PCR-Restriction Fragment Length Polymorphism Analysis of Genes Coding for 16S rRNA in *Veillonella* spp.

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Restriction fragment length polymorphism analysis of PCR-amplified 16S ribosomal DNA (16S rDNA PCR-RFLP) was used to generate restriction profiles of the American Type Culture Collection type strains of the genus *Veillonella*, i.e., *V. atypica*, *V. caviae*, *V. criceti*, *V. dispar*, *V. parvula*, *V. ratti*, and *V. rodentium*. Whole-cell protein profiles were obtained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis for comparative purposes. The 16S rRNA genes were amplified by PCR, and RFLP analysis of the 16S rDNA was performed with *MnII* and *Sau3AI*. *MnII* produced six RFLP patterns for seven type strains, since the patterns for *V. atypica* and *V. caviae* were the same. RFLP patterns with *Sau3AI* could distinguish between *V. atypica* and *V. caviae*. The type strains of *Veillonella* species were easily distinguished by 16S rDNA PCR-RFLP.

The genus *Veillonella* consists of small, nonfermentative, anaerobic, gram-negative cocci that normally inhabit the mouths and respiratory and intestinal tracts of humans and animals (9, 10). Since *Veillonella* species obtain their energy by fermenting organic acids, e.g., pyruvate and lactate, this metabolism has the potential to remove a potent, dental-carries-producing acid. Thus, the presence of *Veillonella* cells in dental plaque samples may reduce the caries-producing potential of the organisms in the plaque (10).

Veillonella species, i.e., *V. atypica*, *V. caviae*, *V. criceti*, *V. dispar*, *V. parvula*, *V. ratti*, and *V. rodentium*, can be distinguished by DNA-DNA hybridization (5, 9), and some of these, i.e., *V. atypica*, *V. dispar*, *V. parvula*, and *V. ratti*, have also been

distinguished by 16S rDNA sequence comparison (11, 12). Conventional phenotypical and biochemical tests have been unable to distinguish *Veillonella* species (9). The protein profiles of *Veillonella* species are distinct, and determination of protein profiles is the recommended method for identifying the species of isolates within this genus (4, 10); this is consistent with the results of this study. In this study, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analyses were performed as described previously (7), and Fig. 1 shows that SDS-PAGE can be used to distinguish *Veillonella* species.

In this study, restriction fragment length polymorphism analysis of PCR-amplified 16S ribosomal DNA (16S rDNA PCR-RFLP) was used to generate restriction profiles of the type strains of the genus *Veillonella*, because this method can be used to identify species within some genera (1, 6, 8, 13, 14).

V. atypica ATCC 17744^T, *V. dispar* ATCC 17748^T, *V. parvula* ATCC 10790^T, *V. caviae* ATCC 33540^T, *V. criceti* ATCC 17747^T, *V. rodentium* ATCC 17743^T, and *V. ratti* ATCC 17746^T were obtained from the American Type Culture Collection. Twenty-eight fresh clinical strains, i.e., A6, A7, A30, A33, D76, D77, D78, D79, D81, D91, D92, G1, G2, G3, G4, G5, G6, G7, G8, G9, G10, G11, G12, G13, G14, G15, G16, and G17, were isolated from dental plaque from 19 volunteers and patients. They were identified to the species level by morphological and biochemical tests, including bacterial end product analysis by gas chromatography (3) and API ZYM (Rapid ID 32A) anal-

M 1 2 3 4 5 6 7

FIG. 1. SDS-PAGE profiles of type strains of *Veillonella* species. Lanes: 1, *V. atypica* ATCC 17744^T; 2, *V. parvula* ATCC 10790^T; 3, *V. dispar* ATCC 17748^T; 4, *V. rodentium* ATCC 17743^T; 5, *V. criceti* ATCC 17747^T; 6, *V. ratti* ATCC 17746^T; 7, *V. caviae* ATCC 33540^T; M, molecular mass markers (95, 68, 39, 29, 20.4, and 14 kDa).

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M 1 2 3 4 5 6 7 M

FIG. 2. Amplified 16S rDNA from type strains of *Veillonella* species digested with *MnII*. Lanes: 1, *V. atypica* ATCC 17744^T; 2, *V. dispar* ATCC 17748^T; 3, *V. parvula* ATCC 10790^T; 4, *V. caviae* ATCC 33540^T; 5, *V. criceti* ATCC 17747^T; 6, *V. rodentium* ATCC 17743^T; 7, *V. ratti* ATCC 17746^T; M, molecular size markers (2,000, 1,500, 1,000, 700, 525, 500, 400, 300, 200, 100 and 50 bp; Bio-Marker EXT; BioVentures).

FIG. 3. Amplified 16S rDNA from type strains of *Veillonella* species digested with *Sau3AI*. Lanes are as defined in the legend for Fig. 2.

ysis and SDS-PAGE analysis (7). As a result, all of the 28 fresh isolates in this study were identified as *V. parvula*. All strains were cultured on brain heart infusion-yeast extract-blood (sheep) agar plates (2) and incubated at 37°C for 3 days in an anaerobic glove box (model AZ-Hard; Hirasawa, Tokyo, Japan) containing 80% N₂, 10% H₂, and 10% CO₂.

