T value at each dilution was higher than that of the equivalent sample obtained from GPY. These results suggest that wine contains substances that inhibit PCR, leading to an underestimation of cell number. This effect was stronger in the case of red wine.

Finally, the reproducibility of the method was tested in both sweet and red wines. For this purpose, four standard curves were obtained from independent experiments for each matrix. As a result, parallelism of the linear regression analysis was observed for each wine (data not shown) and the confidence interval values calculated by Student's *t* test for the intercept and slope of each standard curve were comparable in all but two cases (Table 2).

(ii) Authentic spoiled wine samples. The real-time PCR system was applied to 8 red wine samples (50% Bobal, 50% Monastrell) provided by a Spanish winery, 7 bottles and 1 bag in a box of red wine, suspected of being spoiled by yeast because they showed signs of gas production. DNA was extracted from 10-ml aliquots and then subjected to amplification by real-time PCR. In all cases, we obtained a melting curve of 78.5°C, corresponding to the species *S. cerevisiae*, and nonspecific amplifications were absent. This result was confirmed by analyzing 5 colonies of isolates from one of the bottles by restriction fragment length polymorphism of the ribosomal 5.8S-ITS region. As a result, the 5 isolates showed the typical 850-bp 5.8S-ITS amplification product previously described for the *S. cerevisiae* species (10). Moreover, the restriction patterns obtained with three endonucleases (CfoI, 375 + 325 + 150;

TABLE 3. Quantification of *S. cerevisiae* in true spoiled wine samples by plating and real-time PCR

Wine sample designation[a]	Enumeration method[b]	
	Plating	QPCR
B1	1.88×10^5	1.31×10^5
B2	ND[c]	7.80×10^3
B3	ND	6.80×10^2
B4	ND	1.32×10^3
B5	ND	5.19×10^2
B6	ND	2.50×10^2
P1	ND	4.80×10^2

[a] Wine samples (Bobal and Monastrell) were taken from bottles (B) or a bag in a box (P).
[b] Values are in CFU/ml.
[c] ND, growth not detected.

HaeIII, 325 + 230 + 170 + 125; HinfI, 375 + 365 + 110) revealed the identity of these strains to be *S. cerevisiae.*

In a different experiment, the DNA of each sample was used for quantification in real-time PCR. The C_T values determined were extrapolated to the corresponding standard curve of DNA from artificially inoculated wine samples, ranging from 5×10^6 CFU/ml to 50 CFU/ml. The concentrations obtained are summarized in Table 3.

Simultaneously, 10 ml of each contaminated sample was centrifuged and pellets were plated for subsequent standard counts. Surprisingly, cell counts revealed the presence of yeast contamination in only one of the samples analyzed (B1). In this case, the correlation between the number of CFU determined by plating (1.8×10^5 CFU/ml) and by PCR (1.31×10^5 CFU/ml) was excellent. The noncorrelation between cell count and real-time PCR for the rest of the samples would indicate the presence of nonculturable or dead *S. cerevisiae* cells.

DISCUSSION

Until now, real-time PCR has been applied to detect quickly and sensitively, and also often to quantify, the great many bacteria associated with foods. In contrast, there are few works concerning such studies with yeasts, probably because for decades yeasts have been considered innocuous to human health. This is the case with *S. cerevisiae*, the yeast species most commonly used in the food industry to produce foods and beverages. For this reason, the PCR systems developed for *S. cerevisiae* detection focus on monitoring inoculated and natural fermentations at the strain level (6, 15, 20). But *S. cerevisiae* is also able to cause spoilage in foods and drinks, especially in alcoholic beverages and soft drinks (5), and can even cause infections in human (19). The interest in detecting spoilage yeasts is increasing because alteration in food and drink causes important economic losses (for a review, see reference 17). In these cases, methods for identification at species level are necessary. Systems based on the PCR technique have been reported as suitable for detecting this yeast species. Examples of this are restriction fragment length polymorphism of the ribosomal 5.8S-ITS region (10, 13) and the gene MET2 (18) and amplification of intron splice sites (7). However, they do not tackle yeast quantification through real-time PCR, and this is the main reason why we thought the development of such a system would be of special interest. In addition, real-time PCR has the advantage over these techniques in that it avoids the digestion and electrophoresis steps, which are time-consuming, thus offering a faster system. Another novelty of the real-time PCR technique, compared to the aforementioned techniques, is that DNA extraction can be performed directly from wine, without prior culture isolation, contributing to reduced detection time. This is possible because real-time PCR is more sensitive and smaller amounts of initial DNA can be detected than with traditional PCR techniques. As a result, only a short time is needed to obtain the final result in real-time PCR, no more than 5 h (between 1 and 2 h for DNA extraction and precipitation depending on the number of samples and 3 h for the real-time PCR). Therefore, this would allow effective interventions to be carried out when necessary in the food industry.

