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Rapid, Specific, and Sensitive Detection of Spoilage Molds in Orange Juice Using a Real-Time Taqman PCR Assay

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ABSTRACT

The outgrowth of spoilage organisms, including molds and yeasts, results in significant financial loss to the food industry and wastes natural resources. The objective of this study was to develop a rapid, specific, and sensitive real-time PCR method for detecting spoilage molds during screening of raw materials and final product quality control analysis. The 18S rRNA gene was used to develop PCR primers and probe. With this set of primers and probe, less than 1,000 mold cells per milliliter of orange juice (10 cells per reaction) were detected with the real-time PCR system within 6 to 7 h. No cross-reactivity was found with other common foodborne bacteria, yeasts, or food ingredients. This technique is significantly faster than current detection and identification procedures, which take from days to weeks.

Microbial spoilage is a major challenge to the food industry. Up to 25% of the total food supplies are lost due to spoilage *(12).* Molds are particularly problematic in products with low pH and low water activity, conditions under which most other microorganisms cannot grow. The conventional plate counting method for molds often takes days to weeks to obtain results, which is not feasible for products with limited shelf life. Timely detection of these organisms with high specificity and sensitivity is important for both quality assessment of raw materials and quality assurance of the final product.

Immunological and DNA-based approaches such as enzyme-linked immunosorbent assays and various DNA hybridization and nonhybridization methods have been used for rapid detection of molds *(5, 8, 15, 16).* Of these methods, PCR detection is rapid and sensitive *(10).* However, certain shortcomings, particularly the high rate of false-positive results and the need for postamplification analysis, have limited the application of this technique in industrial settings. Recent advancements in PCR technology such as development of real-time PCR devices and the incorporation of various new detection chemicals have overcome some of these problems. Consequently, real-time PCR is emerging as a potential alternative to the standard plate counting method in both clinical and industrial settings. The simplest type of real-time PCR assay utilizes a fluorescent dye that binds to nucleic acids and returns a signal to the optical module on the thermal cycler. A commonly used fluorescent dye is SYBR Green I, which has been used in assays designed to detect many different targets *(6).* Although SYBR Green I assays can provide useful quantitative information from the mixture of dye, a pair of PCR primers, DNA template, and the PCR cocktail, the dye can bind to any double-stranded DNA molecules in the reaction, including nonspecific PCR products or primer dimers. Therefore, a high false-positive rate is still the main drawback of this approach, although melting curve analysis can help differentiate various products after amplification. Additional detection chemicals are needed in real-time PCR assays to achieve specific detection. For instance, the specificity of real-time PCR detection can be improved considerably by use of a fluorescent-labeled oligonucleotide probe, such as those used in molecular beacon or the Taqman real-time PCR, with sequence complementary to the target gene fragment to detect the presence of the PCR amplicons *(6).* In such systems, the false-positive rate for detection is significantly reduced because signal detection depends on both the annealing of primers to their complementing sequences for PCR amplicon synthesis and the annealing of the oligonucleotide probe to the amplification products. The closed-tube format eliminates the need for postamplification analysis and the associated contamination problems, and the robust operation makes the application of PCR on a large number of samples possible.

Sequences of the 18S rRNA gene have been used extensively to analyze the taxonomic schemes of eukaryotes *(1, 13).* The 18S rRNA genes of molds are sufficiently conserved across taxa that they can be used to design primers and probes that are specific for this group of organisms. Because there are multiple copies of the 18S rRNA gene in the genome, real-time PCR assay targeting this gene also should have improved sensitivity.

The objective of this study was to develop the methodology for rapid detection of spoilage molds in orange juice using a Taqman-based real-time PCR. This technique involves identifying the 18S rRNA gene in *Byssochlamys fulva* and *Penicillium digitatum,* which are common spoilage fungi in food-processing environments, and developing a primer-probe set suitable for the above real-time PCR system. The specificity of the primer-probe set to molds,

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yeasts, and bacteria commonly associated with foods and to selected fruits (eukaryotic raw food materials) was examined. Because foods often contain ingredients and additives that could interfere with the PCR, the applicability of each real-time PCR system must be verified and optimized for individual foods. The applicability and sensitivity of this mold detection assay were assessed in orange juice.

