Long PCRs of Transposons in the Structural Analysis of Genes Encoding Acquired Glycopeptide Resistance in Enterococci


ABSTRACT

Glycopeptide-resistant enterococci (GRE) associated with multiple antibiotic resistance present a major challenge to clinical practice and infection control due to limited or nonexistent antimicrobial treatment options. The genes encoding VanA- and VanB-type glycopeptide resistance have been shown to reside on transposons Tn1546 and Tn1547, respectively. These transferable genetic elements may carry the resistance determinants between and within different ecological niches. Molecular epidemiological studies of nosocomial outbreaks of VanA- and VanB-type GRE have revealed genomic heterogeneity in a significant number of isolates as well as cross-transmission of clonal strains (15,19). These observations indicate horizontal transfer of glycopeptide resistance genes as an important mechanism for the spread of GRE. To target infection control and better understand the epidemiology of GRE, outbreak investigations and molecular epidemiological studies should therefore apply at least two different approaches, i.e., molecular typing methods to analyze bacterial genomic heterogeneity and structural analysis of mobile resistance determinants.

Pulsed-field gel electrophoresis has

INTRODUCTION

Infections with multidrug-resistant bacteria present a major challenge to clinical practice and infection control due to limited or nonexistent antimicrobial treatment options. Glycopeptide-resistant enterococci (GRE) associated with multiple antibiotic resistance have received particular attention (13). Two phenotypes of acquired glycopeptide resistance in enterococci, VanA and VanB, have been described (13). The genes encoding the VanA- and VanB-type glycopeptide resistance have been shown to reside on transposons Tn1546 (2) and Tn1547 (14), respectively, or in closely related transferable genetic elements (10). These mobile genetic elements may carry the resistance determinants between different strains. Molecular epidemiological studies of nosocomial outbreaks of VanA- and VanB-type GRE have revealed genomic heterogeneity in a significant number of isolates as well as cross-transmission of clonal strains (15,19). These observations indicate horizontal transfer of glycopeptide resistance genes as an important mechanism for the spread of GRE. To target infection control and better understand the epidemiology of GRE, outbreak investigations and molecular epidemiological studies should therefore apply at least two different approaches, i.e., molecular typing methods to analyze bacterial genomic heterogeneity and structural analysis of mobile resistance determinants.

Pulsed-field gel electrophoresis has

Figure 1. Agarose gel electrophoresis of long PCR-amplified fragments. (A) A representative agarose gel electrophoresis of Tn1546 amplified fragments from human clinical VanA-type GRE strains of different origins (lanes 2–8). Lane 1: 1-kb ladder (Life Technologies, Gaithersburg, MD, USA) with sizes as given in Panel C; lane 9: negative control (H2O). (B) Restriction fragments length analysis of Tn1546 amplified fragments. BclI/HincII-digested Tn1546 amplified fragments analyzed with agarose gel electrophoresis. Lanes 1–3: independent VanA-type human clinical GRE isolates; lane 4: φX174 DNA HaeIII digest (Promega, Madison, WI, USA). (C) Agarose gel electrophoresis of Tn1547 amplified fragments diluted 1:10. Lane 1: 1-kb ladder (Life Technologies); lane 2: negative control (H2O); lane 3: VanB-type E. faecalis clinical isolate; lane 4: glycopeptide-susceptible E. faecalis clinical isolate; lane 5: VanB-type E. faecalis V583 (16).
been used extensively to determine strain-relatedness of GRE outbreaks (19). To detect and characterize glycopeptide resistance determinants, gene probing and single-gene polymerase chain reactions (PCRs) have been utilized (2,10). Here we describe the development and use of long PCRs (i.e., specific amplification of targets in the 5–25-kb range) (8), also called extended-range PCRs, in the structural analysis of van gene organization in GRE.