The detection limit of a real-time PCR system can change depending on the specificity of the primers designed. We used a fragment of an RAPD product as a target for the detection and quantification of *S. cerevisiae* because this PCR product clearly differentiated this species from the other species associated with fermented foods in the *Saccharomyces* sensu stricto complex. The designed primers, SC1d and SC1r, showed a high specificity for *S. cerevisiae*. The detection limit was studied; concentrations down to 5.6, 3.8, and 5 CFU/ml could be detected in GPY medium, sweet wine, and red wine, respectively. This detection limit is very good, and it is of the same order as that obtained for other microorganisms in similar assays. This is the case for a real-time PCR assay for the detection and enumeration of *D. bruxellensis* in wine (21). These authors report a detection limit of 1 CFU/ml, and they did not observe differences when DNA was extracted from a rich medium, wine, or wine supplemented with *S. cerevisiae*. There are no legal limits for yeast content in wine; there are only recommendations by the OIV (The Office International de la Vigne et du Vin), as Loureiro and Malfeito-Ferreira (16) describe. The OIV recommends that the microbial load should be less than 10^4 to 10^5 CFU/ml for microorganisms producing powdery sediments or less than 10^2 to 10^3 CFU/ml for microorganisms producing flocculent sediments. Below these levels, bottled wine is clear and thus acceptable. Taking these observations into account, the method developed is useful given that the minimum detectable cell number falls below that recommended by the OIV.

The possibility of detecting a low number of cells and thus confirming that *S. cerevisiae* is not present is particularly important in wine to avoid spoilage during storage before commercialization. Initial contamination with a small number of cells could be a potential cause of spoilage. Another aspect regarding food spoilage prevention programs is the potential of quantitative detection to estimate the risk of *S. cerevisiae* contamination in wine samples. No quantitative PCR studies have, until now, been performed for *S. cerevisiae*. The goal of this study was to optimize a molecular technique that would allow quantification. The regression coefficients obtained after the linear regression indicated a good correlation between the amount of template (log input cells) and the amount of product (represented by the C_Ts) in standard curves performed with DNA from GPY medium and wine. As a result, quantification was accurate for concentration values between 5.8×10^6 and 5.8 CFU/ml for GPY medium and from 3.8×10^5 to

3.8 CFU/ml and 5×10^6 to 50 CFU/ml in sweet and red wines, respectively. Nevertheless, the number of cells was underestimated when quantification was assessed in wine samples. This effect was stronger in red wine than in sweet wine, indicating that the amount of PCR inhibitors differs depending on the grape variety. The addition of BSA to the PCR mixture as an amplification facilitator did not eliminate the problem, despite the known ability of BSA to relieve inhibition by binding inhibitors (24). Some authors have reported differences when PCR was performed with DNA extracted from pure cultures or directly from food. This is the case with Delaherche et al. (8), who suggested that the contamination level of *D. bruxellensis* that they detected by real-time PCR in wine (10^4 CFU/ml) was due to the presence of polyphenols or tannins. Phister and Mills (21) have shown that dilution of DNA 1/10 in water prior to QPCR reduced variability in red wines, and the resulting QPCR analysis showed a good correlation with plating results. Although this recommendation may help to lower the wine curve to match the rich media curve, this practice raises the lower limit of detection (21). This is why we preferred to construct the standard curves for detection and quantification in authentic wine samples by diluting DNA from *S. cerevisiae* in the same matrix as the samples (i.e., wine). In doing so, we observed that PCR detected the presence of *S. cerevisiae* DNA in all of the spoiled red wine samples provided by a Spanish winery. However, we observed that in 7 of the 8 samples analyzed, growth was not detected through plate count. This result indicated a problem in differentiation between dead and live cells by PCR-based systems, as has been described previously by many authors. Since mRNA detection is considered a better indicator of cell viability, real-time PCR systems based on reverse transcription have been proposed to avoid the problem of false positives as a result of DNA PCR amplification of dead cells (28). Comparison with sequences available in the EMBL nucleotide sequence database showed that the cloned RAPD product corresponds to a coding region, the NMD3 gene that encodes an essential cytoplasmic protein required for stable 60S ribosomal subunits in *S. cerevisiae* (14). Unfortunately, one of the primers selected, SC1d, was homologous to an intergenic region, and thus, it was not useful for reverse transcription. Since SC1d contains part of the 3′ end of the primer used for RAPD amplification (OPA-7), it must be used in the PCR to maintain specificity. Despite this drawback, there is an advantage to real-time PCR versus cultivable methods because detection of the yeast, once dead, is still interesting for estimating the risk of organoleptic alterations, caused by cell activity before their death. In this respect, it could be interesting to establish the minimum concentration of *S. cerevisiae* that causes unpleasant effects in wine, as has been done with other yeast species such as *Brettanomyces*. The typical unpleasant odor appears when the *Brettanomyces* concentration reaches 10^5 CFU/ml or higher (4).