MATERIALS AND METHODS

Microbial strains and growth conditions. *B. fulva* ATCC 24474 and *P. digitatum* ATCC 10030 (American Type Culture Collection, Manassas, Va.) and an environmental mold isolate KW01 were cultured on acidified potato dextrose agar (PDA; Becton Dickinson, Sparks, Md.) and incubated at 25°C for 2 to 5 days. The yeast *Zygosaccharomyces bailii* ATCC 36947 was cultured on a yeast-mold agar plate containing 3 g/liter yeast extraction (Fisher Biotech, Fair Lawn, N.J.), 3 g/liter malt extract (Biochemika, Fluka Chemie, Buchs, Switzerland), 5 g/liter peptone (Fisher), and 10 g/liter glucose (Becton Dickinson) at 25°C for 2 days. Bacterial strains used in this study were cultured on agar plates for 24 h under the conditions indicated: *Alicyclobacillus acidocaldarius* ATCC 43030 on ATCC 573 (http://www.atcc.org/mediapdfs/573.pdf) at 48°C, *Escherichia coli* DH-5α (Invitrogen, Carlsbad, Calif.) on Miller Luria-Bertani agar (Fisher) at 37°C, *Lactococcus lactis* subsp. *lactis* 2301 *(17)* on M17-L agar (Becton Dickinson) at 30°C, *Listeria monocytogenes* V7 *(19)* on tryptic soy agar (TSA; Becton Dickinson) at 37°C, and *Pseudomonas putida* ATCC 49451 on TSA at 30°C. Stocks of all strains were stored in the respective liquid media supplemented with 200 g/liter glycerol and kept at −80°C. Cultures were kept at 4°C and maintained by biweekly transfers in the respective media. Apples, oranges, and grapes were purchased from a local grocery store.

DNA extraction. For DNA extraction, microbial cells from colonies (*Z. bailii* ATCC 36947, 48-h colonies of mold KW01, *B. fulva* ATCC 24474, 5-day colonies of *P. digitatum* ATCC 10030, and 18-h colonies of bacterial strains) were picked using an inoculation loop (10^4 to 10^5 CFU) and resuspended in 1 ml of saline or orange juice. The cells were then recovered from saline or orange juice by centrifugation at 5,400 × *g* for 10 min. Apple, orange, and grape samples were peeled, and the fruit tissues were collected aseptically with sterile disposable knives. Cell pellets or tissues were suspended in 300 μl DNA extraction buffer (10 g/liter cetyltrimethyl ammonium bromide, 1.4 M NaCl, 100 mM Tris, and 20 mM EDTA) and transferred to a 2.0-ml conical screw-cap tube (USA Scientific, Ocala, Fla.) containing 300 μl of glass beads (0.5-μm diameter for mold and yeast cells, 1.0-μm diameter for apple, orange, and grape cells, and 0.1-μm diameter for bacterial cells; Biospec Products, Inc., Bartlesville, Okla.). The sample mixtures were frozen in liquid nitrogen for 2 min and then thawed at 95°C for 3 min, and the freeze-thaw process was repeated once. The samples were then homogenized in a Mini-Bead-Beater-8 (Biospec) for 3 min at maximum speed. Genomic DNA was further purified with a commercial isolation kit (DNeasy Tissue Kit, Qiagen, Valencia, Calif.) and eluted with 100 μl of elution buffer following the manufacturer's instructions.

DNA sequence analysis. A commercial program (MegAlign, DNASTAR, Inc., Madison, Wis.) was used for DNA sequence alignment. Comparison of primers and probes prepared in this study with sequences from the GenBank database was conducted with the BLAST search tool (http://www.ncbi.nlm.nih.gov/BLAST).