From 3-day-old cultures, DNA was extracted with the InstaGene Matrix (Bio-Rad) according to the manufacturer's instructions. The 16S rRNA gene sequences were amplified by PCR with primers 27f and 1492r (6, 13) and *Taq* DNA polymerase (Takara Premix Taq; TaKaRa Biomedicals, Shiga, Japan) according to the manufacturer's instructions. The primer sequences were as follows: 27f, 5'-GTG CTG CAG AGA GTT TGA TCC TGG CTC AG-3'; and 1492r, 5'-CAC GGA TCC TAC GGG TAC CTT GTT ACG ACT T-3'. PCR amplification was performed in a DNA thermal cycler (PTC-100; MJ Research). The temperature profile included 35 cycles, each consisting of a template denaturation step at 94°C for 1 min, a primer annealing step at 52°C for 1 min, and an extension step at 72°C for 2 min. PCR products were detected by 0.8% agarose gel electrophoresis in Tris-acetate-EDTA buffer. The gel was stained with ethidium bromide and photographed under UV light. BioMarker EXT (BioVentures) was used as the molecular size marker.

PCR products were purified with the SpinBind PCR purification system (FMC BioProducts, Rockland, Maine). Purified 16S rRNA genes were digested singly with restriction endonucleases, i.e., *HaeIII*, *MnII*, or *Sau3AI* (New England Biolabs, Beverly, Mass.) according to the manufacturer's instructions. Digestion products were separated by 2% agarose (NuSieve 3:1 agarose; FMC BioProducts) gel electrophoresis in Tris-borate-EDTA buffer. The gel was stained with ethidium bromide and photographed under UV light.

RFLP patterns for the 16S rDNA PCR products from the

TABLE 1. 16S rDNA PCR-RFLP profiles of type strains of the genus *Veillonella*

Strain	16S rDNA PCR-RFLP profiles ^a		
	<i>MnII</i>	<i>Sau3AI</i>	<i>HaeIII</i>
<i>V. atypica</i> ATCC 17744 ^T	A	A	A
<i>V. dispar</i> ATCC 17748 ^T	B	B	B
<i>V. parvula</i> ATCC 10790 ^T	C	C	B
<i>V. caviae</i> ATCC 33540 ^T	A	D	A
<i>V. criceti</i> ATCC 17747 ^T	D	D	A
<i>V. rodentium</i> ATCC 17743 ^T	E	E	B
<i>V. ratti</i> ATCC 17746 ^T	F	D	A

^a 16S rDNA PCR-RFLP profiles were obtained in this study (Fig. 2 to 4).

seven type strains obtained with the three enzymes, i.e., *MnII*, *Sau3AI*, and *HaeIII*, are shown in Fig. 2 to 4. Among the three restriction endonucleases, *MnII* produced six RFLP patterns for seven reference strains, since the patterns for *V. atypica* and *V. caviae* were the same (Fig. 2, lanes 1 and 4). In contrast, RFLP patterns produced with *Sau3AI* could separate the two species (Fig. 3, lanes 1 and 4). *Sau3AI* produced five patterns, since the patterns for *V. caviae*, *V. criceti*, and *V. ratti* were the same (Fig. 3, lanes 4, 5, and 7). *HaeIII* produced two patterns, since the patterns for *V. atypica*, *V. caviae*, *V. criceti*, and *V. ratti* were the same (Fig. 4, lanes 1, 4, 5, and 7) and those for *V. dispar*, *V. parvula*, and *V. rodentium* were the same (Fig. 4, lanes 2, 3, and 6). The combination of the results of 16S rDNA PCR-RFLP using *MnII* and *Sau3AI* allowed the seven American Type Culture Collection type strains to be distinguished from one another (Table 1).

Twenty-eight fresh clinical strains, identified as *V. parvula* by conventional methods, were assigned to *V. parvula* (data not shown). Further studies, including large-scale studies with more clinical isolates, to confirm the usefulness of this 16S rDNA PCR-RFLP method are clearly required.

The 16S rRNA gene sequences from strains of three species of *Veillonella*, i.e., *V. atypica* DSM 20739, *V. dispar* DSM 20735, and *V. parvula* DSM 2008, were accessed from the GenBank. The expected sizes of the restriction fragments (at least 100 bp long) digested with *MnII* are as follows: *V. atypica*, 260, 205, 190, 135, 126, and 107 bp; *V. dispar*, 316, 260, 205, 135, and 107 bp; and *V. parvula*, 354, 316, 205, and 107 bp. For digestion with *Sau3AI*, they are as follows: *V. atypica*, 659, 308, 216, 155, and 116 bp; *V. dispar*, 1,026, 216, and 155 bp; and *V. parvula*, 368, 308, 216, 172, 155, and 116 bp. For digestion with *HaeIII*, they are as follows: *V. atypica*, 459, 392, 191, 133, and 121 bp; *V. dispar*, 459, 354, 191, 133, and 121 bp; and *V. parvula*, 459, 354, 191, 133, and 121 bp. The RFLP patterns obtained in this study (Fig. 2 to 4) are in accordance with the restriction profiles expected, except for those for *V. atypica* and *V. parvula* digested with *Sau3AI*.

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FIG. 4. Amplified 16S rDNA from type strains of *Veillonella* species digested with *HaeIII*. Lanes are as defined in the legend for Fig. 2.

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