Note

Modified Multiplex PCR Methods for Comprehensive Detection of Pectinatus and Beer-Spoilage Cocci

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Specific PCR primers were designed based on the 16S rRNA genes of recently proposed beer-spoilage species, Pectinatus haikarae, Megasphaera suecicensis, and M. paucivorans, and two sets of our previously reported multiplex PCR methods for Pectinatus spp. and beer-spoilage cocci were reconstructed. Each modified multiplex PCR method was found specifically to detect beer-spoilage species of Pectinatus and cocci, including new species.

Key words: Pectinatus haikarae; Megasphaera suecicensis; Megasphaera paucivorans; multiplex PCR

Beer is a microbiologically stable beverage due to the presence of antibacterial alcohol and hop compounds, as well as low pH and the depletion of dissolved oxygen and nutrients.2) Thus, only a limited number of bacterial species, principally belonging to the genera Lactobacillus, Pediococcus, Pectinatus and Megasphaera, are known to spoil beer. As described in our previous report, we divided these beer-spoilers into three groups, Lactobacillus (Gram-positive rods), Pectinatus (Gram-negative rods), and Pediococcus and Megasphaera (Gram-positive and Gram-negative cocci) on the basis of morphological and Gram-staining features, and constructed three sets of multiplex PCR methods to detect beer-spoilage species in each group.2) These multiplex PCR methods were designated L. multiplex (Lactobacillus), P. multiplex (Pectinatus), and C. multiplex (cocci) respectively.2) However, four new beer-spoilage species, Lactobacillus backi, Pectinatus haikarae, Megasphaera suecicensis, and M. paucivorans, have recently been proposed.3,4) Hence, we investigated the applicability of our multiplex PCR methods to determine whether these new species can be detected by them. In the event, among these new species, only L. backi was detected by the L. multiplex method. L. backi is described as a species closely related to Lactobacillus coryniformis, and, was found to have DNA sequences corresponding to the L. multiplex primers specific to L. coryniformis. On the other hand, P. haikarae, M. suecicensis, and M. paucivorans were not detected by P. multiplex and C. multiplex respectively. Hence, in the present study, we attempted to improve the previously described multiplex PCR methods, P. multiplex and C. multiplex, in order to include the new beer-spoilage species, P. haikarae, M. suecicensis, and M. paucivorans.

PCR primers to detect the new beer-spoilers were designed based on the nucleotide sequences of the 16S rRNA genes, which were registered under accession nos., DQ223729, DQ223730, and DQ223731 (Table 1).5) For P. haikarae, a species-specific primer pair was designed. In the cases of M. suecicensis and M. paucivorans, a primer pair simultaneously detecting both species was designed, in view of the more than 99% similarity in the 16S rRNA gene sequences between these two species.6) In addition, the common primer pair had the advantage of reducing the number of primers in the multiplex PCR methods. The primer pair for P. haikarae and the common primer pair for M. suecicensis and M. paucivorans were added to the existing primer mix of the corresponding multiplex PCR, P. multiplex and C. multiplex respectively. In Table 1, the primer sequences and predicted sizes of each PCR product are listed.

Specificity, the reactivity and sensitivity of the each modified primer mix were evaluated by conducting multiplex PCR. For the template of multiplex PCR, two different DNA extraction methods were adopted. In evaluating specificity, DNA was extracted from a pure culture of each test-strain with a MagExtractor DNA Preparation reagent (Applied Biosystems, Foster City, CA). The DNA extraction was conducted according to the manufacturer’s instructions, followed by 5-fold

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dilution with sterile distilled water. This extraction method was adopted in actual applications due to its simplicity and rapid results. PCR was set up in a 50 μl reaction volume, containing 25 μl of PerfectShot Ex Taq (TakaraBio, Shiga, Japan), 0.8 μM of each primer (Table 1), and 5 μl of DNA solution.

PCR was performed using the GeneAmp PCR system 9700 (Applied Biosystems). The amplification profile was 94 °C for 2.5 min, followed by 30 cycles consisting of 15 s at 94 °C, 15 s at 55 °C and 30 s at 72 °C. The last extension step was 3 min. Five μl of the reaction mixture was analyzed by agarose gel (2%) electrophoresis, and the amplified PCR products were visualized with SYBR Green I Nucleic Acid Gel Stain (Invitrogen, Carlsbad, CA). The 100 bp DNA ladder (TakaraBio) was used for the molecular size marker.