MATERIALS AND METHODS

Preparation of PCR Template

DNAs from 2–3 bacterial colonies (≤24 h old) of each GRE isolate dissolved in 10 µL 0.9% NaCl were purified by Dynabeads® DNA DIRECT™Kit (Dynal, Oslo, Norway) according to the instructions from the manufacturer and resuspended in 30 µL TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The extraction procedure yielded 8–18 ng bacterial DNA/µL.

General PCR Conditions

For each PCR, 3–5 µL DNA extract were used. PCR amplifications were performed in a GeneAmp® PCR System 9600 or 2400 (Perkin-Elmer, Norwalk, CT, USA). Contamination precautions cited by Kwok were followed (12). Aliquots (15 µL) of amplification and restriction enzyme digestion products were analyzed on TBE agarose gels with ethidium bromide (0.5 µg/mL).

Tn1546 Long PCR

A long PCR (Tn1546 PCR) for the vanA gene cluster covering 10,414 bp of the 10,851-bp transposon Tn1546 described in the VanA reference strain Enterococcus faecalis BM4147 (2) was established. Table 1 presents details on primer location and characteristics. A master mixture was made with components from GeneAmp XL PCR Kit (Perkin-Elmer). The PCR was carried out in a 70-µL reaction volume including 1.2× XL buffer, 1.1 mM Mg(OAc)₂, 0.23 mM of each dNTP, 0.23 µM of each primer, 4 U rTth DNA Polymerase (Perkin-Elmer) and 3 µL bacterial DNA extract. Using hot-start PCR, optimized amplification conditions were 94°C initially for 1 min, 94°C for 15 s and 68°C for 6 min for 27 cycles and a final 15-min extension period at 72°C.

RESULTS

Tn1546 Long PCR

A representative agarose gel electrophoresis picture of Tn1546 amplified fragments is shown in Figure 1A. Amplified fragments of the expected size were detected in lanes 3 and 5–8. Samples in lanes 2 and 4 yielded negative results. The specificity of Tn1546 amplified fragments was examined by restriction enzyme digestion in comparison with the published information (2). A 5-µL sample of each PCR amplified fragment was digested with 1 U HincII (New England Biolabs, Hitchin, Hertfordshire, England, UK) and 1 U BclI (New England Biolabs) for 2 h in a 20-µL reaction volume in accordance with the instructions of the manufacturer. Figure 1B presents representative HincII/BclI digests of Tn1546 amplified fragments. Lanes 1 and 3 show a restriction fragment length pattern (RFLP)-designated type A in accordance with the published Tn1546 sequence (2). Figure 1B, lane 2 shows an RFLP-designated type B, with an approximately 1500-bp insertion in fragment 7 covering the vanX-vanY region.

<table>
<thead>
<tr>
<th>Target Region</th>
<th>Direction (Name)</th>
<th>5'-3' Sequence (Positiona)</th>
<th>Tm b</th>
<th>Amplified Fragment Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tn1546</td>
<td>Forward (trnpF)</td>
<td>AAC CTA AGG GCG ACA TAT GGT G (nt 164–185)</td>
<td>67.4</td>
<td>10,414</td>
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<tr>
<td></td>
<td>Reverse (vanZR11)</td>
<td>GGT ACG GTA AAC GAG CAA TAA TAC G (nt 10577–10573)</td>
<td>68.2</td>
<td></td>
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<tr>
<td>Tn1547</td>
<td>Forward (XLBF1)</td>
<td>GTT TGA TGC AGA GGC AGA CGA CT (nt 450–472)</td>
<td>69.9</td>
<td>5,959</td>
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<tr>
<td></td>
<td>Reverse (XLBR1)</td>
<td>ACA AGT TCC CCT GTA TCC AAG TGG (nt 6408–6385)</td>
<td>69.9</td>
<td></td>
</tr>
</tbody>
</table>

a The nucleotide positions given are from the published sequences of the vanA (2) and vanB gene cluster (7).
b Melting temperature calculated according to the nearest-neighbor method (4).
(2) of the Tn1546.