Gene cloning, DNA sequencing, and strain identification. To determine the target gene sequences of *B. fulva* ATCC 24474 and *P. digitatum* ATCC 10030, a set of universal primers targeting the mold 18S rRNA gene was developed following an established approach *(18).* The 18S rRNA gene sequences from several eukaryotes were aligned: *Aspergillus candidus* (GenBank accession no. AB008396), *Eurotium amstelodami* (AB002076), *Emericella nidulans* (AB008403), *Penicillium chrysogenum* (AF548087), *Candida colliculosa* (X98119), *Gallus gallus* (AF173612), *Triticum aestivum* (TAE272181), and *Vitis* sp. Soltis and Soltis 2519 (AF207053). The conserved DNA sequences were identified and used to design the universal primers 18SrRNAup (5′-TGCATGGCCGTTCTTAGTTGG-3′) and 18SrRNAlow (5′-GTGTGTACAAAGGGCAGGG-3′). The 18S rRNA gene fragments were amplified by conventional PCR with this set of primers and the genomic DNA of *B. fulva* ATCC 24474 and *P. digitatum* ATCC 10030 as templates. PCR conditions were one cycle at 95°C for 3 min, 30 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s, and a final extension at 72°C for 7 min in a thermal cycler (iCycler, Bio-Rad, Hercules, Calif.). PCR products were purified with a commercial purification kit (QIAquick, Qiagen) following the manufacturer's instructions. Purified PCR products were cloned into pCR 2.1 vectors and transformed into *E. coli* INVαF′-competent cells with a cloning kit (TA Cloning, Invitrogen). Recombinant plasmids were recovered with a miniprep kit (QIAprep, Qiagen). DNA sequences were determined with a DNA analyzer (ABI PRISM 3700, Applied Biosystems, Foster City, Calif.) at the Plant Genome Sequence Facility (Ohio State University).

To determine the phylogenetic identity of the mold KW01, a second set of universal primers, 18SrRNAtotal5 (5′-GAAACT GCGAATGGYTC-3′) (Y = C+T) and 18SrRNAtotal3 (5′-GTACAAAGGGCAGGGACG-3′), were designed based on the DNA sequence alignment from the same eukaryotes. A longer 18S rRNA gene fragment was amplified by conventional PCR with this set of primers and the genomic DNA from KW01 as template. PCR conditions were one cycle at 95°C for 3 min, 30 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 2 min, and a final extension at 72°C for 7 min in the iCycler. PCR product purification, cloning, and sequencing were carried out following the procedures used with the other primers. The phylogenetic identity of KW01 was determined by comparing homologous sequences in the National Center for Biotechnology Information (NCBI; Bethesda, Md.) using the BLAST search tool and by evaluating morphological features of the colonies.

Development of mold-specific primer-probe set. The strategies and criteria described previously *(3, 9)* for primer-probe development suitable for the Taqman real-time PCR system were followed. The primers were synthesized by Sigma-Genosys (The Woodlands, Tex.). The probe was labeled at the 5′ end with the reporter dye FAM (6-carboxyfluorescein) and at the 3′ end with the quencher dye Black Hole Quencher (BHQ) dye I and was synthesized by Biosearch Technologies (Novato, Calif.).

Real-time PCR conditions. For the real-time PCR assay, the reaction was conducted in thin-wall microcentrifuge tubes (Bio-Rad). The reaction mixture contained 25 μl of the premixed commercial PCR reagent iQ Supermix (100 mM KCl, 40 mM Tris-HCl pH 8.4, 1.6 mM dNTPs, 50 units/ml iTaq DNA polymerase, 6 mM $MgCl_2$, and stabilizers; Bio-Rad), 1 μl of 10 μM primers, 1 μl of 10 μM probe, 1 μl of genomic DNA extract, and double-distilled water for a final volume of 50 μl. The PCR was performed for one cycle at 95°C for 3 min followed by 40 cycles of