The specificity of the modified primer mix of P. multiplex was determined, using six closely related species of *Pectinatus* and 14 frequent brewery isolates, in addition to the three target species. It was found that only the target species could be detected without false positive results for the non-target species (Table 2). Reactivity was investigated using 54 strains of the target species of *Pediococcus* and *Ped. inopinatus*, and all of the strains investigated were detectable (data not shown). These results indicate that the modified primer mix of *C. multiplex* had enough specificity and reactivity. Then sensitivity was investigated in a manner identical with P. multiplex. Because the detection limits of the modified primer mix were found to be comparable with those of the simplex primer pairs, the sensitivity of the modified C. multiplex method was considered to be suitable for practical application. Furthermore, a more than 10³-fold diluted DNA solution of each species was sufficiently detected by the modified primer mix. Taken collectively, the modified primer mix of C. multiplex showed practical specificity, reactivity and sensitivity.

In this study, we improved on the previously reported multiplex PCR methods, P. multiplex and C. multiplex, by developing specific primers to detect newly assigned beer-spoilage species, *P. haikarae*, *M. sueciensis*, and *M. paucivorans*. These specific primers were found to work well in a modified multiplex primer mix. Consequently, one can cover three species of beer-spoilage *Pectinatus* by the advanced P. multiplex PCR method, and six species of beer-spoilage *Pediococcus* and

### Table 1. Primers for the Modified Multiplex PCR

<table>
<thead>
<tr>
<th>Method</th>
<th>Target species</th>
<th>Primers</th>
<th>Direction</th>
<th>Sequence (5'→3')</th>
<th>Target DNA</th>
<th>Predicted product size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. multiplex</em></td>
<td><em>Pectinatus cerevisiophilus</em></td>
<td>16C-F</td>
<td>Forward</td>
<td>CGTATGCAGAGATGCAATT</td>
<td>16S rDNA</td>
<td>621</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IC-R</td>
<td>Reverse</td>
<td>CACTCTTTACAAGTAGTAC</td>
<td>ITS region</td>
<td></td>
<td>6</td>
</tr>
<tr>
<td><em>Pectinatus frisingensis</em></td>
<td></td>
<td>16F-F</td>
<td>Forward</td>
<td>CGATCCAGAGATGGATATT</td>
<td>16S rDNA</td>
<td>708, 883</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IF-R</td>
<td>Reverse</td>
<td>CCATCTTCTGAAAAACCTC</td>
<td>ITS region</td>
<td></td>
<td>6</td>
</tr>
<tr>
<td><em>Pectinatus haikarae</em></td>
<td></td>
<td>Pf1</td>
<td>Forward</td>
<td>AATACCGGAATTGTGAAGAG</td>
<td>16S rDNA</td>
<td>508</td>
<td>this study</td>
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<td></td>
<td></td>
<td>Pfr2</td>
<td>Reverse</td>
<td>CTTCTCCTGCCACTCAGACAT</td>
<td>16S rDNA</td>
<td></td>
<td>this study</td>
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<tr>
<td><em>C. multiplex</em></td>
<td><em>Pediococcus damnosus</em> and <em>Ped. inopinatus</em></td>
<td>PIDEF1</td>
<td>Forward</td>
<td>ACCGAATACGATCTAAG</td>
<td>16S rDNA</td>
<td>566</td>
<td>2</td>
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<td></td>
<td></td>
<td>PIDR8</td>
<td>Reverse</td>
<td>TAAAGACCGACCTTACCAG</td>
<td>16S rDNA</td>
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<tr>
<td><em>Pediococcus clausenii</em></td>
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<td>PCLAF3</td>
<td>Forward</td>
<td>TGTGAGAGTAACTGCTCATG</td>
<td>16S rDNA</td>
<td>462</td>
<td>2</td>
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<tr>
<td></td>
<td></td>
<td>PCLAR3</td>
<td>Reverse</td>
<td>ACGCCCTATTCTTGGTTA</td>
<td>16S rDNA</td>
<td></td>
<td>2</td>
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<tr>
<td><em>Megasphaera cerevisiae</em></td>
<td></td>
<td>Mc-f4</td>
<td>Forward</td>
<td>CATTTCGGTTAAAAGAATCA</td>
<td>16S rDNA</td>
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<td></td>
<td></td>
<td>Mc-e4</td>
<td>Reverse</td>
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<td>16S rDNA</td>
<td></td>
<td>2</td>
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<tr>
<td><em>Megasphaera sueciensis</em> and <em>M. paucivorans</em></td>
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<td>Msp-f</td>
<td>Forward</td>
<td>TATGGCCAATACCCATAGAT</td>
<td>16S rDNA</td>
<td>155</td>
<td>this study</td>
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<tr>
<td></td>
<td></td>
<td>Msp-r</td>
<td>Reverse</td>
<td>CACTTTTAAGACAGACTGGA</td>
<td>16S rDNA</td>
<td></td>
<td>this study</td>
</tr>
</tbody>
</table>