Direct detection of *S. cerevisiae* in wine by real-time PCR using the primer set designed in this study was very sensitive and reproducible and, perhaps more importantly, allows winemakers to enumerate *S. cerevisiae* in a short period of time (4 to 5 h). National or international regulations do not exist, but the detection limit is sensitive enough to detect the levels set by the recommendations of the OIV and the technique meets the needs of the wine industry. Although the method outlined in this paper has been performed on wine samples, it could be adapted to other kinds of food where *S. cerevisiae* could cause spoilage.

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REFERENCES

1. **Bialek, R., M. Weiss, K. Bekure-Nemariam, L. K. Najvar, M. B. Alberdi, J. R. Graybill, and U. Reischl.** 2002. Detection of *Cryptococcus neoformans* DNA in tissue samples by nested and real-time PCR assays. Clin. Diagn. Lab. Immunol. **9:**461–469.
2. **Bleve, G., L. Rizzotti, F. Dellaglio, and S. Torriani.** 2003. Development of reverse transcription (RT)-PCR and real-time RT-PCR assays for rapid detection and quantification of viable yeasts and molds contaminating yoghurts and pasteurized food products. Appl. Environ. Microbiol. **69:** 4116–4122.
3. **Casey, G. D., and A. D. Dobson.** 2004. Potential of using real-time PCR-based detection of spoilage yeast in fruit juice—a preliminary study. Int. J. Food Microbiol. **91:**327–335.
4. **Chatonnet, P., D. Dubourdieu, J. N. Boidron, and M. Pons.** 1992. The origin of ethylphenols in wines. J. Sci. Food Agric. **60:**165–178.
5. **Deak, T., and L. R. Beuchat.** 1996. Yeasts in specific types of foods, p. 61–95. *In* T. Deak and L. R. Beuchat (ed.), Handbook of food spoilage yeast. CRC, Boca Raton, Fla.
6. **de Barros Lopes, M., A. Soden, P. A. Henschke, P. Langridge.** 1996. PCR differentiation of commercial yeast strains using intron splice site primers. Appl. Environ. Microbiol. **62:**4514–4520.
7. **de Barros Lopes, M., A. Soden, A. L. Martens, P. A. Henschke, and P. Langridge.** 1998. Differentiation and species identification of yeasts using PCR. Int. J. Syst. Bacteriol. **48:**279–286.
8. **Delaherche, A., O. Claisse, and A. Lonvaud-Funel.** 2004. Detection and quantification of *Brettanomyces bruxellensis* and "*ropy*" *Pediococcus damnosus* strains in wine by real-time polymerase chain reaction. J. Appl. Microbiol. **97:**910–915.
9. **Draper, N. R., and H. Smith.** 1981. Applied regression analysis, 2nd ed. John Wiley & Sons, Inc., New York, N.Y.
10. **Esteve-Zarzoso, B., C. Belloch, F. Uruburu, and A. Querol.** 1999. Identification of yeasts by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers. Int. J. Syst. Bacteriol. **49:**329–337.
11. **Fernández-Espinar, M. T., E. Barrio, and A. Querol.** 2003. Genetic variability among species of the *Saccharomyces sensu stricto*. Yeast **20:**1213–1226.
12. **Fernández-Espinar, M. T., V. López, D. Ramón, E. Bartra, and A. Querol.** 2001. Study of the authenticity of commercial wine yeast strains by molecular techniques. Int. J. Food Microbiol. **70:**1–10.
13. **Fernández-Espinar, M. T., B. Esteve-Zarzoso, A. Querol, and E. Barrio.** 2000. RFLP analysis of the ribosomal internal transcribed spacer and the 5.8S rRNA gene region of the genus *Saccharomyces*: a fast method for species identification and the differentiation of "flor" yeasts. Antonie Leeuwenhoek **78:**87–97.
14. **Ho, J. H., and A. W. Johnson.** 1999. *NMD3* encodes an essential cytoplasmic protein required for stable 60S ribosomal subunits in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **19:**2389–2399.
15. **López, V., M. T. Fernández-Espinar, E. Barrio, D. Ramón, and A. Querol.** 2002. A new PCR-based method for monitoring inoculated wine fermentations. Int. J. Food Microbiol. **81:**63–71.
16. **Loureiro, V., and M. Malfeito-Ferreira.** 2003. Spoilage yeasts in the wine industry. Int. J. Food Microbiol. Rev. **86:**23–50.
17. **Loureiro, V., and A. Querol.** 1999. The prevalence and control of spoilage yeasts in foods and beverages. Trends Food Sci. Technol. **10:**356–365.
18. **Masneuf, I., M. Aigle, and D. Dubordieu.** 1996. Development of a polymerase chain reaction/restriction fragment length polymorphism method for *Saccharomyces cerevisiae* and *Saccharomyces bayanus* identification in enology. FEMS Microbiol. Lett. **138:**239–244.
19. **Murphy, A., and K. Kavanagh.** 1999. Emergence of *Saccharomyces cerevisiae* as a human pathogen implications for biotechnology. Enzyme Microb. Technol. **25:**551–557.
20. **Ness, F., F. Lavallée, D. Dubordieu, M. Aigle, and L. Dulau.** 1993. Identification of yeast strains using the polymerase chain reaction. J. Sci. Food Agric. **62:**89–94.
21. **Phister, T. G., and D. A. Mills.** 2003. Real-time PCR assay for detection and enumeration of *Dekkera bruxellensis* in wine. Appl. Environ. Microbiol. **69:** 7430–7434.