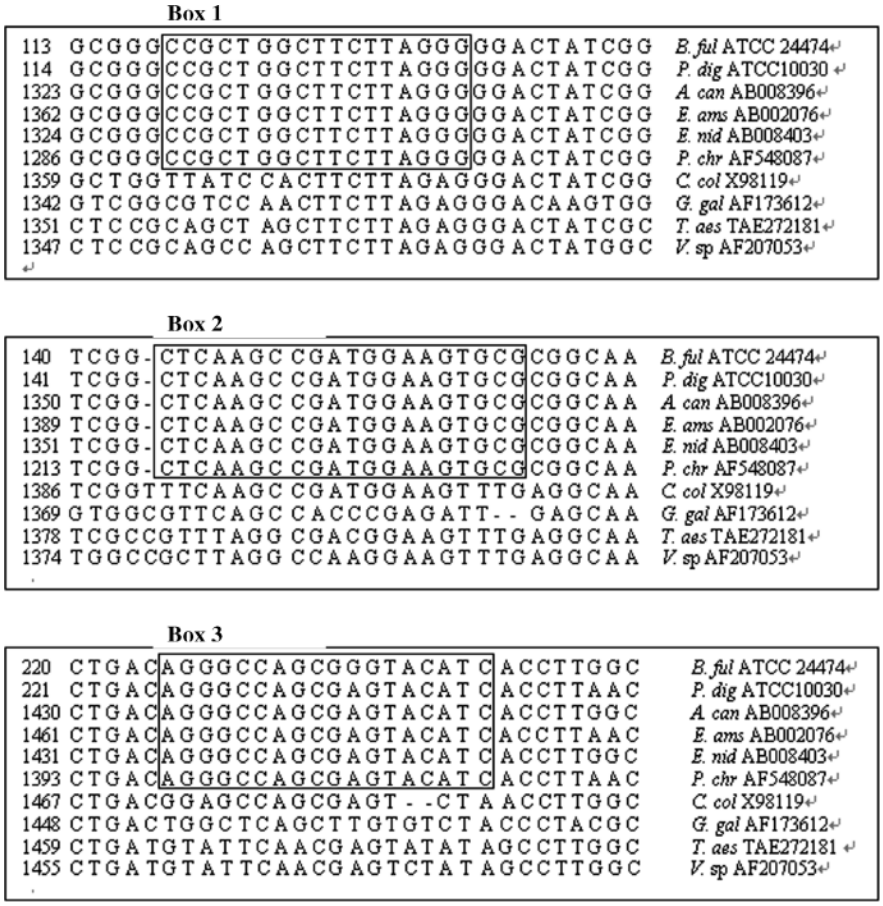

FIGURE 1. *Alignment of 18S rRNA gene fragments from* Byssochlamys fulva *(*B. ful*) ATCC 24474*, Penicillium digitatum *(*P. dig*) ATCC 10030*, Aspergillus candidus *(*A. can*) AB008396*, Eurotium amstelodami *(*E. ams*) AB002076*, Emericella nidulans *(*E. nid*) AB008403*, Candida colliculosa *(*C. col*) X98119*, Gallus gallus *(*G. gal*) AF173612*, Triticum aestivum *(*T. aes*) TAE 272181, and* Vitis *sp. (*V. *sp.) Soltis and Soltis 2519 AF207053. Boxed nucleotide sequences were used to design PCR primers and probe. Box 1 refers to the primer MoldFP; box 2 refers to the probe MoldTP; and box 3 refers to the complement reverse fragment of primer MoldRP.*

95°C for 30 s and 55°C for 45 s with the real-time PCR system (iCycler).

Specificity analysis. Specificity of the detection system was tested with the spoilage molds *B. fulva* ATCC 24474 and *P. digitatum* ATCC 10030. The common bacteria *A. acidocaldarius* ATCC 43030, *E. coli* DH-5α, *L. lactis lactis* 2301, *L. monocytogenes* V7, and *P. putida* 49451 and fruit tissue samples were tested for cross-reactivity. Genomic DNA of the organisms was extracted following the same protocols. A 1-μl aliquot of the 100-μl DNA elute was used as a template, and the real-time PCR amplification was carried out under the previously described conditions.