**Tn1547 Long PCR**

A representative agarose gel electrophoresis picture of Tn1547 amplified fragments is shown in Figure 1C. The specificity of Tn1547 amplified fragments was analyzed by restriction enzyme digestion as described for the Tn1546 amplified fragments. EcoRV RFLP (New England Biolabs) of the Tn1547 amplified fragments, shown in Figure 1C, lanes 3 and 5, revealed fragment sizes (2494, 1176, 2093 and 196 bp) in accordance with the published vanB gene cluster sequence (7) (data not shown).

**DISCUSSION**

The utility of long PCR is dependent upon its ability to reproducibly amplify the target region with high specificity and fidelity (8). To promote PCR specificity, we focused on hot-start PCR and optimal primer design using the nearest-neighbor algorithm for selection of primers with small differences in high melting temperatures during amplification standardization. A primer design tool (9), which is available through the Internet ([http://alces.med.umn.edu/webprimers.html](http://alces.med.umn.edu/webprimers.html)), was used for primer selection. The yield of amplification product in extended-range PCRs is also dependent upon the quality and concentration of the template (8). We were not able to detect amplified fragments using a crude boiled bacterial template, which has been successfully used for the amplification of single genes in the van gene clusters (data not shown). However, the use of uniform, supermagnetic, polymer particles (Dynabeads DNA DIRECT Kit), originally developed for rapid isolation of PCR-ready eukaryotic DNA, repeatedly provided high-quality DNA for our long PCRs. The successful use of Dynabeads biomagnetic separation technology for template preparation might be because of its removal of PCR inhibitors in addition to providing high-integrity DNA.

The Tn1546 and Tn1547 PCRs have been used to amplify van gene clusters from GRE isolates of several different geographical origins (data not shown). All VanB-type resistance elements have so far been successfully amplified using Tn1547 PCR protocol. A subgroup of VanA-type resistance elements have yielded negative results in the Tn1546 PCR (see Figure 1A, lanes 2 and 4). These negative results seem to be due to rearrangements and/or deletions in the target region of the forward primer (data not shown). The application of long PCR in the characterization of the vanA gene cluster has been pursued be-
fore (20). It would be interesting to compare these two long-PCR protocols in molecular epidemiological studies of Tn1546 elements.

The international spread of antimicrobial resistance is due to dissemination of resistant bacterial clones and mobile genetic elements carrying antimicrobial resistance determinants. Horizontal gene transfer of transposons conferring the van gene cluster has been suggested as a mechanism for spread of GRE (2). Although the origins of the vanA and vanB gene clusters are unknown, several reservoirs of VanA-type GRE have been identified recently. VanA-type GRE have been isolated from pet animals (17), animal food (6), feces of community patients (11,18), farm animals (1,3) and uncooked chicken, pork and beef in European countries (5,11).

These observations indicate that food and pet animals could be reservoirs for human GRE colonization and infections. The vanA gene has been identified by PCR in GRE strains from these reservoirs, but little is known about the structural elements conferring the vanA gene. Earlier studies have used Southern blot hybridization, cloning and DNA sequencing to analyze the organization of the structural elements in the vanA gene clusters (2,10). Restriction-enzyme digestion of extended-range, PCR-amplified fragments might be a more convenient method to characterize van gene cluster relatedness. Application of long PCR for molecular epidemiological studies of GRE strains from different ecological and geographical origins could reveal reservoirs and transmission lines. Specific sub-fragments of interest can then be targeted by specific PCRs and DNA sequencing to evaluate the possible horizontal transmission of glycopeptide-resistance determinants between and within different ecological niches. In a more general perspective, analysis of extended-range, PCR-amplified fragments might be a simple and efficient approach for the analysis of antimicrobial resistance determinants for molecular and epidemiological purposes.

REFERENCES


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