*Internal transcribed spacer region of 16S-23S rDNA.*
Target species for P. multiplex PCR method
Pectinatus cerevisiophilus DSM 20467<sup>T</sup>  
Pectinatus frisingensis DSM 6306<sup>T</sup>  
Pectinatus haikarae DSM 16980<sup>T</sup>

Closely related species of Pectinatus
Megasphaera cerevisiae DSM 20462<sup>T</sup>  
Megasphaera sueciensis DSM 17042<sup>T</sup>  
Megasphaera paucivorans DSM 16981<sup>T</sup>  
Selenomonas lactificex DSM 20757<sup>T</sup>  
Zymophilus paucivorans DSM 20756<sup>T</sup>  
Zymophilus raffinosus DSM 20765<sup>T</sup>

Table 2. Specificity Evaluations of the Multiplex PCR Methods

<table>
<thead>
<tr>
<th>Multiplex PCR method</th>
<th>P.</th>
<th>C.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Target species for P. multiplex PCR method</td>
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<td>+</td>
</tr>
<tr>
<td>Pectinatus cerevisiophilus DSM 20467&lt;sup&gt;T&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Pectinatus frisingensis DSM 6306&lt;sup&gt;T&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Pectinatus haikarae DSM 16980&lt;sup&gt;T&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Closely related species of Pectinatus</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Megasphaera cerevisiae DSM 20462&lt;sup&gt;T&lt;/sup&gt;</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Megasphaera sueciensis DSM 17042&lt;sup&gt;T&lt;/sup&gt;</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Megasphaera paucivorans DSM 16981&lt;sup&gt;T&lt;/sup&gt;</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Selenomonas lactificex DSM 20757&lt;sup&gt;T&lt;/sup&gt;</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Zymophilus paucivorans DSM 20756&lt;sup&gt;T&lt;/sup&gt;</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Zymophilus raffinosus DSM 20765&lt;sup&gt;T&lt;/sup&gt;</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Closely related species of Pediococcus and Megasphaera
Pediococcus acidilactici JCM 5885<sup>T</sup>
Pediococcus dextrinus JCM 5887<sup>T</sup>
Pediococcus pentosaceus JCM 5890<sup>T</sup>
Pediococcus parvulus JCM 5889<sup>T</sup>
Lactococcus lactis JCM 5805<sup>T</sup>
Leuconostoc mesenteroides JCM 6124<sup>T</sup>
Leuconostoc paramesenteroides NCIB 8033<sup>T</sup>
Enterococcus casseliflavus HC268
Pectinatus cerevisiophilus DSM 20467<sup>T</sup>
Pectinatus frisingensis DSM 6306<sup>T</sup>
Pectinatus haikarae DSM 16980<sup>T</sup>
Selenomonas lactificex DSM 20757<sup>T</sup>
Zymophilus paucivorans DSM 20756<sup>T</sup>
Zymophilus raffinosus DSM 20765<sup>T</sup>

Frequent brewery isolates
Lactococcus lactis HC311
Clostridium beijerinckii HC350
Serratia marcescens HC367
Cedroacter freundii HC417
Enterobacter cloacae HC432
Staphylococcus warneri HC437
Propionibacterium acnes HC440
Bacillus thuringiensis HC442
Pantoaea agglomerans HC453
Paenibacillus anothyophilus HC459
Paenibacillus jaellae HC466
Staphylococcus epidermidis HC475
Klebsiella oxytoca HC534
Sporolactobacillus ruminicic HC566

<sup>a</sup> + indicates a positive reaction; – indicates a negative reaction.

**Megasphaera** by it. Hence, these advanced multiplex PCR method should contribute to microbiological quality control of breweries as a simple and accurate method, leading to a reduction in the risk of microbiological incidence and providing high-quality beer products for consumers.

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**References**