Sensitivity analysis. Sensitivity of the real-time PCR assay was tested with the mold KW01, which was isolated from a spoiled soy product. Cells collected from acidified PDA plates were suspended in 1 ml of 8.5 g/liter saline solution or shelf-stable reconstituted orange juice (MinuteMaid, Coca-Cola Inc., Atlanta, Ga.). These original cell suspensions were further serially diluted in saline or orange juice, respectively. Cells from 1 ml of each dilution were collected by centrifugation, and the DNA of collected cells was extracted following the previously described procedures. A 1-μl aliquot of the eluted DNA was used as a template, and real-time PCR amplification was carried out as previously described. To compare the real-time PCR output with results of conventional plate counting approach, 100 μl of the 10^{-1} to 10^{-3} dilution of cell suspension was plated on acidified PDA plates. Plates were incubated at 25°C for 48 h, and then colonies were counted.

RESULTS

Primer-probe set. With the universal primer set 18SrRNAup and 18SrRNAlow and the genomic DNA from *B. fulva* ATCC 24474 and *P. digitatum* ATCC 10030, two 18S rRNA gene fragments (400 and 393 bp, respectively) were amplified by conventional PCR. The two fragments were cloned, and the DNA sequences were determined. Both 18S rRNA sequences were deposited in the GenBank database (accession nos. AY960109 and AY960108, respectively).

To develop a primer-probe set within the 18S rRNA gene region that would be suitable for the Taqman real-time PCR detection assay and specific for molds, DNA sequences from several eukaryotes, including *B. fulva* ATCC 24474 and *P. digitatum* ATCC 10030, were aligned. Three conserved oligonucleotides in molds were derived: the forward primer MoldFP 5′-CCGCTGGCTTCTTAGGG-3′, the reverse primer MoldRP 5′-GATGTACTCGCTGGC CCT-3′ flanking an amplicon of 110 bp, and the probe MoldTP 5′-CTCAAGCCGATGGAAGTGCG-3′ (Fig. 1). The primers and the FAM- and BHQ-labeled probe were used in the Taqman real-time PCR assay.

Specific detection of molds. Real-time PCR assays were performed to determine the specificity of the 18S rRNA gene-targeting primers and probe against spoilage molds *B. fulva* ATCC 24474 and *P. digitatum* ATCC 10030. Representative strains of selected bacteria common-

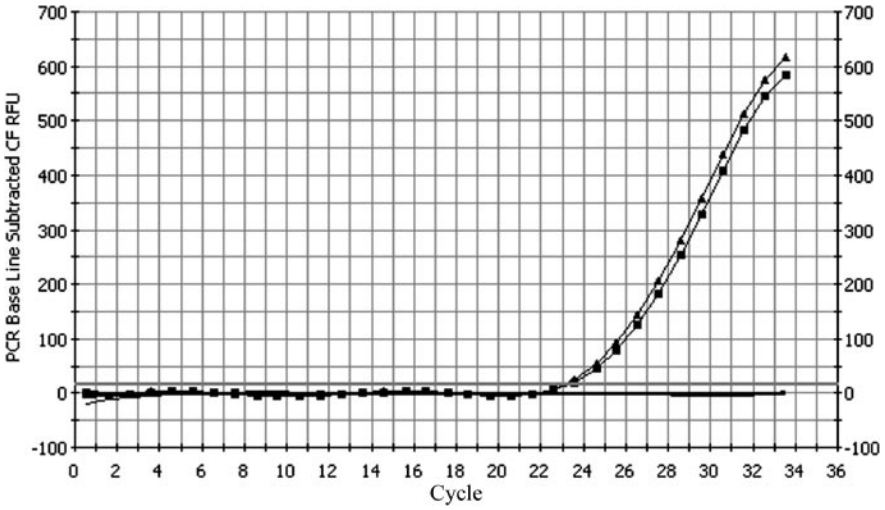

FIGURE 2. *Real-time PCR amplification of 18S rRNA gene fragment with the mold-specific primer-probe set.* ■, Byssochlamys fulva *ATCC 24474;* ▲ Penicillium digitatum *ATCC 10030.* Alicyclobacillus acidocaldarius *ATCC 43030,* Escherichia coli *DH-5α,* Lactococcus lactis lactis *2301,* Listeria monocytogenes *V7,* Pseudomonas putida *49451, grape, orange, and apple gave no response (horizontal line). CF RFU, curve fit relative fluorescence units.*