22. **Pryce, T. M., I. D. Kay, S. Palladino, and C. H. Heath.** 2003. Real-time automated polymerase chain reaction (PCR) to detect *Candida albicans* and *Aspergillus fumigatus* DNA in whole blood from high-risk patients. Diagn. Microbiol. Infect. Dis. **47:**487–496.
23. **Querol, A., E. Barrio, T. Huerta, and D. Ramón.** 1992. Molecular monitoring of wine fermentations conducted by active dry yeast strains. Appl. Environ. Microbiol. **58:**2948–2953.
24. **Rådström, P., C. Löfström, M. Lövenklev, R. Knutsson, and P. Wolfss.** 2003. Strategies for overcoming PCR inhibition, p. 149–161. *In* C. W. Dieffenbach and G. S. Dveksler (ed.), PCR primer. A laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
25. **Redzepovic, S., S. Orlic, S. Sikora, A. Majdak, I. S. Pretorius.** 2002. Identification and characterization of *Saccharomyces cerevisiae* and *Saccharomyces paradoxus* strains isolated from Croatian vineyards. Lett. Appl. Microbiol. **35:**305–310.
26. **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.
27. **Selvarangan, R., U. Bui, A. P. Limaye, and B. T. Cookson.** 2003. Rapid identification of commonly encountered *Candida* species directly from blood culture bottles. J. Clin. Microbiol. **41:**5660–5664.
28. **Sheridan, G. E. C., C. I. Masters, J. A. Shallcross, and B. M. Mackey.** 1998. Detection of mRNA by reverse transcription-PCR as an indicator of viability in *Escherichia coli* cells. Appl. Environ. Microbiol. **64:**1313–1318.
29. **White, P. L., A. Shetty, and R. A. Barnes.** 2003. Detection of seven *Candida* species using the Light-Cycler system. J. Med. Microbiol. **52:**229–238.