ly associated with foods and cells from selected fruits were included in the study to test the possibility of cross-reactivity by the primer-probe set. Assays were performed in triplicate, and a representative real-time PCR chart is shown in Figure 2. According to the output charts, positive curves were identified in samples containing *B. fulva* ATCC 24474 and *P. digitatum* ATCC 10030. Common foodborne microorganisms, including the yeast *Z. bailii* ATCC 36947, did not cause cross-reactivity. Although only a few representative strains were used in the laboratory specificity studies, a computer-based specificity search was conducted covering all the deposited DNA sequences available through NCBI. BLAST search results revealed that all homologous sequences with 88% or higher identify (88% for the 17-mer MoldFP, 83% for the 18-mer MoldRP, and 80% for the 20-mer probe MoldTP) were from molds. These results suggested that under properly stringent conditions, the combination of the sequences of the oligonucleotide primers and probe used in the study would be distinctive enough to detect mold without cross-reactivity with other microorganisms or the eukaryotic fruit cells.

Identification of the environmental mold isolate KW01. KW01 was isolated from an environmental sample and formed large single colonies on acidified PDA after 48 h of incubation at 25°C. The cells were positive by real-time PCR with the mold-specific primer-probe set (data not shown).

Using the primer pair 18SrRNAtotal5 and 18SrRNAtotal3 and the genomic DNA from KW01, a 1,431-bp 18S rRNA gene fragment was amplified by conventional PCR. This fragment was sequenced, and the sequence was deposited in GenBank (accession no. AY960110). DNA sequence analysis revealed that this mold strain is mostly homologous to *Penicillium* spp. After 72 h of incubation on PDA, the colony had a diameter about 0.5 cm with gray-green color in the front and yellow color from the back of the agar medium. Under the microscope, the conidia were globose, and conidiophores had two-stage or three-stage branches. These observations were consistent with the identification of *Penicillium* sp.

Detection limit in orange juice and saline. Experiments were conducted to determine the detection sensitivity for targeted organism using the newly developed real-time PCR assay. Because *Penicillium* sp. KW01 formed large single colonies without heavy filament structure on acidified PDA after 48 h of incubation at 25°C, this mold was selected for the study. All experiments were repeated at least three times. KW01 was detected at less than 10^3 CFU/ml, corresponding to 10 CFU per reaction, in all replicates. A representative detection chart is presented in Figure 3. Orange juice containing KW01 at no less than 4.3×10^2 CFU/ml, corresponding to 4.3 CFU per reaction, tested positive by the real-time PCR method (Fig. 3). The time elapsed before appearance of a positive signal corresponded to the cell density in the juice. More thermal cycles were needed to produce detectable amplicons from samples containing low populations than from those with high populations. Similar limits of detection were obtained when KW01 cells were suspended in both saline and orange juice (data not shown), indicating no significant inhibition to the reaction by factors in orange juice.

DISCUSSION

Reliable detection of microorganisms in a timely manner has been a major challenge to the food industry, and real-time PCR technology has the potential to address this issue. Molds, yeasts, and aciduric bacteria are main spoilage agents in acid foods, including fruit juices. Rapid and sensitive detection of these spoilage agents in both the raw materials and final products are essential for product quality control. A major advantage of the Taqman procedure is that it is flexible in terms of sequence matching between the probe and target sequences. Therefore design of detection probes with a broader spectrum of organism coverage becomes possible. Based on this feature, we developed two real-time systems to detect the presence of spoilage *Alicyclobacillus* and a few closely related species in selected foods *(3, 9).*

In this study, a primer-probe set specific for molds targeting the 18S rRNA gene was developed. With this prim-

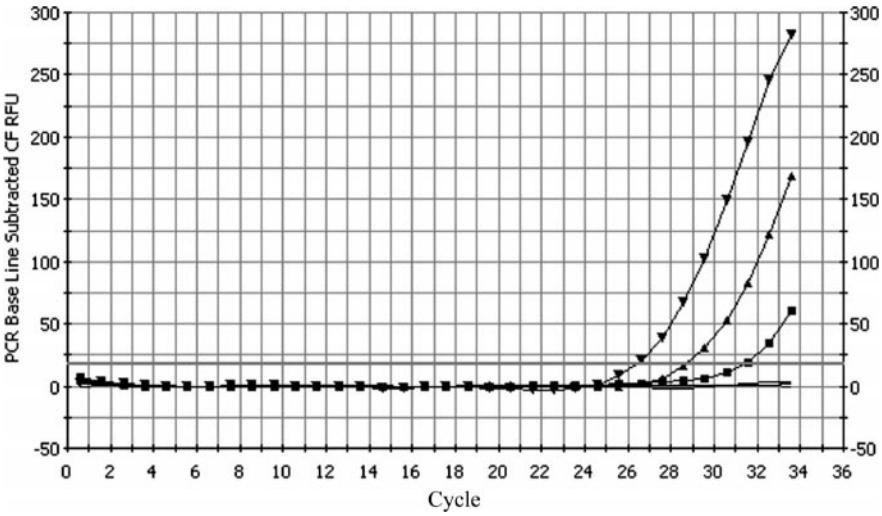

FIGURE 3. *Sensitivity of detection for real-time PCR product obtained with the environmental mold KW01 diluted in orange juice.* ▼, *4.3* $\times$ 10^2 *CFU per reaction;* ▲, *4.3* $\times$ 10^1 *CFU per reaction;* ■, *4.3 CFU per reaction. The uninoculated orange juice (blank control) gave no response (horizontal line). CF RFU, curve fit relative fluorescence units.*

er-probe set, the presence of the common spoilage molds *B. fulva* ATCC 24474 and *P. digitatum* ATCC 10030 can be detected within 6 to 7 h. This time scale is a major improvement compared with the current mainstream industrial practices, which are based on total plate counting and biochemical analyses that take at least 48 h. Because of its design, this primer-probe set for mold detection can be used to screen for a broad range of mold strains. Results from both the laboratory specificity study and the computer-based specificity search further suggest that this primer-probe set is specific for molds, with no cross-reactivity against fruit and bacterial cells.

A certain cell density is required to conduct serial dilutions for the sensitivity analysis. However, most molds will form filament structures at high density, and accurate cell counting becomes difficult. Although conidia and ascospores have been counted with counting chambers *(7)*, we used a different approach. Mold strain KW01, which formed clear single colonies on acidified PDA, was isolated from the environment and used in the sensitivity analysis. With the newly developed real-time PCR system, the presence of KW01 at less than 10^3 CFU/ml, corresponding to 10 CFU per reaction, was detected without cross-reactivity with bacteria or other eukaryotes without any enrichment procedures. This result is comparable to those obtained from previous studies with real-time PCR detection limits of 1.0×10^2 to 1.0×10^1 CFU per reaction *(2, 11)*. This sensitivity is also comparable to or better than that achieved by the conventional culturing methods (20 to 30 CFU per plate). Detection sensitivity of this real-time PCR system can be further improved by concentrating the microbial cells from a larger sample, which would be important for analysis of industrial samples that range from 25 ml to 1 liter. Although in various studies the sensitivity was reduced in real food matrices, similar limits of detection in saline and orange juice were achieved in the current study. This result indicated that components of the orange juice did not cause significant inhibition of the real-time reaction.

Compared with extraction of bacterial DNA, extraction of genomic DNA from molds is much more complex and the efficiency of the process is directly related to the detection sensitivity of the real-time PCR method. Among various extraction approaches, the glass beads method was chosen because of its simplicity and the consistency of the results. The detergent in the extraction buffer further improved the efficiency of DNA extraction *(4, 10, 14)*. After cell lysis, the detergent was removed from the DNA during the genomic DNA purification process. The detection procedure, including DNA extraction and real-time PCR, can be completed within 6 to 7 h.

This newly developed real-time PCR assay can specifically detect spoilage molds in orange juice, with a detection sensitivity comparable to that of conventional approaches. The new approach significantly improved the detection time (6 to 7 h versus 48 h or longer). Such a system should be very useful for industrial applications from screening of raw materials to quality control of the final product.

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REFERENCES

1. Berbee, M. L., and J. W. Taylor. 1992. Detecting morphological convergence in true fungi, using 18S rRNA gene sequence data. *Biosystems* 28:117–125.
2. Bleve, G., L. Rizzotti, D. Franco, and T. Sandra. 2003. Development of reverse transcription (RT)-PCR and real-time RT-PCR assays for rapid detection and quantification of viable yeasts and molds contaminating yogurts of pasteurized food products. *Appl. Environ. Microbiol.* 69:4116–4122.
3. Connor, C. J., H. Luo, B. B. McSpadden-Gardener, and H. H. Wang. 2005. Development of a real-time PCR-based system targeting the 16S rRNA gene sequence for rapid detection of *Alicyclobacillus* spp. in juice products. *Int. J. Food Microbiol.* 99:229–235.
4. Danilevich, V. N., and E. V. Grishin. 2001. A new approach to the isolation of genomic DNA from yeast and fungi: preparation of DNA-containing cell envelopes and their use in PCR. *Bioorg. Khim.* 28:136–146.
5. Farkas, J. 2003. Rapid detection of microbial contamination. Avail-

able at: http://www.flair-flow.com/industry-index.html. Accessed 17 March 2005.
6. Hanna, S. E., J. C. Christopher, and H. H. Wang. 2005. Real-time polymerase chain reaction for the food microbiologist: technologies, applications, and limitations. *J. Food Sci.* 70:49–53.
7. Haugland, R. A., M. Varma, L. J. Wymer, and S. J. Vesper. 2004. Quantitative PCR analysis of selected *Aspergillus, Penicillium* and *Paecilomyces* species. *Syst. Appl. Microbiol.* 27:198–210.
8. Li, S., R. R. Marquardt, and D. Abramanson. 2000. Immunochemical detection of molds: a review. *J. Food Prot.* 63:281–291.
9. Luo, H., A. E. Yousef, and H. H. Wang. 2004. A real-time polymerase chain reaction–based method for rapid and specific detection of spoilage *Alicyclobacillus* spp. in apple juice. *Lett. Appl. Microbiol.* 39:376–382.
10. Pedersen, L. H., P. Skouboe, M. Boysen, J. Soule, and L. Rossen. 1997. Detection of *Penicillium* species in complex food samples using the polymerase chain reaction. *Int. J. Food Microbiol.* 35:169–177.
11. 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.
12. Ray, B. 2004. Fundamental food microbiology, 3rd ed. CRC Press, Boca Raton, Fla.
13. Sogin, M. L., and J. H. Gunderson. 1987. Structural diversity of eukaryotic small subunit ribosomal RNAs. Evolutionary implications. *Ann. N.Y. Acad. Sci.* 503:125–139.
14. Vanburik, J.-A. H., R. W. Schreckhise, T. C. White, R. A. Bowden, and D. Myerson. 1998. Comparison of six extraction techniques for isolation of DNA from filamentous fungi. *Med. Mycol.* 36:299–303.
15. Van der Vossen, J. M., and H. Hofstra. 1996. DNA based typing, identification and detection systems for food spoilage microorganisms: development and implementation. *Int. J. Food Microbiol.* 33: 35–49.
16. Van der Zee, H., and J. H. Huis in't Veld. 1997. Rapid and alternative screening methods for microbiological analysis. *J. AOAC Int.* 80: 934–949.
17. Walsh, P. M., and L. L. McKay. 1981. Recombinant plasmid associated with cell aggregation and high-frequency conjugation of *Streptococcus lactis* ML3. *J. Bacteriol.* 146:937–944.
18. Wang, H., K. Baldwin, D. J. O'Sullivan, and L. L. McKay. 2000. Identification, gene cloning, nucleotide sequencing, and expression of pyruvate carboxylase in fast milk coagulating *Lactococcus lactis* subsp. *lactis* C2. *Appl. Environ. Microbiol.* 66:1223–1227.
19. Yousef, A. E., E. T. Ryser, and E. H. Marth. 1988. Methods for improved recovery of *Listeria monocytogenes* from cheese. *Appl. Environ. Microbiol.* 54:2643–2649.